

## Antioxidant and Angiotensin 1 Converting Enzyme Inhibitory Functions from Chicken Collagen Hydrolysates

Olugbenga P. Soladoye<sup>1\*</sup>, Jordi Saldo<sup>1</sup>, Lluís Peiro<sup>2</sup>, Arnau Rovira<sup>2</sup> and Montserrat Mor-Mur<sup>1</sup>

<sup>1</sup>Universitat Autònoma De Barcelona, 08193, (Bellaterra) Cerdanyola de Valles, Spain

<sup>2</sup>Gallina Blanca Star, Bach 40 (Pol Ind Can Jardi), 08191 Rubí, Spain

### Abstract

Chicken collagen was explored for its possible ACE inhibitory and antioxidant activities. Flavourzyme, Neutrase and Alcalase were employed for hydrolysis of chicken collagen at predetermined times with optimal conditions for corresponding enzymes. Flavourzyme hydrolysate showed the highest antioxidant activity as measured by ORAC-FL assay (20942  $\mu\text{mol TE}/100\text{ g}$ ) followed by Neutrase (19207  $\mu\text{mol TE}/100\text{ g}$ ) and Alcalase (14352  $\mu\text{mol TE}/100\text{ g}$ ). Further purification by size exclusion chromatography showed that lower molecular weight fractions (between 170-776 Dalton) have highest antioxidant capacity (52787 and 44093  $\mu\text{mol TE}/100\text{ g}$  for Flavourzyme and Neutrase fractions respectively). The ACE inhibitory activity of collagen hydrolysates also appeared to be higher with low molecular weight fractions (between 1200-450 Dalton) having  $\text{IC}_{50}$  value of about 47.2 and 59.7  $\mu\text{g}/\text{ml}$  for Flavourzyme and Neutrase respectively. The present study suggests collagen as an effective candidate for both ACE inhibitory and antioxidant activity which can be employed in functional food formulations.

**Keywords:** Functional foods; Antioxidant; Angiotensin converting enzyme inhibitor; Chicken collagen; Hydrolysate

### Introduction

The worldwide increase in chronic lifestyle related diseases (CLRD) has demanded more concerted efforts from all relevant fields including the scientific community to discover cheap, yet effective remedies for this deplorable human health status. According to the World Health Organization (WHO), cardiovascular diseases (CVDs) are the number one cause of death globally, 80% of which occurs in lower and middle income countries with an estimated 23.6 million people likely to die of CVDs, mainly heart disease and stroke, by 2030 [1]. About 14.9% of the world wide population is currently estimated to have high blood pressure and some 500 million more may be diagnosed by 2025 [2], most of which can be implicated on diets and lifestyle.

Oxidation is an important process in the physiology of all living organisms as oxidative metabolisms are important for cells survival. However, its side effect is the production of free radicals. Reactive Oxygen Species (ROS) such as superoxide anion radicals ( $\text{O}_2^{\cdot-}$ ), hydroxyl radicals (OH), non-free radical species such as hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and singlet oxygen ( $^1\text{O}_2$ ) are formed during these normal body reactions [3]. Fair enough however, human bodies possess several mechanisms to eradicate or control these oxidation products. These include varieties of enzymatic antioxidants such as catalase, superoxide dismutase and glutathione peroxidase. On the contrary, when these mechanisms are overpowered either due to excess of free radical production or inability to adequately eradicate them, they can attack the closest molecules causing destructive and lethal cellular effect by oxidizing lipids, proteins, DNA and enzymes in the body [4]. This process can lead to several human diseases including cancer, diabetes, stroke, arteriosclerosis, Alzheimer's and heart diseases among others [3-5].

Furthermore, blood pressure has been found to be influenced by environmental factors (e.g. pollutants, stress), life style related factors (e.g. smoking, type of diet, alcohol, physical inactivity), and natural tendencies (including heredity, age, or gender), most of which lead to alterations by endogenous enzymes that act on the regulation of vasoconstriction (e.g. angiotensins) [2,6]. Angiotensin II is a

powerful vasoconstrictor which causes among other things, increase in sympathetic activities, along with increased contraction of cardiac muscle and arteriovenous tone thereby elevating blood pressure. Several mechanisms have been proposed showing the link between oxidative stress and hypertension. Zhou et al. and Di Bernardini et al. [3,6] had explained a prior physiological process that may contribute to the increase in blood pressure. They observed that oxidation and disulphide bridge formation between the thiol (SH) groups of  $\text{C}_{18}$  and  $\text{C}_{138}$  residues of angiotensinogen, result in some conformational changes which enhances its cleavage to precursor of hormone angiotensin. And this structural alteration seems to be largely contributed to by ROS. Branday and Lokhandwala [7] also demonstrated that oxidative stress leads to  $\text{AT}_1$  receptor upregulation, subsequently leading to increase in blood pressure. On the contrary, other authors Grossman [7] have also suggested another link between oxidative stress and hypertension, postulating that oxidative stress is a consequence of hypertension. In any case, both physiological disorders have been found to be related although controversies exist on their relationships [8].

Having the understanding that antioxidants are beneficial to human health as they shield the body against the reactive oxygen and nitrogen species, Erdmann et al. [9] have added that consumption of antioxidant laden food products appears to provide further benefits to the endogenous defense mechanisms with fighting oxidative stress. Many plant and animal sources have been explored for natural antioxidant activities since synthetic antioxidants (including butylated hydroxytoluene, butylated hydroxyanisole and propyl galate) have been associated with potential health risks *in vivo* [10]. Explorations of

**\*Corresponding author:** Olugbenga P. Soladoye, Universitat Autònoma De Barcelona, 08193, (Bellaterra) Cerdanyola de Valles, Spain, Tel: +14033070175; E-mail: [philip.soladoye@usask.ca](mailto:philip.soladoye@usask.ca)

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peptides generated from various protein sources with high antioxidant activity may also be a step in the right direction. Also, bioactive peptides which can interact with ACE either as pro drug or inhibitory peptide thereby reducing blood pressure by relaxing the arteries when the formation of Angiotensin II is inhibited may also be explored. Many of these peptides of about 2-20 amino acid units have been identified from different sources including animal and plant proteins, some of which have been found effective for ACE inhibition, antioxidant, among other functions [11-13].

