BIOCATALYST AND BIOPROCESS ENGINEERING FOR THE SYNTHESIS OF AMINOPOLYOLS BY ENZYMATIC OXIDATION AND ALDOL ADDITION

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Bellaterra, September 2012.
Title of the thesis: Biocatalyst and bioprocess engineering for the synthesis of aminopolyols by enzymatic oxidation and aldol addition

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ACKNOWLEDGEMENTS

“If something’s hard to do, it’s not worth doing it”- these are the words of wisdom said by Homer J. Simpson. Anyway, in life, and especially when one is dedicating to research, this should definitely not be the motto. Now that this thesis is finally ready for the defence, and when I look back and remember all the hard work and dedication that it required, I really feel that it was worth it!!!

So, it is finally the moment to thank to all the people that in some way made it possible.

At first place I would like to thank to my mentors. To Goyo for his experienced guiding of my work, for availability and kindness. To Carmen for her 100% dedication, for all help and understanding, and for friendship. Thanks a lot to Josep, and the rest of group of aldolases: Gloria Caminal, Gloria Gonzales, Dolors and Carles for giving me the opportunity to be the part of the group and for all the help and advices they gave me when I needed them.

To Ines, Max and Rossmery, for sharing the experiences and doubts, and for bringing the good mood to laboratory. Thanks to Margot, for her availability, will to help whenever I needed, and for all her friendly smiles. To the rest of aldolaseros: Cristina, JuanMi, Jordi, Alfred, Dani, Marcela and Martina for all the shared time at labs.

To Carlos and Isaac for becoming such close friends and sharing all the good and bad moments with me, for their love and support. To Cynthia for her friendship and positivity. To Mabel, Yolanda, Lara, Mariangel, Juliana, Angelica, Andrea, Hoque, Bartroli, Mar, and the rest of the department for making such a friendly ambience at UAB.

To Jovanka for being the best friend in Barcelona, for her ability to make me laugh no matter what. To another Serbian friend Bojana, for her friendship and great help at the beginning of my life here.

Thanks a lot to Nataša and Nikola (my nephew, hehehe) for all the love and the unforgettable time we shared in Barcelona and Belgrade. For professor Zoran Vujčić for giving me the opportunity to carry out my research stay in his laboratory, for
professional approach and a lot that I feel I had learned from him, and for great sense of humor that helped me to enjoy my stay. To the rest of the group from Belgrade: Biljana, Sojka, Mira, Marica, Marinela, Anja and Barbara for their kindness and help. Hvala vam svima puno!!!!

I would never manage to get through this without the support, faith in me and love from my parents, my brother, and the rest of the family and friends from Serbia. And, finally thanks a lot to David. For sharing the life and for all his love.
RESUME

Nowadays, there is a growing interest for the application of the enzymes as process catalysts due to the environmental issues and the unique properties that they provide: high selectivity and specificity, and high catalytic activity while working under mild operational conditions. In this doctoral thesis, the enzymatic synthesis of the valued product Cbz-aminopolyol was achieved in a complex multienzymatic system by coupling the enzyme catalyzed oxidation of an amino alcohol to the corresponding amino aldehyde and enzyme catalyzed aldol addition to dihydroxyacetone phosphate (DHAP). Cbz-ethanolamine was selected as a model amino alcohol.

First of all, the enzymatic oxidation of Cbz-ethanolamine to Cbz-glycinal was studied by applying chloroperoxidase (CPO) from Caldariomyces fumago as a biocatalyst. The reaction was performed successfully using tert-butyl hydroperoxide as an oxidant. Peroxide addition strategy had to be optimized in order to minimize the peroxide dependent inactivation. Cbz-glycinal yield of 39.1% was reached when the peroxide was added at the rate of 3 mM/h. Furthermore, different reaction media were analyzed looking for the way to increase substrate concentration while favoring reaction rate and Cbz-glycinal production. Use of dioxane in the concentration of 5% (v/v) resulted in 6-fold improved Cbz-glycinal production compared to the value reached in aqueous reaction (production of 47.6 mM in cosolvent system containing 5% dioxane compared to 7.8 mM in aqueous one).

Then, with the aim of further improving of the stability of CPO, chemical modifications of CPO were carried out. Side-chain selective modifications of amino groups of Lys residues, and carboxyl groups of Asp and Glu residues, as well as crosslinking and periodate oxidation of sugar moiety were performed. The stability of modified CPOs was evaluated at different pH values, temperatures, and in the presence of tert-butyl hydroperoxide. Effect of modification of CPO on the performance of the reaction of Cbz-ethanolamine oxidation was studied as well. Those modifications that involved carboxyl groups via carbodiimide coupled method and the periodate oxidation of the sugar moiety produced better catalysts than native CPO in terms of stability and activity at elevated pH values and temperatures. At the temperature of 50°C and
peroxide addition rate of 12 mM/h, yields of Cbz-glycinal were improved from 16.1%, the value reached when using native CPO, to 21.5-22.1% when using modified CPOs. Even so, the problem of rapid irreversible inactivation of CPO by peroxides remained unsolved.

Expecting a more drastic improvement in CPO stability, different immobilization methods were studied: ionic adsorption, covalent attachment by carbodiimide coupled method, and covalent attachment of oxidized enzyme on monoaminoethyl-N-aminoethyl (MANA) agarose gels as well as covalent attachment on Eupergit® C. Conditions for each immobilization method were optimized in order to maximize the immobilization yields and minimize the enzyme inactivation during the immobilization process. Then, the presence of diffusion limitations of the immobilized enzyme preparations as well as the stability of immobilized enzymes in the conditions at which the reaction of interest takes place were tested. The most stable immobilized enzyme system, covalent attachment on MANA-agarose via carbodiimide coupled method was finally applied as a biocatalyst for the synthesis of Cbz-glycinal reaching higher total conversion (59.9%) than in the reaction catalyzed by soluble enzyme (47.9%).

Finally, the reaction of oxidation of Cbz-ethanolamine catalyzed by CPO was successfully coupled in one-pot reactor to the aldol addition of the amino aldehyde with DHAP catalyzed by recombinant rhamnulose-1-phosphate aldolase (RhuA) from *Escherichia coli* yielding Cbz-aminopolyol ((3R)-5-[[Benzyl oxy] carbonyl] amino)-5-deoxy-1-O-phosphonophenylpent-2-ulose) as a final product. The effect of the immobilization of the enzymes, reactor configuration and reaction medium were studied in order to improve Cbz-aminopolyol production. The production of Cbz-aminopolyol, when catalyzed by immobilized enzymes in presence of 5% (v/v) dioxane reached the value of 86.6 mM (31 g/l).
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>I</td>
</tr>
<tr>
<td>RESUME</td>
<td>III</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>VII</td>
</tr>
<tr>
<td>1  INTRODUCTION</td>
<td>3</td>
</tr>
<tr>
<td>1.1  <strong>Enzymes as biocatalysts</strong></td>
<td>3</td>
</tr>
<tr>
<td>1.2  <strong>Enzyme stabilization strategies</strong></td>
<td>7</td>
</tr>
<tr>
<td>1.2.1 Chemical modification of the enzymes</td>
<td>7</td>
</tr>
<tr>
<td>1.2.2 Enzyme immobilization</td>
<td>12</td>
</tr>
<tr>
<td>1.2.2.1 Diffusion limitations</td>
<td>14</td>
</tr>
<tr>
<td>1.2.2.1.1 Internal diffusion limitations</td>
<td>15</td>
</tr>
<tr>
<td>1.2.2.1.2 External diffusion limitations</td>
<td>17</td>
</tr>
<tr>
<td>1.3  <strong>Enzymatic reactors</strong></td>
<td>19</td>
</tr>
<tr>
<td>1.3.1 Immobilized enzyme reactors</td>
<td>19</td>
</tr>
<tr>
<td>1.3.2 Characterization of non-ideal reactors</td>
<td>21</td>
</tr>
<tr>
<td>1.3.3 Engineering of the reaction media</td>
<td>24</td>
</tr>
<tr>
<td>1.3.4 Multi-enzyme cascade reactions</td>
<td>26</td>
</tr>
<tr>
<td>1.4  <strong>Enzymatic stereoselective synthesis of aminopolyols</strong></td>
<td>27</td>
</tr>
<tr>
<td>1.4.1 Aldolases</td>
<td>28</td>
</tr>
<tr>
<td>1.4.1.1 DHAP-dependent aldolases</td>
<td>30</td>
</tr>
<tr>
<td>1.4.1.2 Aldol addition of DHAP to N-protected amino aldehydes catalyzed by DHAP-dependent aldolases</td>
<td>34</td>
</tr>
<tr>
<td>1.4.2 Oxidation of alcohols to aldehydes</td>
<td>35</td>
</tr>
<tr>
<td>1.4.3 Peroxidases</td>
<td>37</td>
</tr>
<tr>
<td>1.4.3.1 Chloroperoxidase</td>
<td>39</td>
</tr>
<tr>
<td>2  OBJECTIVES</td>
<td>45</td>
</tr>
<tr>
<td>3  MATERIALS AND METHODS</td>
<td>49</td>
</tr>
<tr>
<td>3.1  <strong>Materials</strong></td>
<td>49</td>
</tr>
<tr>
<td>3.2  <strong>Synthesis of L-rhamnulose-1-phosphate</strong></td>
<td>49</td>
</tr>
<tr>
<td>3.2.1 Qualitative determination of L-lactaldehyde and L-rhamnulose-1-phosphate</td>
<td>52</td>
</tr>
<tr>
<td>3.2.2 Quantification of L-lactaldehyde</td>
<td>52</td>
</tr>
<tr>
<td>3.3  <strong>Purification of rhamnulose-1-phosphate aldolase on IMAC</strong></td>
<td>53</td>
</tr>
<tr>
<td>3.4  <strong>Enzyme activity assays</strong></td>
<td>54</td>
</tr>
<tr>
<td>3.4.1 Chloroperoxidase activity assays</td>
<td>54</td>
</tr>
<tr>
<td>3.4.1.1 Chlorination activity assay</td>
<td>54</td>
</tr>
<tr>
<td>3.4.1.2 Peroxidation activity assay</td>
<td>55</td>
</tr>
<tr>
<td>3.4.2 Rhua activity assay</td>
<td>56</td>
</tr>
<tr>
<td>3.5  <strong>Quantification of reaction substrates and products</strong></td>
<td>57</td>
</tr>
<tr>
<td>3.5.1 Quantification of CBZ-ethanolamine, CBZ-glycinal, CBZ-glycine and CBZ-aminopolyol</td>
<td>57</td>
</tr>
</tbody>
</table>
4 ENGINEERING OF THE REACTION MEDIA FOR CHLOROPEROXIDASE CATALYZED
OXIDATION OF CBZ-AMINO ALCOHOLS TO CBZ-AMINO ALDEHYDES

4.1 INTRODUCTION

4.2 METHODS
4.2.1 CPO STABILITY
4.2.2 OXIDATION OF CBZ-ETHANOLAMINE TO CBZ-GLYCINAL CATALYZED BY CPO

4.3 RESULTS AND DISCUSSION
4.3.1 CHLOROPEROXIDASE CATALYZED OXIDATION OF CBZ-ETHANOLAMINE IN AQUEOUS MEDIUM
4.3.1.1 Choice of the oxidant and optimization of the addition frequency
4.3.1.2 Effect of elevated temperature on the oxidation of Cbz-ethanolamine by CPO catalysis
4.3.1.3 Use of polyethyleneimine (PEI) as a stabilizing agent
4.3.1.4 Reaction with repeated addition of CPO
4.3.2 CHLOROPEROXIDASE CATALYZED OXIDATION OF CBZ-ETHANOLAMINE IN BIPHASIC MEDIUM
4.3.3 CHLOROPEROXIDASE CATALYZED OXIDATION OF CBZ-ETHANOLAMINE IN AQUEOUS MEDIUM IN PRESENCE OF COSOLVENTS
4.3.3.1 Choice of the cosolvent and the rate of t-BuOOH addition
4.3.3.2 Optimization of the temperature using dioxane as a cosolvent
4.3.3.3 CHLOROPEROXIDASE CATALYZED OXIDATION OF CBZ-ALANINOL

4.4 CONCLUSIONS

5 CHEMICAL MODIFICATIONS OF CHLOROPEROXIDASE FOR ENHANCING STABILITY AND ACTIVITY

5.1 INTRODUCTION

5.2 METHODS
5.2.1 CHEMICAL MODIFICATIONS OF CPO
5.2.1.1 Modification of amino groups of CPO
5.2.1.2 Modification of carboxyl groups of CPO
5.2.1.3 Crosslinking of CPO with DMS
5.2.1.4 Periodate oxidation of CPO
5.2.2 DETERMINATION OF THE DEGREE OF MODIFIED AMINO GROUPS
5.2.3 ISOELECTRIC FOCUSING AND ZYMOGRAM
5.2.4 STABILITY OF CHEMICALLY MODIFIED CPOs
5.2.5 OXIDATION OF CBZ-ETHANOLAMINE TO CBZ-GLYCINAL CATALYZED BY NATIVE AND MODIFIED CPOs

5.3 RESULTS AND DISCUSSION
5.3.1 CHEMICAL MODIFICATIONS OF CPO
5.3.2 OXIDATION OF CBZ-ETHANOLAMINE TO CBZ-GLYCINAL CATALYZED BY CHEMICALLY MODIFIED CPOs
5.3.3 EFFECT OF pH ON THE STABILITY OF MODIFIED CPOs AND THE REACTION OF OXIDATION OF CBZ-ETHANOLAMINE
5.3.4 EFFECT OF THE TEMPERATURE ON THE STABILITY AND REACTION OF OXIDATION OF CBZ-ETHANOLAMINE CATALYZED BY MODIFIED CPOs

5.4 CONCLUSIONS

6 IMMOBILIZATION OF CHLOROPEROXIDASE


6.1 INTRODUCTION

6.2 METHODS
6.2.1 STABILITY OF SOLUBLE CPO 125
6.2.2 PREPARATION OF MANA-agarose SUPPORT 125
6.2.3 DETERMINATION OF MAXIMAL ENZYMATIC LOAD ON MANA-agarose 127
6.2.4 PERIODATE OXIDATION OF CPO 127
6.2.5 IMMobilization of CPO by ADSorption on MANA-agarose gels 128
6.2.6 IMMobilization of CPO by COVALENT ATTACHMENT WITH EDAC on MANA-agarose gels 128
6.2.7 IMMobilization of oxidized CPO by COVALENT ATTACHMENT ON MANA-agarose gels 128
6.2.8 IMMobilization of CPO on Eupergit® C 129
6.2.9 MONITORING OF IMMobilization process 129
6.2.10 STUDIES OF DIFFUSION LIMITATIONS OF IMMobilized PREparations of CPO 129
6.2.11 PEROxIDE DEPENDENT INACTIVATION OF IMMobilized PREparations of CPO 130
6.2.12 Oxidation of CBZ-ETHANOLAMINE TO CBZ-GLYCINAL CATALYZED BY SOLUBLE AND IMMobilized CPO 130
6.2.12.1 Reaction with soluble CPO 130
6.2.12.2 Reaction with immobilized CPO 131

6.3 RESULTS AND DISCUSSION
6.3.1 PH STABILITY OF CHLOROPEROXIDASE 131
6.3.2 IMMobilization of CPO ON MANA-agarose 132
6.3.2.1 Maximal enzymatic load on MANA-agarose gels 134
6.3.2.2 Ionic adsorption of CPO on MANA-agarose gels 135
6.3.2.3 Multipoint COVALENT ATTACHMENT OF CPO ON MANA-agarose gels 137
6.3.2.4 Multipoint COVALENT ATTACHMENT OF oxidized CPO ON MANA-agarose gels via its sugar moiety 142
6.3.3 IMMobilization of CPO ON Eupergit® C 145
6.3.4 DIFFUSION LIMITATIONS OF THE IMMobilized PREparations of CPO ON MANA-agarose 146
6.3.5 STABILITY OF THE IMMobilized PREparations of CPO 147
6.3.6 Oxidation of CBZ-ETHANOLAMINE TO CBZ-GLYCINAL CATALYZED BY IMMobilized CPO 149

6.4 CONCLUSIONS

7 SYNTHESIS OF AMINOPOLYOLS IN ONE-POT MULTienzyme OXIDATION AND ALDOL ADDITION

7.1 INTRODUCTION 157
7.2 METHODS 158
7.2.1 Preparation of the SUPPORTS FOR IMMobilization 158
7.2.1.1 Preparation of glyoxyl- and MANA-agarose gels 158
7.2.1.2 Preparation of Co-IDA 158
7.2.2 IMMobilization of ENZYMES 159
7.2.2.1 IMMobilization of CPO by COVALENT ATTACHMENT ON MANA-agarose 159
7.2.2.2 IMMobilization of RhuA ON Eupergit® C 159
7.2.2.3 IMMobilization of RhuA by ADSorption on MANA-agarose 159
7.2.2.4 IMMobilization of RhuA by COVALENT ATTACHMENT ON MANA-agarose 159
7.2.2.5 IMMobilization of RhuA ON glyoxyl-agarose 160
7.2.2.6 IMMobilization of RhuA ON Co-IDA 160
7.2.3 DEGRADATION OF DHAP 160
7.2.4 ONE-POT REACTION 160
7.2.5 RESIDENCE TIME DISTRIBUTION 163
7.2.6 IDENTIFICATION OF CBZ-AMINOPOLYOL BY LC-MS 164

7.3 RESULTS AND DISCUSSION 164
7.3.1 ENZYMATIC REACTION COUPLING FOR THE SYNTHESIS OF CBZ-AMINOPOLYOL 164
7.3.2 EFFECT OF ENZYMATIC IMMOBILIZATION ON THE SYNTHESIS OF CBZ-AMINOPOLYOL 170
7.3.2.1 Coupled enzymatic reaction catalyzed by immobilized CPO and soluble RhuA 170
7.3.2.2 Immobilization of RhuA 172
7.3.2.3 Coupled enzymatic reaction catalyzed by immobilized CPO and RhuA 177
7.3.3 EFFECT OF THE REACTOR CONFIGURATION ON THE SYNTHESIS OF CBZ-AMINOPOLYOL 179
7.3.4 EFFECT OF REACTION MEDIUM ON THE SYNTHESIS OF CBZ-AMINOPOLYOL 183
7.4 CONCLUSIONS 186

CONCLUSIONS 189

REFERENCES 193
CHAPTER 1

INTRODUCTION
Chapter 1.
1 INTRODUCTION

1.1 Enzymes as biocatalysts

Some chemical reactions occur spontaneously at good reaction rates and others require catalysts, molecules which have the ability to reduce the magnitude of energy barrier which has to be overcome so that the reactants can be chemically converted into products (Illanes 2008). Enzymes are proteins having catalytic activity and are involved in virtually every transformation which occurs in vivo. Therefore, they catalyze a wide variety of the synthesis of biologically important molecules, even those which do not occur in vivo (Whitesides and Wong 1985). The main goal of biocatalysis is to use these physiological catalysts as process catalysts which will be able to act under reaction conditions of an industrial process (Illanes 2008).

Enzymes are environmentally friendly (they are biodegradable; besides they produce a small amount of waste) and work on mild conditions which is one of the benefits of enzymes as industrial biocatalysts.

Apart from this, enzymes are highly efficient catalysts, with reaction rates up to $10^{17}$-fold higher compared to those of uncatalyzed reactions, which is even more remarkable considering the mild conditions under which they act, lowering that way significantly the consumption of the energy.

One of the most important properties of the enzymes which distinguishes them from chemical catalysts is their high specificity (i.e. relative activity of the enzyme towards different types of substrates) and selectivity (i.e. type of reaction catalyzed by enzyme). Their specificity can be divided into four groups (Rozzell 1999):

- **Substrate specificity**: the ability to recognize and act on a certain kind of compounds within a larger group of chemically related compounds.
- **Stereospecificity**: the ability to accept only one enantiomer or diastereoisomer.
- **Regiospecificity**: the ability to act on one location in a molecule in a selective way.
Chapter 1.

Functional group specificity: the ability to act on a single functional group selectively, even in a presence of other equally or more reactive functional groups.

The usually strict substrate specificity and selectivity of enzymes were traditionally considered as a limiting factor for their synthetic application. In fact, high specificity may be a drawback when the purpose is to create a general synthetic method; but enzyme selectivity and specificity can also be highly beneficial if the interest is the particular transformation which the enzyme catalyzes, as it is the case in pharmaceutical products and fine chemicals (Whitesides and Wong 1985). Apart from this, better selectivity means better production of single stereoisomers, fewer side reactions, easier separation of products, and less pollution— all the benefits which can lower the cost of the process. Additionally, their high enantiospecificity permits the resolution of racemic mixtures.

While all these unique properties of enzymes make their use highly advantageous, many of the traditionally considered drawbacks are being solved over the last few decades through research and development in different areas. For example, enzymes were believed not to be able to work in organic solvents; nowadays, many examples of biotransformations catalyzed by enzymes performed in organic solvents can be found (Vulfson et al. 2001). Enzyme instability at in vitro conditions represents another synthetic limitation. The problem of instability can be in many cases overcome by simple choice of appropriate experimental conditions. Besides, several strategies for the stabilization of the enzymes have been developed, including chemical modifications of enzymes, immobilization, aggregation, etc. Furthermore, limited availability of the enzymes with desired substrate specificity and activity and their instability can be resolved thanks to the development of recombinant DNA technology; protein engineering (by in vitro evolution or rational design) permits the modification of catalytic properties of the enzymes (e.g. substrate specificity, stability, etc.). Besides, optimization of recombinant expression systems allowing the high-level expression in different host cells importantly facilitates the obtaining of the enzymes and decreases their cost (Illanes 2008; Schoemaker et al. 2003).

The characteristics of enzymes as biocatalysts are consequences of their complex molecular structure. Being proteins, their molecules are composed of a great number
of amino acid residues that range from tens to several thousands. Their biologically active three-dimensional structure is called *native structure* and is the result of interactions of amino acid residues. Apart from this, proteins can be associated with other molecules (prosthetic groups) which can be carbohydrates (in case of glycoproteins), lipids (in case of lipoproteins), nucleic acids (in case of nucleoproteins) or metal ions (metalloproteins). The enzyme can lose its catalytic activity by unfolding its tertiary structure (*denaturation*) or due to chemical changes in its molecule (*inactivation*).

As the stability and the activity of the enzyme often have opposite trends (e.g. low stability and high activity of enzymes at elevated temperatures), in order to achieve the good reaction performance with minimal activity losses there is a trade-off between these two important properties of the enzyme.

All the so far known enzymes are classified according to the guidelines of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB) into six families. The classification was made based on the chemical reaction that they catalyze (Figure 1.1.1).

**Figure 1.1.1** Classification of the enzymes according to UIBMB.
Finally, it is important to point out that even though biocatalysis has not yet gained the status of a first-line alternative in chemical industry, there is no doubt that it represents a powerful technology due to its unique properties. Thanks to applying and combining different methods of enzyme engineering, such as the improvement of enzyme properties via chemical modifications or techniques of molecular biology, via immobilization and finally via reaction and reactor engineering, there is an increasing trend of the use of enzymes in the industry. In fact, industrial applications represent more than 80% of the global market of enzymes (Illanes 2008). The global market for industrial enzymes was valued at $3.1 billion in 2009 and reached about $3.6 billion in 2010. The estimated market for 2011 was about $3.9 billion (BCC Research, Market Forecasting). BCC projects this market to grow at a compounded annual growth rate (CAGR) of 9.1% to reach $6 billion by 2016, the enzymes applied in the food and beverage industries representing the largest sector of the industrial enzymes industry, followed by the sector of technical enzymes (Figure 1.1.2). Some of the enzymes widely used in industry and their applications are listed in Table 1.1.1 (Gavrilescu and Chisti 2005).

Figure 1.1.2 Global revenue of industrial enzymes market in the period of 2009-2016 (BCC Research, Market Forecasting).
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Application industry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteases</td>
<td>Detergents, food, pharmaceuticals, chemical synthesis</td>
</tr>
<tr>
<td>Carbohydrases</td>
<td>Food, feed, pulp and paper, sugar, textiles, detergents</td>
</tr>
<tr>
<td>Lipases</td>
<td>Food, effluent treatment, detergents, fine chemicals</td>
</tr>
<tr>
<td>Pectinases</td>
<td>Food, beverage</td>
</tr>
<tr>
<td>Cellulases</td>
<td>Pulp, textile, feed, detergents</td>
</tr>
<tr>
<td>Amylases</td>
<td>Food</td>
</tr>
</tbody>
</table>

1.2 **Enzyme stabilization strategies**

Enzyme stabilization under process conditions is one of the major issues in biocatalysis. In some cases, the enzyme stability can be enhanced by careful choice of the operation conditions; in other ones application of different stabilization strategies is necessary. Some of the simplest and most commonly used strategies involve the stabilization of enzymes via the methods of chemical modification or immobilization.

1.2.1 **Chemical modification of the enzymes**

Methods of chemical modification of the enzymes have been developed as a major tool in the elucidation of the enzymatic properties and were therefore used to determine the nature of active site residues and/or to differentiate the amino acids which are participating in the catalytic act to those that take part in substrate binding process (Kaiser et al. 1985). Chemical modifications of enzymes allow the introduction of an almost unlimited variety of functional groups (De Santis and Jones 1999; Diaz-Rodriguez and Davis 2011; Feeney et al. 1982; Glazer 1970; Roig and Kennedy 1992). Taking into account the tight connection between the structure of the enzyme and its catalytic properties, it might be surprising that the chemical modification of their structure can be beneficial. In spite of this, by careful selection of the chemical modification method, it has been
possible to achieve a variety of improvements of their properties, such as stability, catalytic activity and selectivity (Roig and Kennedy 1992). Thus, chemical modification of the protein as a tool for searching improved enzymes represents an alternative to site-directed mutagenesis and directed evolution (De Santis and Jones 1999).

The kinetic properties of an enzyme can be modified, in order to tailor its binding capacity for a particular substrate, which is usually carried out by modifying the residues which constitute the secondary binding sites. The specificity of an enzyme can be altered by chemical modification due to different factors. Some chemical modifications allow the change of optimum pH for the reaction catalyzed by enzymes, other ones change their specificity, or even more, some of them alter their selectivity (Roig and Kennedy 1992).

There is a variety of specialized reagents which have been used in order to chemically modify the enzymes: affinity labels, and other specifically designed site-directed reagents, group-selective reagents which react exclusively with one particular type of amino acid side chain, and many others that react nonspecifically with a number of different side chains (Means and Feeney 1990). Furthermore, some reagents have been designed to preserve and others to alter the electrostatic charge of the enzyme. Site-specific modifications are usually performed by using the affinity labels. Their strong affinity for a particular site concentrates a reactive group at that particular site, where its reaction with a nearby amino acid of the side-chain is promoted by their mutual proximity. Thus, the methods of affinity labeling are one of the most important methods for the identification of amino acid residues in the active site of the enzymes (Means and Feeney 1990; Roig and Kennedy 1992).

Side-chain selective modifications involve the use of side chain selective reagents, i.e. those compounds that react, under certain specified conditions, with a single or a limited number of side-chain groups in a rather predictable way (Means and Feeney 1990). Table 1.2.1 shows some of the most commonly used methods for side chain selective modifications of the proteins. Under determined conditions each reagent reacts mainly with the indicated side chain. But, depending on the nature and the amount of the reagent, the protein and the conditions used for modification, the complete modification of all such side chains is not always achieved. Selection of the conditions and the amount of the reagent used is limited by the stability of the enzyme.
at these conditions, and therefore cannot be always optimized for a complete modification. Hence, it is very convenient to have an appropriate method for the quantification of the extent of modification. For example, in case of modification of amino groups, the quantification can be achieved by the indirect measurement of the remained amino groups by the reaction with 2,4,6-trinitrobenzenesulfonic acid (TNBS) (Fields 1972).

The loss of enzymatic activity after such modification is often taken as an evidence for the essentiality of the groups modified though it can be also caused by the procedure used for modification (pH, temperature, presence of reagents etc.) or by alteration of the active conformation of the enzyme.

Many of these modifications involve the ε-amino groups of Lys and carboxyl groups of Asp and Glu, since these amino acids are usually abundantly found on the protein surface. For the acylation of ε-amino groups of Lys, a variety of acidic anhydrides have been used. Dicarboxylic anhydrides (e.g. maleic or phthalic) react specifically with ε-amino groups of Lys and introduce the negative charge to the protein molecule (Butler et al. 1969; Means and Feeney 1990). Modifications of carboxyl groups of the protein (Glu and Asp) commonly involve the activation of the carboxyl group by a water-soluble carbodiimide and the subsequent reaction of the activated carboxyl group with a nucleophile (Hoare and Koshland 1967; Ma and Nakai 1980). Water-soluble carbodiimides are standard vias for modifications of carboxyl groups of proteins due to the relatively mild conditions used (Feeney et al. 1982; Ma and Nakai 1980). Carbodiimide when used in the absence of suitable nucleophile may also act as zero-length cross-linking agent allowing the formation of amide linkages between carboxyl groups of Asp and Glu and the ε-amino groups of Lys side chain (La Rotta Hernandez et al. 2005).
Table 1.2.1 Commonly used side chain modifications (Means and Feeney 1990).

<table>
<thead>
<tr>
<th>Side chain or group</th>
<th>reaction and/or reagent</th>
<th>optimum reaction pH</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amino Lys</strong></td>
<td>amidation/ethyl acetimidate</td>
<td>9</td>
<td>no side reaction with other residues in case of amidation and reductive alkylation; side reactions with Tyr in case of acylation</td>
</tr>
<tr>
<td></td>
<td>reductive alkylation/ formaldehyde+ NaBH₃CN or NaBH₄</td>
<td>7 or 9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>acylation/acetic anhydride</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td><strong>Carboxyl Asp + Glu</strong></td>
<td>water soluble carbodiimide +nucleophile</td>
<td>4.5-5</td>
<td>possible side reactions with thiol groups or Tyr; many different nucleophiles can be used</td>
</tr>
<tr>
<td><strong>Guanidine Arg</strong></td>
<td>dicarbonyls</td>
<td>≥ 7</td>
<td>reaction promoted in borate buffer; reversible upon dialysis</td>
</tr>
<tr>
<td><strong>Imidazole His</strong></td>
<td>diethyl pyrocarbonate</td>
<td>4-5</td>
<td>side reaction with Lys</td>
</tr>
<tr>
<td><strong>Indole Trp</strong></td>
<td>N-bromosuccinimide</td>
<td>≤ 4</td>
<td>oxidation of thiol groups</td>
</tr>
<tr>
<td><strong>Phenol Tyr</strong></td>
<td>iodation</td>
<td>≥ 8</td>
<td>many different procedures and reagents; side reactions with His</td>
</tr>
<tr>
<td></td>
<td>tetratinromethane</td>
<td>8</td>
<td>oxidation of thiol groups</td>
</tr>
<tr>
<td><strong>Thiol Cys</strong></td>
<td>carboxymethylation</td>
<td>7</td>
<td>no effect on other residues</td>
</tr>
<tr>
<td></td>
<td>N-ethylmaleimide</td>
<td>≥ 6</td>
<td>side reactions with Lys or His</td>
</tr>
<tr>
<td></td>
<td>5,5’-dithiobis(2-nitrobenzoic acid) (Ellman’s reagent)</td>
<td>≥ 7</td>
<td>no side reactions with other side chains</td>
</tr>
<tr>
<td><strong>Thioether Met</strong></td>
<td>oxidation (peroxides)</td>
<td>≥ 1</td>
<td>side reactions with thiol groups</td>
</tr>
</tbody>
</table>

Even though side-chain selective modifications have been used and evaluated as a very useful tool for the enhancement of the stability and functionality of the enzymes (Bund and Singhal 2002; Kotormán et al. 2009; Liu and Wang 2007; Ma and Nakai 1980; Mossavarali et al. 2006; Sangeetha and Abraham 2006), these results are often unpredictable and difficult to explain due to the little or no mechanistic insight.

Crosslinking of the proteins as a method of chemical modification has been widely used for the enhancement of their stability (Govardhan 1999). In this method, the molecule is braced with chemical crosslinkers (bi- or poly-functional; homo- or
heterofunctional reagents) intramolecularly or intermolecularly to other species in order to stabilize its active structure due to enhanced rigidity of the protein molecule. Various chemicals have been used for this purpose. Some of the most commonly used homobifunctional crosslinkers are glutaraldehyde, dimethyl suberimidate (DMS), disuccinimidyl suberate (DSS) (Davies and Stark 1970), etc.

The chemical crosslinking is an easy and inexpensive method to enhance the stability of an enzyme and increase the tolerance to organic solvents, but it is difficult to determine the extent and the exact location of the modification. Therefore, it is often difficult to deduce which changes caused the improvement in stability. Besides, relatively high activity losses usually occur during the crosslinking process.

Finally, the modification of the carbohydrate component of the glycoproteins represents another useful method for the alteration of the properties of the protein. The sugar chains play important roles as signals for cell-surface recognition phenomena in multi-cellular organism, but they are not directly involved in catalysis. Besides, the sugar moieties provide important physical properties of the glycoproteins (conformational stability, charge and waterbinding capacity) (Kobata 1992), and therefore the chemical modification, such as periodate oxidation may alter their stability. Possible formation of Schiff base bond between the protein amino groups and sugar aldehyde groups formed after the periodate oxidation of the protein might result in intermolecular cross-linking, though these linkages are usually present in a very low amount (Gerber et al. 1977; Kozulic et al. 1987). Besides, by this way, a large number of reactive aldehyde groups can be formed on the protein surface, which gives the opportunity for the direct enzyme immobilization on supports containing amino groups (Fleminger et al. 1990).

The main disadvantage of these methods is the lack of control with respect to the extent and regiochemistry of the reaction. This can be overcome by site-selective chemical modification using combined site-directed mutagenesis and chemical modification approach, which has emerged as a rapid, controlled, and versatile strategy for obtaining well characterized enzymes with improved and predictable properties (Davis 2003; De Santis and Jones 1999; Diaz-Rodriguez and Davis 2011).
1.2.2 Enzyme immobilization

Immobilization is the conversion of enzymes from a water-soluble, mobile state to a water-insoluble, immobile state (Klibanov 1983). The mayor benefits of enzyme immobilization are the easy separation of the enzyme from the reaction mixture, their reuse or use in continuous reactors, and their potential stabilization. Apart from this, immobilization enables the use of enzymes in one-pot multienzyme processes by improving the compatibility of the enzymes. Therefore, enzyme immobilization was developed with the idea of full exploitation of the technical and economical advantages of biocatalysis based on isolated enzymes. The immobilization method has to be chosen in that way to provide a good overall performance which includes high immobilization yields with minimal activity losses, low mass transfer limitations and improved operational stabilities.

