Purification and characterization of casein kinase I from broccoli

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Casein kinase I from broccoli was purified ~ 65000-fold by chromatography on phosphocellulose, phenyl-Sepharose, CM-Sephacel, and affinity chromatography on N-(2-aminoethyl)-5-chloroisoquinoline-8-sulphonamide (CKI-7)–Sepharose. The catalytic subunit of casein kinase I was identified as a 36–38 kDa polypeptide doublet by using the technique of activity gel assay after SDS/PAGE with casein as a gel-incorporated substrate. A silver-stained polypeptide doublet of the same molecular mass constituted at least 95 % of the protein in the final preparation, corresponding to a specific activity of ~ 1800 nmol/min per mg of protein. The enzyme was found to be a monomer by gel filtration and glycerol gradient sedimentation; the native molecular mass was calculated to be 34.2 kDa. These characteristics, as well as other essential features of plant casein kinase I activity, such as substrate specificity and sensitivity to inhibitors, were found to be similar to those established for animal casein kinase I. Broccoli casein kinase I showed weak immunological cross-reactivity with antibodies raised against bovine casein kinase I.

INTRODUCTION

Casein kinase I is a ubiquitous multifunctional protein kinase present in animal, yeast and plant cells (for review see Tuazon and Traugh, 1991). The casein kinase I forms isolated from various biological sources share many physical and biochemical properties and appear to be encoded by a family of conserved genes (Rowles et al., 1991; Robinson et al., 1992; Wang et al., 1992). Of particular biological interest is the enzyme's partial localization to the nucleus as well as the range of its actual and potential physiological substrates, which encompass translational factors, certain regulatory metabolic enzymes, and many nuclear proteins involved in DNA replication and RNA transcription (Tuazon and Traugh, 1991). The last group of substrates, together with the presence in the nucleus, indicate for this enzyme a role in the regulation of gene expression.

In contrast with the abundant information on the animal and yeast casein kinases I, much less is known about their plant counterparts. In the most systematic study to date, Dobrowolska et al. (1987) performed separation of maize seedling casein kinases into distinct casein kinase I- and II-like activities and established the similarity of major characteristics of plant casein kinase I to those of the animal enzyme. Since then, plant casein kinase I has not yet been purified, which impedes progress in more detailed characterization of this enzyme, in particular the identification of physiological substrates and studies of its structural and functional relatedness to the animal and yeast enzymes, as well as its possible modes of regulation.

In this paper, we describe the purification to near homogeneity of cytosolic casein kinase I from broccoli. We identify the enzyme as a monomer of 36–37 kDa and present its major characteristics. We also present data on the immunological relatedness of the plant enzyme.

EXPERIMENTAL

Plant material

Broccoli (*Brassica oleracea* var. *italica*) was purchased in a fresh state from local wholesale distributors.

Chemicals

$[\gamma\text{-}^{32}P]ATP$ (sp. radioactivity 3000 Ci/mmol) was obtained from Amersham; N-(2-aminoethyl)-5-chloroisoquinoline-8-sulphon-amide (CKI-7) was from Seikagaku America; phosphocellulose was from Whatman; Sephacryl S-200, CNBr-activated Sepharose, phenyl-Sepharose and CM-Sephacel were from Pharmacia; casein fractions, histone IIIS and phosvitin were from Sigma; pre-stained protein molecular-mass markers were from Bio-Rad; other chemicals were analytical grade.

Antibodies

Rabbit polyclonal antiserum against bovine casein kinase I was kindly provided by Dr. Michael Dahmus (University of California, Davis). Affinity-purified goat anti-rabbit IgG (H + L) antibody conjugated with horseradish peroxidase was purchased from Bio-Rad.

Protein kinase assay

Protein kinase activity was assayed in 20 μl of an assay solution containing 50 mM Tris/HCl, pH 8.0, 10 mM $MgCl_2$, 10 μM ATP, 1 μCi of $[\gamma\text{-}^{32}P]ATP$ and 5 μg of partially dephosphorylated casein (Sigma, C4032). After adding up to 2 μl of enzyme preparation, the assays were incubated for 10 min at room temperature. The samples were spotted on 2 cm × 2 cm squares of Whatman 3MM filter paper, which were subsequently washed for 3 × 10 min in 10 % trichloroacetic acid/1 % sodium pyro-phosphate, rinsed in ethanol, dried, and counted for Čerenkov radiation in a scintillation counter. One unit of activity is defined as the amount of enzyme incorporating 1 nmol of phosphate into casein per 1 min incubation time, and is referred to as 1 nmol/min.

Synthesis of CKI-7–Sepharose

CKI-7 was immobilized on to CNBr-activated Sepharose as described by Chijiwa et al. (1989).