Collagen is the main fibrous protein in bones, cartilages and skin, accounting for about 25-35% of the whole body protein content of mammalian and avian species [14]. Its vast and cheap nature in various animal byproducts has increased its exploration interest for nutritional and pharmaceutical applications. Outbreaks of mad cow diseases and the banning of collagen from pigs due to religious restrictions in some regions has further made it necessary to find a more acceptable, safe and healthy collagen source for various industrial applications of which poultry source represents a good option. Collagenous materials from poultry industries are normally discarded in large amount constituting cost for the industries and also a source of environmental pollution that may jeopardize the health of human [12]. The utilization of these wastes in a more valuable products with nutritional and health contributing tendency will be a landmark achievement. As hypertension and many of these CLRDs constitute great source of spending for various developed countries and many of the affected individuals are unaware of their physiological disorder, exploration of remedies of food origin will not only be a cheap and effective source to tackle this problem, it will also help cut government spending in this regard.

The scarcity of researches with collagen from chicken source coupled with its unique sequence caught our interest. Its rich source of hydrophobic amino acids (e.g Proline, Alanine among others) which have been reported in several researches as having ACE inhibitory and antioxidant activities when appropriately located in peptide sequence, further contributed to our interest in this component. Chicken soups have widely been acclaimed as medicinal due to its healing power and peptides fractions may be parts of the functional parts of this commonly consumed delicacy. Apart from these, studies that focused on the effects of oral intake in both animal and human models have revealed an efficient absorption and metabolism of hydroxyproline containing peptides. Possible bioactivity of these sequences has also been reported [15,16] with their ability to bypass gut digestion [17].

Hence, this study aims to identify possible bioactive peptides fractions from chicken collagen hydrolysates with either antioxidant or/and ACE inhibitory function and specifically; the activity of different enzymes at various time periods in generating bioactive peptides was observed and the degree of hydrolysis and its influence on activities of the derived peptides fractions including antioxidant and ACE inhibitory functions was also examined.

## Materials and Methods

### Raw material description

Avian collagen (AC) (partially hydrolyzed, with ~ 97% protein content, Table 1) from Ingredis Distribución Ingredientes, Reus, Spain was used in this experiment. Preliminary assessment of the degree of hydrolysis of this material shows between 10 -14% degree of hydrolysis (TNBS, 2, 4, 6-Trinitrobenzene-1-sulfonic acid). This material is supplied as water solvable, light yellow colored powder about 0.3 mm granule size.

### Enzymes and chemicals

Three different enzymes were assayed in this experiment: Flavourzyme<sup>®</sup>, Neutrase<sup>®</sup> and Alcalase<sup>®</sup>, all purchased from Novozyme A/S, Bagsvaerd, Denmark. 2, 2'-Azobis (2-methylpropanamide) dihydrochloride (AAPH, 98%) and Fluorescein were supplied by Acros Organic, New Jersey, US and Sigma Aldrich, Co., UK respectively. O-aminobenzoylglycyl-p-nitro-L-phenylalanine-L-proline (Abz-Gly-Phe-(NO<sub>2</sub>)-Pro-OH) and Angiotensin Converting Enzyme (ACE) (EC 3.4.15.1) from rabbit lung supplied by Bachem AG, Hauptstasse, Bubendorf and Sigma Aldrich, USA respectively. 2,4,6-trinitrobenzene sulfonic acid (TNBS) (picrylsulfonic) was supplied by G-Bioscience, USA. All corresponding buffers were prepared to appropriate pHs and all other reagents were of analytical grades.

### Enzymatic hydrolysis of avian collagen

A 10% (w/v) avian collagen reacting mixture was made with double distilled water. The pH was adjusted and equilibrated to the optimal range for the corresponding enzyme. About 1% enzyme to substrate (E/S) ratio was employed in this experiment. The hydrolysis was carried out in a round bottom reaction glass equipped with a temperature regulating water bath and continuous titration (702 SM Tritino, Metrohm, Herisau, Switzerland) for pH control. Samples were withdrawn at 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, and 5 h time intervals for each treatments. These were cooled and pH adjusted to around 7 to cater for subsequent analyses which require around neutral and inactivation ensued at 90°C for more than 20 minutes.

### Degree of hydrolysis (DH)

Degree of hydrolysis was carried out by adapting the TNBS method of Jens [18] and Spellman et al. [19] to a 96-well microplate reader method. Fifty microliters of hydrolysate sample was mixed with 950 µl of 1% Sodium dodecyl sulfate (SDS). This mixture was then diluted with phosphate buffer (pH 8.25, 0.2 mM) at the ratio of 1:100. TNBS (0.01%) was prepared immediately prior to the analysis in the same phosphate buffer. One twenty five microlitre of each diluted samples or the leucine standard solution were introduced into wells of the transparent microplate in triplicates. Sixty two micro litres of TNBS solution was later added to each well, properly mixed and incubated for 1 h at 50°C in the dark (Vacuum oven, WTB Binder). HCl (30 µl

Amino acid	% composition
Asparagine	5.72
Threonine	1.74
Serine	2.76
Glutamic acid	10.18
Proline	11.80
Glycine	22.85
Alanine	10.12
Cysteine	0.08
Valine	2.58
Methionine	0.80
Isoleucine	1.25
Leucine	2.97
Tyrosine	0.72
Phenylalanine	2.49
Lysine	3.82
Histidine	0.56
Arginine	7.31
Hydroxyproline	8.80

Table 1: Amino acids content of the Avian collagen used as raw material.

of 1N) was added to each well to stop the reaction. Calibration curve was prepared with 0.05-2.5 mM leucine solutions. The endpoint absorbance measurement was recorded at 350 nm and compared to the standard curve from which the amount of free amino group was estimated, representing the amount of peptide bound cleaved during the hydrolysis.