During the immobilization process some of the enzyme molecules are immobilized, while others might remain unbound. The parameter which describes which percentage of the enzyme is immobilized is called immobilization yield. Apart from this, the activity of the immobilized enzyme (retained activity) is usually lower than the expected one, which can be due to the enzyme inactivation by the binding procedure, and can be further reduced by mass transfer limitations or steric hindrance. Both parameters depend on the immobilization method itself, and the amount of the soluble enzyme used.

There are many different immobilization methods and they can be generally divided into five groups. The classification and the main features of each group are presented on the Figure 1.2.1.
The technique of cross-linking of soluble enzymes is considered as the method of chemical modification (Section 1.2.1), while the cross-linking of enzyme crystals (CLECs) and enzyme aggregates (CLEAs) represent methods of immobilization.

The properties of immobilized enzyme preparation depend on the properties of both the enzyme and the carrier material. There is no universally applicable method for immobilization, because every enzyme is different, but there are many parameters that can help in a search for the most appropriate one.

Characteristics of enzyme which have to be taken into account are numerous, and the most important are the following ones: molecular mass, surface functional groups, specific activity, enzyme stability against pH, temperature, agents, etc. On the other side in order to choose an appropriate support for immobilization, the following aspects must be considered: its chemical basis and composition, functional groups, chemical stability, pore size, particle diameter, and the maximal enzyme load that can be immobilized. As a result, the immobilized enzyme will have different chemical, biochemical, mechanical and kinetic properties (Tischer and Wedekind 1999).
Most immobilization methods involve a step of activation (derivatization) of the support and/or enzyme and a contacting step in which the activated species interact with each other, producing the immobilized enzyme. Optimization of the immobilization process is a rather complicated task, since there are many variables involved in both steps, such as, the necessary amount of the activating agent(s), the amount of the enzyme used per support volume, pH, temperature, time of contact, etc. (Illanes 2008).

Finally, the stability of the immobilized enzyme depends on many factors, such as the number and the nature of the bonds formed between the enzyme and the support, the degree of confinement of enzyme molecules in the support and the microenvironment (Cao 2005).

### 1.2.2.1 Diffusion limitations

When immobilized enzymes are used, the reaction is taking place on the surface or inside the biocatalyst particle, therefore in different conditions than those in the bulk reaction medium, mainly regarding the substrate and product concentrations. Its kinetics can differ from that one obtained when catalyzed by soluble enzyme, due to two main effects: those which are the result of structural changes produced on the enzyme after immobilization or conformational effects (alteration of its three-dimensional structure and steric effects due to its close proximity to the surface of the support), and mass transfer effects or diffusion limitations (Illanes 2008).

Diffusion limitations can be external or internal. External diffusion limitations are the result of a layer of stagnant liquid which surrounds the solid immobilized enzyme particle, across which the substrate transport occurs only by molecular diffusion, not by convection. Internal diffusion limitations or porous diffusion exist when the enzyme is placed inside a porous particle. In that case, the transport of substrates and products by diffusion within the particle is even slower than in a liquid medium. Therefore, the rate of the reaction catalyzed by immobilized enzyme depends not only on the reaction itself, but also on the transport of the substrates and the products to and from the interior of the particle (Figure 1.2.2).
Figure 1.2.2 Schematic representation of the diffusion steps and reaction occurring in the interior of immobilized enzyme particle.

1.2.2.1 Internal diffusion limitations

In stirred tank reactors, where vigorous mixing is applied, external diffusion can be neglected. In that case, the reaction rate will depend on the internal diffusion and the reaction itself. When the diffusion rate through the particle is high enough, the substrate concentration inside the particle will be equal to the one in the bulk reaction medium. In that case, no diffusion limitations are present. On the contrary, when the reaction rate exceeds the rate of diffusion of the substrates through the particle, the effective reaction rate is limited by the latter, and therefore there is a concentration gradient of the substrates inside the particle (Levenspiel 1999). There is a relation between reaction and diffusion rates. The diffusion rate depends on the gradient of the concentrations. The bigger the concentration gradient, the faster the diffusion will be. On the other side, the rate of the reaction also depends on the substrate concentration, being faster when higher concentrations are present. Therefore, the concentration gradient favors the diffusion, but not the reaction.

Effectiveness factor $\eta$ is usually used to express the degree of mass transfer control (Tischer and Wedekind 1999):
Chapter 1.

\[ \eta = \frac{\nu'(rate \ of \ the \ reaction \ catalyzed \ by \ immobilized \ enzyme)}{\nu(rate \ of \ the \ reaction \ catalyzed \ by \ soluble \ enzyme)} \]

Another important parameter when studying the porous diffusion limitations is the Thiele modulus \( \Phi \) which represents the ratio between the reaction rate and the rate of the diffusion. In case of Michaelis-Menten kinetics, this parameter is expressed as:

\[ \Phi^2 = \frac{\nu_{\text{max}}}{K_M \cdot D_{\text{eff}}} \left( \frac{R}{3} \right)^2 \]

Where \( \nu_{\text{max}} \) is the maximal reaction rate (mol/l·s); \( K_M \) is Michaelis-Menten constant (mol/l), \( D_{\text{eff}} \) is the effective diffusion coefficient of the substrate inside the particle (cm\(^2\)/s) and \( R \) is the radium of the particle (cm).

Effectiveness factor and Thiele modulus can be related empirically (Figure 1.2.3). As it can be seen, the efficiency factor has its maximal value for low values of the Thiele modulus, meaning that in these conditions no diffusion limitations are present. Therefore, taking into account the definition of the Thiele modulus, it can be concluded that in order to achieve the maximal effectiveness of the reaction catalyzed by immobilized enzyme, the two factors which have to be optimized are maximal reaction rate, which depends on the enzyme loading per volume of the support, and the particle size.
1.2.2.1.2 External diffusion limitations

In practice, the effect of the external diffusion in many cases has to be taken into account. In order to facilitate the explanations it will be assumed that an enzymatic reaction takes place at the surface and that follows the Michaelis-Menten kinetics. External diffusion rate can be expressed as:

\[
\nu_{\text{diff}} = k_L \cdot ([S_b] - [S_s])
\]

Where \( k_L \) represents the external mass transfer coefficient (cm/s), \( S_b \) and \( S_s \) are the concentrations of substrate in a bulk reaction medium and at the surface, respectively (mol/cm\(^3\)).

Damkoehler number is the dimensionless parameter which serves to determine the significance of the effect of external mass transfer limitations, and is defined as:

\[
Da = \frac{\text{maximal reaction rate}}{\text{maximal diffusion rate}} = \frac{\nu_{\text{max}}}{k_L \cdot [S_b]}
\]
Therefore, two limiting cases are possible. When the Damkoehler number is sufficiently large, then the substrate concentration on the surface is very low \([S_s] = 0\) since the reactivity of the surface is so high that all the substrate molecules which are transferred to the surface are immediately converted. As a result, the overall reaction rate is equal to the maximal possible diffusion rate, i.e. the reaction is limited by the external diffusion. On the contrary, when Da is very small, the bulk concentration is practically equal to the surface concentration and the overall reaction rate is equal to the actual rate of the reaction.

When external diffusion and chemical reaction have the same rate in steady-state conditions, the overall reaction rate depends on both of them (Horvath and Engasser 1974).

The external effectiveness factor of the process can be expressed as:

\[
\eta_{ex} = \frac{\nu'(rate\ of\ the\ reaction\ catalyzed\ by\ immobilized\ enzyme)}{\nu(rate\ of\ the\ reaction\ catalyzed\ by\ soluble\ enzyme)}
\]

It is represented on the Figure 1.2.4 as a function of the Damkoehler number.

**Figure 1.2.4** Variation of the external efficiency factor \(\eta_{ex}\) as a function of the Damkoehler number, Da, for the different values of dimensionless concentration \(\beta\) defined as \(\beta=[S_b]/K_M\) for an enzymatic surface reaction (Horvath and Engasser 1974).
Finally, it is important to mention that the external mass transfer coefficient, $k_L$, is a function of the velocity of the fluid flowing around the particle and the particle size, and it decreases as the particle size decreases and/or fluid velocity increases. Therefore, in order to avoid the presence of the external diffusion limitations, these two parameters have to be optimized. The effects of internal and external diffusion limitations explained so far were showed separately. However, in practice, these two effects often go together, especially when fixed-bed reactors containing immobilized enzymes are used. Overall reaction rate is therefore determined by all the three factors: external and internal diffusion and catalytic reaction itself.

### 1.3 Enzymatic reactors

While the reaction yield and selectivity are mostly determined by the biocatalyst, the productivity is often determined by the process itself, including reactor configuration, mode of operation, reaction conditions and reaction media used. Hence, in order to accomplish the good overall performance of the enzymatically catalyzed reaction, a careful choice of all the process parameters has to be made. This is a rather complicated task, because they are often dependent on each other at some degree, and at the same time the properties of the biocatalyst itself have to be taken into account.

#### 1.3.1 Immobilized enzyme reactors

The biocatalysis employing soluble enzymes is mostly limited to the use of batch stirred tank reactors and continuous membrane reactors containing ultrafiltration membranes which allow the retention of the enzyme within reactor. On the contrary, batch stirred tank reactors (STR), continuous stirred tank reactors (CSTR), packed-bed reactors (PBR), fluidized bed reactors (FBR) are main reactor types which can be used for the process applying immobilized enzymes (Pitcher 1978). Further, all these types can be combined or modified (e.g. addition of recirculation loop).
The choice of the reactor configuration depends on the demands of the process itself and immobilized enzyme system used. For example, when enzymes are very sensitive to the temperature or pH, control of these parameters is one of the main requirements for the good reactor performance. Besides, process efficiency is tightly correlated to the mass transfer limitations. As it was already explained in the previous Section, the only way to avoid the presence of internal diffusion limitations is the good adjustment of enzyme loading and particle size. Therefore, if already present, they cannot be eliminated by the operational conditions. On the other side, external diffusion limitations are among other factors, also dependent on the operational conditions.

The most applied configurations are stirred tank reactors (both batch and continuous), due to the simplicity in operation, and the packed-bed column reactors where the immobilized enzyme is fixed within the reactor while the reaction mixture passes through, due to their generally high efficiency and ease of operation. Use of stirred tank reactors permits easy control of temperature and pH. Another important advantage of the use of this reactor configuration is that harsher mixing conditions can be applied in order to eliminate the external mass transfer limitations. But the vigorous mixing may damage the immobilized enzyme particles and also affect the enzymatic stability at some degree (Pitcher 1978). Therefore stirred tank reactors are inadequate when immobilized enzyme particles used are sensitive to shear forces.

Besides, in STR there may be dead zones arising from stagnation of the reaction mixture near the walls, in which there is little or no exchange of fluid with the “active zone” (the central part of the reactor near the stirrer).

Use of packed bed reactors allows the better preservation of immobilized enzyme particles, which also allows the more facile reuse of immobilized enzymes. However, there are as well several operational problems for the use of PBR with immobilized enzymes: difficult control of temperature and pH (especially in a large scale production), compression of the bed, formation of preferential ways, the existence of dead zones that reduce the effective volume of the reactor and the appearance of external diffusion limitations. In order to assure the good performance of this type of reactor, the choice between small particles with low diffusion resistance and high
pressure drops and larger particles which cause diffusion limited reactions, but lower pressure drops, has to be made (Pitcher 1978).

Apart from this, maybe the most important parameter to optimize when working with packed bed reactors is the flow rate. At low flow rates diffusion limitations in packed bed reactor lead to lower reaction rates and productivities compared to those in stirred tank reactor. The flow rates should be increased in order to minimize the external diffusion limitations. On the other side, high flow rates can lower the conversion in case of insufficient time of contact between the enzyme and substrate, and can additionally cause high pressure drops (Buchholz 1982; Richter et al. 1996).

In expanded or fluidized bed reactors, the enzyme particles are retained by a hydrodynamic balance between the gravity and drag forces promoted by the upflow inlet stream (Illanes 2008). The main advantage of the use of fluidized bed reactor includes low pressure drop. But the flow rates required for the fluidization may result in low residence times, and therefore insufficient contact time, which can cause low conversions. For particles with similar density to the one of the reaction media, the problem of washing out may appear.

1.3.2 Characterization of non-ideal reactors

There are two main types of ideal reactors: batch stirred tank reactors (complete mixing) and plug flow reactors (no axial mixing). The behavior of real, non-ideal reactors falls in between these two ideal models.

In general, reactor performance depends on the kinetics of the reaction, the flow pattern, and the mixing characteristics within the reactor. Therefore, in order to understand the behavior of a non-ideal reactor, it is very useful to analyze its flow pattern, and compare to which extent they resemble to one of the ideal reactor models. For this purpose, the analysis of residence time distribution (RTD) represents a very useful method. This method permits determine how long individual molecules stay in the vessel (the molecules taking different routes through the reactor will take different time to pass through it) and therefore, to diagnose the reactor behavior (e.g. channeling, dead zones, etc). The operational problems, such as channeling and bypassing, are highly undesired since they can lower importantly the performance of
Chapter 1.

the reactor. Therefore, by diagnosing them using RTD analysis they can be further avoided by the choice of the different operational conditions or reactor configuration. The RTD function, $E(t)$, also called the exit age distribution, has the units of $\text{time}^{-1}$. Effects of different non-ideal behaviors of the reactor (stagnation, dispersion, and channeling) on RTD function are shown on Figure 1.3.1.

![Figure 1.3.1 Examples of nonideal flow in STR and PBR, and effect of some features of nonideal flow on $E(t)$ function (Missen et al. 1999).](image)

The experimental measurement of the RTD is usually carried out by the stimulus-response experiments, by adding a tracer at the inlet (stimulus or signal), and monitoring its concentration at the outlet (response). The tracer can be added as a pulse or step-change signal. A unit pulse at $t = 0$ is represented by the Dirac delta function $\delta(t = 0) = \delta(t)$, having that the area of the pulse is unity ($\int_{0}^{\infty} \delta(t) dt = 1$). Knowing the pulse amount ($m_0$, kg), it can be represented as:
\[(m_o / q_o) \cdot \delta(t) = c_o\]

Where \(q_o\) is the steady-state flow rate of the fluid and \(c_o\) the initial concentration of the tracer in the reactor.

Then, the area of the pulse is given as:

\[
\int_0^\infty c_0dt = \int_0^\infty \frac{m_0}{q_0} \cdot \delta(t)dt = \frac{m_0}{q_0}
\]

The area under response function must have the same value, if the amount of the tracer is completely accounted for by a material balance having that:

\[
m_o / q_o = \int_0^\infty c_{out}(t)dt
\]

Where \(c_{out}(t)\) is the concentration of the tracer leaving the reactor.

Therefore, having this condition satisfied, it is possible to check the accuracy of the pulse-tracer experiment (Levenspiel 1999; Missen et al. 1999).

Furthermore, the RTD function, \(E(t) (\int_0^\infty E(t)dt = 1)\) can be calculated as:

\[
E(t) = c(t)/(m_o / q_o) = c(t)/(\int_0^\infty c_{out} (t)dt)
\]

Finally, once the function \(E(t)\) is determined, the mean residence time, \(\tau_{mean}\), and the variance of the distribution, \(\sigma^2\), can be calculated using the following equations:

\[
\tau_{mean} = \int_0^\infty t \cdot E(t)dt
\]

\[
\sigma^2 = \int_0^\infty t^2 \cdot E(t)dt - \tau_{mean}^2
\]
1.3.3 Engineering of the reaction media

Aqueous medium was traditionally thought to be the only suitable reaction medium for the application of enzymes. The major drawback of the aqueous biocatalysis is the fact that most of the organic substrates have low solubility in water, and therefore, low volumetric productivities can be accomplished. But, once proved that many enzymes can function even under nearly anhydrous conditions and that in addition exhibit a number of synthetically useful properties (e.g. enhanced stability, different selectivity, etc.), this extended importantly the scope of their application in organic synthesis (Vulfson et al. 2001).

Nevertheless, when using the enzyme in an organic medium, the biocatalyst is influenced by a number of factors that can alter its structure and function. Non-aqueous solvents can affect the enzyme performance by altering the chemical activity of the substrate, enzyme conformation or the local environment of the active site. The inactivation of the enzyme is mainly caused by dissolved solvent molecules and by contact with the interface between the aqueous and organic phases. Therefore, the solvent used must be chosen in order to change the catalytically active conformation of the enzyme in a minor degree (Ross et al. 2000; Ryu and Dordick 1992; Tan and Lovrien 1972; Torres and Castro 2004).

Biocatalysis in organic medium can be homogeneous, when the reaction medium consists of a mixture of water and water miscible solvents; and heterogeneous in which the second phase is formed due to the presence of water-immiscible organic solvent.

In a homogeneous system, use of cosolvents enables the improvement of the solubility of the substrates, while depending on the properties of an enzyme and the solvent used, it might have a low impact on enzyme stability. To accomplish this, moderate concentrations of the cosolvent must be used, since they tend to penetrate the aqueous microenvironment of the enzyme, altering its native conformation (Illanes 2008).

Biphasic medium permits working with even higher concentrations of organic substrates. Most of the organic substrates and products are partitioned between the phases, being mainly dissolved in the organic one, but since enzyme is dissolved in the
aqueous phase, the biocatalysis always occurs in the later phase. Therefore, another advantage is the possible shifting of chemical equilibrium towards higher reaction yields (Semenov et al. 1987). But, the interface can impose diffusion restrictions to the substrates, and therefore can reduce the reaction rate. Besides this, enzyme inactivation can be promoted by dissolved solvent molecules and by the presence of the interface. Enzyme inactivation by the interfacial mechanism consists in structural rearrangement in the enzyme molecule, due to the interactions of the enzymatic hydrophobic core and the hydrophobic interface, resulting in partial or complete unfolding of the enzyme molecules (Ross et al. 2000).

During the last decades, two new classes of non-conventional solvents have been developed: ionic liquids and supercritical fluids. Ionic liquids are a class of highly polar non-aqueous solvents with non-molecular, ionic character, composed usually by a rather simple anion and a complex organic cation, the salts which are liquid at room temperature (Garcia-Junceda et al. 2004). Ionic liquids are often described as green substitutes for organic solvents mostly because of their low vapor pressure, no flammability, recyclability and low toxicity (Parvulescu and Hardacre 2007; Soares et al. 2007). In spite of this there are several difficulties which have to be solved when applying ionic liquids, such as their purification, control of water activity and pH, high viscosity and problematic product recovery (Illanes 2008). Supercritical fluids are materials above their critical temperature and pressure having the properties in between the properties of liquids and gases. The densities of supercritical fluids are comparable to those of liquids, while the viscosities are comparable to those of gases. The main advantages of the system are the high diffusion rates which facilitate transport phenomena and can increase the bioconversion rate. Further, supercritical fluids such as carbon dioxide are non-toxic and can be removed easily after the reaction. The main drawback of supercritical reaction media is that the process requires reactors and auxiliary equipment that can withstand high pressures, which increases process costs (Cantone et al. 2007).
1.3.4 Multienzyme cascade reactions

Biocatalytic cascade represents a reaction system in which two or more transformations are carried out concurrently employing at least one biocatalyst, and therefore it can be chemo-enzymatic or multi-enzymatic (Ricca et al. 2011). Multienzyme cascade one-pot reactions - the combination of several enzymatic reactions in concurrent process occurring in the same reaction vessel - is a strategy for the enhancement of a synthesis in which a substrate is subjected to successive reactions in just one reactor, avoiding the steps of separation and purification of the intermediate product. By this way the operating time and costs, use of auxiliary reagents and energy and waste production can be drastically reduced. Moreover, in case of chemical equilibrium of the first reaction in the sequence, this can be shifted towards higher yields if the product is further converted in the following reaction in the sequence. This leads to better yields, synthetic efficiencies and reduced side reactions (Ricca et al. 2011; Santacoloma et al. 2010). In fact, in the cells of living organisms, a huge variety of enzymatic reactions are carried out in a common reaction medium - the cytosol. Therefore, when implementing the one-pot multienzyme synthesis in vitro, such enzymatic cascade reactions are tried to be reproduced and applied in organic synthesis.

The main difficulty which has to be overcome in order to perform successful one-pot multistep synthesis is the compatibility of the enzymes in the operating conditions. Conditions for the one-pot reaction (i.e. reaction medium, concentrations of the substrates, pH, temperature, etc.) should allow simultaneous reactions in order to prevent the formation of undesired side product and therefore should represent a compromise between the optimal conditions for each individual reaction. Enzyme immobilization is often essential for multienzyme one-pot applications. It can be beneficial in terms of process productivity, due to increased compatibility and stability of the enzymes in common reaction medium (Bruggink et al. 2003; Lopez-Gallego and Schmidt-Dannert 2010; Ricca et al. 2011; Santacoloma et al. 2010; Zhou 2010).

A great number of multienzyme cascade reactions have been developed in past decades. Some of the most important ones are those implementing the carbon-carbon bond forming enzymes into cascade systems (Babich et al. 2011; Sanchez-Moreno et
al. 2009; Schoevaart et al. 2000; Van Herk et al. 2006; Wong and Whitesides 1983) and those for the production of optically active alcohols in which the cascade system allows the regeneration of the cofactor (Ricca et al. 2011).

1.4 **Enzymatic stereoselective synthesis of aminopolyols**

The synthesis of enantiomerically pure compounds is of increasing interest, since it is well known that the biological activity of enantiomers can differ drastically in its kind and intensity because of the chiral nature of life processes (Fessner and Walter 1997). During the last few decades, the synthesis of enantiomerically pure compounds became one of the most important fields of organic chemistry. Use of enzymes for this purpose as an alternative to classical methods of organic chemistry is beneficial due to the fact that the enzymes are *per se* chiral, selective and evolutionarily optimized for high catalytic efficiencies.

One group of such compounds are iminocyclitols (polyhydroxy-\(N\)-heterocycles or azasugars). These compounds are potential inhibitors of glycosidases and glycosyltransferases, and therefore, they can find wide application as new antibiotics, antimetastatic, antihyperglycemic, or immunostimulating agents (Calveras et al. 2009). Iminocyclitols can be easily obtained by cyclating or isomerising corresponding aminopolyols, which are complex molecules containing two chiral \(C\)-atoms (Figure 1.4.1).

![Figure 1.4.1 Obtainment of iminocyclitols from aminopolyols.](image-url)
Therefore, the key step in the synthesis of iminocyclitols is the obtainment of corresponding enantiomerically pure aminopolyols.

1.4.1 Aldolases

Aldol addition reactions nowadays represent one of the most powerful tools for the carbon-carbon bond formation and obtainment of enantiopure multifunctional molecules with up to two new chiral centres out of small and simple precursors (Clapes et al. 2010; Samland and Sprenger 2006). Aldolases are a class of lyases that catalyze the stereoselective C-C bond formation by the aldol addition between an aldehyde (electrophile) and a ketone (nucleophile) (Figure 1.4.2). Although they commonly show strict specificity for the donor substrate (ketone), they can use a broad range of acceptor aldehydes, and therefore can be used for the synthesis of various aldol products (Clapes et al. 2010; Dean et al. 2007; Takayama et al. 1997). Another important advantage is their stereoselectivity, which allows the formation of only one stereoisomer with highly predictable conformation.

![Figure 1.4.2 Scheme of the aldol addition reaction.](image)

Aldolases are classified into two groups based on their catalytic mechanism. Class I aldolases activate the substrate via formation of Schiff base intermediate between the carbonyl group of the donor substrate and an essential lysine residue, whereas class II aldolases use a divalent metal ion (e.g. Zn$^{2+}$) as a Lewis acid activating the nucleophile for the formation of new C-C bond (Breuer and Hauer 2003; Dean et al. 2007). The mechanism of the catalysis of class I and class II aldolases is represented on the Figure 1.4.3.
Based on their donor substrate specificity, aldolases are classified into five groups (Clapes et al. 2010; Fessner 1998; Samland and Sprenger 2006):

- **Dihydroxyacetone phosphate (DHAP) dependent aldolases**- aldolases which show strict specificity for DHAP, but accept a relatively broad range of acceptor aldehydes. Four class II aldolases belong to this group: D-fructose-1,6-biphosphate aldolase (FruA), D-tagatose-1,6-biphosphate aldolase (TagA), L-rhamnulose-1-phosphate aldolase (RhuA), and L-fuculose-1-phosphate aldolase (FucA). Rabbit muscle fructose-1,6-biphosphate aldolase (RAMA) is a class I aldolase member of this group. These enzymes catalyze in vivo the reversible asymmetric aldol addition of DHAP to D-glyceraldehyde-3-phosphate or L-lactaldehyde.

- **Dihydroxyacetone (DHA) dependent aldolases**- D-fructose-6-phosphate aldolase (FSA) is an enzyme of great potential for selective aldol addition of DHA, hydroxyacetone (HA), hydroxybutanone (HB), and glycoaldehyde (GA) while
accepting a large variety of acceptor aldehydes (Castillo et al. 2006; Concia et al. 2009; Schurmann and Sprenger 2001; Sugiyama et al. 2007).

- **Acetaldehyde dependent aldolases** - this group contains only one member: 2-deoxy-D-ribose-5-phosphate aldolase (DERA). In fact, so far this is the only known aldolase that catalyzes C-C bond formation between two aldehydes. Besides acetaldehyde, propanal, acetone and fluoroacetone can serve as donors. Hence, this enzyme can catalyze an elongation by two or three C-atoms.

- **Glycine dependent aldolases** - group of enzymes which catalyzes the reversible formation of β-hydroxy-α-amino carbonic acids. These enzymes require the presence of pyridoxal phosphate (PLP) as a cofactor. The best studied aldolases from this group are threonine aldolase and serine hydroxymethyltransferase.

- **Pyruvate and phosphoenolpyruvate (PEP) dependent aldolases** - large group of aldolases almost exclusively of microbial origin applied in the synthesis of α-keto acids. These enzymes catalyze an elongation by three C-atoms. The best studied member of this group of aldolases is N-acetylneuraminic acid (NeuAc) aldolase (or sialic acid aldolase), exploited in the synthesis of D- and L-sialic acid analogues.

### 1.4.1.1 DHAP-dependent aldolases

The class of aldolases of the greatest importance in synthetic applications is the class of DHAP-dependent aldolases. DHAP-dependent aldolases create two new chiral centers (C-atoms 3 and 4) with excellent enantio- and diastereo-selectivities. Existence of two chiral centers gives the possibility for the formation of four stereoconfigurations. Each one of the four microbial aldolases from this class shows strict selectivity for the formation of one stereoisomer (Figure 1.4.4). The exception is tagatose-1,6-biphosphate aldolase which catalyzes mainly the formation of 3S,4S stereoisomer, but with no strict selectivity, and therefore tends to form the mixture of all stereoisomers. Therefore, it is less used for synthetic applications.
Within class I DHAP-dependent aldolase, only FruA (RAMA) and TagA are known. They are usually found in mammalian, and less commonly in microbial organisms. Class II involves all four DHAP-dependent aldolases which are typically found in bacteria. In general, class II aldolases are much more stable than class I aldolases, having half lives of several weeks or even months, compared to half lives of class I aldolases of only a few days (Fessner and Walter 1997). These enzymes also show a very high tolerance to elevated proportions of organic solvents (up to 50%) which is a property of importance for the synthesis involving non polar substrates with low solubility in water.

Rhamnulose-1-phosphate aldolase (RhuA; E.C. 4.1.2.19) is a class II DHAP-dependent aldolase. This enzyme was isolated from *Escherichia coli* (Chiu and Feingold 1969) and was characterized as a metalloprotein containing Zn\^{2+} ions (Schwartz et al. 1974). RhuA is a homotetramer and consists of 274 amino acid residues with total molecular weight of 120 kDa (Kroemer et al. 2003; Kroemer and Schulz 2002).
RhuA catalyzes the cleavage of L-rhamnulose-1-phosphate to DHAP and L-lactaldehyde, the reaction which in reverse direction represents C-C bond formation, creating two new chiral centers. So far, RhuA was proved to be a very useful biocatalyst for the preparation of a variety of rare or unnatural sugars having a (3R, 4S)-trans stereochemistry, accepting wide range of substrates with relatively high reaction rates. Apart from this, RhuA is the DHAP-dependent aldolase with the greatest tolerance towards sterically congested acceptor aldehydes (Fessner and Walter 1997). On the other side, anionically charged aldehydes (e.g. glyceraldehyde phosphates) which are preferable substrates for FruA, are not accepted by RhuA.

Increasing interest over past decades for the application of DHAP-dependent aldolases for biosynthesis purposes stimulated greatly the investigation of this class of enzymes. By using simple aldehydes, short-chain sugar phosphates can be obtained. Apart from this, a variety of heteroatoms can be introduced (with the condition that it does not cause the steric hindrance on the molecule of the acceptor aldehyde), allowing the synthesis of sugars or sugar analogues containing heteroatoms (e.g. amino, nitro, azido, phosphono, etc.)

Even though the DHAP-dependent aldolases have such a great potential for synthetic applications, their use is still quite limited by few factors. The first limitation lies on the fact that the aldol addition of DHAP leads to phosphorylated products. Since for most applications, the unphosphorylated product is the desired one, the additional step of dephosphorylation is required. The most serious limitation is the instability and cost of DHAP.

DHAP is very unstable in solution and suffers chemical decomposition via an enediol intermediate into methylglyoxal and inorganic phosphate or reversible isomerization to D-glyceraldehyde phosphate (Fessner and Walter 1997) (Figure 1.4.5). Chemical decomposition and isomerisation were showed to be minimized by performing the aldol addition reaction at low temperatures (Suau et al. 2006). Apart from this, it was demonstrated by Suau et al. that type II aldolases can also catalyze the reaction of decomposition of DHAP to methylglyoxal and inorganic phosphate via the formation of the same enediol intermediate.
There are several reported methods of chemical or enzymatic synthesis of DHAP (Schumperli et al. 2007). Still, none of the existing methods can serve for the scalable route to DHAP. The chemical methods have the disadvantage of the use of toxic and expensive chemicals, and the obtaining of relatively low yields of DHAP. With most enzymatic methods described, a low productivity of DHAP can be accomplished. Apart from this, some described routes require complicated multienzymatic systems, which increase importantly the cost of the synthesis. Therefore, the problem of instability and cost of DHAP still remains the important issue to be investigated looking for more effective biotransformations catalyzed by DHAP-dependent aldolases.

Finally, even though it was generally believed that all DHAP aldolases have strict specificity for DHAP, recently it was reported that RhuA can accept also DHA at reasonable reaction rates, when the reaction is performed in borate buffer (Garrabou et al. 2011; Sugiyama et al. 2006). The given explanation is that DHA-borate ester formed in situ mimics DHAP and therefore is accepted by RhuA. This method offers great potential for synthetic applications, since RhuA creates chiral centers with different configuration to that of FSA.
1.4.1.2 Aldol addition of DHAP to N-protected amino aldehydes catalyzed by DHAP-dependent aldolases

As already mentioned in the previous Section, DHAP-dependent aldolases have been proven to be very useful biocatalysts in synthesis, since they catalyze the aldol addition of DHAP accepting a variety of aldehydes. This reaction yields a product with two new chiral centers, being its stereochemistry dependent mainly on the enzyme employed. As explained at the beginning of the Section 1.4, aminopolyols are the precursors of iminocyclitols, compounds of a great therapeutic potential. Aminopolyols can be obtained by the aldol addition of DHAP to amino aldehydes catalyzed by DHAP-dependent aldolase (Figure 1.4.6) (Espelt et al. 2005; Espelt et al. 2003; Suau et al. 2006). In order to prevent the formation of the internal Schiff base bond between the amino and aldehyde groups, a protective group, which is typically carbobenzoxy group (Cbz), must be added to the N-terminal of amino aldehyde. For this purpose in previous works, the protected amino aldehydes were synthesized chemically (Ardao et al. 2006; Calveras et al. 2006; Calveras et al. 2009; Suau et al. 2008).

![Figure 1.4.6 General scheme of aldol addition of DHAP to Cbz-amino aldehydes catalyzed by DHAP dependent aldolases.](image)

Iminocyclitols can be easily obtained from Cbz-aminopolyols using acid phosphatase for the removal of the phosphate group and posterior hydrogenation (Figure 1.4.7) (Espelt et al. 2003).
1.4.2 Oxidation of alcohols to aldehydes

As it was explained in the Section 1.4.1, the key step for obtaining iminocyclitols is the aldol addition of amino aldehydes to DHAP which can be catalyzed by DHAP-dependent aldolases. Aldehydes are generally important and versatile compounds widely used in organic synthesis. Among them, α-amino aldehydes belong to the group of one of the most synthetically versatile ones due to the presence of both amino and carbonyl groups (Jurczak and Golebiowski 1989; Jurczak et al. 2003). These compounds are widely applied to the construction of important pharmaceuticals and natural products (Myers and Kwon 2005; Reetz 1999; Soderquist et al. 2008) and were also found to be able to function as transition state analogue inhibitors for various proteases (Andersson et al. 1982; Fairlie et al. 2000).

Conventional methods of organic synthesis for the selective oxidation of alcohols to aldehydes are not easily performed due to the difficulties to prevent the further oxidation of resulting aldehydes to carboxylic acids. Some of the methods that have been reported for the synthesis of aldehydes from alcohols, such as Swern, Jones and Dess-Martin oxidations have several disadvantages: they use toxic compounds for the synthesis, are environmentally unsuitable due to the large amount of waste produced, and in many cases have high costs of oxidizing agents and auxiliaries (Lenoir 2006). Additionally they are characterized by low selectivity and thus the lack of control and predictability of the structure of the products obtained (Burton 2003). Transition metals have been used as catalysts in order to achieve selective oxidations of alcohols using oxygen as an oxidant, but their use is restricted by their high costs and the facts
that they are often commercially unavailable and potentially toxic (Lenoir 2006). Therefore, the oxidation of alcohols to aldehydes is advantageous to be performed via enzymatic biocatalysis.

