CAPÍTOL I

Identificació de dues formes moleculars diferents de la proteïna quinasa CK2 d'*Arabidopsis thaliana*.

Resum dels resultats presentats a l'article "Identification of two different molecular forms of *Arabidopsis thaliana* casein kinase II". Plant Science (1997) 124, 131-142.

En aquest treball es va presentar l'aïllament, per cromatografia líquida, de la proteïna quinasa CK2 d'*Arabidopsis thaliana*, i la caracterització bioquímica de dues isoformes d'aquesta proteïna.

El material de partida eren plantes d'*Arabidopsis* en estadi de roseta, crescudes indistintament en terra o en medi líquid MS (Murashige and Skoog). Mitjançant la combinació de diversos tipus de reïnes cromatogràfiques, es va establir un protocol de separació i purificació parcial de la CK2, que es resumeix a continuació.

L'extracte cru de proteïnes es precipitava amb sulfat amònic i, a continuació, s'aplicava a una reïna de bescanvi iònioc en DEAE-cel.lulosa. Es van detectar tres pics d'activitat "caseïna quinasa" al llarg del perfil de la cromatografia (Fig 1, A). La primera de les activitats es detectava en el rentat de la reïna i no era inhibida per heparina, per la qual cosa, es va identificar com activitat CK1. Els altres dos pics es detectaven en aplicar un gradient linear salí (0-1M). El primer pic eluïa a una concentració de 0.1M NaCl i es va anomenar CKIIB, mentre que el segon pic eluïa a 0.3M NaCl i es va anomenar CKIIA.

Les fraccions corresponents a les formes CKIIA i CKIIB, es van reunir i es van cromatografiar per separat en una reïna de bescanvi iònic en fosfocel.lulosa, que uneix fortament la CK2 i, per tant és molt utilitzada en els procediments de purificació. Contràriament al que s'esperava, la forma CKIIB no es va unir a la reïna, en cap de les condicions de força iònica experimentades. La forma CKIIA es va unir eficaçment a la reïna, d'on n'eluïa a una concentració de 0.6M de NaCl (Fig 1, B).

Per tal de purificar la forma CKIIB, les fraccions obtingudes en la cromatografia en DEAE-cel.lulosa es van aplicar a una reïna d'afinitat en heparina agarosa. Malgrat que la CKIIB s'unïa a aquesta reïna, el grau de purificació que s'assolia era molt baix, raó per la qual es va omitir aquesta cromatografia del protocol general de purificació. Com a pas final de la purificació de la forma CKIIB, es va realitzar una cromatografia líquida a alta pressió amb una columna de bescanvi aniònic Protein-Pak Q AP-1. La proteïna s'unïa completament a la reïna, d'on n'eluïa en un únic pic, en aplicar el gradient salí (Fig 1, C).

A les Taules 1 i 2 es presenta un sumari del resultat de l'aïllament de les formes CKIIA i CKIIB d'*Arabidopsis*. Les activitats específiques després de la purificació foren de 855 U/mg per a la CKIIA, i de 91 U/mg per a la CKIIB. Els graus de purificació assolits foren de 87 vegades la CKIIA i 78 vegades la CKIIB.

A continuació, es va determinar l'estructura polipeptídica de les formes CKIIA i CKIIB. 5 μg de cada isoforma continguts a la fracció que mostrava una activitat catalítica més alta en l'última etapa de purificació, es van separar en una electroforesi SDS-PAGE i es van processar per a un experiment de Western blot. Les membranes es van incubar amb anticossos policlonals preparats contra la CK2 oligomèrica de timus de vedella (cedits pel Dr. M. Dahmus, Universitat de California, CA, USA) i que reconeixien específicament les dues subunitats de l'enzim. Les dues isoformes crosreaccionaven amb els anticossos, si bé el patró de bandes que es detectava era diferent (Fig 2). En la mostra corresponent a la isoforma CKIIB, els anticossos reconeixien una única banda d'aproximadament 43 KDa, que concordaria amb la massa molecular deduïda dels dos cDNAs aïllats per a la subunitat α de la CK2 d'*Arabidopsis* (Mizoguchi, 1993). En canvi, en la mostra corresponent a la isoforma CKIIA es detactaven dues bandes, d'aproximadament 43 i 27 KDa. La banda de 43 KDa podria correspondre a la subunitat α, mentre que la banda de 27 KDa concordaria amb la massa molecular deduïda dels cDNAs aïllats per a la subunitat β d'*Arabidopsis* (Collinge, 1994). A partir d'aquests resultats es podia concloure que la forma CKIIB es composava únicament de subunitat α, mentre que la forma CKIIA contenia els dos tipus de polipèptids, α i β.