### Oxygen radical absorbance capacity (ORAC-FL) assay

This analysis was based on the method of Franka and Dell, [19] with slight modifications. The radical was prepared in phosphate buffer (pH 7.4, 10 mM) and fluorescein (10 nM) was prepared in the same buffer. Trolox in concentrations from 12.5  $\mu$ M-200  $\mu$ M was used for standard curve. Exactly 25  $\mu$ l of each diluted sample or standard were pipetted into a black opaque 96-well microplate in triplicate. One hundred and fifty microlitre of 10 nM fluorescein solution was added to each well and incubation followed for about 30 minutes at 37°C. After the incubation, 25  $\mu$ l of AAPH radical (250 mM) were automatically added to the wells and reading started immediately in FLUORstar OPTIMA reader with fluorescence measured at excitation and emission wavelength of 485 nm and 520 nm respectively. The equipment was set at kinetic mode such that reading was carried out every 3 minutes during the 30 cycles (3 minutes/cycle). The ability of the antioxidant in the sample (or in the standard) to protect the fluorescein probe was measured by the net area under the kinetic curve (AUC). Antioxidant capacity was expressed as  $\mu$ mol TE/100 g of protein.

### ACE (Angiotensin-converting enzyme) inhibiting activity

This was based on the method of Sentandreu and Toldrá, [20] with slight modifications. Three different reaction buffers were prepared prior to the analysis (Buffer A: 150 mM Tris base buffer, (pH 8.3) with 0.1  $\mu$ M ZnCl<sub>2</sub>; Buffer B: 150 mM Tris base buffer (pH 8.3) with 1.125 M NaCl) and Buffer C: 150 mM Tris base buffer (pH 8.3) and were stored at about 2-8°C. O-aminobenzoylglycyl-p-nitro-L-phenyl-L-proline (Abz-Gly-Phe-(NO<sub>2</sub>)-Pro-OH) (Bachem) was the substrate peptide used and ACE (Angiotensin Converting Enzyme) from rabbit lung was also employed.

Prior to the experiment, from 0.25 U enzymes supplied, 0.08 U/ml ACE stock solution was prepared by adding 3.125 ml of 50% glycerol (prepared with buffer A). ACE working solution (0.04 U/ml) was prepared immediately prior to analysis by diluting the stock solution half way with buffer A. The solution was agitated to ensure homogeneity. Working solution of the peptide (substrate) was also prepared by diluting 5 mg of it in 25 ml of buffer B.

Forty microliter of the collagen hydrolysate to be tested was introduced into each well of the 96-well microplate in triplicate; 40  $\mu$ l of ACE working solution was also added. The reaction started when 160  $\mu$ l of the substrate peptide solution was added. The microplate was shaken gently and read off in fluorimeter set at excitation and emission wavelength of 350 and 420 nm respectively. The incubation (T<sup>a</sup>= 37°C) continues for about 40 minutes in the fluorimeter chamber and kinetic measurements were taken every 5 minutes. The difference in the slope of the positive control and that of the sample measurement was used as the % inhibition and the concentration that causes 50% inhibition was also calculated as the IC<sub>50</sub> value.

### Size exclusion chromatography (SEC)

Selected fractions with highest activities of interest were separated by Gel filtration Chromatography equipped with 17-5176-01 Superdex™ Peptide 10/300 GL column (GE Healthcare, Buckinghamshire, UK).

The column was equilibrated with 0.05 M phosphate buffer at pH 7.4 and samples which were previously filtered through 0.2  $\mu$ m filter were loaded into the column. Elution was done with the same phosphate buffer at flow rate of 0.4 ml/min, running temperature 25°C and pressure of 15 bar. Signal was monitored at 215 nm and peaks were collected into different vials for subsequent analysis at different retention times. Molecular weights of the fractions were calculated with their corresponding retention times using the regression line of the generated standard curve plotted using Aprotinin (6512), Ribonuclease A (13700), Cobalamin (1355), and leu- Enkephalin (556)

### Statistical analysis

StatgraphicsPro version 5.1 was used for the data processing. All analyses were done in triplicates and results were expressed as the mean  $\pm$  SD. The effect of change in hydrolysis time was observed with different enzymes and significant differences between hydrolysis times were declared at P < 0.05 using LSD.