Enzymatic synthesis of α-amino aldehydes catalyzed by alcohol dehydrogenase from horse liver was previously accomplished by Andersson and Wolfenden (Andersson and Wolfenden 1982). However, by this method, α-amino aldehydes were obtained as aldehyde semicarbazones. Therefore, additional step to obtain the free aldehydes was required. Another disadvantage of this approach is the necessity of expensive cofactor $\text{NAD}^+$ required for the activity of the enzyme, or an effective system for its regeneration.

Therefore, there is a great interest for the search of new enzyme catalyzed method for the oxidation of amino alcohols to amino aldehydes, with no or minimal further oxidation to amino acid (Figure 1.4.8). Peroxidases are the enzymes from the group of oxidoreductases which can oxidize a wide range of organic compounds with no requirement of expensive cofactor. Chloroperoxidase (CPO) from *Caldariomyces fumago* is an enzyme from this group found to oxidize primary alcohols to aldehydes in a very selective way (Kiljunen and Kanerva 2000).

![Figure 1.4.8 Selective oxidation of α-amino alcohols to α-amino aldehydes.](image-url)
1.4.3 Peroxidases

Application of oxidoreductases as biocatalysts allows the development of sustainable, highly efficient and environmentally friendly processes, due to the fact that these enzymes are selective and have high redox potentials (Xu 2005). Peroxidases are enzymes from the group of oxidoreductases that use hydrogen peroxide (H$_2$O$_2$) or organic peroxides (R-OOH) as oxidants. Unlike other enzymes from the group of oxidoreductases, peroxidases can catalyze selective oxidations with no requirement of expensive cosubstrates or cofactors, and most of them can catalyze a wide range of reactions accepting a great variety of the substrates. Apart from this, this class of enzymes is ubiquitously found in all domains of life. Peroxidases are usually named after their sources (e.g. horseradish, soybean, peanut, lacto- or myeloperoxidases) or their substrates (e.g. bromo-, chloro-, lignin, citochrome c- and manganese peroxidase) (Adam et al. 1999; Colonna et al. 1999; van De Velde et al. 2001; van Deurzen et al. 1997c). Special group of peroxidases are haloperoxidases which have capacity to halogenate organic substrates in the presence of halide ions and peroxide.

Peroxidases are classified into three groups depending on the structure of their active center:

- **Heme-peroxidases**, which contain ferric protoporphyrin IX as the prosthetic group
- **Vanadium peroxidases**, containing vanadate ion as the prosthetic group
- **Non-metal peroxidases**, those peroxidases which exhibit their catalytic activity only when used in acetate or propionate buffers, via the formation of the peracetic acid in the active site due to the acylation of a serine, and subsequent reaction with hydrogen peroxide.

The majority of peroxidases are heme-proteins with the ferric protoheme group associated to the prosthetic group (Figure 1.4.9). In the native enzyme iron (III) is coordinated by the four nitrogen atoms of the heme group. The fifth axial ligand in most peroxidases is a histidine, except for chloroperoxidase (CPO) from *C. fumago*.
which has cysteine as the axial ligand, similar to P450. In fact, during over 50 years, CPO was only known as heme-thiolate peroxidase (Hofrichter et al. 2010).

![Ferric protoporphyrin IX prosthetic group](image)

**Figure 1.4.9** Ferric protoporphyrin IX prosthetic group (Adam et al. 1999).

The catalytic cycle of oxygen transfer processes catalyzed by heme-peroxidases is shown in Figure 1.4.10. Peroxidase reaction occurs via oxidation of the native enzyme by peroxide, forming the iron (III) hydroperoxide intermediate, which after the release of the water molecule forms an active intermediate known as Compound I. Compound I is an iron (V) oxo species, and is also present as an iron(IV) oxo porphyrin radical cation species. It is unstable and prone to self-destruction in the presence of an excess of peroxide although the exact mechanism is still unclear.

If further following the classical peroxidase chemistry (Figure 1.4.10 (2a and 2b)), Compound I takes out an electron from the substrate forming the Compound II. The enzyme returns to its native state by the second one-electron transfer. In case of an oxygen transfer reaction (Figure 1.4.10 (3)), Compound I is reduced in only one-electron oxidation step to the native state.
Figure 1.4.10 Reaction cycle of heme-peroxidases. (1) Formation of Compound I. (2a and 2b) Two one-electron oxidations (classical peroxidase reaction). (3) Two-electron oxidation (oxygen-transfer reaction). (4) Catalase reaction. S represents the substrate (van Deurzen et al. 1997c).

Reaction catalyzed by peroxidases can be divided into four groups:

1. **Oxidative dehydrogenation or classical peroxidase reaction**, reaction catalyzed mainly by heme-peroxidases.
2. **Oxidative halogenation**, reaction characteristic only for haloperoxidases.
3. **Catalase reaction** or disproportionation of hydrogen peroxide to molecular oxygen and water.
4. **Oxygen transfer reactions**.

### 1.4.3.1 Chloroperoxidase

Chloroperoxidase (CPO; EC 1.11.1.10.) from the filamentous fungus *C. fumago* was firstly isolated and characterized over 45 years ago (Morris and Hager 1966). Molecular weight of this enzyme is 42000 Da. CPO is rich in aspartic acid, glutamic acid, serine and proline; these four amino acids constitute about 45% of the total amino acid content. It has 321 amino acids with predominantly acidic residues and a pI in a range of 3.2-4.0. Besides, CPO is a glycoprotein with a carbohydrate content of 25-30%, in which major constituents are glucosamine and arabinose.
CPO is commercially available as fine chemical from different providers. Production of wild-type CPO was accomplished in continuous and semi-continuous flow bioreactors (Blanke et al. 1989) and more recently in agitated culture flasks containing immobilized mycelium (Kaup et al. 2008).

CPO is the most versatile enzyme from heme-peroxidases family. This enzyme can catalyze the formation of halogen-carbon bond in presence of a suitable acceptor substrate and peroxide using chloride, bromide and iodide, but not fluoride ions (Hager et al. 1966). Besides this biological function as a peroxide-dependent chlorinating enzyme, CPO can also catalyze the typical reactions for traditional heme-peroxidases, catalase, and cytochromes P450 monooxygenases (oxygen insertion activity).

Halogenation reactions are catalyzed by acidic form of CPO (pH<3), while non-halogenating reactions are catalyzed by neutral form of CPO (pH=5-6). Within the class of non-halogenating reactions CPO was found to catalyze enantioselective sulfoxidations (Colonna et al. 1990), epoxidations (Geigert et al. 1986; Lakner and Hager 1996), benzylic hydroxylation (Miller et al. 1995), oxidation of amino into nitroso group (Corbett et al. 1978) and oxidation of primary alcohols to aldehydes (Geigert et al. 1983; Kiljunen and Kanerva 2000). Catalase activity of CPO takes place in the absence of halide ion and organic substrate and results in the disproportionation of H$_2$O$_2$ to molecular oxygen and water (Sun et al. 1994).

The reason for which CPO is able to exhibit such multiple activities lies in the structure of its active site. CPO bears cystein (Cys29) like cytochrome P450, while other peroxidases have histidine as fifth axial ligand, but its environment is significantly different (Blanke and Hager 1988; Torres and Ayala 2010). The distal heme pocket in CPO, which is responsible for binding of the peroxide, was determined to contain polar amino acids, which is typical for peroxidases, in contrast with P450s. However, distal amino acid, which acts as acid-base catalyst, is not histidine, like in other heme-peroxidases, but glutamic acid (Glu183) (Yi et al. 1999).

Due to its structural and functional similarities with both heme-peroxidases and cytochrome P450, CPO is often described as “heme-peroxidase-cytochrome P450
functional hybrid” (Sundaramoorthy et al. 1995) or a “Janus enzyme”* (Manoj and Hager 2008) because of its diverse catalytic properties, catalyzing one-electron oxidations and two-electron oxidations coupled with halid oxidation and simple oxygen transfer.

Even though CPO is such a promising enzyme for synthetic applications, its practical use is limited by its low operational stability in the presence of peroxides. The inactivation of CPO by peroxides is caused by the destruction of the heme group (Ayala et al. 2011; Park and Clark 2006). Exact molecular mechanism of CPO inactivation is still unclear, but it was proved that free radicals formed during dismutation of peroxides play a central role (Park and Clark 2006). Some authors had previously reported that apart from this, oxidation of cisteine associated to the heme group caused by peroxide is likely to inactivate CPO (Grey et al. 2007).

Different strategies for the improvement of the operational stability of CPO were studied so far: stabilization via immobilizations (Petri et al. 2004; Zhang et al. 2009), chemical modifications (Liu and Wang 2007), addition of stabilizing agents (Andersson et al. 2000; Spreti et al. 2004) or antioxidants (Grey et al. 2008); addition of the peroxide by simple continuous or step-wise addition using syringes and pumps (Lakner and Hager 1996) or controlled feed-on-demand addition, using peroxide-stat (Seelbach et al. 1997; Van Deurzen et al. 1997b); and by in situ generation of hydrogen peroxide catalyzed by glucose oxidase (Jung et al. 2008; van de Velde et al. 2000).

* Mythical Roman god Janus was the god of doors and gates; he was represented with multiple faces.
CHAPTER 2

OBJECTIVES
2 OBJECTIVES

The general objective of the thesis was the enzymatic obtainment of amino aldehydes from corresponding amino alcohols, and their further aldol addition to dihydroxyacetone phosphate (DHAP) to yield aminopolyols. The enzymes selected for this purpose were: chloroperoxidase (CPO) from Caldariomyces fumago for the first and recombinant rhamnulose-1-phosphate aldolase (RhuA) from Escherichia coli for the second reaction. Cbz-ethanolamine was chosen as the model amino alcohol.

For the completion of this objective, other partial objectives were proposed:

I. **Optimization of the reaction conditions for the oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by CPO** consisting in the choice of an oxidant, optimization of the strategy of the oxidant addition and use of stabilizing agents (e.g. polyethyleneimine) in order to minimize the peroxide dependent inactivation, and reaction media engineering in order to improve the reaction productivity. Within this objective, studying the possibility of CPO accepting other amino alcohols (e.g. Cbz-alaninol) was proposed as well.

II. **Improvement of the catalytic properties of CPO as biocatalyst by chemical modification** consisting in the choice of the chemical modification methods that provide the best stability improvements compared to the native CPO and their evaluation as biocatalysts for the oxidation of Cbz-ethanolamine to Cbz-glycinal.

III. **Improvement of the catalytic properties of CPO as biocatalyst by immobilization** consisting in the choice of the immobilization methods and the optimization of the immobilization process in order to achieve high immobilization yields with minimal activity losses, study of the presence of diffusion limitations of the immobilized enzyme preparation, as well its stability in the presence of peroxide and the performance of the reaction of Cbz-ethanolamine oxidation catalyzed by the immobilized CPO.
Finally, the last objective was the coupled synthesis of aminopolyols from amino alcohols consisting in the choice of the operational conditions in which the coupled enzymatic synthesis can be carried out in a consecutive or simultaneous manner by using soluble and/or immobilized enzymes, reaction media engineering and finally the operation of enzymatic reactor.
CHAPTER 3

MATERIALS AND METHODS
Chapter 3.
3 MATERIALS AND METHODS

3.1 Materials

CPO from *C. fumago* was obtained from Chirazyme Labs (Greenville, NC, USA) as a solution of partially purified enzyme (11.6 mg protein/ml), with a specific activity of 1400 U/mg. RhuA was expressed in *E. coli* as a fusion protein containing a hexahistidine tag (Ruiz et al. 2009). 10% crosslinked agarose beads (10 BCL) was purchased from Iberagar (Coim, Portugal). Fast flow chelating sepharose was purchased from Amersham Biosciences (Uppsala, Sweden). Bis(cyclohexylamine) rhamnulose-1-phosphate was synthesized according to reported procedure (Fessner et al. 1993). Monochlorodimedone (1,1-dimethyl-4-chloro-3,5-cyclohexanedione), Cbz-ethanolamine (benzyl-N-(2-hydroxyethyl)-carbamate), Cbz-glycine, tert-butyl hydroperoxide (t-BuOOH, 70% (w/w) in water) and dihydroxyacetone phosphate hemimagnesium salt hydrate were purchased from Sigma Aldrich (St. Louis, MO, USA). Cbz-glycinal was obtained from Sunshine Chemlab, Inc (Downingtown, PA, USA). All the other reagents were obtained from diverse commercial suppliers and were of the highest available purity and of analytical grade.

3.2 Synthesis of L-rhamnulose-1-phosphate

L-rhamnulose-1-phosphate was synthesized as a salt of cyclohexylamine by aldol addition of dihydroxyacetone phosphate (DHAP) and L-lactaldehyde catalyzed by rhamnulose-1-phosphate aldolase (RhuA) (Figure 3.2.1) and posterior neutralization with cyclohexylamine, according to the reported procedure (Fessner et al. 1993) adopted by Ardao (Ardao 2009).
Figure 3.2.1 Enzymatic synthesis of L-rhamnulose-1-phosphate from dihydroxyacetone phosphate (DHAP) and L-lactaldehyde catalyzed by rhamnulose-1-phosphate aldolase (RhuA).

For the purpose of synthesis of L-rhamnulose-1-phosphate, L-lactaldehyde was synthesized from D-threonine and ninhydrin according to the reported method (Zagalak et al. 1966) and modified by Ardao (Ardao 2009) (Figure 3.2.2).

Figure 3.2.2 Synthesis of L-lactaldehyde from D-threonine and ninhydrin.

25 mmol of D-threonine and 51.1 mmol of ninhydrin were dissolved in 400 ml of 0.05 M sodium citrate buffer at pH 5.4 and incubated in a glycerol bath at 100ºC using vigorous magnetic stirring. A condenser coil was applied in order to retain the NH₃ which is liberated during the reaction. After 2 h of reaction, the mixture was left to cool down and then vacuum filtrated (10-16 μm). The reaction mixture had to be purified, in order to remove the purple side product of the reaction of ninhydrin with NH₃ (Figure 3.2.3).
Figure 3.2.3 Side reaction of the synthesis of L-lactaldehyde.

L-lactaldehyde was purified by ion exchange chromatography. First, anionic exchange resin (Dowex 1x8, HCO\textsuperscript{3−} form) was added until pH of the reaction mixture was adjusted to the value of 6.5, and left on vigorous magnetic stirring for approximately 3 h. Then, the resin was separated by filtration and the reaction mixture was further treated with cationic exchange resin (Dowex 50x8, H\textsuperscript{+} form), added in amount required to obtain pH 4.0. After 1 h of vigorous stirring, the resin was separated by filtration and the liquid mixture was vacuum concentrated to obtain the volume of 50-100 ml. The treatment with the ion exchange resins had to be repeated until complete elimination of the purple color. Finally, the solution of L-lactaldehyde was vacuum concentrated to a volume of 25 ml, and was stored at temperature of 4ºC, pH 4.0 and in the inert atmosphere.

L-lactaldehyde was quantified by indirect method. For that, it was reduced with potassium borohydride (KBH\textsubscript{4}) and the resulting 1,2-propanodiol was quantified by HPLC.

Then, 10 mmols of DHAP and synthesized L-lactaldehyde were dissolved in 250 ml of Milli Q H\textsubscript{2}O and the pH was adjusted to 7.5 with KOH. 10 U of RhuA were added and left to react at temperature of 35ºC and mild magnetic stirring. The samples were withdrawn periodically, incubated for 1 minute at 100ºC in order to denaturate the enzyme and stop the enzymatic reaction, and DHAP, L-lactaldehyde and L-rhamnulose-1-phosphate were quantified. When no further consumption was detected, the reaction was stopped by incubating the reaction mixture at the temperature of 100ºC. L-rhamnulose-1-phosphate was purified by ion exchange chromatography. Firstly, the reaction mixture was filtrated through carbon coal in order to remove the possible organic impurities. Then, the filtrated reaction mixture was loaded to the
chromatography column containing 140 ml of the anion exchange resin (Dowex 1x8, HCO3⁻ form), and then washed with 2 volumes of Mili Q H₂O. The fractions of 50 ml of the eluted liquid were collected and the content of L-lactaldehyde and L-rhamnulose-1-phosphate were measured. Then, L-rhamnulose-1-phosphate retained on the resin was eluted with a solution of 0.2 M NaHCO₃. Fractions of 50 ml were collected during the elution and analyzed on HPLC in order to determine the content of L-rhamnulose-1-phosphate. Then the column was washed with the 0.7 M solution of NaHCO₃ in order to recuperate the sugar that could have possibly remained on the column. Then, the fractions containing L-rhamnulose-1-phosphate were lyophilized and dissolved in 100 ml of Mili Q H₂O and treated with cationic exchange resin (Dowex 50x8, H⁺ form) leaving them on vigorous magnetic stirring in order to eliminate remained bicarbonate. Once no release of CO₂ was observed, the solution was vacuum filtrated (10-16 μm) in order to separate the resin, then neutralized with cyclohexylamine and lyophilized. The salts of cyclohexylamine were stored at -20ºC in the inert atmosphere.

3.2.1 Qualitative determination of L-lactaldehyde and L-rhamnulose-1-phosphate

Qualitative determination of L-lactaldehyde and L-rhamnulose-1-phosphate was carried out by analytical HPLC Dionex UltiMate 3000 with IR Waters 2410 detector employing a reversed-phase column X Bridge C18, 5 μm, 4.6x250 mm from Waters (Wexford, Ireland) at 30ºC. The solvent system consisted of 5 mM H₂SO₄ in Mili Q H₂O. The solvents were eluted at a flow rate of 0.6 ml/min and the peaks of L-lactaldehyde and/or rhamnulose-1-phosphate were detected by using index refraction detector.

3.2.2 Quantification of L-lactaldehyde

The quantification of L-lactaldehyde was carried out by an indirect method consisting in the reduction of L-lactaldehyde in the presence of KBH₄ to 1,2-propanediol and its posterior quantification using HPLC. The reduction of L-lactaldehyde was carried out by adding the stoichiometric quantity of KBH₄ considering the yield of the reaction of synthesis to be 100% to 2 ml of the
reaction sample. The mixture was incubated at room temperature at magnetic stirring for 3 h. 1,2-propanediol was quantified on HPLC and the result corresponded to the concentration of L-lactaldehyde initially present in the sample.
Concentration of 1,2-propanediol was estimated by analytical HPLC Dionex UltiMate 3000 with IR Waters 2410 Detector employing a reversed-phase column X Bridge C18, 5 μm, 4.6x250 mm from Waters at 30ºC. The solvent system consisted of 5 mM H$_2$SO$_4$ in Mili Q H$_2$O. The solvent was eluted at a flow rate of 0.6 ml/min and peaks were detected using index refraction detector. Quantitative analysis of 1,2-propanediol was performed from peak areas by the external standard method by means of a prior calibration with samples of known concentration.

3.3 Purification of rhamnulose-1-phosphate aldolase on IMAC

Immobilized metal-chelate affinity chromatography (IMAC) is the purification method based on the differences in affinity of the proteins for metal ion bound to a metal-chelating substance which is immobilized on the chromatographic resin. The affinity between imidazole rings from the histidine residues and the ions immobilized on the chromatographic resin was profited in the procedure developed by Ardao (Ardao 2009), for the purification of recombinant RhuA from *E. coli* containing hexa-histidine tag.
First, 0.84 l of the fermentation broth was centrifuged (Beckman J2-21, 9700 rpm, 35 min, 4ºC), and the obtained supernatant (0.74 l) was discarded. The pellet was resuspended and homogenized in the lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole, pH 8.0) to the final volume of 0.88 l to reach the cell density OD=80. Then, the cell disruption was performed in constant cell disruption system (21 kPa, 4ºC) and was repeated until the value of cell density was less than 10% of the initial one. The insoluble debris was removed by centrifugation (Beckman J2-21, 9700 rpm, 35 min, 4ºC), and supernatant (0.86 l) was loaded to a chromatographic column (Amersham Biosciences XK 50/20) containing 400 ml of high density cobalt-chelate affinity resin, Co-IDA (GE Healthcare 17-0575-02). The loading was performed using FPLC system (fast protein liquid chromatography) which consisted of peristaltic pumps
(Pharmacia LKB Pump P-500), UV detector adjusted to 280 nm (Pharmacia Monitor UV-M) and fractions collector (Amersham Biosciences Frac-200). After the loading, various successive washing of the column with the lysis buffer were performed, in order to eliminate other proteins. Then, the elution of RhuA was performed using eluting buffer (50 mM sodium phosphate, 300 mM NaCl, 300 mM imidazole, pH 8.0). The fractions containing proteins were tested by RhuA activity assay, and those showing activity were precipitated with \((\text{NH}_4)_2\text{SO}_4\) at the concentration of 0.4 g/ml, centrifuged (Beckman J2-21, 9700 rpm, 35 min, 4ºC), and after removing the supernatant the precipitated enzyme was resuspended in the 0.4 g/ml solution of \((\text{NH}_4)_2\text{SO}_4\). The enzyme suspension was stored at 4ºC.

The commercially available metal-chelate support did not contain the metal ions. Therefore, the resin had to be loaded with Co\(^{2+}\) ions by the following procedure. First, the resin was packed into the column and washed with 3 volumes of Mili Q H\(_2\)O, then 3 volumes of 0.2 M CoCl\(_2\) pH 4.7 were passed through the column at a flow rate of 1 ml/min. Finally, the column was washed with 5 volumes of Mili Q H\(_2\)O, equilibrated with 20% (v/v) ethanol and stored.

3.4 **Enzyme activity assays**

The change in the absorbance in the activity assays used for the determination of the enzymatic activity was measured in UV-Visible Spectrophotometer Cary 50 (Varian, Palo Alto, CA, USA) using quartz cuvettes.

In order to measure the immobilized enzyme activity in the suspension samples, magnetic stirring was applied to the spectrophotometric cuvette.

3.4.1 **Chloroperoxidase activity assays**

3.4.1.1 **Chlorination activity assay**

The spectroscopic assay was based on the decrease in absorbance \((A_{278})\) for a solution of monochlorodimedone, MCD \((\epsilon_{278 \text{ nm}}=25500 \text{ M}^{-1}\text{cm}^{-1})\) as it was converted to
dichlorodimedone, DCD ($\varepsilon=120 \text{ M}^{-1}\text{cm}^{-1}$) (Figure 3.4.1), by CPO according to the method described (Hager et al. 1966).

![Figure 3.4.1 Chlorination of MCD to DCD catalyzed by CPO.](image)

0.05 ml of the sample were added to the assay mixture that contained 100 mM potassium phosphate buffer pH 2.75, 20 mM KCl, 2 mM hydrogen peroxide and 0.16 mM MCD in the final assay volume of 1 ml. The absorbance was measured at 25ºC at a wavelength of 278 nm. One unit of CPO was defined as the enzyme activity required to catalyze the conversion of 1 µmol of MCD to DCD per minute at 25ºC.

**3.4.1.2 Peroxidation activity assay**

Peroxidative activity of CPO was assayed by the guaiacol method by measuring the increase in absorbance ($A_{436}$) as it was converted to tetraguaiacol ($\varepsilon_{436 \text{ nm}}=25.5 \text{ M}^{-1}\text{cm}^{-1}$) (Figure 3.4.2) in a 3 ml assay mixture containing 18 mM guaiacol, 0.3 mM hydrogen peroxide and 100 mM acetate buffer pH 5.0, at 25ºC. The reaction was initiated upon the addition 0.1 ml of the enzyme and monitored by increase in absorbance at 436 nm. One unit of peroxidative activity is defined as the amount of the enzyme required to catalyze the conversion of 1 µmol of guaiacol per minute at a temperature of 25ºC.
Figure 3.4.2 Peroxidation of guaiacol to tetraguaiacol catalyzed by CPO.

3.4.2 RhuA activity assay

The activity of RhuA was measured indirectly by following the decrease of the absorbance at 340 nm caused by NADH consumption ($\varepsilon_{340\,nm} = 6220\,\text{M}^{-1}\cdot\text{cm}^{-1}$) using a coupled enzymatic assay. In the first step, rhamnulose 1-phosphate is cleaved to L-lactaldehyde and DHAP catalyzed by RhuA; in the second step, DHAP is reduced using rabbit muscle glycerol 3-phosphate dehydrogenase ($\alpha$-GDH) and NADH (Figure 3.4.3). The second reaction in the sequence was performed with high activity in the assay mixture in order to be practically instantaneous. Therefore, the observed reaction rate corresponds to the rate of DHAP formation catalyzed by RhuA.

0.01 ml of the sample was added to the assay mixture that contained 50 mM TrisHCl buffer pH 7.5, 0.15 mM NADH, 2 mM bis(cyclohexylamine) rhamnulose 1-phosphate and 2.5 U/ml GDH. The absorbance was measured at 25°C at a wavelength of 340. One unit of RhuA activity is defined as the amount of enzyme required to convert 1 μmol of rhamnulose 1-phosphate to DHAP per minute at 25°C and pH 7.5.
Materials and Methods

Figure 3.4.3 Enzymatic assay for the measurement of the activity of rhamnulose-1-phosphate aldolase (RhuA).

3.5 Quantification of reaction substrates and products

3.5.1 Quantification of Cbz-ethanolamine, Cbz-glycinal, Cbz-glycine and Cbz-aminopolyol

Concentrations of Cbz-ethanolamine, Cbz-glycinal, Cbz-glycine and Cbz-aminopolyol were estimated by analytical HPLC Dionex UltiMate 3000 with UltiMate 3000 Variable Wavelength Detection (Sunnyvale, USA) employing a reversed-phase column X Bridge C18, 5 μm, 4.6x250 mm from Waters (Wexford, Ireland) at 30ºC. The solvent system consisted of solvent A, composed of 0.1% v/v trifluoroacetic acid (TFA) in H₂O, and solvent B, composed of 0.095% v/v TFA in H₂O/CH₃CN 1:4. The solvents were eluted at a flow rate of 1 ml/min using a gradient from 20% B to 36% B over 24 min and peaks were detected at 200 nm. Quantitative analysis of products was performed from peak areas by the external standard method by means of a prior calibration with samples of known concentration.
3.5.2 Determination of DHAP concentration

Dihydroxyacetone phosphate concentration was determined spectrophotometrically. DHAP is reduced using rabbit muscle glycerol 3-phosphate dehydrogenase (GDH) and reduced nicotine adenine dinucleotide (NADH). 0.01 ml of the DHAP sample was added in a 1 ml quartz cuvette that contained the assay mixture composed of 0.2 mM NADH in 100 mM Tris HCl buffer pH 7.5 and maintained at 25ºC. The assay was initiated upon addition of α-GDH in the final activity of 0.5 U/ml. The time course of the reaction was followed measuring the decrease of NADH absorbance at 340 nm ($\varepsilon_{340\text{ nm}}=6220$ M$^{-1}$·cm$^{-1}$) before (Abs1) and after (Abs2) GDH addition in UV-Visible Spectrophotometer Cary 50 (Varian). The DHAP concentration can be obtained using the equation: 

$$[\text{DHAP}] (\text{mM}) = \frac{(\text{Abs1}-\text{Abs2})}{(6.22 \text{ mM}^{-1}\cdot \text{cm}^{-1})} \times 100.$$
CHAPTER 4

ENGINEERING OF THE REACTION MEDIA FOR CHLOROPEROXIDASE CATALYZED OXIDATION OF CBZ-AMINO ALCOHOLS TO CBZ-AMINO ALDEHYDES

Publication:
Chapter 4.
4 ENGINEERING OF THE REACTION MEDIA FOR CHLOROPEROXIDASE CATALYZED OXIDATION OF CBZ-AMINO ALCOHOLS TO CBZ-AMINO ALDEHYDES

4.1 Introduction

The great interest for obtaining amino aldehydes lies on their applicability as precursors of important pharmaceutical compounds. Enzymatic synthesis of these compounds can result very advantageous against other synthetic methods in terms of toxicity and costs, as it was pointed out in the Section 1.4.2. Chloroperoxidase (CPO) was previously demonstrated to oxidize primary alcohols to corresponding aldehydes by using hydrogen peroxide or tert-butyl hydroperoxide as electron acceptors (Geigert et al. 1983; Kiljunen and Kanerva 2000). Nevertheless, enzymatic synthesis of amino aldehydes was so far reported only by using horse liver alcohol dehydrogenase (HL-ADH) (Andersson and Wolfenden 1982), but this procedure presents several drawbacks, mainly concerning cofactor requirements, which were explained in the same section. Thus, in this chapter, the oxidation of amino alcohols to amino aldehydes was proposed to be carried out by using CPO.

The main limitation for the synthetic application of CPO is its fast irreversible inactivation in the presence of peroxides. Therefore, the choice of the peroxide and the strategy of its addition have to be the first steps for the optimization of the CPO catalyzed reaction. Simple continuous or step-wise addition using syringes and pumps (Lakner and Hager 1996) and controlled feed-on-demand addition, using peroxide-stat (Seelbach et al. 1997; Van Deurzen et al. 1997b) are the reported strategies of the peroxide addition which enabled the improvement of the operational stability of CPO and therefore overall reaction performance.

In this chapter, the enzymatic oxidation of an amino alcohol to yield the amino aldehyde using the enzyme CPO was performed. As the amino and aldehyde groups of the amino aldehyde tend to react between them forming an internal Schiff base, a
protective group must be added to the amino terminal. In the selection of the reaction of interest, the substrate specificity of CPO for the oxidation of alcohols to aldehydes was considered. As it was previously shown that branched-chain alcohols were not substrates for CPO, due to its sterically constrained active site (Kiljunen and Kanerva 2000), ethanolamine containing carbobenxoxy protective group was selected as a model substrate (Figure 4.1.1). The effects of nature and addition frequency of peroxide, presence of stabilizing agent polyethyleneimine (PEI), nature of reaction media (by using water miscible and immiscible organic solvents) and finally temperature were studied in order to increase amino aldehyde production while preserving enzymatic activity. Once the Cbz-ethanolamine oxidation optimized, the possibility of the oxidation of Cbz-alaninol by CPO catalysis was studied.

Figure 4.1.1 Oxidation of Cbz-ethanolamine to Cbz-glycinal and Cbz-glycine by CPO catalysis.

4.2 Methods

4.2.1 CPO stability

Effect of high temperature on CPO stability was studied at the temperature of 50°C by incubating 20 U of CPO in 10 ml of 100 mM sodium acetate buffer pH 5.0. Effect of biphasic medium and presence of water miscible cosolvents on CPO stability was analyzed by incubating 20 U of CPO in 10 ml of the reaction medium composed of 100 mM sodium acetate buffer pH 5.0 containing 5 and 15% of a water miscible organic solvent (acetone, acetonitrile, dioxane, dimethylformamide (DMF)) or 50% of water immiscible organic solvent (ethyl acetate) at room temperature (20-23°C). In all cases, after different times of incubation aliquots of the enzyme solution were withdrawn and enzymatic activity was measured using MCD assay (Section 3.4.1.1).
4.2.2 Oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by CPO

For reactions performed in aqueous medium 20 mM Cbz-ethanolamine was dissolved in 100 mM acetate buffer at pH 5.0 in a final volume of 10 ml. For reactions performed using PEI as a stabilizing agent Cbz-ethanolamine was dissolved at the same concentration in 100 mM acetate buffer at pH 5.0 containing 0.1-1% PEI. For reactions in biphasic or cosolvent systems between 150 and 500 mM Cbz-ethanolamine was dissolved in the same concentration of the buffer containing between 5 and 15% of a water miscible organic solvent (acetone, acetonitrile, dioxane, DMF) or 50% of water immiscible organic solvent (ethyl acetate). Between 500 and 1100 U/ml of CPO were added to the reaction medium. The reaction was started by adding hydrogen peroxide or tert-butyl hydroperoxide continuously, in two different manners: (i) manual addition of one pulse (10-200 µl) per hour during the first 8 hours of the reaction which was stopped and restarted after 24 h in case that CPO activity was detected; (ii) non-stop addition at a rate of 18 µl/h by using a single-syringe automatic microburette (Crison Instruments, Spain). Concentration of peroxide stock solutions varied between 1.5 and 6.7 M in order to reach peroxide addition rates within the range of 1.5-50 mM/h. Reaction samples were withdrawn periodically and analyzed by liquid chromatography in order to quantify Cbz-ethanolamine and Cbz-glycinal concentrations (Section 3.5.1); CPO activity was also analyzed following the method described in Section 3.4.1.1. Each measurement was carried out in duplicate.

Pseudo half life of CPO was determined experimentally and represents the time at which CPO activity decays to the half of the activity initially added. Conversion and yields of the products were defined respectively as the percentage of the consumed Cbz-ethanolamine or produced products, respect to initially added concentration of Cbz-ethanolamine. These values were additionally corrected by the dilution factor which was calculated for each set of measurements taking into account the volume of the peroxide added in pulses and the volume of the reaction medium withdrawn for the analysis. Initial reaction rate represents the rate of the Cbz-ethanolamine consumption during the period of linear change in concentration. Volumetric productivity was defined as the concentration of Cbz-glycinal produced per time unit during the overall reaction time.
4.3 Results and discussion

4.3.1 Chloroperoxidase catalyzed oxidation of Cbz-ethanolamine in aqueous medium

Reaction of the oxidation of alcohols to aldehydes catalyzed by CPO belongs to the group of halide independent reactions which occur at pH values in a range of 5.0-6.0. As CPO shows better activity and stability at lower pH values (Thomas et al. 1970), pH 5.0 was chosen as optimal. Additionally, in the preliminary studies, the oxidation of Cbz-ethanolamine was performed by using CPO activity of 100 U/ml resulting in a very low yield of Cbz-glycinal. Therefore, the used activity of CPO was increased to 500 U/ml.

Besides, even though CPO was reported to produce the aldehydes from corresponding alcohols in a very selective manner, the further oxidation of aldehydes to acids was observed as well (Kiljunen and Kanerva 2000). Therefore, the reaction conditions for the oxidation of Cbz-ethanolamine to Cbz-glycinal have to be chosen trying to avoid or minimize the undesired further oxidation to Cbz-glycine.

4.3.1.1 Choice of the oxidant and optimization of the addition frequency

The reactions of oxidation of alcohols to corresponding aldehydes catalyzed by CPO occur via direct oxygen transfer reaction using peroxide (typically hydrogen peroxide and tert-butyl hydroperoxide) as electron acceptor, although an excess of peroxide is likely to cause enzyme inactivation (Wariishi et al. 1988). Trying to combine a high reaction rate with a minimal loss of enzymatic activity, the continuous addition of peroxide was proposed by other authors (Lopez et al. 2004; Mielgo et al. 2003). The selection of the oxidant for the reaction of Cbz-ethanolamine to Cbz-glycinal using CPO as well as the continuous addition frequency were studied trying to maximize Cbz-glycinal production and minimize peroxide dependent inactivation of CPO.