Les dades immunològiques sobre l'estructura de la forma CKIIB, es van confirmar mitjançant una cromatografia de gel filtració en una columna protein Pak 125, per a HPLC. La mesura de l'activitat enzimàtica de les diferents fraccions recollides va permetre determinar una massa molecular aparent de la CKIIB de 55 KDa (Fig 3). Aquesta dada concordava amb la hipòtesi de què la forma CKIIB tenia estructura monomèrica, composta únicament de subunitat α.

Per tal de caracteritzar a nivell bioquímic les dues formes aïllades i establir possibles diferències entre elles, es van estudiar algunes propietats enzimàtiques de la CK2, com la utilització de GTP com a donador de fosfat, la inhibició per heparina, l'estimulació per polipèptids bàsics, la fosforilació de substrats acídics i l'autofosforilació de la subunitat β. A

la taula 3 i també a continuació, es resumeixen els resultats obtinguts en aquesta caracterització.

Les dues formes tenien en comú tres propietats de la CK2: la utilització de GTP, a més d'ATP, com a donador de fosfat, la inhibició de l'activitat enzimàtica per heparina i la fosforilació del pèptid específic RRRDDDSDDD. No obstant, es van observar algunes diferències entre les dues isoformes. Les constants cinètiques per al substrat caseïna, Km i Vmax, eren 0.068 mg/ml i 3.7 U/ml, respectivament, per a la CKIIA, i 0.175 mg/ml i 18.2 U/ml, per a la CKIIB. En presència de polipèptids bàsics, com la polilisina, l'activitat de la CKIIA s'estimulava 2 vegades, mentre que la CKIIB, ho feia en 25 vegades, sota les mateixes condicions experimentals.

Un dels substrats de la CK2 més ben estudiats és la calmodulina. Aquesta proteïna només pot ser fosforilada eficientment i en absència d'efectors, per la subunitat α sola. L'holoenzim necesita de polipèptids bàsics per poder-la fosforilar. Per tal d'explorar l'efecte de la subunitat β en l'especificitat de substrat, es va mesurar l'activitat de les formes CKIIA i CKIIB sobre la calmodulina, en presència i en absència de polilisina. D'acord amb aquesta premisa, la forma CKIIB es comportava com una proteïna monomèrica ja que era capaç de fosforilar la calmodulina en absència de polilisina. En canvi, la fosforilació de calmodulina per part de la CKIIA era gairebé insignificant en absència de polilisina, però s'observava una estimulació molt gran en presència del polipèptid (Fig 4).

L'última característica que es va estudiar en les formes CKIIA i CKIIB fou l'autofosforilació de les subunitats α i β. Les poliamines estimulen l'autofosforilació a la subunitat β, mentre els polications, com la polilisina o la protamina, estimulen la fosforilació a la subunitat α, en detriment de la subunitat β. Els assatjos realitzats amb les formes CKIIA i CKIIB, en presència de diferents efectors, van mostrar que les subunitats de la CK2 d'*Arabidopsis* no s'autofosforilen. No obstant, amb la forma CKIIB, es va detectar la fosforilació d'una banda d'aproximadament 15 KDa, que copurificava amb la proteïna (Fig 5). La fosforilació d'aquest substrat endogen era específica de CK2, ja que la incorporació de fosfat s'inhibia per heparina, però cap dels altres moduladors assajats afectava la reacció.

L'estudi de l'expressió de la subunitat CK2β a nivell transcripcional en diferents òrgans de la planta d'*Arabiodopsis* va demostrar que aquesta subunitat no s'expressa per igual als diferents òrgans analitzats, sinó que és molt més elevada en flors i en fulles (Fig 6).