## Results and Discussion

### Degree of hydrolysis with time progression

Figure 1 shows the DH of the 3 enzyme hydrolysates measured by TNBS analysis. Flavourzyme appeared to produce the highest DH up to about 26%, followed by Neutrased (24%) and then Alcalase (20.6%). The nature of the enzyme for instance, Flavourzyme being an protease/peptidase complex of fungal origin (*Aspergillus oryzae*), containing both endoprotease and exopeptidase activities and Neutrased, being an endoprotease of *Bacillus amyloliquefaciens* origin and also containing a non-standardized amount of beta-glucanase with particularly high affinity for hydrophobic amino acid pairs, may also be responsible for their unique activities with collagen at the low level employed. The first few minutes of hydrolysis seem to be very important in whole hydrolysis process. Neutrased seems to be very active at the first 30 minutes of hydrolysis attaining about 20% DH. This value was constant till about the third hour of the hydrolysis before increasing slightly again afterward. In all cases, up to 50% of the whole DH achieved seemed to occur in the first 1 hour of hydrolysis. This result agrees with that of Alemán et al. [15] who found that the hydrolysis rate of marine gelatin were fast in the initial stage of hydrolysis and then gradually decreased until reaching a stationary phase. Considering this gradual progression in hydrolysis, collagen may be an appropriate substrate for the 3 enzymes here included as protease specificities, according to Gómez-Guillén et al. [12] and Ariyoshi, [11], affect peptide size and

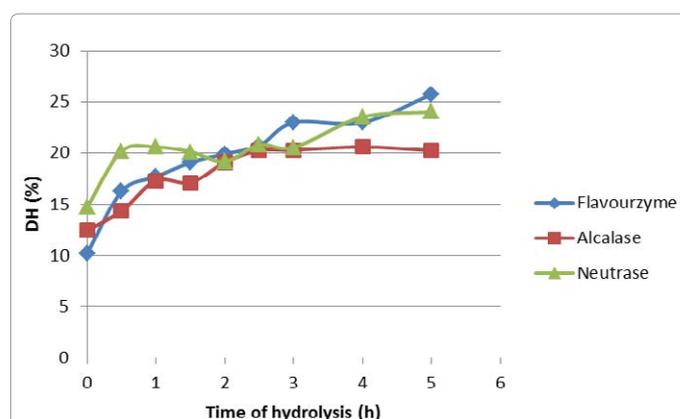


Figure 1: Degree of hydrolysis of 3 selected enzymes with TNBS analysis.

sequence alongside the amount and composition of free amino acid all of which largely affect the biological activities of the hydrolysates.

### ORAC-FL analysis

This assay is based on hydrogen atom transfer (HAT) mechanism. The appropriateness of this assay has been emphasized by different authors since it makes use of oxidants (Peroxyl radicals) that are common in biological systems (ROO·, OH·) and are of pathological significance [21,22]. So, among all chemical assays, it has often been considered the most accurate to simulate what will happen *in vivo*.

All fractions obtained from the three enzymes employed in this study showed consistent increase in antioxidant activity as hydrolysis progresses after which a maximum activity was attained at some point and a subsequent decline appeared with time of hydrolysis (Table 2). Flavourzyme hydrolysate appeared to be the most effective, attaining a maximum value of about 21000 µmol of Trolox Equivalence/100 g of protein at 3 h. Neutrase and Alcalase hydrolysates had their highest ORAC value between 1.5 to 2 h of hydrolysis (19207 and 14400 µmol TE/100 g respectively). These respective ORAC values are relatively high compared to values from previous studies. Wei and Shioh earlier reported values between 188 and 2230 µmol TE/100 g for some selected medicinal herbs, much lower compared to that of our study. The values between 235 and 9218 µmol TE/100 g were also reported in the same study for some culinary herbs, all of which were much below that of the hydrolysates in our present study. Samaranayaka and Li-Chan [23] have also reported ORAC value of about 22500 µmol TE /100 g of fish protein hydrolysate (Pacific fish hake) which is comparable to our present study and in turn much higher than results obtained in strawberries, blueberries and raspberries with antioxidant capacities of 15000, 20000 and 10000 µmolTE/100 g [24]. To our knowledge, antioxidant capacity assessment of collagen from chicken sources using ORAC assay is very scarce in the literatures. Comparing our ORAC values to the USDA database for the Oxygen Radical Absorbance Capacity of selected foods ([http://www.orac-info-portal.de/download/ORAC\\_R2.pdf](http://www.orac-info-portal.de/download/ORAC_R2.pdf)), it is obvious that collagen hydrolysate as observed in this present study, can be an effective antioxidant candidate although controversies exist on the use of this assay.

In a bid to explore other possible antioxidant mechanism of action of Collagen hydrolysate, ABTS ((2,2'-azino- bis (3-ethylbenzothiazoline-6-sulphonic acid)) analysis, which is based on single electron transfer mechanism, was carried out on the crude hydrolysates (result not shown). However, very weak significant effect of the enzyme hydrolysis treatment was observed with time progression. This may suggest that the mechanism of action of this substrate is not electron transfer or

that higher peptide concentration is needed for this method to detect peptides' activity.

Our present results lend support to the fact that collagen from chicken sources can be a source of antioxidant in food system. As previously mentioned peroxy radicals are common in biological system and could be formed in lipid peroxidation systems which can be precursor for various physiological diseases in humans. These hydrolysates (which can be one of the ingredients in chicken soups) or their further purified fractions/peptides could help to scavenge the peroxy radicals in order to terminate the free radical chain reaction of lipid peroxidation [21].

### Angiotensin 1 converting enzyme (ACE) inhibitory activities

The ACE inhibitory activities of the three hydrolysates considered in this study do not improve significantly with hydrolysis time until later during the hydrolysis and the results do not seem to vary significantly from enzyme to enzyme either (results not shown). Moreover, the raw material employed in this experiment seems to possess some level of ACE inhibiting activity (see time 0) and this value improves gradually but not significantly with time for most of the enzyme. Neutrase at 4 hours seems to give the lowest IC<sub>50</sub> (949 µg/ml) and this was significantly different from the value at time 0. Flavourzyme and Alcalase also produced a significantly different ACE inhibitory activity at time 5 h compared to time 0 h with values of 961 µg/ml and 995 µg/ml respectively. It has to be explored if the rather slow increase in the inhibitory activities of the hydrolysates could be improved by increasing the E/S ratio.