When hydrogen peroxide is used as an oxidant for the CPO catalyzed oxidation of Cbz-ethanolamine to Cbz-glycinal, very low conversions were obtained (Figure 4.3.1 A). Nevertheless, the enzyme remained almost completely stable during the reaction time.
(24 h), retaining around 90-100% of its initial activity depending on the concentration of H$_2$O$_2$ added in pulses. This could be explained by a very high catalase activity of CPO towards H$_2$O$_2$ which totally decomposes the peroxide added releasing molecular oxygen and water (Manoj and Hager 2001). On the other hand, when t-BuOOH was added in pulses (Figure 4.3.1 B), the conversion of the Cbz-ethanolamine to Cbz-glycinal was accomplished although a rapid enzyme inactivation was observed resulting in loss of complete initial activity during the reaction time. This is an expected result as there is evidence that with this oxidant the enzyme has a very low catalase activity, so that it is available for enzymatic oxidation (Manoj and Hager 2001). The percentage of the conversion was around 50% in all cases and apart from Cbz-glycinal as a main product, the amino acid Cbz-glycine was produced (6.5-10.8% of Cbz-glycine yield), as the result of the further oxidation of amino aldehyde catalyzed by CPO.

In fact, for the synthetic applications of CPO, H$_2$O$_2$ is being used with success mainly for the chlorination and sulfoxidation reactions, since these reactions occur fast, and therefore can compete with the catalase reaction for hydrogen peroxide utilization (Manoj and Hager 2001; Ortiz-Bermúdez et al. 2003; van Deurzen et al. 1997a; Vargas et al. 1999; Vazquez-Duhalt et al. 2001). For other reactions which are not so fast, such as reactions of oxidation of alcohols and reactions of epoxidation, t-BuOOH is being used instead of H$_2$O$_2$ (Kiljunen and Kanerva 1999; Park and Clark 2005; Samra et al. 1999).
Figure 4.3.1 Optimization of the peroxide addition frequency on the oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by soluble CPO: Cbz-ethanolamine conversion (%), black bars, yield of Cbz-glycinal (%), grey bars and remaining CPO activity (%), white bars) using H_2O_2 (A) or t-BuOOH (B) as oxidant. The reaction medium contained 20 mM of Cbz-ethanolamine and CPO activity of 500 U/ml in 100 mM acetate buffer pH 5.0. All the experiments were performed at room temperature (20-23ºC) for 24 h.

Therefore, t-BuOOH resulted as a more appropriate oxidant for CPO catalyzed oxidation of Cbz-ethanolamine. The time-course of the reactions catalyzed by the two oxidants tested is represented on the Figure 4.3.2.
Figure 4.3.2 Time course for the oxidation of Cbz-ethanolamine (●) to Cbz-glycinal (○) and Cbz-glycine (▼) catalyzed by CPO (% remaining activity, □) using hydrogen peroxide as oxidant in addition frequency of 24 mM/2 h (A) and tert-butyl hydroperoxide as oxidant in addition frequency of 3 mM/h (B) at the temperature of 20-23ºC. CPO activity of 500 U/ml was added to the reaction mixture that contained 20 mM of Cbz-ethanolamine in 100 mM acetate buffer pH 5.0.
CPO requires one mol of peroxide to oxidize one mol of Cbz-ethanolamine to Cbz-glycinal (Colonna et al. 1999; van Deurzen et al. 1997c). If peroxide is added at very low rate, the reaction is limited by peroxide and it proceeds under non-optimal conditions. Otherwise, when the rate of peroxide addition significantly exceeds the reaction rate, accumulation of peroxide leads to fast CPO inactivation. The half life of the enzyme can be significantly prolonged if the peroxide addition rate is adjusted to the initial reaction rate. When adding t-BuOOH at 3 mM/h (Figure 4.3.1 B) the initial reaction rate was calculated as 1.8 mM/h, which is considerably lower than the maximal value that could be reached (3 mM/h) (Figure 4.3.3). Trying to adjust peroxide addition to reaction rate, the oxidation was performed by adding 1.5 mM/h of t-BuOOH, but even lower initial reaction rate was observed (1.1 mM/h) and CPO did not result to be more stable than for the addition of 3 mM/h of t-BuOOH. Therefore, lower yields of product were obtained. On the other side, when 6 mM/h of t-BuOOH were added, a slightly higher initial reaction rate was achieved (2.2 mM/h), but it was not beneficial in terms of stability of CPO, and consequently, final reaction yield, as it is observed in Figure 4.3.1 B.

<table>
<thead>
<tr>
<th>Peroxide addition rate (mM/h)</th>
<th>Initial reaction rate (mM/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
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<tr>
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<td>5</td>
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<tr>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>

**Figure 4.3.3** Variation of initial reaction rates of the Cbz-ethanolamine oxidation catalyzed by CPO using different tert-butyl hydroperoxide addition rates. The reaction medium contained 20 mM of Cbz-ethanolamine and CPO activity of 500 U/ml in 100 mM acetate buffer pH 5.0. All the experiments were performed at room temperature (20-23°C) for 24 h.

68
Hence, from the results obtained in this section, it can be concluded that for the reaction of oxidation of Cbz-ethanolamine in the aqueous medium, tert-butyl hydroperoxide resulted as a more appropriate oxidant than hydrogen peroxide. The best reaction performance was achieved when it was added at the rate of 3 mM/h.

4.3.1.2 Effect of elevated temperature on the oxidation of Cbz-ethanolamine by CPO catalysis

Increase of the temperature in the reaction catalyzed by CPO can have two major effects. On one side, enzyme will suffer at some extent thermal denaturation, but on the other side, the increase of the temperature might be desired in order to accelerate the reaction, and therefore the consumption of the peroxide. The lower accumulation of the peroxide will hence, have a positive effect on the stability of CPO. Therefore, the overall reaction performance will depend on the ratio of these two factors.

The reaction of oxidation of Cbz-ethanolamine in aqueous medium was performed at temperature of 50ºC with peroxide addition rates of 3, 6, 9 and 12 mM/h in order to reassure that the reaction is not limited by the peroxide concentration. Time courses for the synthesis of Cbz-glycinal and inactivation of CPO are represented in Figure 4.3.4.

Very fast loss of activity of CPO was observed in all the reactions studied at this temperature. Only slightly higher initial reaction rates could be obtained compared to those obtained at room temperature, and they were not adjusted to the peroxide addition rate (Table 4.3.1). Consequently, the inactivation of CPO was faster for the higher rates of peroxide addition. Nevertheless, the final yield of Cbz-glycinal reached the highest value (16.8%) for the peroxide addition rate of 12 mM/h.

These results suggest that the high temperature affected more the enzyme stability than the reaction rate, and therefore, having slightly improved initial reaction rates, much lower yields were obtained than in the reactions catalyzed by CPO at room temperature.
Figure 4.3.4 Time course for Cbz-glycinal synthesis (A) and inactivation of CPO (B) in the reactions of oxidation of Cbz-ethanolamine catalyzed by CPO using tert-butyl hydroperoxide as oxidant in addition rates of 3 (●), 6 (○), 9 (□) and 12 mM/h (Δ) at temperature of 50°C. CPO activity of 500 U/ml was added to the reaction mixture that contained 20 mM of Cbz-ethanolamine in 100 mM acetate buffer pH 5.0. Data points represent the average of duplicate measurements; error bars represent the standard deviation.
Table 4.3.1 Comparison of reactions of Cbz-ethanolamine oxidation catalyzed by CPO using different addition rates of t-BuOOH. CPO activity of 500 U/ml was added to the reaction medium that contained 20 mM of Cbz-ethanolamine in 100 mM acetate buffer pH 5.0. All the experiments were performed at temperature of 50ºC. The end of the reaction was considered when no CPO activity was detected.

<table>
<thead>
<tr>
<th>t-BuOOH addition rate (mM/h)</th>
<th>Initial reaction rate (mM Cbz-ethanolamine/h)</th>
<th>Pseudo half life, $t_{1/2}$ (h)*</th>
<th>Yield of Cbz-glycinal (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>1.8</td>
<td>0.51</td>
<td>14.7</td>
</tr>
<tr>
<td>6</td>
<td>3.1</td>
<td>0.34</td>
<td>15.2</td>
</tr>
<tr>
<td>9</td>
<td>3.7</td>
<td>0.27</td>
<td>16.6</td>
</tr>
<tr>
<td>12</td>
<td>6.7</td>
<td>0.22</td>
<td>16.8</td>
</tr>
</tbody>
</table>

Finally, in order to estimate the degree at which the CPO activity loss was due to the thermal denaturation and the peroxide dependent inactivation, the stability of CPO was studied at temperature of 50ºC with no addition of peroxide and the results were further compared to the ones obtained in the reaction performed at room temperature (Figure 4.3.5). It can be seen that the inactivation of CPO in the reaction performed at temperature of 50ºC represents approximately the sum of the thermal inactivation at 50ºC and the inactivation caused by the presence of peroxide which occurred in the reaction catalyzed at room temperature. Besides, the thermal inactivation has stronger influence by causing the loss of almost 50% of the initial activity after only one hour of incubation.

* The half life of the enzyme in the reaction was determined experimentally and represents the time at which CPO activity decays to the half of the activity initially offered. Since the conditions in the reaction medium were changing along the reaction (basically, peroxide accumulation, which was proved to be the main reason for the enzyme inactivation) the decay of the activity was not following regular inactivation pattern. Therefore, any mathematical treatment of the data could not be used in order to calculate this value. The word “pseudo” was added meaning an apparent half life.
4.3.1.3 Use of polyethyleneimine (PEI) as a stabilizing agent

It was previously reported that the stability of CPO in the presence of peroxides can be dramatically improved by the addition of polyethyleneimine (PEI) (Andersson et al. 2000). This polymer exerts a stabilizing effect by protecting the enzyme lowering the concentration of the peroxide near its surface. Table 4.3.2 shows that in a reaction medium containing PEI at concentration of 1% when the oxidant was added in pulses at a concentration of 6.1, 12.2 and 24 mM/2 h, the half life of CPO was around 24 h in all cases. Therefore, having lower exposure of the enzyme to the peroxide led to a higher half life of CPO, but at the same time resulted in lower reaction rates, due to the limiting concentration of the peroxide nearby the enzyme. The increase of
peroxide concentration resulted in improved yield of Cbz-glycinal up to an addition of 50 mM of peroxide each 2 h. Further increase to the addition of 100 mM/2h of peroxide caused faster deactivation of CPO and complete loss of its activity within 24 h; hence lower yields were obtained despite the higher initial reaction rates. At the concentration of PEI of 0.1% the enzyme was more exposed to the peroxide, facilitating the conversion of the substrate although causing almost complete deactivation of the enzyme within 8 h. Since the rate of deactivation of the enzyme increases with time as peroxide accumulates in the reaction medium, the addition of 0.05% PEI together with the peroxide pulses was performed with the purpose of maintaining the enzyme active and therefore improving the substrate conversion. Even though this did not improve the half life of the enzyme, it was noticed that the enzyme remained active during longer time period, retaining around 20% of its initial activity after 8 h of reaction comparing to almost 100% of the activity loss after the same reaction time, when 0.1% of PEI were initially added with no further addition in pulses. Even so, initial reaction rate of 1.11 mM/h and conversion of 36.1% which were the maximal values accomplished with this strategy, are lower values than those obtained in the absence of PEI (initial reaction rate of 1.8 mM/h and conversion of 53.7%).
Table 4.3.2 Oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by soluble CPO using tert-butyl hydroperoxide as oxidant in the presence of PEI; CPO activity of 500 U/ml was added to the reaction medium that contained 20 mM of Cbz-ethanolamine in 100 mM acetate buffer pH 5.0. All the experiments were performed at room temperature (20-23°C).

<table>
<thead>
<tr>
<th>t-BuOOH addition frequency</th>
<th>Reaction time (h)</th>
<th>PEI (%)</th>
<th>Loss of CPO activity (%)</th>
<th>Pseudo half life, t_1/2 (h)</th>
<th>Conversion (%)</th>
<th>Yield of Cbz-glycinal (%)</th>
<th>Yield of Cbz-glycine (%)</th>
<th>Initial reaction rate (mM Cbz-ethanolamine/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1 mM/2h</td>
<td>24</td>
<td>1</td>
<td>38.9</td>
<td>24</td>
<td>5.3</td>
<td>5.3</td>
<td>0.0</td>
<td>0.12</td>
</tr>
<tr>
<td>12.2 mM/2h</td>
<td>24</td>
<td>1</td>
<td>42.4</td>
<td>24</td>
<td>10.4</td>
<td>10.4</td>
<td>0.0</td>
<td>0.28</td>
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<tr>
<td>24 mM/2h</td>
<td>26</td>
<td>0.1</td>
<td>100</td>
<td>5</td>
<td>19.1</td>
<td>16.6</td>
<td>2.5</td>
<td>0.86</td>
</tr>
<tr>
<td>24 mM/2h</td>
<td>24</td>
<td>1</td>
<td>40</td>
<td>24</td>
<td>15.9</td>
<td>15.1</td>
<td>0.8</td>
<td>0.52</td>
</tr>
<tr>
<td>50 mM/2h</td>
<td>24</td>
<td>1</td>
<td>94.1</td>
<td>8</td>
<td>26.4</td>
<td>23.9</td>
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<td>100 mM/2h</td>
<td>24</td>
<td>1</td>
<td>96.8</td>
<td>5</td>
<td>25.0</td>
<td>21.5</td>
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<td>1.11</td>
</tr>
<tr>
<td>25 mM/2h</td>
<td>24</td>
<td>0.1 % +</td>
<td>97.4</td>
<td>5</td>
<td>36.1</td>
<td>23.4</td>
<td>12.7</td>
<td>1.00</td>
</tr>
</tbody>
</table>
4.3.1.4 Reaction with repeated addition of CPO

Possible increment of the conversion and product yield was studied by means of the repeated addition of CPO in pulses at the moments when rapid decrease of the CPO activity was detected as a result of accumulation of peroxide in the reaction medium. For comparison, a parallel reaction was performed, containing the same amount of CPO as the overall amount added during the overall time of the first reaction. The results are shown in Table 4.3.3.

Increment of CPO activity from 500 U/ml (third set of bars in Figure 4.3.1 B) to 1100 U/ml (Reaction I in Table 4.3.3) applied in identical reaction conditions resulted in increased conversions, from 53.7 to 81.7%. However, this benefit was reflected more in the increment of the production of Cbz-glycine than the one of Cbz-glycinal. The yield of Cbz-glycinal reached its maximum at 8 h (Figure 4.3.6) with volumetric productivity of 1.1 mM/h. The accumulation of aminoaldehyde accelerated its further conversion to amino acid, resulting in lower yield of Cbz-glycinal and 2-fold lower ratio Cbz-glycinal/Cbz-glycine at 29 h than that at 8 h. No improvement was observed when CPO was added in some pulses along the reaction (Reaction II in Table 4.3.3).
Table 4.3.3 Oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by soluble CPO using tert-butyl hydroperoxide as oxidant in addition rate of 3 mM/h. In the reaction I CPO activity of 1100 U/ml was added to the reaction medium that contained 20 mM of Cbz-ethanolamine in 100 mM acetate buffer pH 5.0. In the reaction II the initial activity of CPO was 500 U/ml and the activity of 150 U/ml was added in 4 pulses each 2 h. Both experiments were performed at room temperature (20-23°C). The end of the reaction was considered when no CPO activity was detected.

<table>
<thead>
<tr>
<th></th>
<th>CPO activity (U/ml)</th>
<th>Reaction time (h)</th>
<th>Conversion (%)</th>
<th>Yield of Cbz-glycinal (%)</th>
<th>Yield of Cbz-glycine (%)</th>
<th>Volumetric productivity (mM/h)</th>
<th>Cbz-glycinal/Cbz-glycine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction I</td>
<td>1100</td>
<td>8</td>
<td>69.0</td>
<td>45.0</td>
<td>24.0</td>
<td>1.1</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>29</td>
<td>81.7</td>
<td>39.6</td>
<td>38.9</td>
<td>0.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Reaction II</td>
<td>500+4 pulses of 150/2 h</td>
<td>8</td>
<td>64.4</td>
<td>44.1</td>
<td>20.3</td>
<td>1.1</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>29</td>
<td>82.5</td>
<td>39.1</td>
<td>38.1</td>
<td>0.3</td>
<td>1.0</td>
</tr>
</tbody>
</table>
4.3.2 Chloroperoxidase catalyzed oxidation of Cbz-ethanolamine in biphasic medium

Poor solubility of Cbz-ethanolamine in aqueous media is one of the main reasons for relatively low productivity in the synthesis of Cbz-glycinal catalyzed by CPO. Biphasic medium permits working with higher concentrations of organic substrates, which can increase the reaction rate and yield higher productivities (Semenov et al. 1987). CPO was previously shown by Kiljunen and Kanerva (Kiljunen and Kanerva 2000) to successfully catalyze the oxidation of alcohols to aldehydes using a biphasic medium of hexane or ethyl acetate in a concentration of 50% (v/v) in sodium acetate buffer at pH 5.0.
The solubilities of Cbz-ethanolamine in 50% (v/v) hexane:buffer and 50% (v/v) ethyl acetate:buffer were evaluated and resulted to be around 50 and 500 mM respectively, while the one in aqueous medium is around 20 mM. Based on this, 50% (v/v) ethyl acetate was chosen as suitable medium for the purpose of oxidation of Cbz-ethanolamine. In this system distribution coefficient of Cbz-ethanolamine between the two phases expressed as the ratio of its concentration in organic (ethyl acetate) and aqueous phase was determined using a concentration of 20 mM and resulted to be around 22.0.

CPO stability was studied in three different systems: biphasic medium, aqueous medium and biphasic medium with continuous peroxide addition. Figure 4.3.7 shows that the main part of the CPO deactivation in biphasic medium was caused by the presence of t-BuOOH, while the activity decrease in biphasic medium in absence of peroxide hardly reached 30% after 5 h.

![Graph showing CPO stability](image)

**Figure 4.3.7** Stability of soluble CPO in aqueous (●) and biphasic media (Δ), in the absence of t-BuOOH and substrate, and in biphasic medium (■) with the addition of 3 mM/h of t-BuOOH in pulses in the absence of the substrate. Aqueous medium consisted in 100 mM acetate buffer pH 5.0, while the biphasic medium contained ethyl-acetate/100 mM acetate buffer pH 5.0 (1/1). Experiments were performed at room temperature (20-23°C) with shaker agitation. Data points represent the average of duplicate measurements; error bars represent the standard deviation.
In general, organic solvents can affect the stability and catalytic activity of the enzymes due to the dissolved solvent molecules in the aqueous phase by causing conformational changes, by decreasing its conformational flexibility, by altering the chemical reactivity of the substrate, by changing the access of the substrate to the active site of the enzyme and finally by interfacial inactivation, which is often the main reason for the enzyme inactivation in biphasic media (Cooney and Hueter 1974; Doukyu and Ogino 2010). Hence, the observed activity loss could be due to the contact of the enzyme with the aqueous-organic interface (Ryu and Dordick 1992; Torres and Castro 2004), which causes structural rearrangement in the enzyme molecule and consequently partial unfolding (Ross et al. 2000). On the other hand, although some authors described that t-BuOOH is partitioned between the phases (Kiljunen and Kanerva 2000), we observed that its concentration nearby the enzyme is still high enough to cause its fast deactivation.

A reaction was performed using a substrate concentration of 500 mM, which ensures a concentration close to the value of solubility in the aqueous phase, where the enzyme is entirely dissolved (Table 4.3.4). Even though a very low yield of Cbz-glycinal was obtained (3.0% after 28 h), the concentration of the aldehyde produced was around 2 fold improved, having 7.8 mM of Cbz-glycinal produced in the reaction performed in aqueous medium and 15 mM in the biphasic one. On the other side, the initial reaction rate value of 0.66 mM/h was much lower than the one in aqueous reaction (1.8 mM/h) due to the partition of t-BuOOH between the phases and thus a decrease in concentration in the aqueous phase. Concerning enzymatic activity, pseudo half life of the enzyme was improved from 4 h for the reaction performed in aqueous medium to 8 h for the reaction in biphasic system (Figure 4.3.8). This fact points out that the increase in activity loss caused by the interface of the biphasic system is overcompensated by the activity preservation due to a decrease in t-BuOOH concentration nearby the enzyme.

Experiments with peroxide addition rates of 6 and 12 mM/h were performed and Cbz-glycinal yields of 0.6 and 0.2% respectively were obtained. Apart from decreasing the reaction rate, the high addition of peroxide caused a very fast CPO inactivation, being the pseudo half lives of 2.5 h for the t-BuOOH addition rate of 6 mM/h and 1.8 h for the one of 12 mM/h. Consequently the Cbz-glycinal yield was strongly reduced.
Figure 4.3.8 Inactivation of CPO in the biphasic reactions for different addition rates of t-BuOOH: 3 mM/h (Δ), 6 mM/h (●) and 12 mM/h (○). The biphasic medium contained 500 mM Cbz-ethanolamine in ethyl-acetate/100 mM acetate buffer pH 5 (1/1). Experiments were performed at room temperature (20-23ºC) with shaker agitation. Data points represent the average of duplicate measurements; error bars represent the standard deviation.
Engineering of the reaction media for CPO catalyzed oxidation of amino alcohols to amino aldehydes

Table 4.3.4 Oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by soluble CPO at different t-BuOOH addition rates in the biphasic reaction composed of ethyl acetate:100 mM acetate buffer pH 5.0 (50:50) at room temperature (20-23°C), with added CPC activity of 500 U/ml. Concentration of Cbz-ethanolamine in all cases was 500 mM. The end of the reaction was considered when no CPO activity was detected.

<table>
<thead>
<tr>
<th>t-BuOOH addition rate (mM/h)</th>
<th>Reaction time (h)</th>
<th>Pseudo half life, t½ (h)</th>
<th>Conversion (%)</th>
<th>Yield of Cbz-glycinal (%)</th>
<th>Yield of Cbz-glycine (%)</th>
<th>Initial reaction rate (mM Cbz-ethanolamine/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>28</td>
<td>8.0</td>
<td>3.8</td>
<td>3.0</td>
<td>0.0</td>
<td>0.66</td>
</tr>
<tr>
<td>6</td>
<td>24</td>
<td>2.5</td>
<td>0.6</td>
<td>0.6</td>
<td>0.0</td>
<td>0.13</td>
</tr>
<tr>
<td>12</td>
<td>8</td>
<td>1.8</td>
<td>0.2</td>
<td>0.2</td>
<td>0.0</td>
<td>0.09</td>
</tr>
</tbody>
</table>
4.3.3 **Chloroperoxidase catalyzed oxidation of Cbz-ethanolamine in aqueous medium in presence of cosolvents**

4.3.3.1 **Choice of the cosolvent and the rate of t-BuOOH addition**

The use of cosolvents enabled the work with higher concentrations of substrate. The solubility of Cbz-ethanolamine was determined in the presence of the different water miscible organic solvents used in different concentrations (Table 4.3.5). The use of very low concentrations of the cosolvents (5% (v/v)) allows the 10-12.5 fold improvement of the solubility of the substrate, which gives the possibility for an important improvement of the reaction productivity.

**Table 4.3.5** Maximal solubility of Cbz-ethanolamine at room temperature (20-23°C) at 100 mM acetate buffer pH 5.0 containing different proportions of water miscible organic solvents.

<table>
<thead>
<tr>
<th>Cosolvent</th>
<th>Concentration of the cosolvent, %(v/v)</th>
<th>Maximal solubility of Cbz-ethanolamine, mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>-</td>
<td>20</td>
</tr>
<tr>
<td>Ethanol</td>
<td>15</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>&gt; 1000</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>5</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>400</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>&gt; 1000</td>
</tr>
<tr>
<td>Acetone</td>
<td>15</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>DMF</td>
<td>5</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Dioxane</td>
<td>5</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>250</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>
The use of CPO in the presence of miscible organic solvents was reported by various authors (Cooney and Hueter 1974; Loughlin and Hawkes 2000; Torres and Aburto 2005; van Deurzen et al. 1997a). In this work, the effect of different water miscible solvents on the stability of CPO was evaluated in preliminary studies in order to establish which solvents could be potentially useful for CPO catalyzed biotransformation. Use of ethanol as a cosolvent was discarded since it was reported that it is a substrate for CPO catalyzed oxidation (Geigert et al. 1983). The solvents tested were acetonitrile, dimethylformamide (DMF) and dioxane (Figure 4.3.9). In all of the cases studied, the stability of CPO was reduced compared to the one in pure buffer (pseudo half lives within the ranges of 1.5-3 h for solvents used at concentrations within the range of 5-10% (v/v) in 100 mM sodium acetate buffer pH 5.0 and 4 h for pure buffer), except for the dioxane used in concentration of 5% (v/v), when the stability of CPO was the same as in pure buffer. Generally, higher cosolvent content results in faster CPO activity loss.

![Figure 4.3.9](image_url)

**Figure 4.3.9** Values of pseudo half life of CPO in the presence of miscible organic solvents. The inactivation was monitored in 100 mM sodium acetate buffer pH 5.0 containing CPO activity of 2 U/ml with the addition of t-BuOOH in pulses at the rate of 3 mM/h and room temperature (20-23°C).
Chapter 4.

The reaction of oxidation of Cbz-ethanolamine was performed in the presence of the cosolvents selected based on the results of stability of CPO and solubility of Cbz-ethanolamine (acetone, acetonitrile and dioxane) using substrate concentration of 150 mM which represents a value 7.5-fold higher than the one used in aqueous medium (Table 4.3.6). As result of the increased substrate concentration in the reaction medium, the initial reaction rates of the oxidation of Cbz-ethanolamine increased compared to that of aqueous medium, and higher reaction rates resulted in higher pseudo half lives. Diminished accumulation of peroxide due to the higher reaction rate can be the reason for the improved values of pseudo half life of CPO compared to the pseudo half life of 4 h for the aqueous medium reaction in the same conditions. Exception was the reaction in presence of 15% (v/v) acetonitrile in which a very fast deactivation of enzyme was caused by the dissolved solvent molecules. Accordingly with the preliminary studies of the stability of CPO in the presence of cosolvents, when the reaction was performed in 5% (v/v) of dioxane the pseudo half life of CPO was almost 8-fold higher than the one in aqueous reaction.
Table 4.3.6 Oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by soluble CPO using tert-butyl hydroperoxide as oxidant with addition rate of 3 mM/h in the presence of organic cosolvents in 100 mM acetate buffer pH 5 at room temperature (20-23°C), with initially added CPO activity of 500 U/ml. Concentration of Cbz-ethanolamine in all cases was 150 mM. The end of the reaction was considered when no CPO activity was detected.

<table>
<thead>
<tr>
<th>Cosolvent</th>
<th>Concentration % (v/v)</th>
<th>Reaction time (h)</th>
<th>Pseudo half life, $t_{1/2}$ (h)</th>
<th>Conversion (%)</th>
<th>Yield of Cbz-glycinal (%)</th>
<th>Yield of Cbz-glycine (%)</th>
<th>Initial reaction rate (mM Cbz-ethanolamine/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>15%</td>
<td>25</td>
<td>6</td>
<td>10.8</td>
<td>10.8</td>
<td>0.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Acet:onitrile</td>
<td>15%</td>
<td>6</td>
<td>2</td>
<td>2.7</td>
<td>2.7</td>
<td>0.0</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>5%</td>
<td>25</td>
<td>6</td>
<td>16.0</td>
<td>16.0</td>
<td>0.0</td>
<td>2.4</td>
</tr>
<tr>
<td>Dioxane</td>
<td>5%</td>
<td>54</td>
<td>30</td>
<td>38.3</td>
<td>31.7</td>
<td>5.1</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>10%</td>
<td>48</td>
<td>24</td>
<td>32.1</td>
<td>25.1</td>
<td>3.3</td>
<td>3.1</td>
</tr>
</tbody>
</table>
In presence of 5% dioxane the Cbz-glycinal yield was 31.7%, which represents a value slightly lower than that in aqueous medium (39%), although the synthesized product concentration was almost 6-fold higher (47.6 mM in 5% of dioxane compared to 7.8 mM in aqueous medium) (Figure 4.3.10). Improvement in the reaction productivity was achieved also in all the other reaction media studied, except for the one containing 15% (v/v) acetonitrile in which very low conversion was obtained due to a very fast inactivation of the enzyme. Hence, 16, 24 and 38 mM were the concentrations of the product at the end of the reactions containing 15% acetone, 5% acetonitrile and 10% dioxane, respectively.

For reactions performed in dioxane, the initial reaction rate reached 3 mM/h, which corresponds to the value of peroxide addition rate. Therefore, due to no accumulation of peroxide during the first four hours of the reaction, the enzyme retained 98% of its activity after this period, compared to the aqueous reaction with the same addition rate of peroxide in which after 4 h CPO retained only 54% (Figure 4.3.2 B).

**Figure 4.3.10** Time course for oxidation of Cbz-ethanolamine (●) to Cbz-glycinal (○) and Cbz-glycine (▼) catalyzed by CPO (% remaining activity, □) using tert-butyl hydroperoxide as oxidant in addition rate of 3 mM/h at the temperature of 20-23ºC. CPO activity of 500 U/ml was added to the reaction mixture that contained 150 mM of Cbz-ethanolamine in 5% (v/v) dioxane in 100 mM acetate buffer pH 5.0. Data points represent the average of duplicate measurements; error bars represent the standard deviation.
This fact points out that the peroxide addition could be limiting the reaction rate. To avoid this possibility, the increase of the addition rate was studied. For the reaction with 5% (v/v) of dioxane, when the addition of t-BuOOH was augmented to 6 mM/h, the initial reaction rate of Cbz-glycinal production was equal to this value, the corresponding activity retention after the first four hours was 96.7%, and the final yields of Cbz-glycinal and Cbz-glycine obtained were similar to those reached when using 3 mM/h (Table 4.3.7). Further increase of the peroxide addition to 12 mM/h could no longer be considered as optimal since the initial reaction rate was lower than this value; the accumulation of the unconsumed peroxide caused the deactivation of 52% of the initial CPO activity in the first four hours and consequently lower yields of product.

All the reactions described so far were performed by manual addition of peroxide, which requires the stop during the night. In fact, the peroxide was added by one pulse per hour for periods of 8 hours per day. When it was not being added, the enzyme was using the excess of the peroxide to continue the oxidation, but when all accumulated peroxide was consumed, the reaction stopped. This can be avoided by use of continuous addition of peroxide using a microburette (Table 4.3.7). The main disadvantage is the lower stability of CPO and slightly decreased product yields. On the other hand, the automatic addition of peroxide simplifies the operation and shortens the reaction time. Moreover, the non-desired oxidation of Cbz-glycinal to Cbz-glycine, which is favored by high concentrations of aldehyde and time, occurs in less extension. Thus, the ratio Cbz-glycinal/Cbz-glycine improved from 6.2 to 8.1 when peroxide was added by microburette.
**Table 4.3.7** Effect of t-BuOOH addition rates on the oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by soluble CPO in the presence of 5% dioxane in 100 mM acetate buffer pH 5.0 at room temperature (20-23°C) with initially added CPO activity of 500 U/ml. Concentration of Cbz-ethanolamine in all cases was 150 mM. The end of the reaction was considered when no CPO activity was detected.

<table>
<thead>
<tr>
<th>Addition of t-BuOOH</th>
<th>Reaction time (h)</th>
<th>Pseudo half life t ½ (h)</th>
<th>Conversion (%)</th>
<th>Yield of Cbz-glycinal (%)</th>
<th>Yield of Cbz-glycine (%)</th>
<th>Initial reaction rate (mM Cbz-ethanolamine/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pulses 3 mM/h</td>
<td>54</td>
<td>30</td>
<td>38.3</td>
<td>31.7</td>
<td>5.1</td>
<td>3.2</td>
</tr>
<tr>
<td>pulses 6 mM/h</td>
<td>32</td>
<td>8</td>
<td>34.5</td>
<td>30.8</td>
<td>5.1</td>
<td>6.2</td>
</tr>
<tr>
<td>pulses 12 mM/h</td>
<td>26</td>
<td>4</td>
<td>29.3</td>
<td>25.1</td>
<td>4.2</td>
<td>10.2</td>
</tr>
<tr>
<td>3 mM/h with microburette</td>
<td>24</td>
<td>15</td>
<td>26.2</td>
<td>25.1</td>
<td>3.1</td>
<td>3.1</td>
</tr>
</tbody>
</table>
Further advance of the reaction performance was studied in the presence of dioxane at concentration of 10% which allowed work with even higher concentrations of substrate. The reaction performed using 250 mM of ethanolamine and continuous addition of peroxide (using microburette) at the rate of 3 mM/h, resulted in a final yield of Cbz-glycinal of 12.9%. The strategy of the peroxide addition was not the same as in the reaction performed in 10% dioxane using Cbz-ethanolamine concentration of 150 mM (Table 4.3.6), but the comparison of these two results can help estimation of the possible gain of using higher substrate concentrations. It can be seen that in both reactions very similar productions of Cbz-glycinal were achieved (concentrations of 38 and 33 mM produced by using 150 and 250 mM of the substrate, respectively). Both values were much lower than those achieved when using 5% dioxane, due to the faster inactivation of the enzymes in the presence of higher cosolvent concentration.

### 4.3.3.2 Optimization of the temperature using dioxane as a cosolvent

Effect of the temperature on the oxidation of Cbz-ethanolamine catalyzed by CPO might be important. The temperature should be chosen in that way to be more beneficial for the increase of reaction rate than for thermal inactivation of the enzyme. The reaction in presence of 5% dioxane with constant addition (using microburette) of 3 mM/h of peroxide was chosen as a model reaction, and the effect of temperature was studied at 11, 20 and 37ºC (Table 4.3.8). At 11ºC the reaction had a very low rate comparing to the one at 20ºC and to the peroxide addition rate, hence CPO is deactivated due to the accumulation of the peroxide in the reaction medium. Despite the lower enzymatic activity and conversion, the ratio of Cbz-glycinal and Cbz-glycine produced was improved to 13.3 comparing to the value of 8.1 for 20ºC, suggesting that at lower temperatures, synthesis of amino acid was more delayed than the one of amino aldehyde. At 37ºC rapid thermal deactivation was detected, causing low reaction rates, therefore the slow consumption of t-BuOOH, which caused the additional deactivation of CPO. The temperature of 20ºC can be considered as optimal, since at this temperature thermal deactivation is not appreciable, and it allows good reaction rate and the highest Cbz-glycinal yield minimizing the deactivation of CPO by peroxide.
Table 4.3.8 Effect of temperature on the oxidation of Cbz-ethanolamine to Cbz-glycin catalyzed by soluble CPO. tert-butyl hydroperoxide was used as oxidant in addition rate of 3 mM/h via a microburette in the presence of 5% of dioxane in 100 mM acetate buffer pH 5, with initially added CPO activity of 500 U/ml. Concentration of ethanolamine in all cases was 150 mM. The end of the reaction was considered when no CPO activity was detected.