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Identification of two different molecular forms of *Arabidopsis thaliana* casein kinase II

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Abstract

We have isolated and identified two different molecular forms of casein kinase II (CKIIA and CKIIB) from *Arabidopsis thaliana*. The use of heterologous antibodies that react with both catalytic and regulatory subunits in animal systems, revealed that CKIIB contains a single type of polypeptide with a molecular mass of 43 000 Da, whereas CKIIA is composed of two different polypeptides of 43 000 and 27 000 Da, respectively. Both forms show enzymatic characteristics typical of CKII activity, such as phosphorylation of the acidic peptide RRRDDDSDDD, use of GTP as phosphate donor, inhibition of their activity by low concentrations of heparin and stimulation by polylysine. K_m values for β-casein are 0.068 mg/ml for CKIIA and 0.175 mg/ml for CKIIB. Calmodulin, a well-characterized substrate for casein kinases II, is significantly better phosphorylated by the CKIIB form than by the CKIIA form, suggesting an inhibitory effect of β-subunit on the enzymatic activity for this substrate. However, no autophosphorylation of the 27-kDa polypeptide has been detected, which can be explained by the lack of the conserved β-autophosphorylation site in *Arabidopsis thaliana*, as deduced from its cDNA sequence (Collinge and Walker, 1994, Plant Mol. Biol. 25, 649-658). In contrast, CKIIB form, devoid of regulatory subunit, co-purifies with a protein substrate of 15-kDa and efficiently phosphorylates it. A distinct pattern of mRNA accumulation for the catalytic and regulatory subunits suggests a different distribution of the molecular isoforms in the adult plant, that may reflect a functional specialization. © 1997 Elsevier Science Ireland Ltd.

Keywords: *Arabidopsis*; Casein kinase II; Molecular isoforms; Plant kinases

1. Introduction

Casein kinase II (CKII) is an ubiquitous Ser/Thr kinase in eukaryotic cells. It phosphorylates a number of substrates involved in multiple cellular

Abbreviations: CKII, casein kinase II; TCA, trichloroacetic acid; DTT, dithiothreitol.

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regulatory pathways, such as DNA replication, DNA transcription, gene expression or cell division (for a review, see [1]). The enzyme purified from animals and yeast is composed of two different subunits, showing a characteristic tetrameric structure, $\alpha_2\beta_2$. The catalytic subunit is the α-subunit, with a molecular mass ranging between 37 and 44 kDa, and coded in many species by two genes, α and α'. The β-subunit, with a molecular mass of 24–26 kDa in most cases, plays a regulatory role, stabilizing the catalytic subunit and determining the substrate specificity of CKII. The β-subunit is coded by a single gene, except in the case of yeast where two genes, β and β', have been shown to code for functional β-subunits, one of them with a very unusual molecular mass (41 kDa) [2].

For plants, different activities with enzymatic properties resembling those of CKII have been reported for many years [3,4]. However, there are few conclusive data regarding its molecular structure. In contrast to animal CKII, plant monomeric forms, composed only of catalytic subunits, have been isolated from several species, such as wheat [3], maize [5] and broccoli [6]. These monomeric forms coexist with higher molecular weight forms (presumably tetrameric, according to its molecular size) in broccoli and maize [6,7], although the biochemical nature of the polypeptides associated with the catalytic subunit has not been demonstrated in either case. In pea seedlings, just an oligomeric CKII form has been reported [8], with a structure composed of catalytic and regulatory subunits as in the animal systems.

Molecular cloning has confirmed the existence of catalytic and regulatory subunit homologues in plants. One cDNA encoding the catalytic α-subunit in maize [9] and two cDNAs in *Arabidopsis thaliana* [10] have been reported. These cDNAs show up to 76% identity to α-subunits from yeast and animals. Moreover, two cDNAs corresponding to regulatory subunit homologues have been cloned from *Arabidopsis thaliana* [11], being the first indication of a similar structure and regulation of CKII in plants and animals. Expression in bacteria of one catalytic and one regulatory subunit from *Arabidopsis* resulted in the reconstitution of an oligomeric active form, demonstrating the ability of the β-subunit to interact with the α-subunit and modulate its activity to some extent [12].

In this work we have isolated and characterized the CKII from *Arabidopsis thaliana* showing that two isoforms coexist and that they have different biochemical properties. According to its cross-reactivity with antibodies against whole bovine CKII, one form is composed only of catalytic subunits, while the other contains both catalytic and regulatory subunits. This finding supports the notion that monomeric forms of CKII is a general rule in the plant kingdom, in contrast to its apparent absence in the animal systems. The main biochemical properties of these two forms are discussed in comparison to those of CKII from other species.

2. Materials and methods

2.1. Plant material

Arabidopsis thaliana ecotypes Columbia and Landsberg erecta were grown in the greenhouse under a 16-h light/8-h dark regime, at 22°C. For in vitro germination, seeds were surface sterilized and grown in liquid MS medium [13] for 2 weeks under continuous illumination.