### Antioxidant capacities and ACE relationship with degree of hydrolysis

The DH, which is the percentage of peptide bonds cleaved with respect to the total number of peptide bond available in the substrate, does not seem to always have a direct relationship with the antioxidant and ACE activity as observed in this present study. An extent of relationship exists between ORAC-FL antioxidant values in Flavourzyme hydrolysed substrate where increase in DH leads to a corresponding increase in ORAC values. Moreover, this relationship seems to occur in two linear phases with the first phase (0-1h) having a linear relationship of about 98.6% and the second phase (1.5 -3 h) about 99.4% relationship between DH and ORAC value. Maximum antioxidant capacity was attained at about 23% DH and subsequent increase in DH leads to its decline. The increase in the DH treatment at the early period of hydrolysis appears to make the most significant impact on antioxidant capacity measured with ORAC-FL and subsequent increase in DH negatively affected antioxidant activity. The highest activity appears to be between 1.5-2 h of hydrolysis with both Neutrase and Alcalase at DH of about 20% in both cases (Figure 1). Further increase above this level seems to lead to corresponding decreases in antioxidant capacities.

Some researchers have found relationship between DH and antioxidant capacity. Although this statement may sound ambiguous and the comparison between studies may be difficult as different assays might have been employed in assessing DH and antioxidant activities. Thiansilakuli et al. [25] observed that higher degree of hydrolysis led to increase in antioxidant activity in fish protein hydrolysate. This was proposed to be due to presence of high amount of low molecular weight peptides in the hydrolysate fraction. Alemán et al. [15] also reported an increased antioxidant capacity with corresponding increase DH in squid gelatin hydrolysate (with Alcalase) which authors thought to be due to enhancement of radical scavenging activity. This result seems to

Time (h)	Flavourzyme	Alcalase	Neutrase
0	10873.1 ± 1005.5 <sup>a</sup>	10571.4 ± 1322.7 <sup>a</sup>	11956.6 ± 492.8 <sup>a</sup>
0.5	11936.3 ± 1183.3 <sup>ab</sup>	13858.3 ± 336.9 <sup>c</sup>	12587.0 ± 351.2 <sup>a</sup>
1	12404.2 ± 1449.3 <sup>ab</sup>	14352.2 ± 304.7 <sup>c</sup>	13133.9 ± 519.9 <sup>a</sup>
1.5	13077.0 ± 896.6 <sup>ab</sup>	14285.7 ± 494.7 <sup>c</sup>	18299.4 ± 1405.7 <sup>b</sup>
2	14388.3 ± 1603.2 <sup>bc</sup>	13494.8 ± 396.6 <sup>c</sup>	19207.2 ± 892.1 <sup>b</sup>
2.5	16753.2 ± 1763.3 <sup>c</sup>	12281.0 ± 817.7 <sup>b</sup>	11758.8 ± 308.0 <sup>a</sup>
3	20941.5 ± 2998.3 <sup>d</sup>	12996.0 ± 714.0 <sup>b</sup>	11949.4 ± 559.5 <sup>a</sup>
4	12925.0 ± 209.3 <sup>ab</sup>	12825.1 ± 1222.1 <sup>b</sup>	12905.6 ± 1401.0 <sup>a</sup>
5	13141 ± 529.2 <sup>ab</sup>	12043.7 ± 405.5 <sup>b</sup>	12173.9 ± 379.8 <sup>a</sup>

Values in the table are means of triplicate values ± standard deviation. Different letters within each column denote significant difference (P<0.05).

**Table 2:** ORAC values (µmol TE/100 g) for the 3 enzyme hydrolysates with time progression.

agree to an extent with our ORAC result, especially with Flavourzyme. However, Pihlanto [4] has reported that there seems not to be any relationship between DH and antioxidant activity from different whey protein hydrolysates. This relationship stands to be fully proven but in any case, the relationship between DH and antioxidant activity seem to be dependent on enzymes type, protein source, type of analysis and the variable conditions employed in the analysis.

Degree of hydrolysis appears to affect ACE inhibitory activity in some of our enzyme treatments although the level of significance is low. Overall, the linear relationship between degree of hydrolysis and ACE activity in Flavourzyme is about 77.4% (result not shown). A strong linear relationship was noticed within the first few periods (120 min) of hydrolysis with Alcalase (99.2%) although this relationship was lost at time point from 2 h. All these results suggest that ACE inhibition activity of avian collagen peptides should be due not only to its molecular weight but also to the amino acid composition and sequence.

### Antioxidant and ACE activities of size exclusion chromatographic (SEC) fractions

**Antioxidant activities of the SEC classified fractions:** Two different hydrolysate fractions (Flavourzyme, 3h and Neutrase, 2h) were selected for further separation into different molecular weights fractions and subsequently, antioxidant capacity of the fractions were assayed. These two fractions were selected based on their highest antioxidant activity measured by ORAC-FL among all the enzyme treatments. With size exclusion chromatography, 11 different fractions were identified and collected for their antioxidant analysis. ORAC assay conducted on these fractions was observed to be higher with low molecular weight fractions. The highest activity were observed particularly with fractions of 170-267 Dalton in Flavourzyme hydrolysates and these fractions appeared to contribute largely to the antioxidant capacity observed in hydrolysate, attaining about 5 fold increase (47048-52787  $\mu\text{mol TE}/100\text{g}$ ) in antioxidant capacity compared to the initial activity of the whole hydrolysate at zero time (crude hydrolysate, Table 2) (Figure 2a and 2b).