<table>
<thead>
<tr>
<th>T (°C)</th>
<th>Reaction time (h)</th>
<th>Pseudo half life, t ½ (h)</th>
<th>Conversion (%)</th>
<th>Yield of Cbz-glycin (%)</th>
<th>Yield of Cbz-glycine (%)</th>
<th>Initial reaction rate (mM Cbz-ethanolamine/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>28</td>
<td>13</td>
<td>21.5</td>
<td>21.2</td>
<td>1.6</td>
<td>0.9</td>
</tr>
<tr>
<td>20</td>
<td>24</td>
<td>15</td>
<td>26.2</td>
<td>25.1</td>
<td>3.1</td>
<td>3.1</td>
</tr>
<tr>
<td>37</td>
<td>8</td>
<td>3.5</td>
<td>6.6</td>
<td>6.6</td>
<td>0.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>
4.3.4 Chloroperoxidase catalyzed oxidation of Cbz-alaninol

Cbz-alaninol is another synthetically useful and versatile amino aldehyde, widely used in previous works in the reactions of aldol addition to DHAP catalyzed by DHAP-dependent aldolases yielding the corresponding aminopolyols (Ardao et al. 2011; Espelt et al. 2005; Espelt et al. 2003; Suau et al. 2008; Suau et al. 2006; Suau et al. 2005).

Therefore, the substrate specificity of CPO was tested by using Cbz-alaninol as a substrate for the synthesis of Cbz-alaninal (Figure 4.3.11).

Figure 4.3.11 Oxidation of Cbz-alaninol to Cbz-alaninal and Cbz-alanine by CPO catalysis.

Oxidation of Cbz-alaninol did not yield any conversion when the same CPO activity as that for the oxidation of Cbz-ethanolamine was used (500 U/ml). Then, 10-fold higher activity of CPO (5000 U/ml) was introduced to the reaction medium, which consisted of 100 mM sodium acetate buffer pH 5.0 containing 5% (v/v) dioxane. The initial concentration of Cbz-alaninol was 20 mM, which is the maximal solubility in this reaction medium composition. However, a very low conversion of Cbz-alaninol was obtained (Figure 4.3.12) and the production of Cbz-alaninal reached the value of 1.2 mM after 30 h of reaction. In this reaction, the reached production per CPO activity unit used was $2.4 \times 10^{-4}$ mM/U, the value almost 400-fold lower than the one obtained with Cbz-ethanolamine as a substrate ($95.2 \times 10^{-3}$ mM/U). It was reported that alcohols which contain ramifications could not be accepted as substrates for CPO, due to the steric hindrance of the active site (Kiljunen and Kanerva 2000; Torres and Ayala 2010; van Rantwijk and Sheldon 2000).
Figure 4.3.12 Time course for oxidation of Cbz-alaninol (●) to Cbz-alaninal (○) and Cbz-alanine (▼) catalyzed by CPO (□) using tert-butyl hydroperoxide as oxidant in addition rate of 3 mM/h at temperature of 20-23ºC. CPO activity of 5000 U/ml was added to the reaction mixture composed of 20 mM Cbz-alaninol in 100 mM acetate buffer pH 5.0 containing 5% (v/v) of dioxane. Data points represent the average of duplicate measurements; error bars represent the standard deviation.
4.4 Conclusions

Chloroperoxidase catalyzed oxidation of Cbz-ethanolamine to Cbz-glycinal was performed using tert-butyl hydroperoxide and hydrogen peroxide as oxidants. Frequency of the addition of the oxidants in aqueous medium was optimized and tert-butyl hydroperoxide resulted as a more appropriate oxidant when added at the rate of 3 mM/h, when yield of Cbz-glycinal reached the value of 39.1%.

Use of PEI was observed to stabilize CPO in the presence of peroxide. In spite of this, it did not result advantageous in terms of reaction productivity, because it exerts a stabilizing effect by lowering the concentration of the peroxide near its surface, and therefore preventing the oxidation reaction.

Improved conversions of Cbz-ethanolamine were achieved by adding more activity at the beginning of the reaction, but it was observed that the accumulation of Cbz-glycinal accelerates its further undesired oxidation to Cbz-glycine.

Looking for benefits in terms of reaction productivity, reaction medium engineering was further studied. Oxidation of Cbz-ethanolamine was investigated in the presence of miscible and immiscible organic solvents. Even though cosolvents and biphasic medium generally affect enzymatic activity, the adjustment between reaction kinetics and peroxide addition rate avoided the peroxide excess in the reaction medium and, consequently, enzymatic stability and reaction productivity were importantly improved. Dioxane added in the concentration of 5% (v/v) was the most appropriate system as almost 6-fold higher concentration of Cbz-glycinal was produced compared to the value obtained in aqueous reaction (47.6 mM in 5% of dioxane compared to 7.8 mM in aqueous medium).

In general, Cbz-glycinal yields were limited by the subsequent oxidation reaction of the aldehyde towards Cbz-glycine.

Finally, Cbz-alaninol was tested as a substrate for CPO, but only very low conversion was obtained, which is in accordance with the previously reported results.

Even though different strategies were successfully applied for the improvement of the overall reaction performance, the inactivation of CPO by peroxide remained the main issue. Hence, its instability stayed as the problem to be resolved, e.g. via the techniques of
Chapter 4.

chemical modification or immobilization, in order to accomplish the further enhancement of the reaction of Cbz-glycinal synthesis.
CHAPTER 5

CHEMICAL MODIFICATIONS OF CHLOROPEROXIDASE FOR ENHANCING STABILITY AND ACTIVITY
Chapter 5.
5 CHEMICAL MODIFICATIONS OF CHLOROPEROXIDASE FOR ENHANCING STABILITY AND ACTIVITY

5.1 Introduction

A chemical modification of enzymes is a useful method for the improvement of their efficiency as biocatalysts. A number of chemical methods have been reported whereby the stability or activity of the enzymes can be changed (Bund and Singhal 2002; Kotormán et al. 2009; Liu and Wang 2007; Sangeetha and Abraham 2006; Szabó et al. 2009). The alterations of the enzyme stability, activity, specificity and selectivity are the result of the conformational changes which occur due to the modifications of its primary structure. Some of the commonly used methods of chemical modifications are side-chain selective modifications, crosslinking and modification of the sugar moiety of the protein.

As it was explained more profoundly in the Section 1.2.1, side chain selective modifications involve the use of side chain selective reagents (those that react, under certain specific conditions, with a single or a limited number of side-chain groups in a rather predictable way) (Means and Feeney 1990). Within this type of chemical modifications, some of the most commonly used are those that involve the modifications of ε-amino groups of Lys and carboxyl groups (Glu and Asp) of the protein because these amino acids are often most frequent in the amino acid content.

Among the variety of the acid anhydrides that specifically react with ε-amino groups, in this work dicarboxylic anhydrides (maleic or phthalic) were used for the modification of CPO (Figure 5.1.1). Even though this type of modification requires alkaline pH values (pH 8.0 and above) (Butler et al. 1969; Means and Feeney 1990), successful modifications by this method on acidic pH values were published as well (Liu and Wang 2007). The improvements of the thermostability, stability in the presence of organic solvents and catalytic activity for both sulfoxidation and oxidation of phenol have been reported for CPO (Liu and Wang 2007).
Figure 5.1.1 Reaction scheme for selective modification of ε-amino groups by using maleic (A) and phthalic anhydride (B).

For the modification of carboxyl groups of CPO (Glu and Asp) the method proposed in this work consisted of mild water-soluble carbodiimide (EDAC) coupling and the subsequent reaction of the activated carboxyl group with a nucleophile (ethanolamine or ethylenediamine). Hence, carbodiimide coupling consists of two steps (Figure 5.1.2). In first step, the carboxyl anion reacts with the protonated carbodiimide molecule, forming the instable intermediate product called pseudourea. In the second step pseudourea reacts with a nucleophile, forming stable covalent bond which is followed by the release of urea molecule (Khorana 1953; Williams and Ibrahim 1981). Carboxyl modifications with EDAC were found to change the properties of some enzymes such as activity, specificity and physicochemical properties (Kochhar and Dua 1984; Ma and Nakai 1980; Mejillano and Himes 1991). Chemical modifications of the carboxyl groups of CPO by carbodiimide and hexamethylenediamine were reported to improve its pH stability, thermostability, as well as tolerance to organic solvents (La Rotta Hernandez et al. 2005).
Figure 5.1.2 Reaction scheme for selective modification of carboxyl groups by EDAC and subsequent reaction with nucleophile (R’= CH₂-CH₂-OH in a case of ethanolamine; R’= CH₂-CH₂-NH₂ in a case of ethylenediamine).

The technique of chemical crosslinking of proteins has been used widely for the enhancement of their stability. The stabilization accomplished by increasing the rigidity of the active structure of the enzyme by inter- or intramolecular crosslinking can be achieved by the use of a variety of bi- or poly-functional crosslinkers. So far use of glutaraldehyde was the only reported method for the successful crosslinking of CPO (La Rotta Hernandez et al. 2005). In this work, the chemical crosslinking of CPO was performed by using dimethyl suberimidate (DMS) as a crosslinker. DMS contains imidoester group at each end of an 8-atom spacer arm and is a highly specific reagent for amino groups in proteins (Davies and Stark 1970) (Figure 5.1.3).
Finally, chemical modifications of sugar moiety of glycoproteins may alter their stability and activity since the sugar moieties provide important physical properties, such as hydrophilicity, conformational stability and charge (Kobata 1992). The most frequently used method for the chemical modifications of sugar moiety is the periodate oxidation (Figure 5.1.4). Possible formation of Schiff base bond between the protein amino groups and sugar aldehyde groups formed after the periodate oxidation of the protein might result in intermolecular cross-linking (Gerber et al. 1977; Kozulic et al. 1987). Since CPO is a glycoprotein with a carbohydrate content of 25-30%, the modification of its sugar moiety by periodate oxidation was proposed.

Hence, in this chapter, the effects of chemical modifications of CPO by maleic anhydride (MA), phthalic anhydride (PA), carbodiimide (EDAC) in the presence of ethanolamine (EA) or ethylenediamine (EDA), dimethyl suberimidate (DMS) and sodium periodate on the thermostability, pH stability, and tolerance to tert-butyl hydroperoxide as well as on the reaction performance for the oxidation of Cbz-ethanolamine to Cbz-glycinal were investigated.
5.2 Methods

5.2.1 Chemical modifications of CPO

5.2.1.1 Modification of amino groups of CPO
The mixtures containing 1.66 mg/ml of CPO and 2 mg/ml of maleic or phthalic anhydride in 100 mM sodium acetate buffer at pH 5.0 in the final volume of 0.5 ml were incubated at the temperature of 4ºC. In case of modification with phthalic anhydride, 0.02 ml of DMF were added (corresponding to the concentration of 4% (v/v)) in order to dissolve it. After 1 h of incubation the mixtures were desalted using Sephadex G-25 column and stored at the temperature of 4ºC.

5.2.1.2 Modification of carboxyl groups of CPO
Modification of carboxyl groups of CPO was carried out by following this procedure: the mixtures containing 1 mg/ml of CPO, 5 mM of EDAC and 25 mM of ethanolamine (EA) or ethanoldiamine (EDA) in 100 mM MES buffer at pH 5.0 were incubated during 1.5 h at room temperature. Then 100 μl of 0.5 M sodium acetate buffer pH 5.0 was added in order to stop further reaction. Finally, the mixtures were desalted using Sephadex G-25 column and stored at the temperature of 4ºC.

5.2.1.3 Crosslinking of CPO with DMS
The mixture containing 1.66 mg/ml of CPO and 4 mg/ml of DMS in 100 mM sodium acetate buffer at pH 5.0 in the final volume of 0.5 ml was incubated at the temperature of 4ºC for 1 h and finally desalted using Sephadex G-25 column and stored at the temperature of 4ºC.

5.2.1.4 Periodate oxidation of CPO
For the modification with sodium periodate, the mixture containing 1.66 mg/ml of CPO and 25 mM NaIO₄ in 50 mM acetate buffer at pH 5.0 in the final volume of 0.5 ml was incubated for 1 h at 4ºC in dark. Then 10 μl of 5 M ethylene glycol was added in order to stop the further oxidation. Finally, the mixture was dialyzed in 20 mM acetate buffer pH 5.0.
5.2.2 Determination of the degree of modified amino groups

The degree of modification of amino groups was determined as a percentage of those amino groups in native enzyme, and it was measured spectrophotometrically at 420 nm with 2,4,6-trinitrobenzenesulphonic acid (TNBS) (Kwan et al. 1983). 0.225 ml of 0.2 M borate buffer, pH 9.2, and 0.125 ml of TNBS (1.18 mg/ml) were added to 0.05 ml of the sample containing native or modified CPO in the concentration of 0.74 mg/ml. The reaction was stopped after 30 min by adding 0.125 ml of 2 M NaH$_2$PO$_4$ and 18 mM Na$_2$SO$_3$, and the absorbance (A$_{420\text{nm}}$) was measured using a Philips UV-VIS-NIR PU 8630 spectrophotometer. The percentage of modified amino groups was calculated as a percentage of A$_{420\text{nm}}$ obtained for the modified CPO respect to that value for native CPO. The experiments were performed in duplicate.

5.2.3 Isoelectric focusing and zymograme

Isoelectric focusing was performed using Multiphor II electrophoresis system (Pharmacia-LKB Biotechnology) according to manufacturer’s instruction. Focusing was carried out on 7.5% acrylamide gel with ampholytes in a pH range 3.5–5.2 (Bio-Rad, USA), at 7 W constant powers for 1.5 h at 10ºC. Low pI kit (GE Healthcare) was used as isoelectric point (pI) markers. After the run, native and modified chloroperoxidases were detected using in-gel peroxidation activity staining (zymograme detection) based on guaiacol assay using a solution that contained 8.5 mM guaiacol and 0.95 mM hydrogen peroxide in 100 mM sodium acetate buffer pH 5.0. CPO activity appeared as orange bands on clear background. After the zymograme detection, the CBB (Coomassie Brilliant Blue) staining was carried out.

5.2.4 Stability of chemically modified CPOs

Effect of pH on CPO stability was studied over the pH range 5.0–7.5. The reaction medium (sodium acetate, that was used for the pH values in the range of 5.0-6.0 or potassium phosphate, that was used for the pH values in the range of 6.5-7.5, at concentration of 50
Chemical modifications of CPO

M) containing the modified or native enzyme was incubated at room temperature (25ºC). Effect of temperature on CPO stability was studied at the temperatures of 4ºC, 25ºC and 50ºC by incubating native or modified CPO in 50 mM sodium acetate buffer pH 5.0. Effect of the presence of t-BuOOH was studied by incubating native or modified CPOs in 50 mM acetate buffer pH 5.0 containing 3 mM t-BuOOH at room temperature (25ºC). Added CPO peroxidation activity was 1 U/ml, in all experiments. After different times of incubation aliquots of enzyme solution were withdrawn and enzyme residual peroxidation activity was measured as described in the Section 3.4.1.2.

The inactivation kinetics was studied by plotting time course of inactivation on semi-log plot, and from the slope of linear regression obtained, the half life of the enzyme was calculated. It was observed that the degradation follows first order kinetics; hence from the regression of the type f=a·exp(-b·t), the half life was calculated as t\(_{1/2}\)=ln2/b.

5.2.5 Oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by native and modified CPOs

Cbz-ethanolamine was dissolved at concentration of 20 mM in 100 mM acetate buffer at pH 5.0 or 100 mM phosphate buffer at pH 7.0 in a final volume of 1 ml. Then activity of 500 U/ml (chlorination activity, Section 3.4.1.1) of native or modified CPO were added to the reaction medium. The reaction was started by adding tert-butyl hydroperoxide continuously, by manual addition of one pulse (10.7 µl) per hour during 8 h of reaction. After this time the addition was stopped and restarted after 24 h in case that CPO activity was detected. Concentration of peroxide stock solution was within a range of 0.28-1.12 M in order to reach peroxide addition rate of 3-12 mM/h. The reactions were performed at room temperature (25ºC) or at the temperature of 50ºC using mild agitation. Reaction samples were withdrawn periodically and analyzed by liquid chromatography in order to quantify Cbz-ethanolamine and Cbz-glycinal concentrations and to determine the activity of CPO. Each measurement was carried out in duplicate.
Pseudo half life of CPO was determined experimentally and represents the time at which CPO activity decays to the half of the activity initially added. Conversion and yields of the products were defined respectively as the percentage of the consumed Cbz-ethanolamine.
or produced products, related to initially added concentration of Cbz-ethanolamine. These values were additionally corrected by the dilution factor which was calculated for each set of measurements taking into account the volume of the peroxide added in pulses and the volume of the reaction medium withdrawn for the analysis. Initial reaction rate represents the rate of the Cbz-ethanolamine consumption during the period of linear change in concentration.

5.3 Results and discussion

5.3.1 Chemical modifications of CPO

Chemical modifications of CPO were carried out via six different methods: modifications of Lys residues with maleic and phthalic anhydride, modifications of carboxyl groups with EDAC and EA or EDA, crosslinking with DMS and periodate oxidation. The retained activities for chlorination of MCD (Section 3.4.1.1) and peroxidation of guaiacol (Section 3.4.1.2) of the modified CPOs were measured and compared to those of native enzyme (Figure 5.3.1). The loss of both chlorinating and peroxidative activity was observed for all types of modification, within the range of 39.5 and 56.8% for the chlorinating activity, and 16.4 and 44.5% for peroxidative activity. In all cases, higher losses were observed for chlorinating than for peroxidative activity. This could be due to the fact that CPO possesses at least two different binding sites for the substrates for peroxidation reactions, occurring via different mechanisms (Manoj and Hager 2008).
Figure 5.3.1 Effect of modifications of CPO on the chlorinating (black bars) and peroxidative (grey bars) activity. Each value represents the mean of two measurements. The error bars represent the standard deviation of the measurements.

The degree of the modification was determined by the indirect measurement of the remained amino groups by the reaction with TNBS, for CPO modified with MA, PA, DMS and periodate (Figure 5.3.2). The percentage of modified amino groups had very proximate values in all cases, possibly due to the accessibility of the Lys residues for the chemical modifications and it was more than 70% in all cases, even for the periodate oxidation of CPO. The possible explanation in the latter case is that formed aldehyde groups on the sugar moiety of CPO react with amino groups forming internal Schiff base bonds.
In order to check out whether the modifications had caused the change in the overall charge of the enzyme molecule, the analysis by isoelectric focusing was performed. Results of isoelectric focusing and zymogramre for native and modified CPOs for the pH range of 3.5-5.2 are shown on figure 5.3.3. As it can be seen, no change in pI value was observed after the modifications of CPO with maleic and phthalic anhydrides, DMS and NaIO₄, while for both methods corresponding to the modification of carboxyl groups, a slight increase of pI was detected. Modifications of the Lys residues of the enzyme eliminate the positive charge in the enzyme molecule. Hence, it could have been expected that the enzyme modified in that way will have lower pI value than the native one. However, since in the molecule of CPO, only four Lys residues are present, it is reasonable that no visible change in pI value occurred. On the contrary, the modifications of carboxyl groups eliminate the negative charge, and thus, the increase of the pI of CPO after such a modification could have been expected, since CPO contains a great number of superficial Asp and Glu residues.

**Figure 5.3.2** Percentage of modified amino groups determined by the reaction with TNBS. Each value represents the mean of two measurements. The error bars represent the standard deviation of the measurements.
Figure 5.3.3 Isoelectric focusing of native and modified CPOs for the pH range of 3.5-5.2. (A) zymogram determination of active enzyme was performed with guaiacol assay. (B) CBB staining.
5.3.2 Oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by chemically modified CPOs

Modified CPOs were tested as biocatalysts for the oxidation of Cbz-ethanolamine to Cbz-glycinal and were compared to the native enzyme. Possible improvement of the reaction performance could be either due to the improvement of the stability of CPO in the presence of peroxide or due to the improved affinity for the substrate. Therefore, in the preliminary studies, the stability of all modified CPOs was examined in the presence of 3 mM tert-butyl hydroperoxide. The very fast inactivation was observed in all the cases. The half lives were slightly lower compared to the native enzyme (Figure 5.3.4) indicating that none of the modifications made the heme group less prone to destruction by peroxides.

![Half lives of native and modified CPO in the presence of 3 mM tert-butyl hydroperoxide. The stability experiments were performed in 100 mM sodium acetate buffer pH 5.0 at room temperature.](image)

**Figure 5.3.4** Half lives of native and modified CPO in the presence of 3 mM tert-butyl hydroperoxide. The stability experiments were performed in 100 mM sodium acetate buffer pH 5.0 at room temperature.
When applied in the reaction, native and all modified CPOs lost their activity after only 24 h of reaction. Even so, yields of Cbz-glycinal and conversions of Cbz-ethanolamine were slightly improved for all modified CPOs compared to the native one, except for CPO modified with phthalic anhydride (Table 5.3.1). Similar to the reaction catalyzed by native CPO, in all the experiments, between 8.5 and 13% of Cbz-glycine, the product of further oxidation of the amino aldehyde by CPO, was produced.

Table 5.3.1 Oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by native and modified CPOs using tert-butyl hydroperoxide as oxidant at the addition rate of 3 mM/h. 500 U/ml of CPO measured by MCD assay were added to the reaction medium that contained 20 mM of Cbz-ethanolamine in 100 mM sodium acetate buffer pH 5.0 at room temperature. The end of reaction was considered when no CPO activity was detected, which was after 24 h in all the experiments.

<table>
<thead>
<tr>
<th>Conversion (%)</th>
<th>Yield of Cbz-glycinal (%)</th>
<th>Yield of Cbz-glycine (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native CPO</td>
<td>56.6</td>
<td>45.8</td>
</tr>
<tr>
<td>CPO-MA</td>
<td>59.3</td>
<td>46.4</td>
</tr>
<tr>
<td>CPO-PA</td>
<td>52.4</td>
<td>43.9</td>
</tr>
<tr>
<td>CPO-EDAC-EA</td>
<td>58.7</td>
<td>47.5</td>
</tr>
<tr>
<td>CPO-EDAC-EDA</td>
<td>59.1</td>
<td>47.7</td>
</tr>
<tr>
<td>CPO-DMS</td>
<td>60.2</td>
<td>48.9</td>
</tr>
<tr>
<td>CPO-NaIO₄</td>
<td>60.7</td>
<td>47.5</td>
</tr>
</tbody>
</table>

5.3.3 Effect of pH on the stability of modified CPOs and the reaction of oxidation of Cbz-ethanolamine

Stability of modified CPOs was studied at pHs 5.0, 6.0, 6.5, 7.0 and 7.5 at room temperature. Native CPO and all modified CPOs were almost completely stable at pHs 5.0-6.0 retaining 70-100% of the initially offered activity during 3 days of incubation. As expected, at higher pH values, more rapid deactivation was observed. At pH 6.5 half lives of modified CPOs were within the range of 72 h (for CPO-PA) and 161 h (for CPO modified by periodate). Native CPO at this pH value had the half life of 116 h. Therefore,
the best improvement at this pH value was achieved for CPO modified by periodate, with 1.4-fold improved half life. CPO-MA, CPO-PA and CPO-DMS resulted less stable at pH 7.0 than the native CPO, while all other modified CPOs showed improved stability on this pH value (Figure 5.3.5). The most drastic improvement was observed for CPO-EDAC-EA and periodate oxidized CPO, with half lives 2.1 and 1.5-fold improved, respectively. Finally, at pH 7.5, native and all modified CPOs lost more than 90% of their activity after only half an hour of incubation.

Figure 5.3.5 Half lives of native and modified CPOs in 50 mM sodium phosphate buffer pH 7.0 at room temperature.

Taking into account the main objective of the thesis, which was coupling of the reaction of oxidation of Cbz-ethanolamine catalyzed by CPO to the reaction of aldol addition of DHAP catalyzed by rhamnulose-1-phosphate aldolase (RhuA), improvement of the stability of modified CPOs on pH 7.0 could be beneficial, since the latter reaction takes place optimally at pH 7.5. Therefore, the compatibility of the enzymes might be enhanced. Expecting the improvement in the reaction performance at pH 7.0 by using those modified preparations that showed the highest improvement of the stability at this pH value (CPO-
EDAC-EA, CPO-EDAC-EDA and CPO-NaIO₄), the reaction of oxidation of Cbz-ethanolamine was performed and their efficiency as catalysts on these conditions was compared to the one of native CPO.

All three modified preparations tested resulted to be to some extent better catalysts at pH 7.0 than the native CPO. In all cases, slightly higher yields of Cbz-glycinal were produced at higher reaction rates than in the reaction catalyzed by native CPO (Figure 5.3.6 A). Nevertheless, relatively low yields were obtained in all cases, due to rapid inactivation of the enzyme, resulting in around 90% of activity loss after only 2 hours of reaction (Figure 5.3.6 B).

The Table 5.3.2 gives the comparison of some important results obtained from these reactions. As it can be seen, the yields of Cbz-glycinal were improved from 9.8% (value obtained with native CPO) to more than 11% when modified CPOs were used, with almost no Cbz-glycine produced. The productivity improvement, even being low, was obviously due to the slightly improved stability of the enzyme and the reaction rate when using modified CPOs in these conditions. Even so, from the results of the stability at this pH value, especially for CPO-EDAC-EA where a drastic improvement of the stability was observed, much better reaction performance could have been expected. This is probably due to the fact that the very rapid inactivation observed in all cases was partly caused by the high pH value, but more significantly by the accumulation of the peroxide in the reaction medium which, as it was already shown, has more effect on the modified CPOs than on the native one.
Figure 5.3.6 (A) Yields of Cbz-glycinal in the reaction of oxidation of Cbz-ethanolamine catalyzed by native CPO (●), CPO-EDAC-EA (□), CPO-EDAC-EDA (Δ) and CPO-NaIO₄ (○) and (B) remaining activities of native CPO (% black bars), CPO-EDAC-EA (% light grey bars), CPO-EDAC-EDA (% dark grey bars) and CPO-NaIO₄ (% white bars) at different reaction times using tert-butyl hydroperoxide as oxidant at the addition rate of 3 mM/h. 500 U/ml of CPO were added to the reaction medium that contained 20 mM of Cbz-ethanolamine in 50 mM sodium phosphate buffer pH 7.0 at room temperature. The end of reaction was considered when no CPO activity was detected. Each value represents the mean of two measurements. The error bars represent the standard deviation of the measurements.
**Table 5.3.2** Reactions of oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by native and modified CPOs at room temperature in the reaction medium that contained 20 mM Cbz-ethanolamine in 50 mM sodium phosphate buffer pH 7.0, by using t-BuOOH as an oxidant at the addition rate of 3 mM/h.

<table>
<thead>
<tr>
<th></th>
<th>Conversion (%)</th>
<th>Yield of Cbz-glycinal (%)</th>
<th>Yield of Cbz-glycine (%)</th>
<th>Pseudo half life, $t_{1/2}$ (h)</th>
<th>Initial reaction rate (mM Cbz-ethanolamine/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>native CPO</td>
<td>11.9</td>
<td>9.8</td>
<td>0.8</td>
<td>0.47</td>
<td>1.8</td>
</tr>
<tr>
<td>CPO-EDAC-EA</td>
<td>12.0</td>
<td>11.5</td>
<td>0.7</td>
<td>0.61</td>
<td>2.3</td>
</tr>
<tr>
<td>CPO-EDAC-EDA</td>
<td>12.3</td>
<td>11.3</td>
<td>1.0</td>
<td>0.56</td>
<td>2.2</td>
</tr>
<tr>
<td>CPO-NaIO$_4$</td>
<td>11.3</td>
<td>11.0</td>
<td>0.2</td>
<td>0.55</td>
<td>2.0</td>
</tr>
</tbody>
</table>

5.3.4 **Effect of the temperature on the stability and reaction of oxidation of Cbz-ethanolamine catalyzed by modified CPOs**

Thermostability of native and modified CPOs was studied at the temperatures of 4ºC, 25ºC and 50ºC at pH 5.0. No loss of activity was observed for native or any modified CPO at the temperatures of 4ºC after 3 days of incubation. After the same incubation time, at room temperature, loss of around 20% of activity was observed for CPO-MA, CPO-PA and CPO-DMS, while others remained completely active.

All the modified CPOs resulted to be more stable than the native CPO at 50ºC, those modified with carbodiimide coupled method being the most stable ones, with 1.8-fold improved half lives for the modification with EDA and 4.8-fold for modification with EA, compared to the soluble CPO (Figures 5.3.7 and 5.3.8).
Figure 5.3.7 Remaining activities of native and modified CPOs at the temperature of 50ºC in 100 mM sodium acetate buffer pH 5.0 for different incubation periods: 1 h (% black bars), 4 h (% grey bars) and 6 h (% white bars). Each value represents the mean of two measurements. The error bars represent the standard deviation of the measurements.

Figure 5.3.8 Half lives of native and modified CPOs in 100 mM sodium acetate buffer pH 5.0 at temperature of 50ºC.
The reactions of oxidation of Cbz-ethanolamine were then carried out at the temperature of 50ºC by using those modified preparations that resulted more stable at this temperature (CPO-EDAC-EA, CPO-EDAC-EDA and CPO-NaIO₄). Use of high temperatures may be beneficial due to the generally higher reaction rates, which in case of the reaction catalyzed by CPO means also faster consumption of t-BuOOH, i.e. less accumulation.

t-BuOOH was added at the rate of 3 mM/h in order to assure a low concentration in the reaction medium, and therefore preserve the activity of the enzyme. In these conditions native CPO lost more than 70% of the initial activity after only one hour of reaction, while after the same reaction time the modified ones lost within 54-60% of activity; consequently, higher yields of Cbz-glycinal were produced (Figure 5.3.9 A and B).

The biggest improvement in the conversion and product yield was accomplished when CPO-NaIO₄ was used as a biocatalyst, reaching a final yield of Cbz-glycinal of 21.3%, while the most stable one resulted to be CPO-EDAC-EA (Table 5.3.3). The values of initial reaction rates obtained for the modified CPOs were 3.4 mM/h, 4.5 mM/h and 5.2 mM/h for CPO-EDAC-EA, CPO-EDAC-EDA and CPO-NaIO₄, respectively. All these values exceeded the rate of peroxide addition, which was in this case possible, since due to very high reaction and enzyme inactivation rates, it was measured only in first half an hour of the reaction, while the addition of t-BuOOH was performed by means of one pulse each 2 h. Therefore, the concentration of peroxide was limiting the overall reaction rate.
Figure 5.3.9 (A) Yields of Cbz-glycinal in the reaction of oxidation of Cbz-ethanolamine catalyzed by native CPO (●), CPO-EDAC-EA (○), CPO-EDAC-EDA (△) and CPO-NaIO₄ (●) and (B) remaining activities of native CPO (% black bars), CPO-EDAC-EA (% light grey bars), CPO-EDAC-EDA (% dark grey bars) and CPO-NaIO₄ (% white bars) at different reaction times using tert-butyl hydroperoxide as oxidant at the addition rate of 3 mM/h. 500 U/ml of CPO were added to the reaction medium that contained 20 mM of Cbz-ethanolamine in 100 mM sodium acetate buffer pH 5.0 at temperature of 50ºC. The end of reaction was considered when no CPO activity was detected. Each value represents the mean of two measurements. The error bars represent the standard deviation of the measurements.
Table 5.3.3 Reactions of oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by native and modified CPOs at the temperature of 50°C in the reaction medium that contained 20 mM Cbz-ethanolamine in 100 mM sodium acetate buffer pH 5.0, by using t-BuOOH as an oxidant at the addition rate of 3 mM/h.

<table>
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<td>Native CPO</td>
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<td>13.9</td>
<td>1.6</td>
<td>0.51</td>
<td>1.8</td>
</tr>
<tr>
<td>CPO-EDAC-EA</td>
<td>19.8</td>
<td>18.2</td>
<td>2.9</td>
<td>0.90</td>
<td>3.4</td>
</tr>
<tr>
<td>CPO-EDAC-EDA</td>
<td>21.4</td>
<td>18.3</td>
<td>3.1</td>
<td>0.76</td>
<td>4.5</td>
</tr>
<tr>
<td>CPO-NaIO$_4$</td>
<td>25.8</td>
<td>21.3</td>
<td>4.5</td>
<td>0.80</td>
<td>5.2</td>
</tr>
</tbody>
</table>

In order to avoid the limitation by peroxide and taking into account that native CPO reached the highest yields of Cbz-glycinal at the temperature of 50°C when peroxide was added at the rate of 12 mM/h (results shown in the Section 4.3.1.2), the reactions catalyzed by modified CPOs were performed by adding the peroxide at this rate. Even though almost complete inactivation of CPO was observed after only 1 h of reaction, the Cbz-glycinal yields were further improved. In fact, almost entire conversion that was achieved in these reactions occurred after only 30 minutes of the reaction, the time in which more than 20% of the yields of the product were reached when using modified CPOs, and around 15% when using native one (Figure 5.3.10).
Figure 5.3.10 (A) Yields of Cbz-glycinal in the reaction of oxidation of Cbz-ethanolamine catalyzed by native CPO (●), CPO-EDAC-EA (○), CPO-EDAC-EDA (Δ) and CPO-NaIO₄ (○) and (B) remaining activities of native CPO (% black bars), CPO-EDAC-EA (% light grey bars), CPO-EDAC-EDA (% dark grey bars) and CPO-NaIO₄ (% white bars) at different reaction times using tert-butyl hydroperoxide as oxidant at the addition rate of 12 mM/h. 500 U/ml of CPO were added to the reaction medium that contained 20 mM of Cbz-ethanolamine in 100 mM sodium acetate buffer pH 5.0 at temperature of 50°C. The end of reaction was considered when no CPO activity was detected. Each value represents the mean of two measurements. The error bars represent the standard deviation of the measurements.
In these conditions reaction was not limited by peroxide concentration, but at the same time the presence of peroxide caused more rapid CPO inactivation. Even so, final reaction yields and productivities were improved compared to those obtained when the peroxide was added at the rate of 3 mM/h.