2.2. Protein kinase assays

Casein kinase II assays were performed as described in [14], except that one unit of activity was defined as the amount of enzyme that incorporates 1 pmol of ^{32}P into casein/min of incubation. Samples of 1 mg/ml of dephosphorylated β-casein (Sigma) or 0.32 mM of the specific peptide RRRDDDSDDD (Boehringer) were used as substrates. ^{32}P incorporation into β-casein was measured either by TCA precipitation or by SDS/PAGE followed by autoradiography. ^{32}P incorporation into the peptide was measured by absorption to Whatmann P 81 paper that was washed three times in 75 mM phosphoric acid and once in acetone. All the assays were carried out in duplicate. Phosphorylation of β-casein using GTP as phosphate donor was measured by competition

of unlabelled GTP (1.26 mM) with [γ-^{32}P]ATP (0.126 mM, 2000 c.p.m./pmol). As a control, competition with unlabelled ATP, under the same conditions as above, resulted in no incorporation of radioactivity into casein. The stimulatory activity of basic compounds was measured by preincubation of the catalytic activity with the assayed fractions for 5 min on ice; this was followed by a protein kinase assay with β-casein. Autophosphorylation was assayed in the same conditions as above but omitting the substrates in the reaction medium and using a [γ-^{32}P]ATP specific activity of 2000 c.p.m./pmol. Kinetic constants were determined using dephosphorylated β-casein as substrate. Five substrate concentrations, in duplicate, were assayed within a concentration range of 0.3–300 K_m. Kinetic results were analyzed using a nonlinear regression data analysis program [15]. Assays using calmodulin (Sigma) as substrate were carried out in the same conditions as above, except that 4.8 mM EGTA was included in the reaction mixture.

Casein kinase I activity assays were made using β-casein as substrate. The effect of low concentrations of heparin on enzymatic activity and the utilization of GTP as phosphate donor were used as criteria to distinguish between casein kinase I and casein kinase II activities.

cAMP-dependent protein kinase and p34^{cdc2} protein kinase activities were determined as described [16].

2.3. Electrophoresis and Western blots

Samples were run on 10, 12.5 or 15% SDS-polyacrylamide gels and, for Western blotting, proteins were electrotransferred onto immobilon-P membranes (Millipore). CKII subunits were detected using antisera against oligomeric bovine CKII (kindly provided by M. Dahmus, University of Davis, CA) (dilution 1:500) and the immunocomplexes were visualized using the alkaline phosphatase detection system (BioRad).

2.4. Northern blots

RNA was prepared from different tissues of the plant using a phenol-SDS extraction and LiCl precipitation method (Extract-A-Plant™ RNA Isolation Kit, Clontech, Palo Alto, CA) according to the manufacturer's recommendations. The samples were then electrophoresed on a 1% agarose-formaldehyde gel, transferred onto a Zeta-Probe GT membrane (Bio Rad), and hybridized with the radiolabelled gel-purified CKB1 insert [11] in 0.2 M sodium phosphate/1 mM EDTA, pH 7.2/1% bovine serum albumin/7% SDS at 65°C. The membrane was washed twice in 40 mM sodium phosphate/1 mM EDTA, pH 7.2/1% SDS at 65°C. Equal loading of RNA was verified by ethidium bromide staining of the gel. The results were visualized and quantified using a GS-525 molecular imager system (BioRad).

2.5. Protein purification

Fresh plant tissues (100 g) were homogenized with a mortar and pestle under liquid nitrogen and resuspended in 50 mM Tris/HCl (pH 7.5) buffer, containing 10 mM NaCl, 1 mM DTT, 10% (v/v) glycerol, 1 mM PMSF and 0.25 M sucrose. The homogenate was centrifuged at 10 000 × g for 30 min. After being filtered through glass wool, the supernatant was precipitated by solid ammonium sulphate to reach 60% saturation. The protein precipitate was collected by centrifugation at 10 000 × g for 30 min and redissolved in 50 mM Tris/HCl (pH 7.5) buffer, containing 10 mM $MgCl_2$, 1 mM DTT, 10% (v/v) glycerol and 1 mM PMSF (buffer A). After dialysis against the same buffer, the protein solution was clarified by centrifugation at 10,000 x g for 30 min and applied to a 10-ml column of DEAE-Sepharose CL-6B (Sigma) equilibrated with buffer A. The column was washed until no protein could be detected in the eluate and, afterwards, a 40-ml linear gradient of 0-1.0 M NaCl in buffer A was applied. Two different peaks showing casein kinase II activity were detected at this step and pooled separately for further purification. The two peaks were named CKIIA and CKIIB respectively, according to their molecular structure (see below) and the adopted nomenclature for maize isoforms [7]. The two fractions were dialysed against buffer A + 0.25 M NaCl and loaded onto a phosphocellulose P11 column (Whatman) equilibrated in the same