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Purification of casein kinase I

All operations were performed at 4 °C. Broccoli heads (4 kg) were processed in 1 kg portions by homogenization in 3 litres of 250 mM sucrose in a buffer containing 20 mM potassium phosphate, pH 7.0, 5 mM NaF, 5 mM EDTA and 50 µg/ml phenylmethanesulphonyl fluoride (PMSF) (buffer A). Nuclei were removed by centrifugation at 700 g for 10 min, and poly-(ethylene glycol) (PEG) 6000 was dissolved in the supernatant at a concentration of 4 % and $(NH_4)_2SO_4$ at 0.2 M. After stirring for 30 min, crude membranes and other amorphous material were removed by centrifugation at 9000 g for 15 min to obtain the clarified extract. PEG 6000 was added to the supernatant to a final concentration of 20 %, and after stirring for 30 min the suspension was centrifuged at 6000 g for 10 min. The pellet was redissolved in buffer B (50 mM Tris/HCl, pH 7.0, 5 mM NaF, 5 mM EDTA) with 200 mM KCl and freshly added PMSF (50 µg/ml final concn.) and 10 µM leupeptin (400 ml of buffer/kg of plant material). The solution was clarified by centrifugation at 9000 g for 15 min. The supernatant fraction was subjected to batch adsorption by stirring for 1 h with 100 ml of phospho-cellulose suspension in buffer B. The phosphocellulose was allowed to settle, decanted and washed with 2 × 400 ml of buffer B with 100 mM KCl. The batch was poured into a column (2.8 × 16 cm) and washed with 10 vol. of buffer B with 100 mM KCl. The column was eluted with 8 vol. of a linear gradient of 150–1000 mM KCl in buffer B with 5 mM 2-mercaptoethanol. The fractions containing casein kinase activity were pooled, and loaded on a column of phenyl-Sepharose (1.6 cm × 5 cm) equili-brated with 500 mM KCl in buffer C (50 mM Tris/HCl, pH 7.0, 5 mM 2-mercaptoethanol). More efficient adsorption of the enzyme on hydrophobic columns took place at pH 7.0, whereas elution was facilitated at a slightly higher pH of 8.0. Therefore, Tris/HCl buffer of pH 7.0 was used in the loading solution. This buffer was prepared as a 2 M stock at room temperature, and under the conditions of dilution and temperature used showed sufficient buffering capacity. The column was washed with 10 vol. of equilibration buffer and eluted with 8 vol. of a linear gradient of 500 mM KCl to 60 % ethylene glycol in buffer D (like buffer C, but pH 8.0), followed by further elution with 60 % ethylene glycol in buffer D. The active fractions were pooled, diluted 3-fold with buffer C and loaded on a column of CM-Sepharose (0.5 cm × 12 cm). After washing with 5 vol. of buffer C, the column was eluted with 8 vol. of a gradient of 50–400 mM KCl in buffer C. The active pool was loaded on a CKI-7–Sepharose column (0.5 cm × 4 cm), which was washed with 3 vol. of 1 M KCl in buffer C and then 2 vol. of buffer C, and eluted with 5 vol. of 60 % ethylene glycol in buffer D. The final preparation was stable over several weeks when stored at −20 °C.

Protein electrophoresis

Gel electrophoresis under denaturing conditions was performed in the presence of SDS as described by Laemmli (1970). The stacking and separating gels contained 4 and 10 % (w/v) poly-acrylamide respectively (acrylamide/bisacrylamide, 30:1 w/w). Gel thickness was 0.75 mm, and the running distance 5 cm. The gels were stained with silver by the protocol of Wray et al. (1981).

Immunoblotting

After separation by SDS/PAGE calibrated with pre-stained protein markers, proteins were electrophoretically transferred to nitrocellulose (Towbin et al., 1979). After blocking with non-fat dry milk and binding with primary and secondary antibodies, the blots were developed with diaminobenzidine (Harlow and Lane, 1988).

Detection of protein kinase activity in situ after SDS/PAGE (activity gels)

The activity assay in situ was a slight modification of the procedure described previously (Klimczak and Hind, 1990). Partially dephosphorylated casein was added to the separating gel solution to a final concentration of 500 µg/ml before polymer-ization. Pre-stained molecular-mass markers were used for gel calibration. After electrophoresis, the gel was washed for 4 × 15 min at 4 °C in 30 gel volumes of 50 mM Tris/HCl (pH 8.0)/5 mM 2-mercaptoethanol and incubated for 4 h and 1 h in two changes of 40 vol. of assay buffer (50 mM Tris/HCl, pH 8.0, 10 mM $MgCl_2$, 10 mM dithiothreitol, 200 mg/l BSA and 20 % glycerol). The assay in situ was performed for 5–8 h at room temperature with 50 µCi of [γ-^{32}P]ATP in 1 gel volume of assay buffer contained in a sealed plastic bag. Afterwards, the gel was washed for 4 × 20 min with 20 gel volumes of distilled water, and incubated overnight in the presence of 5 g of Dowex 1-X8 wrapped in one layer of Miracloth. Subsequently, the gel was washed twice with 20 gel volumes of 10 % trichloroacetic acid/1 % sodium pyro-phosphate and equilibrated with distilled water. Finally, the gel was covered with plastic wrap (or dried on to Whatman 3MM paper) and autoradiographed by using Fuji RX X-ray film and Kodak intensifying screens.