Modified CPO resulted better catalysts than the native one in all the aspects (Table 5.3.4): the conversions of Cbz-ethanolamine were improved from 18.5% when native CPO was used to more than 26% for the reactions catalyzed by modified CPOs; yields of Cbz-glycinal were 16.1% and 21.5-22.1% for native and modified CPOs respectively; the values of half lives were higher for modified compared to the native CPO; and finally, around 1.3-fold improvement in reaction rates was achieved for all the modified CPOs tested.

Table 5.3.4 Reactions of oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by native and modified CPOs at temperature of 50ºC in a reaction medium that contained 20 mM Cbz-ethanolamine in 100 mM sodium acetate buffer pH 5.0, by using t-BuOOH as an oxidant at the addition rate of 12 mM/h.

<table>
<thead>
<tr>
<th></th>
<th>Conversion (%)</th>
<th>Yield of Cbz-glycinal (%)</th>
<th>Yield of Cbz-glycine (%)</th>
<th>Pseudo half life, $t_{1/2}$ (h)</th>
<th>Initial reaction rate (mM ethanolamine/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native CPO</td>
<td>18.5</td>
<td>16.1</td>
<td>2.4</td>
<td>0.22</td>
<td>6.7</td>
</tr>
<tr>
<td>CPO-EDAC-EA</td>
<td>26.7</td>
<td>21.6</td>
<td>5.1</td>
<td>0.29</td>
<td>9.9</td>
</tr>
<tr>
<td>CPO-EDAC-EDA</td>
<td>26.2</td>
<td>21.5</td>
<td>4.7</td>
<td>0.30</td>
<td>9.2</td>
</tr>
<tr>
<td>CPO-NaIO₄</td>
<td>26.1</td>
<td>22.1</td>
<td>4.0</td>
<td>0.29</td>
<td>8.9</td>
</tr>
</tbody>
</table>
5.4 Conclusions

In this chapter, different types of chemical modifications were studied in order to improve the properties of CPO as catalyst. While those modifications that involved the amino groups of the enzyme, both by selective reaction with dicarboxylic anhydrides (maleic and phthalic) or by crosslinker DMS had less or no effect on the improvement of the properties of CPO, those which modified carboxyl groups via EDAC coupling and the periodate oxidation of the sugar moiety produced better catalysts than native CPO in almost all the aspects. These modified CPOs showed to be more stable on high pH values and temperatures, and consequently better reaction overall performances for the oxidation of Cbz-ethanolamine to Cbz-glycinal were accomplished. Improvement of the stability in both cases could be due to the intermolecular crosslinking, in the first case by using EDAC as a zero-length crosslinker, and in the second one by the formation of Schiff base bonds between the aldehyde groups formed on the sugar moiety and the amino groups of the protein.

Even though chemical modifications enabled the enhancement of the stability and activity of CPO, the main source of its operational instability, i.e. rapid irreversible inactivation by peroxides, remained unsolved.

As the best results were obtained when modifying CPO by carbodiimide coupling or by periodate oxidation of the sugar moiety, this gives the possibility for the further immobilization of CPO on supports containing amino groups, either by carbodiimide coupling or by direct Schiff base formation between the support and periodate oxidized CPO. In this way, the further improvements of CPO as a biocatalyst for the oxidation of Cbz-ethanolamine might be achieved.
CHAPTER 6

IMMOBILIZATION OF
CHLOROPEROXIDASE

Publication:

6 IMMOBILIZATION OF CHLOROPEROXIDASE

6.1 Introduction

Immobilization of enzymes has been developed rapidly in the past 40 years since its benefits were evaluated and utilized with success in biotechnological, medical and analytical applications. The apparent activity retention upon the process depends mostly on the support characteristics, the enzyme-support interaction and the amount of soluble enzyme used. It may be reduced by the binding procedure, and also by mass transfer effect as a result of diminished availability of enzyme molecules within pores or from slowly diffusing substrate molecules. Nevertheless, this drawback can be compensated by a stability improvement of the immobilized enzymes under working conditions compared to the soluble enzyme, resulting in overall benefit (Cao 2005; Tischer and Wedekind 1999). Several methods for the immobilization of CPO were reported. Immobilization of CPO on silica-based materials has been demonstrated to increase its pH, oxidizing agent and temperature tolerance (Montiel et al. 2007; Petri et al. 2004; Toti et al. 2006). Besides CPO has been immobilized on mesoporous materials by adsorption (Han et al. 2002; Hudson et al. 2007) or covalent attachment (Aburto et al. 2005; Jung et al. 2010), on aminopropyl-glass beads (Kadima and Pickard 1990), talc (Aoun et al. 1998) and celite (Andersson et al. 1999) by adsorption and on magnetic beads (Bayramoğlu et al. 2008) and chitosan membranes (Zhang et al. 2009) by covalent attachment. Generally, the main disadvantage of all the methods for the immobilization by adsorption is the leaching of the enzyme from the support. Concerning covalent attachment, all the reported methods for the immobilization of CPO involved the formation of the bonds between various functional groups on the support surface and amino groups from the lysine residues of CPO. The most probable reason for relatively low immobilization yields reported so far is the presence of only four lysine residues in its amino acid sequence. The methods of immobilization via carboxyl groups on the protein surface may be very suitable since the aspartic and glutamic acid usually constitute one of the major fractions of surface groups on proteins (Fernandez-Lafuente et al. 1993). Monoaminoethyl-N-
aminoethyl (MANA) agarose gels containing very low pK primary amino groups are very suitable supports for immobilization of enzymes at low pH values (Fernandez-Lafuente et al. 1993). The method proposed consists in ionic adsorption of the enzymes to the support in a low-ionic-strength medium, which is a simple and mild immobilization method. However, an important drawback of immobilizing enzymes by adsorption is the problem of leaching of the enzyme from the support which may occur when used in the reaction of synthesis. Therefore, further, by applying a mild coupling method for the activation of carboxyl groups of the enzyme using carbodiimide, ionic adsorption is transformed to covalent attachment.

Another possibility for the successful and simple application of these primary amino supports is the immobilization of glycoproteins via their sugar moieties, previously oxidized with periodate resulting in a high density of aldehyde groups. These aldehydes can further react with amino groups from the resins, yielding Schiff base bonds (Fleminger et al. 1990; Knezevic et al. 2006).

Eupergit® C is another widely applied support for the immobilization of enzymes consisting of macroporous beads, made by copolymerization of \( N,N' \)-methylene-bis-(methacrylamide), glycidyl methacrylate, allyl glycidyl ether and methacrylamide. Due to the high density of epoxy groups on the surface of the beads it is possible to form a stable multipoint covalent attachment with groups that are very abundant in the protein surface. Thus, enzymes are usually covalently immobilized on Eupergit C® through their amino, sulfhydryl, hydroxyl or phenolic groups. At the end of the immobilization process, epoxy groups can be easily blocked by reaction with different thiol or amine compounds (mercaptoethanol, ethanolamine, glycine, etc.) under mild conditions, preventing further uncontrolled reaction between the support and the enzyme (Katchalski-Katzir and Kraemer 2000; Knezevic et al. 2006; Mateo et al. 2000a; Mateo et al. 2002; Mateo et al. 2000b).

In this chapter, CPO was proposed to be immobilized on the support Eupergit® C as well as on MANA-agarose applying three different methods: ionic adsorption, covalent attachment using carbodiimide and covalent attachment of oxidized enzyme. Application of the proposed immobilization methods for the immobilization on MANA-agarose allowed the formation of multipoint linkage between the amino groups of the support and the most abundant amino acid residues of CPO (Asp and Glu) or its sugar moiety.
Furthermore, reaction of oxidation of Cbz-ethanolamine catalyzed by immobilized and soluble CPO was performed.

6.2 Methods

6.2.1 Stability of soluble CPO

Effects of pH on CPO stability were studied over the pH range 5.0-10.0. The reaction media (10 ml of selected buffer –sodium phosphate for pH in a range of 5.0-7.5 or bicarbonate for pH=10.0, in a concentration of 50 mM) containing 20 U of enzyme were incubated at room temperature on a roller. Effect of sodium cyanoborohydride on CPO stability was analyzed using molar excesses of 1:10, 1:100, 1:1000 and 1:10000 (CPO:cyano-borohydride). The reaction media containing 2 U/ml of CPO (1400 U/mg) and 100 mM phosphate buffer pH 6.0 were incubated at 4ºC in a roller. In both cases, after different times of incubation aliquots of enzyme solution were withdrawn and enzymatic activity was measured using the previously described chlorinating assay (Section 3.4.1.1).

6.2.2 Preparation of MANA-agarose support

The amino-agarose support was prepared from glyoxyl-agarose gels following the previously reported method (Fernandez-Lafuente et al. 1993). Glyoxyl-agarose gels were prepared by etherification of 10% crosslinked agarose gels with glycidol, and further periodate oxidation of the resulting glyceryl-agarose (Figure 6.2.1) (Guisán 1988). For that, 15 ml of agarose gels were washed thoroughly with distilled water and the gel was suspended in 11.6 ml of a solution that contained NaOH, NaBH$_4$ and glycidol, in final concentrations of 0.32 N, 5.38 mg/ml and 2.04 M, respectively. The reaction was left on mild agitation in the rotary evaporator for approximately 19 h at room temperature (20-25ºC). Once the reaction was finished, agarose was vacuum-filtered and washed with distilled water (around 10 successive washings) in order to eliminate the rest of reagents. The next step was the oxidation of the resulting glyceryl-agarose with different amounts of sodium periodate (50-300 μmol NaIO$_4$/ml agarose), during approximately 30-45
minutes. Aliquots of supernatant were withdrawn periodically in order to measure the remaining periodate. The formation of aldehyde groups was calculated from the consumed NaIO₄, which was evaluated by colorimetry, detecting the iodine liberated from KI (10% (w/v)) by oxidation with remained NaIO₄ in the presence of saturated solution of NaHCO₃. The values of absorbances were measured using Spectrophotometer Cary 50 (Varian, Palo Alto, CA, USA). After the oxidation, glyoxyl-agarose was vacuum-filtered and washed with distilled water.

Figure 6.2.1 Reaction scheme of the activation of agarose to glyoxyl-agarose.

MANA-agarose gels were prepared by reaction of glyoxyl-agarose with ethylenediamine and further reduction with sodium borohydride. The condensation between single linear aldehyde groups and the diamine was a fast and reversible process, involving the formation of unstable Schiff bases (Figure 6.2.2). Reaction time was relatively short, around 2 hours.

Figure 6.2.2 Reaction scheme for the activation of glyoxyl-agarose to MANA-agarose.

The standard conditions determined by Fernandez-Lafuente et al. (Fernandez-Lafuente et al. 1993) to obtain ‘quantitative aminations’ (around 99% of conversion from aldehyde groups to amino groups in the support) were used. In this procedure, 15 g of highly derivatized glyoxyl-agarose were suspended in 135 ml of 0.1 M bicarbonate buffer at pH
Immobilization of CPO

10.0 with 2 M ethylenediamine. The suspension was gently stirred on a roller for 2 h. Finally, 1.5 g of NaBH₄ (10 mg/ml) was added to the suspension and it was left on mild agitation on a roller for other 2 h in order to finish the reduction. The utilization of such a high concentration of NaBH₄ allowed the complete conversion of Schiff bases into stable secondary amine bonds, complete reduction of almost negligible amount of remaining aldehyde groups into inert hydroxyl ones and complete reduction of a small percentage of immobilized nitro compounds formed as a consequence of the oxidation of ethylenediamine. The reduced gels were vacuum filtered and washed with 150 ml of 0.1 M acetate at pH 5.0 containing 1 M NaCl, with 150 mL 0.1 M bicarbonate at pH 10.0 containing 1 M NaCl, and finally, with 750 ml of distilled water.

6.2.3 Determination of maximal enzymatic load on MANA-agarose

1 ml (1.07 g) of highly derivatized MANA-agarose gel was suspended in 9 ml of 25 mM sodium phosphate buffer pH 6.0. Once mixed, readjusting the pH value was necessary since the presence of high density of amine groups on the support causes an increase of pH. Then, between 50 and 200 mg of bovine serum albumin (BSA) were added and left to immobilize on the support under mild agitation on a roller at room temperature. Aliquots of the supernatant were withdrawn periodically, and the protein concentration was measured by Bradford method.

6.2.4 Periodate oxidation of CPO

CPO solution was previously dialyzed against 0.05 M phosphate buffer pH 5.0 at 4ºC, in order to minimize the presence of impurities that could interfere in the oxidation process. Oxidation of CPO was carried out with an excess of sodium periodate (125 mM) in 0.05 M phosphate buffer pH 5.0 at 4ºC in the dark. Aliquots were taken periodically to determine the periodate consumption. The quantity of consumed NaIO₄ was evaluated by the same colorimetric method that was described for preparation of the glyoxyl agarose support. Once the oxidation was finished, the oxidized enzyme solution was dialyzed against 0.05 M phosphate buffer pH 5.0 at 4ºC.
6.2.5 **Immobilization of CPO by adsorption on MANA-agarose gels**

1 ml (1.07 g) of highly derivatized MANA-agarose gel was suspended in 9 ml of buffer (sodium acetate, sodium phosphate, HEPES or MES) of known concentration (10, 25, 50 or 100 mM) and pH value (5.0 or 6.0). Once mixed and before adding the enzyme, readjusting the pH value was necessary since the presence of high amine groups density on the support causes an increase of pH. Then, between 10 and 10000 U of native CPO were added and left to immobilize on the support under mild agitation on a roller at room temperature.

6.2.6 **Immobilization of CPO by covalent attachment with EDAC on MANA-agarose gels**

For the multipoint covalent attachment of CPO on MANA-agarose, the first step was the ionic adsorption of the enzyme on the support for 15 min, until no activity was detected in the supernatant. At this moment, different amounts of carbodiimide (final concentrations of 25 or 50 mM) in buffer (sodium phosphate or MES) at pH 5.0-6.0 were added and the derivatives were left on roller agitation during different intervals of time (2-3 h). Finally, NaCl was added in final concentration of 1 M in order to eliminate the non-covalently bound enzyme and left on mild agitation during 1 h.

6.2.7 **Immobilization of oxidized CPO by covalent attachment on MANA-agarose gels**

1 ml (1.07 g) of highly derivatized MANA-agarose gel was suspended in 9 ml of sodium phosphate at 25 mM and pH value of 5.0 or 6.0. Once the support mixed with the buffer, readjustment of the pH was necessary. Then, between 10 and 10000 U of oxidized CPO and NaCNBH\(_3\) in molar excess of 1:100 were added and left to immobilize on the support on mild agitation on a roller at room temperature. When no activity was detected in the supernatant, solid NaCl was added (1 M) and left on mild agitation on a roller during 1-2 h.
6.2.8 Immobilization of CPO on Eupergit® C

1 ml of Eupergit® C gel, previously hydrated for 12 h in Mili Q H₂O, was suspended in 9 ml of 0.1 M phosphate buffer with 0.9 M NaCl at pH 6.0 containing 2.22 U/ml of CPO (final activity of 2 U/ml). The mixture was incubated for 96 h at 4°C under mild agitation on roller. Then, the support was vacuum-filtrated and washed. Finally, the excess of epoxide groups on the matrix was blocked by incubation with 0.2 M β-mercaptoethanol for 4 h at 4°C.

6.2.9 Monitoring of immobilization process

All the immobilization processes were carried out under mild stirring conditions. A blank containing water instead of the agarose gel was followed in parallel in the four procedures. In order to study the time course of the immobilization, aliquots of the supernatant and suspension, as well as samples of blank, were withdrawn periodically and enzymatic activity was tested. The retained activities were calculated as the difference between activity of the suspension and the supernatant in relation to the initial activity of the process. The immobilization yields were calculated as the differences between the initial activity and the supernatant activity at the end of the process related to the initial activity. At the end of the immobilization, the immobilized enzyme preparation was vacuum-filtered, washed and its residual activity was measured. Finally, the immobilized enzyme preparations were stored at 4°C.

6.2.10 Studies of diffusion limitations of immobilized preparations of CPO

The significance of diffusion limitations for different enzymatic loads was studied for the immobilized preparation obtained by coupling oxidized CPO on MANA-agarose gels. CPO activities used in the different experiments were: 1, 5, 10, 20, 50 and 100 U per ml of MANA-agarose. Enzyme was left to bind to the support under mild agitation on a roller at room temperature, and when no activity was detected in the supernatant, the residual activity of the suspension was measured using the standard assay for CPO.
6.2.11 Peroxide dependent inactivation of immobilized preparations of CPO

The immobilized CPO preparations, each one obtained by contacting 20 U of CPO per ml of MANA-agarose, were washed with 100 mM acetate buffer and added in ratio 1:10 to the reaction medium containing 100 mM acetate buffer at pH 5.0 in a volume of 10 ml. In case of soluble enzyme, 20 U of soluble CPO were added to the same volume of reaction medium. The reaction started by adding tert-butyl hydroperoxide continuously by non-stop addition at a rate of 18 µl/h using a single-syringe automatic microburette (Crison Instruments, Spain). Concentration of peroxide stock solution was 1.67 M in order to reach peroxide addition rates of 3 mM/h. Aliquots of the suspension and supernatant were withdrawn periodically and the enzymatic activity was measured using the standard assay for CPO, in order to determine the enzymatic activity of the derivative, as well as possible leaching of the enzyme from the support at these conditions.

The inactivation kinetics was studied by plotting time course of inactivation on semi-log plot, and from the slope of linear regression obtained, the half life of an enzyme was calculated. It was observed that the inactivation follows first order kinetics; hence, the half life was as \( t_{1/2} = \frac{\ln(2)}{\text{slope}} \).

6.2.12 Oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by soluble and immobilized CPO

6.2.12.1 Reaction with soluble CPO

5000 U of CPO were added to a 10 ml reaction medium containing 20 mM Cbz-ethanolamine and 100 mM acetate buffer at pH 5.0. The reaction started after by adding tert-butyl hydroperoxide continuously by non-stop addition at a rate of 18 µl/h by using a single-syringe automatic microburette (Crison Instruments, Spain). Concentration of peroxide stock solution was 1.67 M in order to reach peroxide addition rates of 3 mM/h. Samples were taken periodically and analyzed by liquid chromatography in order to quantify Cbz-ethanolamine and Cbz-glycinal concentrations.
6.2.12.2 Reaction with immobilized CPO

CPO immobilized by covalent attachment on MANA-gels in the presence of EDAC was prepared by mixing 10000 U of CPO per ml of MANA-agarose. The immobilized biocatalyst was washed with 100 mM acetate buffer and added in ratio 1:10 (v:v) to a 10 ml reaction medium containing 20 mM Cbz-ethanolamine and 100 mM acetate buffer at pH 5.0. The reactions were performed and monitored in the same way as for the soluble enzyme.

6.3 Results and discussion

6.3.1 pH stability of chloroperoxidase

One of the most limiting factors when choosing an immobilization method is the commonly narrow interval of pH in which the enzymatic stability is preserved. Therefore, in order to prevent CPO inactivation during the immobilization, the pH stability of the enzyme must be evaluated. Although it is known that its stability decreases when increasing pH, it might be possible to maintain it in sufficiently high level during the necessary time to complete the immobilization.

The effect of pH value on the stability of CPO is shown in Figure 6.3.1. CPO resulted to be extremely unstable at alkaline pH values, and it showed slightly slower inactivation at neutral pH value. Even so, at pH 7 the instability of the enzyme was still too rapid having in mind the time required to perform the immobilization processes. Lowering further the pH to slightly acidic values, the stability of CPO importantly increased so that at pH 6.5 it retained about 70% of initial activity after 3 days of incubation, while at both pHs 6.0 and 5.0 CPO retained more than 95%.
Figure 6.3.1 Effect of pH on the activity of soluble CPO after different times of incubation: 5 minutes (●), 1 hour (□), and 3 days (▽). Soluble CPO was incubated at 25°C in 50 mM sodium phosphate buffer at pH values: 5.0, 6.0, 6.5, 7.0, 7.5 or sodium bicarbonate buffer at pH value 10.0. The initial CPO activities were around 2 U/ml and were set to 100% at t=0.

As results about pH stability of CPO implied, the immobilization of this enzyme had to be performed at acidic pH values. MANA-agarose gels present very low pK value and thus are suitable for the enzyme immobilization at low pH. Apart from this, Eupergit® C is an applicable support for enzyme immobilization over a wide range of pH values. Even though it is mostly used on alkaline and neutral pH values, when the covalent bonds are formed between the amino groups of the enzyme and the oxirane groups of the support, it can also bind the enzyme via their sulfhydryl and carboxyl groups in the acidic, neutral and alkaline pH range (Katchalski-Katzir and Kraemer 2000).

6.3.2 Immobilization of CPO on MANA-agarose

MANA-agarose was prepared from glyoxyl-agarose by following the procedure described in the section 6.2.2 (Figure 6.2.2). Glyoxyl-agarose was prepared by etherification of agarose gels with glycidol and further oxidation of the resulting glyceryl-agarose by using
different amounts of periodates (Figure 6.2.1). In experiments with lower quantities of periodate, all periodate was consumed for oxidation of glyceryl-agarose after approximately 30 min, since the number of groups to be oxidized per ml of agarose is higher than the quantity of periodate that was used (Figure 6.3.2). When 300 μmol of periodate per ml of agarose were used, 264 μmol of aldehyde groups per ml of agarose were formed, which corresponds to 88% of the NaIO₄ that had been offered, taking into account that molar ratio aldehyde groups: NaIO₄ is 1:1. This number represents the maximal superficial density of aldehyde groups which can be generated on 10% crosslinked agarose gels.

![Density of aldehyde groups formed on agarose gels by using different amounts of periodate for the oxidation of glyceryl-agarose.](image)

**Figure 6.3.2** Density of aldehyde groups formed on agarose gels by using different amounts of periodate for the oxidation of glyceryl-agarose.

The ‘quantitative amination’ (Figure 6.2.2) of agarose gels with different concentrations of aldehyde groups allows the preparation of aminated agarose with very different concentration of amino groups. For the purpose of this work, the glyoxyxl-agarose prepared with the highest possible degree of activation (264 μmol of aldehyde groups per ml of agarose) was used. Thus, it can be considered that MANA-support with around 264 μmol of amino groups per ml of agarose was formed. Such a high number of formed aldehyde groups allows the formation of multipoint covalent attachment when immobilizing enzyme on this support, which can significantly increase the stability of the enzyme.

10% crosslinked agarose gels were selected taking into account the average pore size and the size of an enzyme. Besides, by using 6% crosslinked agarose gels, the maximal density
of aldehyde groups that can be obtained is around 75 µmol/ml of agarose. Use of 10% crosslinked agarose gels allows the immobilization of higher enzyme loads than 6% agarose (around 100 and 40 mg of enzyme per ml of agarose for 10% and 6% agarose, respectively) (Guisán et al. 1997).

Finally, several options for the immobilization of CPO on this support were considered in this work: simple ionic adsorption, covalent attachment of CPO through its carboxyl groups via carbodiimide coupled method and finally, direct covalent immobilization of CPO through its glycosidic chains previously oxidized with periodate.

6.3.2.1 Maximal enzymatic load on MANA-agarose gels

When optimizing the immobilization process, it is necessary to know the maximal load of the enzyme which can be immobilized per support volume, i.e. the amount of the enzyme which saturates the support. This was determined by adding different amounts of bovine serum albumin (BSA) per known volume of MANA-agarose and leaving it to adsorb to the support. The remained protein concentration in the supernatant was measured at different intervals of time, until no further change of its concentration was detected (Table 6.3.1). When BSA was added at the concentrations of 50 and 100 mg per ml of MANA-agarose, the entire protein added was adsorbed on the support, while for the load of 200 mg/ml, 42.2% of the protein was measured in the supernatant. Therefore, the saturation of MANA-agarose can be considered to occur for the protein load of 115.6 mg per ml of MANA, the value very proximate to that reported by Guisán et al. (Guisán et al. 1997).

<table>
<thead>
<tr>
<th>BSA concentration added (mg/ml MANA-agarose)</th>
<th>Immobilization yield (%)</th>
<th>Load of immobilized BSA (mg/ml agarose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>97.4</td>
<td>48.7</td>
</tr>
<tr>
<td>100</td>
<td>102.8</td>
<td>102.8</td>
</tr>
<tr>
<td>200</td>
<td>57.8</td>
<td>115.6</td>
</tr>
</tbody>
</table>

Table 6.3.1 Determination of the maximal protein load on MANA-agarose. Different concentrations of BSA were added to 25 mM sodium phosphate buffer at pH 6.0 containing MANA-agarose in a ratio 1:10 (support volume: total volume) at 15ºC.
6.3.2.2 Ionic adsorption of CPO on MANA-agarose gels

Immobilization by adsorption is the simplest immobilization method and consists in reversible surface interactions between the enzyme and the support (D’Souza 1999; Tischer and Wedekind 1999). In general, adsorption of CPO on MANA-agarose support occurs via electrostatic interactions between the external charges on the enzyme and the support, and can be performed at pH values in the range of 4.0-6.0 since it favors the development of positive charge on MANA agarose support (pK=6.8) and negative charge of the carboxyl groups of enzyme (Fernandez-Lafuente et al. 1993) (Figure 6.3.3).

Figure 6.3.3 Reaction scheme for ionic adsorption of the enzyme on MANA-agarose gels.

Therefore, the procedure of the immobilization of CPO by adsorption consists in simple mixing together CPO and MANA-gels under suitable conditions of pH and ionic strength for a necessary period of incubation to complete the immobilization, which is followed by the separation of the immobilized enzyme preparation and its extensive washing in order to remove the unbound enzyme. Hence, the parameters studied in order to optimize the conditions for the immobilization were pH value, incubation time, and type and concentration of the buffer (Table 6.3.2).

Depending on the chemical structure of the available surfaces for adsorption and the pI of CPO (around 4.0) alteration in the pH of the medium will have an influence on the support-protein interactions. The pH 5.0 was chosen as optimal, as it favored both positive charge of the support which has pK value 6.8 and negative charge of the enzyme and did not affect the stability of the soluble chloroperoxidase.

Influence of the incubation time was studied at pH 5.0 in sodium acetate at different buffer concentrations. In general, slight increase of the immobilization yield was observed
at longer incubation times, but it was not always advantageous respect to the retained enzymatic activity, as the activity of the suspension decreased with time.

Adsorption performed in sodium phosphate buffer was more successful than the one in sodium acetate buffer using the same conditions, and immobilization processes required much less time. Since low concentrations of the buffers (between 10-100 mM) were used in all cases to ensure low ionic strength conditions and permit the ionic interactions between the enzyme and the support, no significant differences in terms of immobilization yields were observed within that range of buffer concentrations. However, concerning activity retentions, higher values were obtained when using 50 mM phosphate buffer comparing to the ones obtained with 10 or 25 mM, probably due to more effective buffering when higher concentrations were applied.

Use of MES and HEPES buffers at pH 6.0 resulted in a very fast loss of CPO activity in the suspension, although the CPO in blank preserved its entire initially added activity. Considering the results from the stability experiments (Figure 6.3.1), this pH value should not affect the stability of CPO in such short periods of time. Therefore, the most probable reason for this activity loss is the nature and intensity of electrostatic interaction between the enzyme and the support at this pH value.
**Table 6.3.2** Immobilization yield and retained activity of CPO immobilized by adsorption on MANA-agarose. Experiments were performed by mixing 20 U/ml of support in a ratio 1:10 (support volume: total volume) at 20ºC.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>Buffer concentration (mM)</th>
<th>Adsorption time (min)</th>
<th>Immobilization yield (%)</th>
<th>Retained activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium acetate</td>
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<td>58.4</td>
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<td></td>
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<td>41.5</td>
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<td>45</td>
<td>70.0</td>
<td>40.4</td>
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<td></td>
<td></td>
<td>100</td>
<td>90</td>
<td>68.0</td>
<td>35.5</td>
</tr>
<tr>
<td>Sodium phosphate</td>
<td>5.0</td>
<td>10</td>
<td>15</td>
<td>93.8</td>
<td>50.5</td>
</tr>
<tr>
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<td>57.9</td>
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<td></td>
<td></td>
<td>50</td>
<td>15</td>
<td>91.6</td>
<td>76.6</td>
</tr>
<tr>
<td>MES</td>
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<td>25</td>
<td>10</td>
<td>69.3</td>
<td>29.8</td>
</tr>
<tr>
<td>HEPES</td>
<td>6.0</td>
<td>25</td>
<td>25</td>
<td>92.8</td>
<td>9.3</td>
</tr>
</tbody>
</table>

### 6.3.2.3 Multipoint covalent attachment of CPO on MANA-agarose gels

Once the CPO enzyme was ionically adsorbed to MANA-agarose, carbodiimide (EDAC) was added to the suspension as an activating agent of the carboxylic groups enabling the transformation of adsorption to more stable covalent attachment (Figure 6.3.4). The formation of covalent bonds between the enzyme and the support involving pseudourea...
as an intermediate product occurs via the same mechanism as in a case of chemical modification of CPO by EDAC, as it was explained in the Section 5.1.

![Reaction scheme for covalent attachment of enzyme on MANA-agarose gels by using EDAC as an activator of carboxyl groups of the enzyme.](image)

**Figure 6.3.4** Reaction scheme for covalent attachment of enzyme on MANA-agarose gels by using EDAC as an activator of carboxyl groups of the enzyme.

Even though sodium phosphate buffer at the concentration of 50 mM and pH 5.0 was the most appropriate one for the ionic adsorption of CPO to MANA-agarose gels, the use of this buffer for the carbodiimide coupled transformation of ionic interaction to covalent bonds is limited by the instability of EDAC in this buffer (Gilles et al. 1990). The presence of inorganic phosphate was shown to have a very strong influence on decomposition of EDAC and its half life decreases rapidly with the increase of phosphate concentration and decrease of pH value. Half lives of EDAC in 10 mM and 100 mM phosphate buffer at pH 5.0 were 1.43 h and 0.16 h, respectively. Therefore, EDAC had to be added in excess and phosphate buffer used at low concentration. Experimentally, the benefit of EDAC addition, i.e. the covalent bond success, was analyzed by testing the CPO desorption from the support –after immobilization– by addition of 1 M NaCl. From the immobilization
performed in 10 mM phosphate buffer at pH 5.0 (Table 6.3.3) it can be seen that by increasing EDAC concentration from 25 mM to 50 mM and incubating it for 2 h, the desorption from the support was reduced from 10.9 to 7.4% respect to the overall initially added activity. The possibility of desorption was almost completely eliminated by prolonging the incubation time to 3 h employing 50 mM EDAC (Table 6.3.3).

Other buffers were tested for the adsorption and posterior covalent attachment of CPO in MANA-agarose. It was described that EDAC remains stable in 50 mM MES buffer at pH 7.0 (half life of 37 h), while at lower pH it decomposes more rapidly (at pH 6.0 and pH 5.0 its half-life has the value of 20 and 3.9 h respectively) (Gilles et al. 1990). As the buffer MES at pH 6.0 enabled low retained activities for the ionic adsorption of CPO and also for the covalent attachment (Tables 6.3.2 and 6.3.3), the performance of the immobilization in two separated steps for ionic and covalent attachment was considered. In the first one the enzyme was left to adsorb ionically on the support in 25 mM phosphate buffer at pH 5.0. Then, the immobilized enzyme preparation was vacuum-filtered and added to 25 mM MES buffer at pH 6.0. As at these conditions EDAC is quite stable, 25 mM were enough to enable covalent attachment; consequently, no desorption was observed. However, the final retained activity was 31.3% and the major part of activity loss, around 42%, took place in the moment of change of the buffer between the two steps.
Table 6.3.3 Immobilization yield, retained activity and desorption of CPO covalently immobilized on MANA-agarose using EDAC. Experiments were performed by mixing 20 U/ml of support in a ratio 1:10 (support volume: total volume) at 20°C.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>Buffer concentration (mM)</th>
<th>EDAC incubation time (h)</th>
<th>EDAC concentration (mM)</th>
<th>Immobilization yield (%)</th>
<th>Retained activity (%)</th>
<th>Desorption (2) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium phosphate</td>
<td>5</td>
<td>10</td>
<td>2</td>
<td>25</td>
<td>83.4</td>
<td>32.9</td>
<td>10.9</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>50</td>
<td></td>
<td></td>
<td>88.4</td>
<td>44.9</td>
<td>7.4</td>
</tr>
<tr>
<td>MES</td>
<td>5</td>
<td>25</td>
<td>4</td>
<td>10</td>
<td>94.0</td>
<td><strong>54.5</strong></td>
<td>1.0</td>
</tr>
<tr>
<td>Sodium phosphate/MES(1)</td>
<td>5/6</td>
<td>25/25</td>
<td>2.5</td>
<td>25</td>
<td>98.7</td>
<td>31.3</td>
<td>0.0</td>
</tr>
</tbody>
</table>

(1) Buffers for ionic adsorption/covalent attachment with EDAC.

(2) Desorption was analyzed by adding 1 M NaCl at the end of the EDAC incubation time and represents the percentage of the activity desorbed from the support respect to the initial activity.
Observing the time course of the immobilization when adsorption and covalent attachment steps were performed using the same buffer, the major loss of enzymatic activity occurred during the ionic adsorption step (Figure 6.3.5). In parallel with the immobilization, a blank with soluble enzyme at identical conditions but in absence of the support was performed. While the soluble enzyme suffered a deactivation in presence of EDAC, the enzyme in the immobilization suspension showed more stability towards the same concentration of EDAC. Furthermore, enzymatic retentions were a bit higher when using higher EDAC concentrations. These two facts point out an increase of enzymatic stability once the enzyme is covalently immobilized.