buffer. The column was eluted with a 30-ml linear gradient of 0.25-1 M NaCl. Fractions corresponding to peak CKIIB were dialysed against buffer 50 mM Tris/HCl, pH 8.0 + 50 mM NaCl, and submitted to a final chromatographic step in a Protein-Pak Q AP-1 anion exchange column (Waters), equilibrated with the same buffer, and eluted with 20-ml gradient of 0.05-1 M NaCl.

2.6. Gel filtration chromatography

Gel filtration was performed at a rate of 0.5 ml/min through a Protein-Pak 125 column (Waters) equilibrated with 50 mM Tris/acetate (pH 7.5)/50 mM sodium acetate. Fractions of 0.25 ml were recovered and identified by casein kinase II activity assays. Molecular weight reference markers used were: Bovine serum albumin (66 kDa), ovoalbumin (45 kDa), carbonic anhydrase (29 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14 200 Da), from Sigma.

2.7. Protein determination

Protein concentration were determined using the Bio-Rad protein assay kit with BSA as standard.

3. Results

3.1. Isolation of two different molecular forms of CKII from Arabidopsis thaliana

Casein kinase II was purified from *Arabidopsis thaliana*, either grown in soil until the rosette stage, or allowed to germinate in vitro for 2 weeks. Results were similar regardless of the starting material. The crude extract was precipitated with $(NH_4)_2SO_4$ to remove pigments and concentrate the proteins and loaded onto a DEAE-cellulose column, equilibrated at pH 7.5. This step is known to separate CKII from the contaminating CKI activity and in our case was also able to separate the two molecular forms of CKII. We detected three peaks with phosphotransferase activity using β-casein as substrate (Fig. 1A). The activity in the flow-through was not inhibited by