Fairly similar trend was also observed in Neutrase fractions where the highest activity was distributed between peptide fractions of low molecular weight between 347-798 Dalton (41353-44093  $\mu\text{mol TE}/100\text{g}$ ) resulting in about 4 fold increase in antioxidant activity compared with initial time. Alemán et al. [11,12,26] and Gómez-Guillén et al. [27] have emphasized the fact that biological activities of peptides are to a large extent affected by their molecular weight distribution which in turn may be influenced by processing conditions and the enzyme activity. Several other authors Je et al., Samaranayaka and Li-Chan, [13,28] have also stated that majority of the antioxidant peptides derived from food origin have molecular weight ranging between 500 and 1500. These results are confirmed to an extent in the present study as even much lower fractions were found to possess higher antioxidant activity.

Although ABTS analysis does not show a good result with the crude hydrolysate, with the classified fractions, ABTS radical scavenging activities also appear to be higher with low molecular weight fractions (Figure 3) where fractions of molecular weight around 170 and 267 Dalton, just like in ORAC analysis displayed a very high antioxidant activity, up to 72% and 45% radical inhibition respectively for Flavourzyme hydrolysates fractions.

In general, both fractions (10 and 11) of molecular weight between 170 and 267 Dalton in Flavourzyme hydrolysate, showed

strong activity with both ORAC-FL and ABTS assay, indicating the importance of these fractions in the antioxidant activity of the Flavourzyme hydrolysate. The same trend can be noticed in Neutrase hydrolysate fractions although to a lower extent where similar fractions are responsible for observed activity as assessed by both methods. Higher molecular weight fractions above 1300 Dalton and lower fractions below 170 Dalton showed low antioxidant activity. This may respectively be due to low active peptides sequence in the former (high molecular weight fractions) and the presence of only free amino acids in the later fractions which have been generally shown

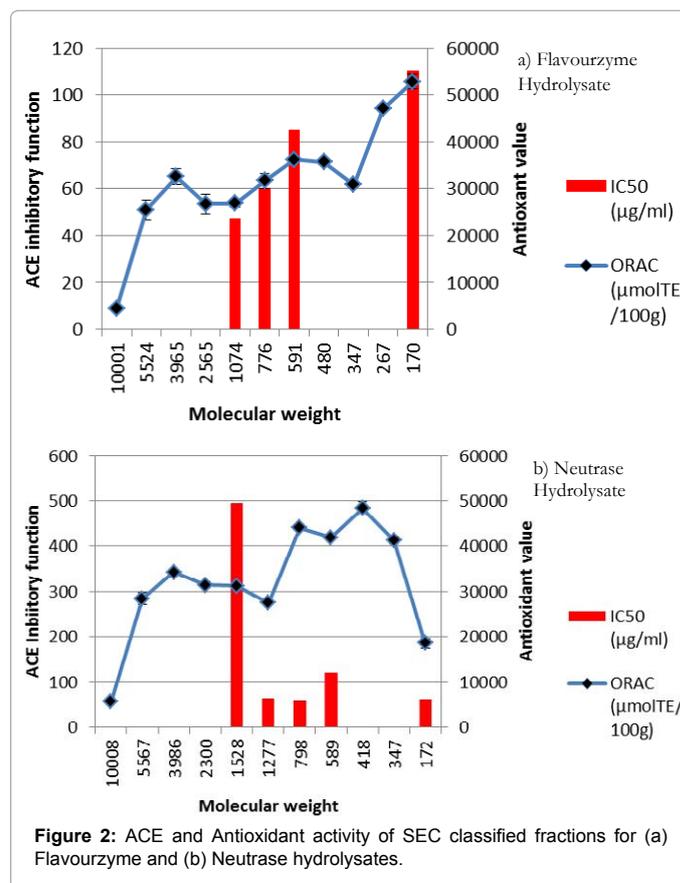


Figure 2: ACE and Antioxidant activity of SEC classified fractions for (a) Flavourzyme and (b) Neutrase hydrolysates.

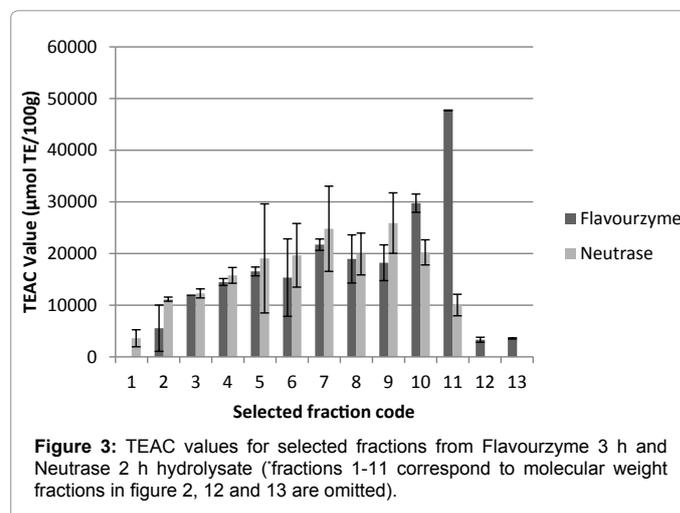


Figure 3: TEAC values for selected fractions from Flavourzyme 3 h and Neutrase 2 h hydrolysate (fractions 1-11 correspond to molecular weight fractions in figure 2, 12 and 13 are omitted).

to have no antioxidant activity [13]. It is also important to note that, the within treatment variations for ORAC analysis as shown by the standard deviation values were lower in the SEC classified hydrolysate fractions (code 1-11) compared to the main hydrolysates. This shows the possibility of more reliable application of these (molecular weight classified) fractions in several food or pharmaceutical applications than the unclassified crude hydrolysates (Figure 2a and 2b).