Figure 6.3.5 Covalent immobilization of CPO (20 U of CPO per ml of the support) on MANA-agarose in 10 mM phosphate buffer pH 5.0 at room temperature (20ºC) using 50 mM EDAC. (I) Ionic adsorption. (II) Carbodiimide coupled formation of covalent bonds between the carboxyl groups of the enzyme and the amino groups of the support. (III) Checking of possible desorption of the enzyme from the support by adding 1 M NaCl. Time course of the immobilization was determined measuring the activities for the whole suspension (●), the supernatant (○) and the blank (▼). Data points represent the average of duplicate measurements; error bars represent the standard deviation.
6.3.2.4 Multipoint covalent attachment of oxidized CPO on MANA-agarose gels via its sugar moiety

Carbohydrate moieties of CPO can be oxidized to form aldehyde groups that can further react with amino groups of MANA-agarose support. However, during the oxidation of the glycoproteins, certain amino acid residues may be oxidized as well, especially cysteine, cystine, methionine, tryptophan, tyrosine, histidine and N-terminal serine and threonine (Clamp and Hough 1965). Whenever these residues are essential for the activity of the enzyme, their oxidation can lead to enzymatic inactivation. The periodate oxidation of CPO was performed in an excess concentration of sodium periodate during 24 h, after which no further consumption of periodate was observed. Upon oxidation, CPO retained around 54% of its initial activity.

Immobilization by multipoint covalent attachment of oxidized CPO on MANA-agarose consisted of two steps. Firstly, oxidized CPO solution and MANA-support were incubated in the presence of sodium cyanoborohydride, which enabled the reduction of Schiff base bonds formed between the enzyme and the support yielding more stable secondary amine bonds. Sodium cyanoborohydride was selected as a reducing agent due to the possibility to perform the reduction step at acidic pH values (Lane 1975) (Figure 6.3.6). As explained before, once immobilization was completed, 1 M NaCl was added in order to determine the possible desorption of the enzyme from the support.

Figure 6.3.6 Reaction scheme for covalent attachment of a periodate oxidized enzyme on MANA-agarose gels.
First of all, the stability of CPO was evaluated in the presence of sodium cyanoborohydride, in order to determine the adequate concentration to prevent its inactivation during the immobilization process (Figure 6.3.7). This reducing agent did not cause any inactivation of CPO after 2 h of incubation when used in molar excess of 1:10 and 1:100 (CPO:cyanoborohydride). However, remaining activities decreased in 38.2 and 92.6% for molar excess of 1:1000 and 1:10000. Therefore, a cyanoborohydride molar excess of 1:100 was selected for the stabilization of Schiff base bonds formed during the immobilization of oxidized CPO on MANA-agarose.

![Remaining CPO activity (\%)](image)

**Figure 6.3.7** Effect of molar excess of sodium cyanoborohydride (µmol sodium cyanoborohydride: µmol CPO) on CPO activity after 2 h of incubation of 2 U/ml of CPO in 100 mM sodium phosphate buffer pH 6.0 and 4ºC. Data points represent the average of duplicate measurements; error bars represent the standard deviation.

Immobilization was carried out at the temperature of 4ºC following the reported methods for the immobilization of oxidized enzymes on amino-supports. Nevertheless, as it was observed that CPO did not suffer inactivation at 20-25ºC, the immobilization was performed at room temperature as well. Complete immobilization of oxidized CPO on MANA-agarose occurred after only 30 min of incubation of the enzyme and the support together with reducing agent (Table 6.3.4 and Figure 6.3.8). Similar retentions of activity were obtained in all the cases studied; the one obtained at pH 5.0 and 4ºC being slightly higher than others (51.4%). Almost no desorption was detected in the experiments after
the addition of 1 M NaCl, even when no sodium cyanoborohydride was used for the reduction of Schiff base linkages. As the reversibility of the Schiff base occurs at very low values of pH, spontaneous breaking of bonds is unlikely and, consequently, reduction of bonds is not required. Besides, the great density of both amino groups on the support surface and aldehyde groups on CPO, enables the formation of multipoint attachment. Therefore, the leakage of the enzyme from the support could occur only in case of breaking all the bonds formed, which is difficult to occur.

**Table 6.3.4** Immobilization yield, retained activity and desorption of oxidized CPO covalently immobilized on MANA-agarose. Experiments were performed by mixing 20 U/ml of support in a ratio 1:10 (support volume: total volume) using 25 mM sodium phosphate buffer.

<table>
<thead>
<tr>
<th>Molar excess of NaCNBH₃</th>
<th>pH</th>
<th>T (ºC)</th>
<th>Immobilization yield(1) (%)</th>
<th>Retained activity(1) (%)</th>
<th>Desorption(2) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:100</td>
<td>6</td>
<td>4</td>
<td>96.2</td>
<td>46.2</td>
<td>1.3</td>
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<td>1:100</td>
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<td>4</td>
<td>97.8</td>
<td>51.4</td>
<td>0.4</td>
</tr>
<tr>
<td>1:100</td>
<td>5</td>
<td>21</td>
<td>99.1</td>
<td>48.0</td>
<td>0.0</td>
</tr>
<tr>
<td>No NaCNBH₃ added</td>
<td>5</td>
<td>21</td>
<td>97.5</td>
<td>47.0</td>
<td>0.3</td>
</tr>
</tbody>
</table>

(1) Values obtained after 30 min of covalent attachment.

(2) Desorption was analyzed by adding 1 M NaCl at the end of the EDAC incubation time.
Figure 6.3.8 Covalent immobilization of oxidized CPO (20 U of CPO per ml of the support) on MANA-agarose with NaCNBH$_3$ added in molar excess 1:100 respect to CPO, in 25 mM phosphate buffer pH 5.0 at room temperature. Time course of the immobilization was determined measuring the activities in the whole suspension (●), the supernatant (○) and blank (▼). Data points represent the average of duplicate measurements; error bars represent the standard deviation.

6.3.3 Immobilization of CPO on Eupergit® C

Immobilization of CPO on Eupergit® C in a solution composed of 0.1 M sodium phosphate buffer and 0.9 M NaCl was performed. High ionic strength is required for the physical adsorption of the enzyme to the support by hydrophobic interactions bringing the enzyme functional groups to close proximity to the oxirane groups of the support and allowing the formation of stable covalent bonds in the next step. The reached immobilization yields were 65.6% after 96 h of incubation at pH 6.0 and 47.8% after 72 h of incubation at pH 6.5. However, very fast loss of CPO activity was observed in these conditions, with final activity retentions of 15.1% (at pH 6.0) and 1.6% (at pH 6.5). Additionally, when β-
mercaptopoethanol was added at the concentration of 0.2 M entire CPO activity was lost. Therefore, this immobilization method was not suitable for the immobilization of CPO.

6.3.4 Diffusion limitations of the immobilized preparations of CPO on MANA-agarose

Kinetics of reactions catalyzed by immobilized enzymes may differ significantly from those using soluble enzymes, as the reaction rate strongly depends on the physical processes of substrates and products transport through the interior of the beads. Substrate diffusion inside the porous medium proceeds simultaneously with the chemical reaction and its effect becomes more significant for relatively high enzyme concentrations in the solid phase because part of the enzyme is not accessible to the substrate (Engasser and Horvath 1973; Horvath and Engasser 1974).

The possible significance of diffusion limitations for CPO immobilized on MANA-agarose was studied for the reaction of CPO enzymatic assay using monochlorodimedone (MCD) as substrate. Oxidized CPO covalently immobilized on MANA-agarose was studied as model immobilized enzyme system. The percentage of apparent retained activity of the immobilized derivative respect to the initially added activity was measured for activities added within the range of 1-100 U/ml of support. In all cases the immobilization yield was close to 100%. It is well known that in the absence of diffusion limitations and when saturation of the support is not occurring, the percentage of the retained activity upon the immobilization does not depend on the activity offered. However, for the immobilized system of oxidized CPO on MANA-agarose the diffusion limitations were present up to the CPO loads of 5 U added per ml of the support, and below this value the measured retained activity remained unchanged (Figure 6.3.9). Therefore, it can be concluded that approximately 33% of the CPO activity loss was due to the immobilization process itself, while the rest of the apparent activity loss is attributed to the presence of diffusion limitations. Hence, for reaction with CPO natural substrate, MCD, it is necessary to work with enzymatic loads lower than 5 U per ml of the support in order to eliminate the limitations by diffusion. Nevertheless, for any other reaction with lower reaction rate than that one with MCD, diffusion limitations will appear at higher enzymatic loads or even will
not appear. Therefore, the real influence of the diffusion limitations on CPO-MANA catalyzed oxidation of Cbz-ethanolamine will be evaluated by comparison of the target reaction performance using soluble and immobilized CPO.

![Graph showing the effect of initially added CPO activity on measured residual activity](image)

**Figure 6.3.9** Effect of the initially added enzymatic activity on the residual activities of the oxidized CPO covalently immobilized on MANA-agarose after 30 minutes of incubation, when no activity was detected in the supernatant. Experiments were performed using a ratio immobilized enzyme volume: total volume of 1:10, in 25 mM phosphate buffer pH 5.0 at room temperature. Data points represent the average of duplicate measurements; error bars represent the standard deviation.

### 6.3.5 Stability of the immobilized preparations of CPO

As it was demonstrated before (Chapters 4 and 5), the main limitation for the use of CPO as a biocatalyst for the oxidation of Cbz-ethanolamine to Cbz-glycinal is its fast inactivation in the presence of peroxides. In order to minimize this inactivation, the reactions were performed under a continuous addition of tert-butyl hydroperoxide, and the addition rate was determined to be 3 mM/h looking for a compromise between reaction rate and activity preservation.
It is well known that mass-transfer controlled reactions appear to be much less sensitive to the decrease of enzyme activity (Tischer and Kasche 1999) due to the fact that the diffusion limitations decrease with the decrease of enzymatic activity. Therefore, for studying the peroxide dependent inactivation of immobilized CPOs, low enzymatic loads were used (20 U/ml of MANA having only approximately 10% of the apparent activity loss due to the diffusion limitations) in order to minimize the possibility for the false impression of stabilization.

The stabilities of the immobilized derivatives of CPO on MANA-agarose were studied at the conditions in which reaction of interest takes place. The activities of soluble and immobilized enzymes were measured under addition of t-BuOOH at the rate of 3 mM/h. In these conditions, soluble CPO lost 96.2% of its activity after only 2 h of incubation (Figure 6.3.10). All of the immobilized enzyme preparations showed significantly improved stability in these conditions, especially CPO immobilized by covalent attachment coupled with EDAC which appeared to retain almost 90% of its activity after 48 h in these conditions. This improvement was reflected in a sharp increase of half-life times, which rose from 0.33 h for soluble CPO to 47.4, 175.7 and 103.6 h for the enzymes immobilized by adsorption, by covalent attachment with EDAC and for oxidized enzyme immobilized by covalent attachment, respectively. Desorption of the enzyme from the support was not observed in these conditions for none of the three immobilized enzyme systems tested.
Figure 6.3.10 Stability of soluble and immobilized CPO under addition of 3 mM/h of t-BuOOH in acetate buffer 100 mM pH 5. Soluble CPO (♦), CPO immobilized by adsorption on MANA-agarose (●), CPO covalently immobilized on MANA-agarose using EDAC (■) and oxidized CPO covalently immobilized on MANA-agarose (▼). The initial CPO activities were around 2 U/ml and were set to 100% at t=0. Data points represent the average of duplicate measurements; error bars represent the standard deviation.

6.3.6 Oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by immobilized CPO

Encouraged by the results of the improved stability of immobilized enzymes, the most stable one, i.e. CPO immobilized by covalent attachment on MANA-agarose using EDAC, was applied as biocatalyst and compared to the soluble enzyme. For this purpose, CPO was immobilized by adding 10000 U per ml of MANA, in order to accomplish the final expected activity of 500 U/ml after adding the immobilized enzyme to the reaction medium in the relation 1:10, taking into account that around 50% of activity loss occurred during the process of immobilization (Table 6.3.3). The complete immobilization was achieved, although the activity measured by MCD assay was only 47.8 U per ml of the support, which is 0.5% of the initially added activity. This result is in accordance with the
high diffusion limitations of this derivative for the reaction of chlorination of MCD, which were demonstrated in the Section 6.3.4.

Experimental conditions, such as continuous addition of tert-butyl hydroperoxide at a rate of 3 mM/h, expected enzymatic activity of 500 U/ml, and reaction medium used were equivalent to those used in the aqueous reaction by using soluble CPO (Section 4.3.1.1). One of the main constraints when trying to oxidize alcohols to aldehydes is the easy further oxidation of aldehydes to acids. The selection of both the enzyme and the reaction conditions is essential to avoid this further oxidation. When catalyzing the oxidation of Cbz-ethanolamine to Cbz-glycinal using CPO immobilized on MANA-agarose with EDAC, around 44.7% of Cbz-glycinal and 17.8% of amino acid Cbz-glycine were obtained (Table 6.3.5 and Figure 6.3.11). The yield of Cbz-glycinal reached its maximum at 30 h. After this time the accumulation of amino aldehyde accelerated its further conversion to amino acid, which became more pronounced with the reaction time, resulting in slightly lower yield of Cbz-glycinal at 48 h than the corresponding one at 30 h. However, the reaction catalyzed by soluble CPO reached a maximum of 39.1% of Cbz-glycinal, whereas much less Cbz-glycine was obtained, as a result of the practically total enzymatic activity loss after 8 h of reaction.
**Table 6.3.5** Oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by soluble CPO and CPO covalently immobilized on MANA-agarose with EDAC, using tert-butyl hydroperoxide as oxidant at the rate of 3 mM/h. The reaction medium contained 20 mM of Cbz-ethanolamine and 500 U/mL of CPO in 100 mM acetate buffer pH 5.0. All the experiments were performed at room temperature (20-23°C).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Reaction time (h)</th>
<th>Activity loss (%)</th>
<th>Cbz-ethanolamine conversion (%)</th>
<th>Cbz-glycin yield (%)</th>
<th>Cbz-glycine yield (%)</th>
<th>Initial reaction rate (mM Cbz-glycin/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble CPO</td>
<td>8</td>
<td>94.2</td>
<td>47.9</td>
<td>39.1</td>
<td>8.8</td>
<td>1.40</td>
</tr>
<tr>
<td>Immobilized CPO</td>
<td>30</td>
<td>34.6</td>
<td>59.1</td>
<td>44.7</td>
<td>17.8</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>53.8</td>
<td>59.9</td>
<td>36.6</td>
<td>21.8</td>
<td></td>
</tr>
</tbody>
</table>
Figure 6.3.11 Profile for the oxidation of Cbz-ethanolamine (●) to Cbz-glycinal (○) and Cbz-glycine (▼) catalyzed by CPO covalently immobilized on MANA-agarose with EDAC, using tert-butyl hydroperoxide as oxidant at the rate of 3 mM/h. The reaction medium contained 20 mM of Cbz-ethanolamine and 1 ml of CPO-MANA (EDAC) in 100 mM acetate buffer pH 5.0 in the final reaction volume of 10 ml and was performed at room temperature (20-23ºC). Data points represent the average of duplicate measurements; error bars represent the standard deviation.

Concerning initial reaction rates, a value of around 1.4 mM/h was obtained when using soluble enzyme, which is around 2.5-fold higher than the value achieved with the immobilized CPO. The main reason could be the high limitations by diffusion of the substrate to reach the immobilized enzyme that is located inside the pores of the support. These limitations had been observed when using the conversion of monocholorodimedone to dichlorodimedone as activity test for activities within the range of 1-100 U/ml support. Despite the oxidation of Cbz-ethanolamine to Cbz-glycinal is much slower, the limitations are also observed due to the high enzymatic load of the support.

Although the reaction catalyzed by the immobilized CPO resulted to be slower, the enzyme was active for a much longer period of time than in the reaction with soluble enzyme. The immobilized CPO withstood the continuous addition of peroxide at the rate of 3 mM/h during 48 h with 53.8% of apparent CPO activity loss, while the soluble enzyme
lost 94.2% of its activity after only 8 h. Even so, it has to be taken into account that the measured remaining activity in the reaction with soluble CPO represents the exact value, while the measured one of immobilized CPO is just an apparent value, due to the presence of very high diffusion limitations. At the moment when no further consumption of Cbz-ethanolamine was detected, conversions of 59.9 and 47.9% of Cbz-ethanolamine were reached for immobilized and soluble enzyme, respectively. This fact highlights one of the benefits of the immobilized CPO, which is the higher stability against addition of peroxide. This advantage can be further exploited by coupling this reaction with the reaction catalyzed by DHAP dependent aldolases, in which once formed, amino aldehyde would be immediately consumed as a substrate for the aldol addition of DHAP. This could possibly result in accelerated reaction of Cbz-ethanolamine oxidation and also could avoid or minimize the further oxidation of Cbz-glycinal to Cbz-glycine. Another important benefit from the immobilization itself and the improved stability of the derivatives is the possibility of enzyme recovery and reuse, as the residual activity at the end of one reaction was high enough to be applied to another enzymatic reaction.

Besides it was observed that, even though the enzyme remained active after 48 h of the reaction, no further conversion occurred. This could be due to the reached reaction equilibrium. The reaction equilibrium could be shifted towards higher yields by coupling this reaction to the reaction of aldol addition of DHAP catalyzed by DHAP-dependent aldolase.
6.4 CONCLUSIONS

Four possibilities were studied for CPO immobilization: three of them applied an aminated support and were based on adsorption and covalent attachment; in the fourth one the epoxy hydrophobic support Eupergit® C was considered for covalent attachment of CPO. The immobilization of CPO on Eupergit® C was shown to be unsuitable due to the complete activity loss; on the contrary, immobilization of CPO on MANA-support allowed good activity retentions (within 51 and 77%). While covalent attachment required oxidation of the enzyme or the presence of coupling or reducing agents, adsorption was performed in milder conditions, and higher retained activities were obtained. However, covalently immobilized enzymes gained much more stability against tert-butyl hydroperoxide addition than adsorbed CPO, and apparent half-life time at these conditions was improved more than 500 fold when using CPO immobilized on MANA-support with EDAC, compared to the soluble enzyme. The immobilized enzymes showed diffusion limitations for the natural reaction of monochlorodimedone chlorination, as well as for oxidation of Cbz-ethanolamine to Cbz-glycinal. In spite of this, the covalently attached CPO using EDAC was revealed as better catalyst than soluble CPO for the Cbz-glycinal synthesis in terms of reaction conversion (59.9%) and remaining activity (46.2%) at the end of the reaction.
CHAPTER 7

SYNTHESIS OF AMINOPOLYOLS IN ONE-POT MULTIENZYME OXIDATION AND ALDOL ADDITION

Publication:
Milja Pešić, Carmen López, Gregorio Álvaro, Josep López-Santín, From amino alcohols to aminopolyols: one-pot multienzyme oxidation and aldol condensation, submitted as full paper to Advanced Synthesis & Catalysis.
7 SYNTHESIS OF AMINOPOLYOLS IN ONE-POT MULTIENZYME OXIDATION AND ALDOL ADDITION

7.1 Introduction

The stereoselective aldol addition reactions catalyzed by aldolases are some of the most useful tools for the synthetic applications due to the fact that they permit the formation of carbon-carbon bonds, generating up to two new chiral centers. The aldol addition of DHAP to amino aldehydes is one of the most interesting applications of DHAP-dependent aldolases, because the product of the reaction, Cbz-aminopolyol, can be easily transformed to iminocyclitols using acid phosphatase for the removal of the phosphate group and posterior hydrogenation as it was shown in the Section 1.4.1.2 (Espelt et al. 2003). Iminocyclitols are glycoprocessing enzyme inhibitors of great therapeutic potential (Calveras et al. 2009; Look et al. 1993; Whalen and Wong 2006). For this purpose, in previous works, the protected amino aldehydes were synthesized chemically (Ardao et al. 2006; Calveras et al. 2006; Calveras et al. 2009; Suau et al. 2008). The capacity of CPO to oxidize the amino alcohol Cbz-ethanolamine to Cbz-glycinal can be integrated with the aldol addition of DHAP catalyzed by DHAP-dependent aldolase obtaining aminopolyols in two enzyme catalyzed steps.

Many examples of multienzyme one-pot synthesis using aldolases can be found in literature. Most of them deal with the production of carbohydrates via in-situ synthesis of DHAP and/or enzymatic dephosphorylation of the aldol adduct, coupled to aldol addition catalyzed by aldolase (Babich et al. 2011; Sanchez-Moreno et al. 2009; Schoevaart et al. 2000; Van Herk et al. 2006; Wong and Whitesides 1983).

In this chapter, the one-pot multienzyme synthesis of Cbz-aminopolyol from Cbz-ethanolamine was studied. The system consisted in two consecutive reactions (Figure 7.1.1). Chloroperoxidase mediated oxidation of Cbz-ethanolamine using tert-butyl hydroperoxide as an electron acceptor produces Cbz-glycinal as a main product and Cbz-glycine as an undesired product of the further oxidation of amino aldehyde. In the next step, the resulting amino aldehyde reacts with DHAP catalyzed by recombinant
His-tagged rhamnulose-1-phosphate aldolase (RhuA) from *Escherichia coli* to provide the phosphorylated aldol product, Cbz-aminopolyol. In order to improve productivity and reduce enzyme consumption, some aspects like enzyme immobilization, reactor configuration and reaction medium were analyzed.

![Enzymatic synthesis of Cbz-aminopolyol.](image)

**Figure 7.1.1** Enzymatic synthesis of Cbz-aminopolyol.

### 7.2 Methods

#### 7.2.1 Preparation of the supports for immobilization

##### 7.2.1.1 Preparation of glyoxyl- and MANA-agarose gels

Glyoxyl- and MANA-agarose gels were prepared according to the procedure described in the Section 6.2.2.

##### 7.2.1.2 Preparation of Co-IDA

Co\(^{2+}\)-charged metal-chelate supports (90 µm mean particle size) were prepared from fast flow chelating sepharose purchased from Amersham Biosciences (Uppsala, Sweden). 20 ml of gel abundantly washed with Mili Q water were incubated for 12 h in 60 ml of 0.2 M CoCl\(_2\) at pH 4.7. Then, the support was abundantly washed with Mili Q water and stored in 20% ethanol at 4ºC.
7.2.2 Immobilization of enzymes

All the immobilization processes were monitored as it was explained in the Section 6.2.9.

7.2.2.1 Immobilization of CPO by covalent attachment on MANA-agarose

Covalent immobilization of CPO on MANA-agarose by carbodiimide coupled method was performed according to the procedure described in the Section 6.2.6 by adding 10000 U of CPO per ml of MANA.

7.2.2.2 Immobilization of RhuA on Eupergit® C

1 ml of Eupergit® C gel was suspended in 9 ml of 1 M sodium bicarbonate buffer at pH 10 containing 1.11 U/ml of RhuA (final activity of 1 U/ml). The mixture was incubated for 24 h at room temperature (20-25ºC) under mild agitation on roller. Then, the support was vacuum-filtered and washed. Finally, the excess of epoxide groups on the matrix were blocked by incubation with 0.2 M β-mercaptoethanol for 4 h at 4ºC.

7.2.2.3 Immobilization of RhuA by adsorption on MANA-agarose

1 ml (1.07 g) of highly derivatized MANA-agarose gel was suspended in 9 ml of 10 mM buffer (sodium phosphate or sodium bicarbonate) of known pH value (5.0-9.0). Once mixed and before adding the enzyme, readjusting the pH value was necessary since the presence of high amine groups density on the support causes an increase of pH. Then, 50 U of RhuA were added and left to immobilize on the support under mild agitation on a roller at room temperature.

7.2.2.4 Immobilization of RhuA by covalent attachment on MANA-agarose

For the multipoint covalent attachment of RhuA on MANA-agarose, the first step was the ionic adsorption of the enzyme (5-10 U per ml of the support) on the support for 0.75-2 h. Then carbodiimide was added in a final concentration of 50 mM and the mixture was left on roller agitation during different intervals of time (3-4 h). Finally, NaCl was added in final concentration of 1 M in order to eliminate the non-covalently bound enzyme and left on mild agitation during 1 h.
Chapter 7.

7.2.2.5 Immobilization of RhuA on glyoxyl-agarose

1 ml (1.07 g) of highly derivatized glyoxyl-agarose gel was suspended in 9 ml of 50 mM sodium bicarbonate buffer at pH 10.0. Then, between 10 and 300 U of RhuA were added and left to immobilize on the support under mild agitation on a roller at room temperature (20-25ºC). Then 1 mg of NaBH₄ was added and left on mild agitation for 30 min.

7.2.2.6 Immobilization of RhuA on Co-IDA

1 ml (1 g) of Co-IDA was suspended in 9 ml of 50 mM sodium phosphate buffer containing 300 mM NaCl and 20 mM imidazole at pH 8. Then, between 4 and 300 U of RhuA were added and left to immobilize on the support under mild agitation on a roller at 4ºC.

7.2.3 Degradation of DHAP

For the study of chemical degradation of DHAP, 20 mM Cbz-ethanolamine and 30 mM DHAP were dissolved in 100 mM acetate buffer and pH value was readjusted to pH 6.0 in a final volume of 5 ml at 4ºC. For the study of enzymatic degradation of DHAP 150 U of soluble RhuA were added in the same conditions and same reactor volume. Aliquots were withdrawn periodically in order to measure DHAP concentration (Section 3.5.2) and RhuA activity (Section 3.4.2).

7.2.4 One-pot reaction

For reactions performed in aqueous medium 20 mM Cbz-ethanolamine and 30 mM DHAP were dissolved in 100 mM acetate buffer and pH value was readjusted to pH 6.0 in a final volume of 20 ml. For reactions performed in cosolvent systems, 100 mM Cbz-ethanolamine and 150 mM DHAP were dissolved in the same concentration of buffer containing 5% (v/v) dioxane. Then, 500 U/ml of CPO and 30 U/ml of RhuA were added. In case of immobilized enzymes, the reaction contained 2 ml of MANA-agarose with CPO (Section 6.2.6) and 2 ml of support with RhuA (prepared by adding 300 U per ml of support). The reactions were performed at 4ºC in case of aqueous reaction or at 8ºC in
case of cosolvent system and started by adding \( t\)-BuOOH continuously at a rate of 18 µl/h by means of a single-syringe automatic microburette (Crison Instruments, Spain). Concentration of \( t\)-BuOOH stock solution was 3.33 M in order to reach peroxide addition rates of 3 mM/h. With the purpose of reuse of immobilized enzymes, after the end of the first reaction, they were separated by vacuum filtration, washed with 100 mM acetate buffer pH 6.0 and introduced to the second reaction.

Discontinuous reactions were performed following two types of reactor configuration. The stirred tank reactor (STR) consisted of a 25 ml glass reactor (Alco S.A., Spain) stirred mechanically (Figure 7.2.1 A and C). The recirculated packed-bed reactor (RPBR) consisted of a 10 ml glass column reactor (Alco S.A., Spain) linked to a stirred reservoir (Figure 7.2.1 B and D). Reaction medium was recirculated through the system using a variable-speed peristaltic pump from Masterflex (Cole-Palmer Instrument Co, Vernon Hills, IL, USA) at a flow rate of 2 ml/min. The column was packed with 4 ml of homogenous mixture of CPO-MANA and RhuA-CoIDA derivatives (2 ml of each one).

Reaction samples were withdrawn periodically and analyzed by liquid chromatography in order to quantify product and substrates concentrations and enzymatic activities. In case of packed-bed reactor, the samples were withdrawn from the reservoir and the measurement of CPO and RhuA activities was performed only at the beginning and the end of the reaction. At the moment when low DHAP concentration was measured, another pulse was added, with the same concentration than the initial one. Each measurement was carried out in duplicate.

Conversion and product yields were defined respectively as the percentage of consumed Cbz-ethanolamine or products, respect to the initial Cbz-ethanolamine concentration.
Figure 7.2.1 Scheme and experimental montage of one-pot reactors with immobilized enzymes: (A) and (C) stirred-tank reactor and (B) and (D) recirculated packed-bed reactor.
7.2.5 **Residence time distribution**

The residence time distribution was analyzed for both reactor configurations (Figure 7.2.2) using immobilized enzymes. For that, the reactors were operated in continuous mode with no addition of t-BuOOH, by using a peristaltic pump (IPC High Precision Multichannel Dispenser, ISMATEC, Germany) which permitted flow rates of the inlet (distilled water) and outlet fluids of 0.08 ml/min (HRT of 250 min) (Figure 7.2.2). The reactors contained 2 ml of each immobilized enzyme preparation in a final reaction volume of 20 ml. The stimulus-response experiments were performed by the addition of 0.8 g of NaCl as a tracer in a single pulse. The conductivity of the fluid leaving the reactor was measured periodically with a Crison Micro CM 2100 conductimeter. The concentration of the tracer was then determined using external standard method by means of a prior calibration with samples of known concentration.

![Scheme of continuous reactors for the determination of RTD with immobilized enzymes: (A) stirred-tank reactor and (B) recirculated packed-bed reactor.](image-url)
7.2.6 Identification of Cbz-aminopolyol by LC-MS

Cbz-aminopolyol ((3R)-5-[[{(Benzyloxy)carbonyl]amino}-5-deoxy-1-O-phosphonopent-2-ulose) was identified by Bruker microOTOF-Q Mass Spectrometer (Bruker Daltonik, Bremen, Germany), equipped with an electrospray ionization source from Bruker and coupled with 1200 RR HPLC from Agilent (Agilent Technologies, Santa Clara, CA, USA). The analytic column was a reversed-phase column X Bridge C18, 5 μm, 4.6x250 mm from Waters (Wexford, Ireland). The solvent system consisted of solvent A, composed of 2.5% v/v formic acid (HCOOH) in H$_2$O, and solvent B, composed of 8% v/v HCOOH in H$_2$O/CH$_3$CN 1:4. The solvents were eluted at a flow rate of 0.7 ml/min using a gradient from 20% B to 36% B over 20 min and peaks were detected at 270 nm. The mass spectrometer was operated both in the positive and negative ion mode.

7.3 Results and discussion

7.3.1 Enzymatic reaction coupling for the synthesis of Cbz-aminopolyol

First difficulty that has to be solved when implementing one-pot multienzymatic synthesis is compatibility of the enzymes. Optimal conditions for the coupled synthesis of Cbz-aminopolyol starting from Cbz-amino alcohol had to be chosen looking for a compromise between the optimal conditions for each reaction. They should allow simultaneous reactions in order to prevent the formation of undesired side products. Chloroperoxidase catalyzes halide independent reactions at pH 5.0-6.0 and is very instable at neutral and alkaline pH values (Colonna et al. 1999; Thomas et al. 1970; van Deurzen et al. 1997c). RhuA has a very sharp pH optimum at pH of 7.5, but it is active at the range of pH 6.0-9.0 (Chiu and Feingold 1969). Therefore, pH 6.0 was chosen as optimal for the coupled reaction. Temperature of 4ºC was selected in order to prevent the chemical degradation of DHAP (Suau et al. 2006). Finally, the addition of t-BuOOH had to be performed in pulses at the rate of 3 mM/h, in order to minimize the inactivation of CPO caused by an excess of peroxide (Chapter 4).
The profile of the one-pot synthesis of Cbz-aminopolyol catalyzed by CPO and RhuA is shown in Figure 7.3.1. The end of reaction was considered when no CPO activity was detected in the reaction medium. Measuring the activity of RhuA during the reaction was not possible, due to the presence of high concentration of DHAP that interfered the activity assay (Section 3.4.2). Therefore, its activity could be measured at the beginning of the reaction before adding DHAP and at the end of reaction when only low concentration of DHAP was present in the reaction medium. At this point RhuA preserved almost 80% of its initial activity.

The reaction coupling resulted in a very low concentration of both intermediate and non-desired subproduct, i.e. Cbz-glycinal and Cbz-glycine, respectively (Experiment 1, Table 7.3.1 and 7.3.2). Besides, a new compound in the HPLC chromatograms was identified as Cbz-aminopolyol by LC-MS using micrOTOF-Q having all the experimental results (isotropic distribution both in negative and positive ion mode) coherent with the exact molecular mass of Cbz-aminopolyol of 363 (Table 7.3.3). At the end of the reaction, major part of the consumed Cbz-ethanolamine was further converted to Cbz-aminopolyol. This fact confirms the success of the reaction coupling, as when no RhuA was presented 8.8% of Cbz-ethanolamine had yielded Cbz-glycine as subproduct instead of Cbz-glycinal (Figure 4.3.2 B from the Section 4.3.1.1). On the other hand, the relatively low initial reaction rate of 0.33 mM/h of Cbz-ethanolamine oxidation was due to the very fast inactivation of CPO, which caused the stop of the reaction at 32.9% of Cbz-ethanolamine conversion. This is probably due to the non-optimal conditions of pH and temperature used in the present reaction (pH 6.0 and 4ºC), as it was previously observed that pH 5.0 and room temperature are more appropriate for the performance of the reaction catalyzed by CPO, and consequently its stability (Chapter 4).

* All the results of the coupled reactions from this Chapter are summarized in Tables 7.3.1 and 7.3.2, in order to facilitate the corresponding comparisons.
Figure 7.3.1 Time course for one-pot oxidation of Cbz-ethanolamine (●) to Cbz-glycinal (■) and Cbz-glycine (▲) catalyzed by soluble CPO adding t-BuOOH at a rate of 3 mM/h and aldol addition of DHAP (○) to Cbz-glycinal to yield Cbz-aminopolyol (▼) catalyzed by soluble RhuA in STR. 500 U/ml of CPO and 30 U/ml of RhuA were added to the reaction mixture containing 20 mM Cbz-ethanolamine and 30 mM DHAP in 100 mM acetate buffer pH 6.0 in a total reaction volume of 20 ml at 4ºC. Data points represent duplicate average measurements; error bars represent the standard deviation (Experiment 1 in Table 7.3.1 and 7.3.2).
Table 7.3.1: Conditions for one-pot synthesis of Cbz-aminopolyol from Cbz-ethanolamine catalyzed by 500 U/ml of CPO and 30 U/ml of RuA. tert-butyl hydroperoxide was added at 3 mM/h and 30 mM or 150 mM of DHAP was added when concentration decreased to zero in aqueous or cosolvent reaction, respectively. The reactions were performed in a final volume of 20 ml at pH 6.0 and 4°C in the case of aqueous reaction or 8°C in the case of cosolvent system.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>CPO support(a)</th>
<th>RhuA support(a)</th>
<th>Reactor configuration(b)</th>
<th>Reaction medium</th>
<th>Cbz-ethanolamine concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>STR</td>
<td>Sodium acetate 100 mM</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>MANA-agarose</td>
<td>-</td>
<td>STR</td>
<td>Sodium acetate 100 mM</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>MANA-agarose</td>
<td>Glyoxyl-agarose</td>
<td>STR</td>
<td>Sodium acetate 100 mM</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>MANA-agarose</td>
<td>Co-IDA</td>
<td>STR</td>
<td>Sodium acetate 100 mM</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>MANA-agarose</td>
<td>Co-IDA</td>
<td>RPBR</td>
<td>Sodium acetate 100 mM</td>
<td>20</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>STR</td>
<td>5% dioxane in sodium acetate 100 mM</td>
<td>100</td>
</tr>
<tr>
<td>7</td>
<td>MANA-agarose</td>
<td>Co-IDA</td>
<td>STR</td>
<td>5% dioxane in sodium acetate 100 mM</td>
<td>100</td>
</tr>
</tbody>
</table>

(a) No support for CPO and/or RuA means that the enzyme was added in soluble form.