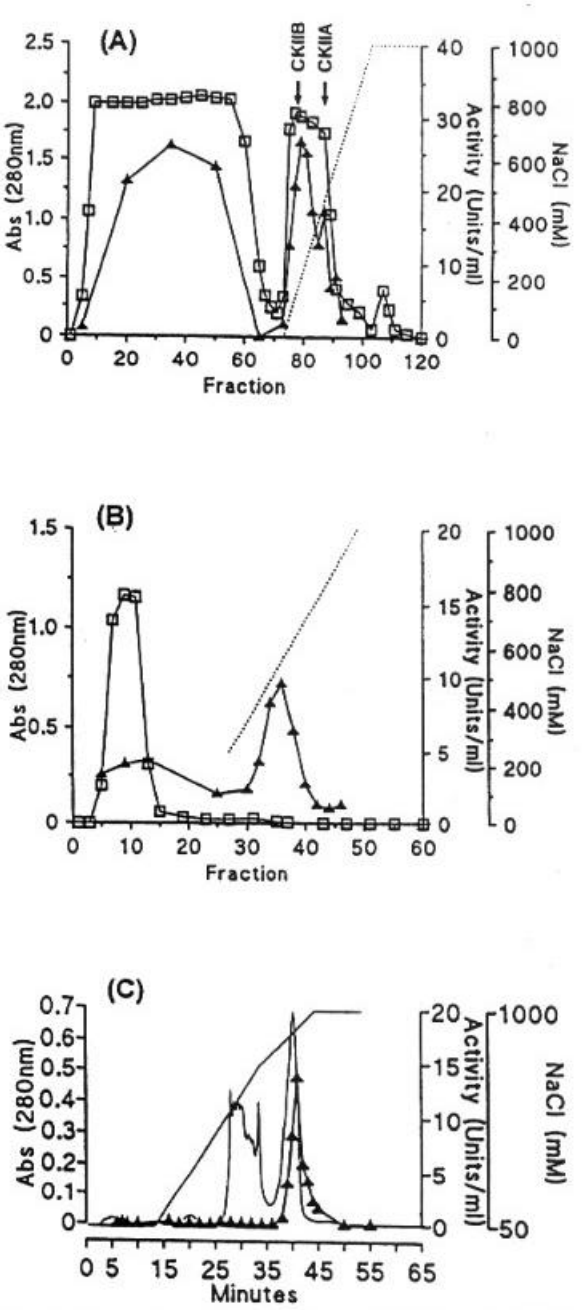

Fig. 1. Chromatographic behaviour of CKIIA and CKIIB isoforms. (A) DEAE-Sepharose chromatography, pH 7.5. (B) Phosphocellulose chromatography, pH 7.5, of CKIIA isoform. (C) Protein-Pak Q chromatography, pH 8.0, of CKIIB isoform. (□) Absorbance at 280 nm. (▲) Phosphate incorporation into β-casein. (···) Linear gradient of NaCl. The chromatographic profile in (C) was obtained using the Millenium 2010 software (Millipore).

Table 1
Summary of the purification scheme of casein kinase IIA from *A. thaliana*

Step	Volume (ml)	Protein (mg)	Activity (U)	Specific activity (U/mg)	Purification-fold
Homogenate	450	999	9765	9.77	1.00
$(NH_4)_2SO_4$ ppt	27	16.2	331.5	20.46	2.09
DEAE-Sepharose	6	1.8	83.5	46.38	4.74
Phosphocellulose	4.5	0.009	7.7	855	87.51

One unit incorporates 1 pmol of phosphate into β-casein in 1 min at 30°C.

heparin (5 μg/ml) and consequently was assigned to the casein kinase I. Two more peaks of activity were eluted from the column at 0.1 and 0.3 M NaCl respectively which were, both, inhibited by heparin, which is a characteristic property of CKII activity. The relative amount of these two peaks remained constant in independent preparations.

The CKII fractions were pooled separately and, since affinity for phosphocellulose is a defining property of CKII, loaded in separate phosphocellulose columns for further purification. Surprisingly, CKIIB did not bind to the resin at a salt concentration of 0.1 M NaCl nor at 0.25 M or in absence of NaCl (result not shown). On the contrary, CKIIA was retained onto the column in the presence of salt (0.1 M or 0.25 M NaCl), and was further eluted at 0.6 M NaCl (Fig. 1B).

In our first attempts to further purify CKIIB form we used a heparin agarose chromatography, based on the affinity of casein kinase II by heparin. However, although CKII activity remained bound to the resin and was eluted after applying a salt gradient (results not shown), this step didn't increase significantly the purification factor of the protein, and therefore, was omitted from the general purification procedure.

As the final step of purification, CKIIB was chromatographied using the strong anion exchanger Protein-Pak Q AP-1 column (Waters) and a major peak of activity was eluted after applying a linear gradient of NaCl (Fig. 1C).

The specific activities of CKIIA and CKIIB after the last step of purification were 855 and 91 U/mg respectively, and the purification factors 87.5 and 78.7 (Tables 1 and 2). Due to the small size of the *Arabidopsis* plant, it is very hard to collect enough tissue to achieve a highly purified preparation of the enzyme. Nevertheless, since all the casein-phosphorylating activity of our preparations was inhibited by heparin, and also efficiently phosphorylated a CKII-specific substrate (see below), we reasoned that the above figures were good enough to allow the determination of the main molecular characteristics of the two forms of *Arabidopsis* CKII. As an additional control, we checked for contaminating kinase activities, such as $p34^{cdc2}$ and protein kinase A. All the purified preparations were free of those interfering activities.

3.2. Structure of Arabidopsis CKIIA and CKIIB

We used polyclonal antibodies raised against oligomeric CKII from calf thymus, kindly provided by M. Dahmus (University of Davis, CA). These antibodies have been shown to recognize both α and β subunits of bovine CKII [17] as well as both subunits of a heterologous system such as Drosophila [18]. Fractions showing maximal CKII activity from the last step of purification of CKIIA and CKIIB forms were precipitated with 15% trichloroacetic acid, washed with 0.1 M ammonium acetate in ethanol, and processed for Western blot analysis as described in Section 2. The antibodies were used at a 1:500 dilution. Our results show that CKIIB fraction cross-react with the antibodies, giving a unique 43 kDa band, which is in agreement with the molecular mass deduced from the cDNAs for the catalytic subunits of *Arabidopsis* CKII [10]. CKIIA fraction also cross-reacts with the antibodies and gives two bands of 43 and 27 kDa, which are in agreement with the expected molecular mass for catalytic