Generally speaking, peptides exhibit their antioxidant nature either by scavenging free radicals, donating electron, and chelating pro-oxidative metal, inactivating reactive oxygen or reducing hydrogen peroxide. The high radical scavenging activities of the low molecular fractions in the present study could be due to the amino acid composition, structure and hydrophobicity of the resulting peptides [28]. The antioxidant activity of peptides has been said to be related to molecular weight and amino acid sequence [29]. Ajibola et al. [30] and He et al. [28] have shown in their study that lower molecular weight hydrolysate (<1kd) fractions possess higher antioxidant activity due to their small peptide size alongside their possession of higher proportion of aromatic and hydrophobic amino acid which will enhance their hydrophobic interaction and hence may improve their radical scavenging activity, hydrogen donating capability and overall antioxidant activity. Suetsuna [31] had shown that the antioxidant activity was higher for peptides with branched chain amino acids such as valine, leucine and isoleucine derived from protease digest of prawn. Saito et al. [32] also observed that peptides containing the amino acids histidine, tyrosine and methionine in their sequence have higher radical scavenging capability. Several authors have also observed that histidine containing peptides have a strong antioxidant activity which was hypothesized to be due to the hydrogen donating, metal chelating and lipid peroxy radical trapping ability of the imidazole ring [32,33]. The indolic and phenolic groups of tryptophan and tyrosine respectively have also been implicated in their hydrogen donating capability [34].

The hydrophobic amino acids constituent has been largely reported to influence the antioxidative activity of peptides [16]. Two aromatic amino acids, tyrosine and phenylalanine have been reported by Ren et al. [33] as having a high antioxidant capacity which was implicated on the special capability of their phenol and indol groups to serve as hydrogen donors. Considering the high content of hydrophobic amino acids (Table 1) and the fairly high content of some aromatic amino acids in collagen which according to Ajibola et al. [34] will increase in low molecular weight hydrolysate fraction could lend support for our observed antioxidant activity with the low molecular weight fractions. Aside from this, observing the molecular weight of the fractions collected in the present study, dipeptide or tripeptides are possibly responsible for most of the antioxidant activities especially if these aforementioned amino acids are to be implicated. However, it has been noted that not only the presence of these amino acids are important but also their correct positioning in peptide sequence [3,12].

**ACE activities of the SEC classified fractions:** Since there was no significant difference between the ACE inhibitory activities of almost all the fractions observed (Table 3), it was decided to use the same fractions used for antioxidant activities to observe possible trend. For both selected samples, it is obvious that the fractions with molecular weight of between 450-1200 Dalton are responsible for the ACE inhibitory activities (fraction 591-1074 in Flavourzyme and 589-1277 in Neutrase) attaining the maximum activity of about 47.2 and 59.7 µg/ml in Flavourzyme and Neutrase respectively. These values are about 20 fold improvement compared to the values of the selected crude hydrolysate fraction. Ruiz-Ruiz et al. [35] have earlier shown

that fraction below 1 kDa expressed the highest ACE inhibitory activity compared to other fractions observed. The same observation was reported by Rui et al. [36] where lower molecular weight hydrolysates showed higher ACE inhibitory activity compared to higher molecular weight fractions with even an enhanced activity with further purified fractions. In the present study, those outside the above stated ranges were unquantifiable as they show no observed activity (Figure 2). This may be due to lack of active sequences in these fractions. However, a fraction with around 172 Da in this experiment showed some level of activity which could be due to the presence of some active dipeptides.

Amino acid composition of peptides from collagen has been noted to be specie specific and their molecular weight can be influenced largely by processing factors [12]. The composition of amino acid from Atlantic salmon collagen as estimated by Rui-Zeng et al. [37] was different from that estimated from Jelly fish collagen in another experiment by Zhang et al. [38] both of which are different from the amino acid composition in our experiment raw material. However, all these collagens possess fairly large amount of proline, glycine, alanine, phenylalanine, glutamine acid and aspartic acid, the majority of which have been previously implicated in ACE inhibition of some peptide fractions. Furthermore, some biological activities in collagen have presumably been associated with the unique Gly-Pro-Hyp sequence in their structure (Kim and Mendis, 2006). The importance of the hydrophobic amino acid residue (including Trp, Tyr, Phe and Pro) at the C terminal positions and Val and Ile at the N-terminus in ACE inhibiting activities of peptides has also been emphasized by several authors [39-41]. Ariyoshi [11] also claimed that the presence of Pro, Tyr or Asp on the C-terminal positions may be very crucial for some peptide inhibitory activities. Several other authors also claimed that peptide's ACE inhibitory activity increases with the presence of Leu in the carboxi terminal extreme [42,43].

Having all these wealth of evidences, we may suggest that the ACE inhibitory activities in our experiment may be due to one or more of these. Gómez-Guillén et al. [12] recently suggested that ACE inhibitory activity described in some collagen derived fractions/hydrolysates may be related to high concentration of hydrophobic amino acid as well as high Pro levels. These amino acids seem to be the most effective in ACE inhibitory activities of peptides and have also been found in naturally occurring peptides. The ACE inhibitory values obtained by Ruiz-Ruiz et al. [35] in hard to cook bean hydrolysates (4596-5400 µg of protein/ml) was about 5 fold higher than our present result suggesting a lower activity in the bean sample.