(b) STR: stirred tank reactor; RPBR: recirculated packed-bed reactor.
Table 7.3.2 Results for one-pot synthesis of Cbz-aminopolyol from Cbz-ethanolamine catalyzed by 500 U/ml of CPO and 30 U/ml of RhuA. Conditions of each experiment are summarized in Table 7.3.1.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Reaction time (h)</th>
<th>Cbz-ethanolamine conversion (%)</th>
<th>Cbz-glycinal yield (%)</th>
<th>Cbz-glycine yield (%)</th>
<th>Cbz-aminopolyol yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>32.9</td>
<td>4.5</td>
<td>0</td>
<td>23.6</td>
</tr>
<tr>
<td>2</td>
<td>72</td>
<td>95.7</td>
<td>5.4</td>
<td>9.1</td>
<td>73.9</td>
</tr>
<tr>
<td>3</td>
<td>72</td>
<td>94.0</td>
<td>7.8</td>
<td>29.3</td>
<td>56.9</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>90.4</td>
<td>7.6</td>
<td>8.7</td>
<td>69.1</td>
</tr>
<tr>
<td>5</td>
<td>192</td>
<td>87.2</td>
<td>2.9</td>
<td>12.3</td>
<td>71.9</td>
</tr>
<tr>
<td>6</td>
<td>48</td>
<td>35.5</td>
<td>1.8</td>
<td>0.3</td>
<td>33.2</td>
</tr>
<tr>
<td>7</td>
<td>72</td>
<td>94.1</td>
<td>2.1</td>
<td>5.4</td>
<td>86.6</td>
</tr>
</tbody>
</table>
Table 7.3.3 Coherency between the experimental and theoric values of the exact molecular mass of Cbz-aminopolyol.

<table>
<thead>
<tr>
<th>Cluster</th>
<th>m/z (theoric value)</th>
<th>m/z (experimental value)</th>
<th>Error (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[M+H]^+</td>
<td>364.0792</td>
<td>364.0788</td>
<td>-1.10</td>
</tr>
<tr>
<td>[M+Na]^+</td>
<td>386.0611</td>
<td>386.0603</td>
<td>-2.07</td>
</tr>
<tr>
<td>[M-H_2O+H]^+</td>
<td>346.0686</td>
<td>346.0685</td>
<td>-0.29</td>
</tr>
<tr>
<td>[M-H]^−</td>
<td>362.0646</td>
<td>362.0655</td>
<td>2.49</td>
</tr>
<tr>
<td>[2M-H]^−</td>
<td>725.1366</td>
<td>725.1345</td>
<td>-2.90</td>
</tr>
</tbody>
</table>

As it is observed in Figure 7.3.1, a decrease of DHAP concentration was more rapid than that of Cbz-ethanolamine, suggesting that one part of it was being degraded at these conditions, either enzymatically or chemically (Figure 1.4.5 in the Section 1.4.1.1). The chemical degradation can be minimized by using low temperatures (Suau et al. 2006), but additionally, DHAP is unstable at neutral and acidic pH values (Sanchez-Moreno et al. 2004). Therefore, the DHAP degradation by chemical and enzymatic pathways was studied (Figure 7.3.2). No chemical degradation of DHAP was observed after 24 h of incubation in these conditions, while the enzymatic degradation catalyzed by RhuA caused the decrease in the concentration of 67% after only 5 h of incubation. Therefore, DHAP had to be added in pulses when rapid decrease in its concentration was observed in the coupled reactions.
Chapter 7.

Figure 7.3.2 Degradation of DHAP in the reaction medium containing 20 mM Cbz-ethanolamine in 100 mM sodium acetate buffer at pH 6.0 by chemical mechanism (○) and by enzymatic mechanism in presence of 30 U/ml of RhuA (●) at 4°C. Data points represent duplicate average measurements; error bars represent the standard deviation.

7.3.2  **Effect of enzymatic immobilization on the synthesis of Cbz-aminopolyol**

Enzymatic stability can be drastically improved by enzyme immobilization on solid supports as it was concluded in Chapter 6. Hence, increased compatibility and stability of the enzymes in the common reaction medium can be achieved, resulting beneficial in terms of process productivity.

7.3.2.1  **Coupled enzymatic reaction catalyzed by immobilized CPO and soluble RhuA**

Immobilization of CPO on MANA-agarose supports was proved to enhance its stability towards the peroxide dependent inactivation and therefore, important improvement of the substrate conversion was achieved when catalyzing the oxidation of Cbz-ethanolamine to Cbz-glycinal, as it was shown in Chapter 6. Even so, the accumulation of Cbz-glycinal in the reaction medium accelerated its further oxidation to Cbz-glycine.
The one-pot coupled reaction catalyzed by CPO immobilized on MANA-agarose and soluble RhuA resulted in almost complete consumption of Cbz-ethanolamine with final yield of Cbz-aminopolyol of 73.9% (Figure 7.3.3). Although it could have been expected that the initial rate of the reaction catalyzed by immobilized CPO should be lower than that obtained with soluble enzyme due to diffusion limitations, it was improved from 0.33 mM/h to 0.65 mM/h of Cbz-ethanolamine, due to the fact that immobilization allowed CPO to preserve the enzymatic activity. As a consequence, a higher conversion was obtained. On the other side, yields of Cbz-glycinal and Cbz-glycine increased to the values of 5.4 and 9.1%, respectively, as a result of the loss of RhuA activity during the prolonged reaction time, and consequently decreased reaction rate of the aldol addition step (Experiment 2 in Table 7.3.1 and 7.3.2). No residual RhuA activity was measured at the end of the reaction time.

![Graph](image)

**Figure 7.3.3** Time course for one-pot oxidation of Cbz-ethanolamine (●) to Cbz-glycinal (■) and Cbz-glycine (▲) catalyzed by CPO-MANA adding t-BuOOH at a rate of 3 mM/h and aldol addition of DHAP to Cbz-glycinal to yield Cbz-aminopolyol (▼) catalyzed by soluble RhuA in STR. 2 ml of CPO-MANA (500 U/ml of reaction) and 30 U/ml of RhuA were added to the reaction mixture containing 20 mM Cbz-ethanolamine and 30 mM DHAP in 100 mM acetate buffer pH 6.0 in a total reaction volume of 20 ml at 4ºC. Data points represent duplicate average measurements; error bars represent the standard deviation (Experiment 2 in Table 7.3.1 and 7.3.2).
7.3.2.2 Immobilization of RhuA

In order to prevent the loss of RhuA activity, the immobilization of RhuA was proposed using different immobilization methods (Tables 7.3.4 and 7.3.5). When RhuA was immobilized on Eupergit® C (Experiment 1 in Table 7.3.4 and 7.3.5), almost complete activity loss was observed after 24 h of incubation. Immobilization on MANA-agarose was studied by two different methods: ionic adsorption and covalent attachment. Ionic adsorption was studied at the pH range of 5.0-9.0 in order to determine the most appropriate conditions which would favor the ionic interactions between the enzyme and the support (Experiments 2-6 in Table 7.3.4 and 7.3.5). However, after 24 h of incubation, very low immobilization yields were accomplished on all pH values studied (within 46 and 56%), with relatively low activity retentions. The best result was obtained at pH 7.0 at which the activity retention reached 35.4%, probably due to the fact that this pH value represents the optimal one for RhuA. When further trying to convert the ionic adsorption to the covalent attachment by coupling the process with carbodiimide activation of carboxyl groups, the immobilization yield was importantly improved (Experiments 7-8 in Table 7.3.4 and 7.3.5), but at the same time almost complete inactivation of the enzyme occurred, probably due to the enzyme inactivation by carbodiimide or due to acidic pH values required for carbodiimide coupling. The medium for the carbodiimide mediated covalent attachment had to be slightly acidic (pH=5.0-6.0) in order to allow the carboxylic groups to act as nucleophylic agents on protonated carbodiimide (Figure 6.3.4 from the Section 6.3.2.3). Immobilization by covalent attachment on glyoxyl-agarose gels occurs via formation of Schiff base bonds between the amino groups on the enzyme surface and aldehyde groups of the agarose (Guisan 1988; Suau et al. 2005). Good immobilization yield (89.7%) with activity retention of 37.2% was reached when RhuA was immobilized by this method (Experiment 9 in Table 7.3.4 and 7.3.5). Finally, RhuA was immobilized by affinity chromatography on agarose support functionalized with iminodiacetic acid (IDA) as chelating agent, and loaded with Co^{2+} ions (Co-IDA) for the attachment of recombinant His-tagged enzymes, by the method developed by Ardao et al. (Ardao et al. 2011) (Experiments 11 and 12 in Table 7.3.4 and 7.3.5). This immobilization method permitted more than 90% immobilization with activity
Synthesis of aminopolyols in one-pot multienzyme oxidation and aldol addition

retention of in a range of 94.7 and 57.4% when adding 4 and 12 U of RhuA per ml of the support. The results of the immobilization of RhuA on Co-IDA were in accordance with those reported by Ardao et al., since it was demonstrated that for the natural reaction, the internal diffusion limitations appeared for enzyme loads higher than 10 U of RhuA per ml of Co-IDA (Ardao et al. 2011).

Table 7.3.4 Conditions for the different immobilization methods used for the immobilization of RhuA.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Support</th>
<th>Immobilization method</th>
<th>T (ºC)</th>
<th>Buffer</th>
<th>pH</th>
<th>Initial activity (U/ml support)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Eupergit® C</td>
<td>Covalent attachment</td>
<td>20-25</td>
<td>1 M sodium bicarbonate</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td>10 mM sodium phosphate</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td>10 mM sodium phosphate</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>MANA-agarose</td>
<td>Adsorption</td>
<td>20-25</td>
<td>10 mM sodium phosphate</td>
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<td>50</td>
</tr>
<tr>
<td>5</td>
<td></td>
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<td></td>
<td>10 mM sodium phosphate</td>
<td>8</td>
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</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td>10 mM sodium bicarbonate</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Covalent attachment</td>
<td></td>
<td>20-25</td>
<td>10 mM sodium phosphate</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>(50 mM EDAC)</td>
<td></td>
<td></td>
<td>10 mM sodium phosphate</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>9</td>
<td>Glyoxyl-agarose</td>
<td>Covalent attachment</td>
<td>20</td>
<td>50 mM sodium bicarbonate</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td>50 mM sodium bicarbonate</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td></td>
<td>50 mM sodium phosphate</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Co-IDA</td>
<td>Affinity attachment</td>
<td>4</td>
<td>50 mM sodium phosphate, 300 mM NaCl, 20 mM imidazole</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>300</td>
<td></td>
</tr>
</tbody>
</table>
Table 7.3.5 Immobilization time, yield and retained activity of RhuA immobilized on different supports. Conditions of each experiment are summarized in Table 7.3.4.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Immobilization time (h)</th>
<th>Immobilization yield (%)</th>
<th>Retained activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24</td>
<td>97.3</td>
<td>4.7</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>50.3</td>
<td>22.2</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td>56.0</td>
<td>26.8</td>
</tr>
<tr>
<td>4</td>
<td>24</td>
<td>47.7</td>
<td>35.4</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>50.3</td>
<td>21.0</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>46.0</td>
<td>13.0</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>75.4</td>
<td>1.4</td>
</tr>
<tr>
<td>8</td>
<td>4.75</td>
<td>95.1</td>
<td>13.9</td>
</tr>
<tr>
<td>9</td>
<td>3.5</td>
<td>89.7</td>
<td>37.2</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>61.8</td>
<td>4.0</td>
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<tr>
<td>11</td>
<td></td>
<td>95.0</td>
<td>94.7</td>
</tr>
<tr>
<td>12</td>
<td>0.75</td>
<td>99.9</td>
<td>57.4</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>98.4</td>
<td>3.1</td>
</tr>
</tbody>
</table>

Then, thermostability of RhuA immobilized by different methods was evaluated, in order to check out which one of the immobilization methods studied produced the most stable immobilized system. The experiments were carried out in the conditions of the reaction of synthesis (100 mM sodium acetate buffer pH 6.0), but by incubating the enzyme at the temperature of 60°C in order to shorten the duration of the experiments, and were compared to the stability of the soluble enzyme at the same conditions (Figure 7.3.4). All the immobilized enzyme systems had improved stability at these conditions compared to the soluble enzyme, except for RhuA immobilized on Eupergit® C. The biggest improvements were achieved for RhuA immobilized on glyoxyl-agarose (Experiment 9 in Table 7.3.5 and Figure 7.3.4) and Co-IDA (Experiment 11 in Table 7.3.5 and Figure 7.3.4), which had 4.25 and 5-fold higher half-lives than the soluble enzyme respectively. The stability improvements in cases of covalently
immobilized RhuA, were probably due to the higher rigidity of the enzyme molecule after the immobilization which prevents its denaturation by unfolding on high temperatures.

Therefore, based on the results of immobilization and stability, two immobilization methods were chosen as the most appropriate ones for the immobilization of RhuA: covalent attachment on glyoxyl-agarose and affinity attachment on Co-IDA.

The method for the immobilization on glyoxyl-agarose had been previously applied in the immobilization of fuculose-1-phosphate aldolase (FucA), which is another enzyme from the group of DHAP dependent aldolases (Suau et al. 2005). The loss of activity during immobilization is caused by the aggressive conditions of the process, as it includes a borohydride reduction of both the Schiff base bonds and the remaining aldehyde groups of the support (Figure 7.3.5) (Alvaro et al. 1990; Guisan 1988; Mateo et al. 2005).
Chapter 7.

Figure 7.3.5 Scheme of enzyme immobilization by covalent attachment on glyoxyl agarose.

On the other side, activity losses when immobilizing enzymes on Co-IDA (Figure 7.3.6) are usually very low since the His-tag is normally located at the terminal end of the sequence, far from the active site of the enzyme (Ardao et al. 2006; Ardao et al. 2011). Both values of activity retention and stability suggest that affinity immobilization on Co-IDA is a more appropriate method for RhuA immobilization.

Figure 7.3.6 Scheme of enzyme immobilization by affinity attachment on Co-IDA.

For the purpose of its use in the coupled reaction, the immobilization via these two methods was carried out by using RhuA activity of 300 U/ml of the support (Experiments 10 and 13 in Table 7.3.4 and 7.3.5), in order to achieve a final activity of 30 U/ml once added to the reaction in the relation 1:10 (immobilized enzyme:total reaction volume).

When RhuA was immobilized on glyoxyl-agarose, the immobilization yield decreased from 89.7% (for 10 U/ml of support) to 61.8% when using a high enzymatic load (300 U/ml support), probably due to the support saturation. When immobilized on Co-IDA high immobilization yields (95.0-99.9%) were obtained for both low and high loads of
Synthesis of aminopolyols in one-pot multienzyme oxidation and aldol addition

enzyme. In both cases, measured retained enzymatic activity decreased drastically at high enzyme loadings because internal diffusion of substrates and products through the interior of the beads limits the global reaction rate (Ardao et al. 2011; Engasser and Horvath 1973; Horvath and Engasser 1974b). Since the diffusion limitations importantly depend on reaction rate, for any other enzymatic reaction with lower reaction rate than the one used for the enzymatic activity measurement, diffusion limitations should appear at higher enzymatic loads or even will not appear.

7.3.2.3 Coupled enzymatic reaction catalyzed by immobilized CPO and RhuA

Practically total conversion of Cbz-ethanolamine was achieved when RhuA on glyoxyldagarose was applied together with CPO immobilized on MANA-agarose (Experiment 3 in Table 7.3.1 and 7.3.2). However, yield of Cbz-aminopolyol was hardly 57% and almost 30% of the subproduct Cbz-glycine was obtained, although RhuA was still partially active. The delay of the aldol addition step compared to the step of Cbz-glycinal synthesis could be due to the corresponding activity loss of RhuA during the process of immobilization (Experiment 9 in Table 7.3.4 and 7.3.5), lower immobilization yields observed for high enzyme loadings (Experiment 10 in Table 7.3.4 and 7.3.5) and additionally by presence of high diffusion limitations for the immobilized RhuA. In a multienzyme reaction where all the enzymes are immobilized, the diffusion of intermediate compounds usually has an important influence on the overall reaction performance, as they are present at low concentrations (Ho and Kostin 1974).

When RhuA was immobilized on CoIDA (Experiment 4 in Table 7.3.1 and 7.3.2), the final yield of Cbz-aminopolyol was further improved to 69.1% while yields of Cbz-glycinal and Cbz-glycine were maintained above 10% after 72 h, which corresponds with the time when no further conversions were detected. The time-course of the coupled reaction catalyzed by CPO-MANA and RhuA-CoIDA is shown on Figure 7.3.7. Having a very fast consumption of DHAP after only 6 h of incubation, another pulse of 30 mM DHAP had to be added in order to avoid limitation of aldol addition step due to low DHAP concentration.
Therefore, taking into account the result of immobilization and the coupled reaction performed with RhuA immobilized via these two methods, it can be concluded that affinity immobilization on Co-IDA definitely represents the most suitable method for the immobilization of RhuA, allowing good immobilization yields and overall reaction performance.

![Figure 7.3.7](image)

**Figure 7.3.7** Time course for one-pot oxidation of Cbz-ethanolamine (●) to Cbz-glycinal (■) and Cbz-glycine (▲) catalyzed by CPO-MANA adding t-BuOOH at 3 mM/h and aldol addition of DHAP (○) to Cbz-glycinal to yield Cbz-aminopolyol (▼) catalyzed by RhuA-CoIDA in STR. 2 ml of CPO-MANA and 2 ml of RhuA-CoIDA were added to the reaction mixture that contained 20 mM of Cbz-ethanolamine and 30 mM DHAP in 100 mM acetate buffer pH 6.0 in a total reaction volume of 20 ml at 4ºC. Data points represent duplicate average measurements; error bars represent the standard deviation (Experiment 4 in Table 7.3.1 and 7.3.2).

Finally, the possibility of the reuse of immobilized enzymes was studied (Figure 7.3.8). However, upon the reuse, a yield of Cbz-aminopolyol of only 6.0% was reached after 70 h of the reaction, with no Cbz-glycinal or Cbz-glycine produced, probably due to the inactivation of the enzymes.
Figure 7.3.8 Conversion (black bars) and yield of Cbz-aminopolyol (grey bars) after the first and the second use of the immobilized enzymes for the coupled synthesis of aminopolyols. 2 ml of CPO-MANA (500 U/ml of reaction) and 2 ml of RhuA-CoIDA (30 U/ml of reaction) were added to the reaction mixture that contained 20 mM of Cbz-ethanolamine and 30 mM DHAP in 100 mM acetate buffer pH 6.0 in a total reaction volume of 20 ml at 4ºC. tert-BuOOH was added at the rate of 3 mM/h. Data points represent duplicate average measurements; error bars represent the standard deviation.

7.3.3 **Effect of the reactor configuration on the synthesis of Cbz-aminopolyol**

In stirred tank reactors (Figure 7.2.1 A), vigorous mixing was applied in order to eliminate the external diffusion limitations. But the vigorous mixing may damage the immobilized enzyme particles and also affect the enzymatic stability (Pitcher 1978). A recirculated packed-bed reactor (RPBR) was designed to overcome this drawback (Figure 7.2.1 B). The configuration consisted in a discontinuous reactor where the immobilized enzyme was confined in a packed bed; the reaction medium containing substrates was forced to pass down through the bed in order to get in contact with both enzymes, which were preserved far from the mixing system and the place where peroxide was being added.
Flow rate through the packed bed represents one of the main factors which affect the external mass transfer rate. Considering that the packed bed contained 4 ml of the immobilized enzymes mixture (2 ml of each one) the maximal flow rate was assayed minimizing the pressure drop and compression of the bed, which could cause the formation of preferential ways or dead zones reducing the effective volume of the reactor (Buchholz 1982; Richter et al. 1996). The flow rate was determined to be 2 ml/min for the total reaction volume of 20 ml and the number of recirculations of overall reaction volume was 6 recirculations per hour.

In order to compare the flux patterns for RPBR and STR, the analysis of residence time distribution (RTD) was performed. This method permits determine how long individual molecules stay in the vessel and therefore, diagnose the flow model and the reactor behavior (e.g. channeling, dead zones, etc) (Levenspiel 1999). The stimulus-response experiments were performed by adapting the system to a continuous operation with an inlet-outlet flow rate of 0.08 ml/min corresponding to a HRT of 250 min (Figure 7.2.2). This flow was chosen in order to allow 25 recirculations of reaction medium in RPBR per one HRT. NaCl (0.8 g) was added as a tracer in a single pulse at the beginning of the experiment in order to reach the maximal concentration in the outlet of 40 g/l, and its concentration was monitored by measuring the conductivity of the outlet fluid. The obtained results and the response functions E(t) for STR and RPBR are shown in Table 7.3.6 and in Figure 7.3.9.

Small peaks on the E(t) curve of RPBR could be due to the existence of dead zones or the recirculation of the fluid through the system. Despite of that, it is obvious that its performance resembles the one of STR, and that both configurations show a behavior which is very proximate to the complete mixing. Apart from this, results of mass balance were consistent for both reactor configurations. The calculated values of HRT were 263 and 249 min for STR and RPBR, respectively.
Table 7.3.6 Results of the stimulus-response experiments for STR and RPBR reactors.

<table>
<thead>
<tr>
<th></th>
<th>STR</th>
<th>RPBR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Response function</td>
<td>$c_{out}(t) = 42.6656 \cdot \exp(-0.2280 \cdot t)$ $R^2 = 0.9945$</td>
<td>$c_{out}(t) = 38.1355 \cdot \exp(-0.2410 \cdot t)$ $R^2 = 0.9935$</td>
</tr>
<tr>
<td>$\tau$ calculated from the regression: $c(t) = c_0 \cdot \exp(-t/\tau)$</td>
<td>263 min</td>
<td>249 min</td>
</tr>
<tr>
<td>mass of the tracer, $m$, calculated from the equation: $m_o / q_o = \int_0^\infty c_{out}(t)dt$</td>
<td>0.887 g</td>
<td>0.864 g</td>
</tr>
<tr>
<td>relative error</td>
<td>9.8%</td>
<td>7.4%</td>
</tr>
<tr>
<td>RTD function</td>
<td>$E(t) = c(t) / (m_o / q_o)$</td>
<td>$E(t) = 0.2280 \cdot \exp(-0.2280 \cdot t)$</td>
</tr>
<tr>
<td>the variance of the distributions, $\sigma^2$ calculated from the equation: $\sigma^2_{\tau} = \int_0^\infty t^2 \cdot E(t)dt - \overline{\tau}^2_{mean}$</td>
<td>19.24 h$^2$</td>
<td>17.21 h$^2$</td>
</tr>
</tbody>
</table>
Figure 7.3.9 Profiles of residence time distribution curves for STR (A) and RPBR (B). The exponential regression is represented with solid line, while the experimental curve is represented with dotted line.
Coupled reaction in a RPBR was performed using CPO attached to MANA-agarose and RhuA immobilized on Co-IDA support (Experiment 5 in Table 7.3.1 and 7.3.2). Cbz-ethanolamine conversion and Cbz-aminopolyol yield were very similar to those obtained for STR (Experiment 4 in Table 7.3.1 and 7.3.2). However, a slightly higher yield of Cbz-glycine was reached and a decrease of reaction rate in RPBR (0.29 mM/h) compared to that value in STR (0.54 mM/h) was observed, resulting in higher reaction times. These facts pointed out the probable presence of external diffusion limitations in the packed bed, which had a higher effect on the aldol addition rate due to the low concentration of Cbz-glycinal in the reaction medium. Additionally, particle Reynolds number was calculated for the packed bed system taking into account an average particle diameter of 150 µm. The value resulted to be $Re_p = 0.06$, which confirmed the presence of very low external mass transfer.

7.3.4 Effect of reaction medium on the synthesis of Cbz-aminopolyol

Finally, in order to improve reaction productivity, use of water-miscible organic solvents was proposed. It was previously demonstrated in the section 4.3.3 (Table 4.3.6 and Figure 4.3.10) that the Cbz-glycinal productivity in the CPO-catalyzed Cbz-ethanolamine oxidation can be drastically improved in a reaction medium containing 5% dioxane (v/v), by working with higher substrate concentrations. Although it could have been expected that the presence of the cosolvent would have a negative effect on CPO, its operational stability was improved. This was showed to be due to the high reaction rate and therefore, fast consumption of $t$-BuOOH with very low accumulation in the reaction medium.

Coupled reactions in the presence of dioxane were performed with soluble and immobilized enzymes in STR (Experiments 6 and 7 in Table 7.3.1 and 7.3.2). The conversion of Cbz-ethanolamine in the presence of dioxane 5% (v/v) was very similar to that in aqueous reaction when soluble enzymes were used, although final yield of Cbz-aminopolyol was improved from 23.6% to 33.2% (Experiments 1 and 6 in Table 7.3.1 and 7.3.2 respectively). However, the production of Cbz-aminopolyol was 7-fold higher in the presence of dioxane, improving from 4.72 mM to 33.2 mM due to the
higher solubility of the substrate in the cosolvent system (solubility in 5% (v/v) dioxane at 8ºC was around 100 mM while in the pure buffer this was around 20 mM). The improvement of the reaction performance in a cosolvent system was even more obvious when the reaction was catalyzed by immobilized enzymes (Figure 7.3.10), with a further improvement of Cbz-aminopolyol yield to 86.6%, and a production of 86.6 mM, which is 6.3-fold higher than that obtained in the aqueous reaction when using immobilized biocatalysts (Experiments 7 and 4 in Table 7.3.1 and 7.3.2 respectively). Besides, thanks to the better matching of the reaction rates at these conditions, concentrations of Cbz-glycinal and Cbz-glycine were maintained at a very low level during the experiment, having final yields of 2.1 and 5.4%, respectively.

![Figure 7.3.10 Time course for one-pot oxidation of Cbz-ethanolamine (●) to Cbz-glycinal (■) and Cbz-glycine (▲) catalyzed by CPO-MANA adding t-BuOOH at 3 mM/h and aldol addition of DHAP (○) to Cbz-glycinal to yield Cbz-aminopolyol (▼) catalyzed by RhuA-CoIDA in STR. 2 ml of CPO-MANA (500 U/ml of reaction) and 2 ml of RhuA-CoIDA (30 U/ml of reaction) were added to the reaction mixture that contained 100 mM of Cbz-ethanolamine and 150 mM DHAP in 100 mM acetate buffer pH 6.0 containing 5% (v/v) dioxane in a total reaction volume of 20 ml at 8ºC. Data points represent duplicate average measurements; error bars represent the standard deviation (Experiment 7 in Table 7.3.1 and 7.3.2).](image-url)
The reuse of immobilized enzymes in this cosolvent system allowed the production of Cbz-aminopolyol in the yield of 19.8% (Figure 7.3.11). The produced concentration of 19.8 mM upon the reuse is 16.5-fold higher than upon the reuse in aqueous reaction (1.2 mM). However, after only 1 cycle of reuse, no residual CPO activity was detected, and therefore, the immobilized enzymes could not be reused in a new cycle.

**Figure 7.3.11** Conversion (black bars) and yield of Cbz-aminopolyol (grey bars) after the first and the second use of the immobilized enzymes for the coupled synthesis of aminopolyols in the reaction medium containing 5% dioxane. 2 ml of CPO-MANA and 2 ml of RhuA-CoIDA were added to the reaction mixture that contained 100 mM of Cbz-ethanolamine and 150 mM DHAP in 100 mM acetate buffer pH 6.0 containing 5% (v/v) dioxane in a total reaction volume of 20 ml at 8°C. tert-BuOOH was added at the rate of 3 mM/h. Data points represent duplicate average measurements; error bars represent the standard deviation.
7.4 CONCLUSIONS

In this chapter, the one-pot multienzyme synthesis of Cbz-aminopolyol was studied. The two-step cascade reaction consisted of oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by CPO, and aldol addition of DHAP to Cbz-glycinal catalyzed by RhuA. The one-pot multienzymatic process favored the simplicity of the reaction performance, as well as a high increase of production and yield due to equilibrium shift. Matching the reaction rates prevented the formation of intermediate or side compounds and activity losses derived from the excess of t-BuOOH in the reaction medium. While the use of soluble enzymes in the cascade reaction yielded only 23.6% of Cbz-aminopolyol due to rapid enzyme inactivation, the use of immobilized ones permitted almost complete consumption of Cbz-ethanolamine reaching yield of Cbz-aminopolyol of 69.1% in stirred-tank reactor. The yield of Cbz-aminopolyol of 71.9% was reached in packed-bed reactor, but at much lower reaction rates compared to that in stirred-tank reactor, which was proved to be due to high external diffusion limitations. Furthermore, the production of Cbz-aminopolyol was 18-fold improved when it was catalyzed by immobilized enzymes in presence of 5% (v/v) dioxane compared to that one catalyzed by soluble enzymes in aqueous medium, reaching a value of 86.6 mM (31 g/l), corresponding to the reaction yield of 86.6%.
CONCLUSIONS
From the results presented in this doctoral thesis, the following conclusions can be made:

- Enzymatic oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by chloroperoxidase (CPO) from fungus *Caldariomyces fumago* can be successfully accomplished by using tert-butyl hydroperoxide as an oxidant in aqueous medium. In order to minimize the peroxide dependent inactivation of the enzyme, the optimization of the peroxide addition velocity had to be carried out searching for a compromise between the reaction kinetics and the inactivation rate of CPO. The best result in terms of final Cbz-glycinal yield was achieved when peroxide was added at the rate of 3 mM/h, the yield of Cbz-glycinal reaching the value of 39.1%.

- Even though the conversion of the reaction was significantly improved by adding CPO in pulses along the reaction, the final yield of Cbz-glycinal was limited by the undesired subsequent oxidation to Cbz-glycine.

- The adjustment between the peroxide addition rate and the initial reaction rate was accomplished by using water miscible and immiscible organic solvents, and therefore, the enzyme remained active during longer periods of time. Dioxane added at the concentration of 5% (v/v) was the most appropriate cosolvent for the oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by CPO. In this system almost 6-fold higher concentration (47.6 mM) of Cbz-glycinal was produced compared to the aqueous reaction (7.8 mM).

- The branched-chain amino alcohol, Cbz-alaninol was not a substrate for CPO, due to its sterically constrained active site.

- CPO chemically modified by carbodiimide coupling and the periodate oxidation of the sugar moiety were showed to be better catalysts than native CPO in almost all the aspects. They showed better stability at elevated temperatures and pH values, and as a consequence better performance of the reaction of oxidation of Cbz-ethanolamine was accomplished compared to the reaction catalyzed by native CPO. But, even though many improvements were achieved by chemical
modifications of CPO, the main problem, i.e. low stability in the presence of peroxides, remained unsolved.

- Immobilization of CPO on MANA-agarose - by ionic adsorption, covalent attachment by carbodiimide mediated activation of carboxyl groups of CPO or covalent attachment of oxidized CPO- allowed good activity retentions (within 51 and 77%).

- Drastic improvements in tolerance to t-BuOOH were achieved for CPO immobilized preparations, CPO immobilized by covalent attachment via carbodiimide coupling being the most stable one. This immobilized enzyme system had around 500-fold improved half life compared to the half life of soluble enzyme in the same conditions.

- CPO immobilized by covalent attachment via carbodiimide coupling was proved as a better catalyst for the oxidation of Cbz-ethanolamine to Cbz-glycinal than soluble CPO in terms of reaction conversion, yield of Cbz-glycinal and residual CPO activity at the end of the reaction.

- Oxidation of Cbz-ethanolamine to Cbz-glycinal catalyzed by CPO was successfully coupled to the reaction of aldol addition of produced Cbz-glycinal to DHAP catalyzed by recombinant rhamnulose-1-phosphate aldolase (RhuA) from *Escherichia coli* in one-pot reactor yielding a valued product Cbz-aminopolyol. Operation in one-pot multienzyme cascade reactor permitted the shift of the equilibrium observed in uncoupled reaction and consequently the enhancements of the production and yield of the final product were accomplished.

- The application of immobilization techniques allowed to preserve the activity of the enzymes, Cbz-aminopolyol yield increasing from 23.6% with soluble enzymes to 69.1% with immobilized ones. In recirculated packed-bed reactor, a slightly higher yield (71.9%) was reached, but at much lower reaction rate due to the high external diffusion limitations.

- Presence of 5% (v/v) dioxane permitted the drastic improvement of the reaction production in stirred-tank reactor using immobilized enzymes. Compared to the aqueous reaction catalyzed by soluble enzyme, 18-fold higher concentration of Cbz-aminopolyol was reached, corresponding to the value of 86.8 mM (31 g/l).
Finally, there are numerous tasks which have to be accomplished in order to further improve the method for the enzymatic synthesis of Cbz-aminopolyol described in this doctoral thesis and to make it applicable at industrial scale. Some of them are:

- Studies of the possibility for the one-pot multienzyme synthesis of Cbz-aminopolyol by using DHA instead of expensive and instable DHAP.
- Operation of the continuous immobilized enzyme reactor, expecting the improvement of CPO stability by avoiding the accumulation of the peroxide in the reaction medium.
- Development the down-stream strategy for the recuperation and purification of Cbz-aminopolyol, its transformation to corresponding iminocyclitols and finally the evaluation of its inhibitory effect on glycoprocessing enzymes.
- Study of the economical feasibility of the overall process and choice of the most profitable strategy for industrial scale application.
- Modeling, simulation, optimization and finally, scale up of the process.

The multienzymatic strategy established in this work served as the basis for the development of other biocatalytic processes, which are currently being carried out in our research group. The methodology and the system described will be further applied for the synthesis of α-aminopolyols by applying different biocatalysts, as well as for the synthesis of β-aminopolyols by applying the same biocatalysts but different substrates. Besides, oxidative enzymes, like chloroperoxidase, are showed to be good catalysts for the reactions of synthesis.
REFERENCES


References


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