The ACE inhibitory activities of chicken collagen have been proven in some previous researches. Saiga et al. (2008) has shown IC<sub>50</sub> of about 130 – 250 µg/ml for chicken leg collagen hydrolyzed with

Time (h)	Flavourzyme	Alcalase	Neutrase
0	1109.6.1 ± 2.8 <sup>a</sup>	1133.8 ± 35.1 <sup>c</sup>	1030.3 ± 13.6 <sup>bcd</sup>
0.5	1091.2 ± 62.1 <sup>ab</sup>	1084.7 ± 14.5 <sup>bc</sup>	1036.6 ± 36.0 <sup>cd</sup>
1	1062.3 ± 92.4 <sup>ab</sup>	1029.8 ± 9.8 <sup>abc</sup>	1066.6 ± 69.7 <sup>cd</sup>
1.5	995.9 ± 42.7 <sup>ab</sup>	1030.6 ± 11.7 <sup>abc</sup>	1080.7 ± 8.5 <sup>d</sup>
2	986.1 ± 112.2 <sup>ab</sup>	1096.4 ± 60.6 <sup>bc</sup>	1047.5 ± 11.7 <sup>cd</sup>
2.5	972.4 ± 91.9 <sup>ab</sup>	1076.1 ± 37.9 <sup>bc</sup>	1002.61 ± 64.2 <sup>abc</sup>
3	1007.7 ± 75.6 <sup>ab</sup>	1047.5 ± 10.7 <sup>abc</sup>	958.8 ± 55.4 <sup>ab</sup>
4	975.4 ± 56.3 <sup>ab</sup>	1018.1 ± 19.8 <sup>ab</sup>	949.0 ± 44.6 <sup>a</sup>
5	960.5 ± 71.0 <sup>b</sup>	995.3 ± 17.7 <sup>a</sup>	1045.6 ± 14.3 <sup>cd</sup>

<sup>a-d</sup>Results with the same letter in the same column are not significant at P>0.05

**Table 3:** ACE inhibitory activities (IC<sub>50</sub>, µg/ml) of enzyme hydrolysate with time progression.

*Aspergillus spp* derived enzymes. Subsequent synthesis of one of these peptides generated a higher  $IC_{50}$  of about 29  $\mu$ M. In their earlier result in chicken breast muscle extract with *Aspergillus* proteases and gastric proteases [44], a strong inhibitory activity ( $IC_{50} = 42 \mu$ M) was observed in the sequenced peptide. These results ascertain the high possibility of obtaining a strong ACE inhibitory peptide from chicken collagen as our results also suggest a positive trend for this activity.

Aside from this, several authors have affirmed the efficient transfer of peptides of chicken origin through the gut epithelium and survive gut digestion thereby increasing their probability of reaching their site of action [12,45]. This advantage makes collagen a good candidate for antihypertensive drugs and functional foods. Evidence also suggested that the activity of chicken derived peptides increased following treatment with digestive enzyme, whereas that of porcine derived peptides decreased when their molecular weight was lowered. This lends more support in chicken derived peptides' ability to bypass the gut digestive system to reach the organs where it will exert physiological activity and sometimes with increased activity due to gastric enzymes' action on the pro- peptides [45-52] although most meat derived peptides have been mostly found to be "true inhibitor" as against "pro-drug inhibitors" or "substrate type" [10]. This hence makes the basic mechanism of action of these peptides to be by either binding to the active site of the ACE enzyme or by binding to an inhibitor active site located on the ACE enzyme which then modifies the protein confirmation, preventing the substrate (Ang I) from binding to the enzyme active site [53].

Finally, our results suggest a strong possibility of obtaining active candidate peptides for ACE inhibition from collagen hydrolysate of chicken origin especially with well purified fractions and this is comparable to several values from various previous researches [54]. Apart from the works of Saiga et al. [44,45] earlier cited, very few works have been done on exploration of ACE inhibitory activities from chicken collagen. Some researchers have shown some comparable results from sequenced peptides from various other sources including Gly-Phe-His-Ile and Gly-Phe-His-Ile-Asn-Gly (117 and 64.3  $\mu$ g/ml respectively) from beef muscle, Asn-Asp (1200 and 8100  $\mu$ g/ml) from head and visceral of sardinelle and the same sequence from porcine meat (3.9 mg/ml) [2]. Nevertheless, comparison from various researches could be complicated as different methods might have been employed in estimating  $IC_{50}$  which might have a very significant effect on the acclaimed activities [55-57].

## Conclusion

Knowing that some peptides possess some multifunctional nature, the present study explores some physiological functions derivable from avian collagen, most of which can be obtained from the waste products of chicken industries. Flavourzyme, Neutrase and Alcalase were used for hydrolysis for varying times at their respective optimal pH and temperature (all at the same E/S ratio). Flavourzyme appears to be the most effective enzyme in producing antioxidant (ORAC-FL value) active peptides, most of which was observed to have been produced with all enzyme treatment at about 2-3 hours hydrolysis. ACE activity also seems to improve with time of hydrolysis although at slower pace. Our results also revealed a weak or no linear relationship between DH and peptide ACE inhibition and antioxidant activity. However, at some point during the hydrolysis, relatively high correlation was noticed between ACE and DH in Neutrase and Flavourzyme [58]. This may suggest a possible relationship between DH and some peptides activity when variable factors are well controlled [59].

Since structure-activity relationship of peptides has not yet been established, their known biological activity may be dependent on molecular weight, amino acid sequence and percentage composition of aromatic or hydrophobic amino acids, all of which may be dependent on enzyme activity and specificity. Our study also confirms the fact that low molecular weight peptides are more active both in antioxidant and ACE inhibitory activity. ORAC-FL values for antioxidant capacity appears to better assess the antioxidant mechanisms by which collagen hydrolysate acts physiologically (probably, hydrogen transfer mechanism) and well classified fractions of hydrolysates may be more appropriate for functional uses as they show low variability in treatments [60]. Finally, incorporating this idea in the production of chicken soups may also help in deriving a tremendous health benefit from this commonly consumed delicacy unconsciously improving the health of consumers and reducing government spending on CLRDS and other related diseases.

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