Determination of native molecular parameters

Gel filtration was performed at a rate of 1.5 ml/h through a column of Sephacryl S-200 (0.7 cm × 50 cm) equilibrated with 400 mM KCl/50 mM Tris/HCl (pH 7.0)/15 % glycerol/5 mM 2-mercaptoethanol. Glycerol-gradient sedimentation was per-formed at 55000 rev./min for 40 h in a Beckman SW60 rotor in a linear gradient of 10–30 % glycerol in 200 mM KCl/50 mM Tris/HCl (pH 7.0)/5 mM 2-mercaptoethanol. Molecular masses and frictional ratios were calculated as described by Siegel and Monty (1966). Partial specific volume was assumed to be 0.725 on the basis of the value calculated for bovine casein kinase I-α (Rowles et al., 1991), by using partial specific volumes of amino acids compiled by Creighton (1984). Determination of axial ratios was performed by interpolation of the data tabulated by Schachman (1959).

Determination of protein concentration

Protein concentration was determined with a commercial reagent from Bio-Rad, by the method of Bradford (1976).

Identification of phosphoamino acids

Radiolabelled phosphoamino acids were obtained by hydrolysis of the phosphorylated protein sample in 6 M HCl at 110 °C for 1 h, and were separated by thin-layer electrophoresis at pH 3.5 and identified by staining with ninhydrin or autoradiography as described by Cooper et al. (1983).

Calculation of kinetic parameters

K_m and V_{max} values were calculated from initial-velocity data by using the program EnzFitter supplied by Elsevier–Biosoft.

RESULTS

Purification of casein kinase I

Most purification protocols developed for mammalian casein

Table 1 Summary of the purification scheme of casein kinase I from broccoli

Step	Volume (ml)	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg)	Yield (%)	Purification (fold)
Crude extract	12000	37200	1056	0.028	100	1
Clarified extract	12000	25200	745	0.030	70	1.1
20% PEG 6000 pellet	1600	10240	596	0.058	56	2.1
Phosphocellulose	350	84	500	5.96	47	213
Phenyl-Sepharose	35	2.45	466	190	44	6790
CM-Sephacel	6	0.84	218	260	21	9290
CKI-7–Sepharose	1.25	0.031	57	1824	5.4	65140
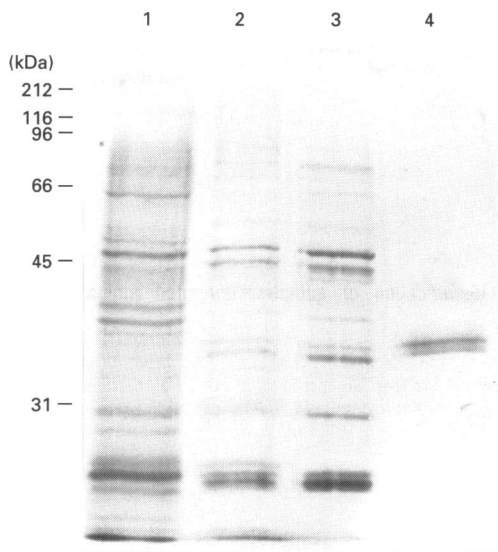
Figure 1 Polypeptide composition of casein kinase I fractions during the final stages of purification

Lane 1, phosphocellulose pool; lane 2, phenyl-Sepharose pool; lane 3, CM-Sephacel pool; lane 4, CKI-7–Sepharose pool. The positions of molecular-mass markers are given in kDa.

kinase I involve five or more chromatographic steps, which result in a relatively low yield (e.g. Dahmus, 1981a; Ahmad et al., 1984; Rowles et al., 1991). Because of the still lower abundance of the plant enzyme, this problem was even more pronounced, and significant losses occurred during classical column procedures to a degree that abolished any resulting purification. Therefore we adapted the affinity procedure on an immobilized casein kinase I-specific inhibitor, CKI-7, developed by Chijiwa et al. (1989).

An efficient initial purification of the plant enzyme was achieved by phosphocellulose chromatography. At this stage casein kinase I is known to co-purify partially with casein kinase II, and indeed, the phosphocellulose eluate contained two peaks of casein kinase: about 80% of the activity centred around 500 mM KCl and 20% centred around 700 mM KCl. These two casein-phosphorylating activities could be completely separated by subsequent DEAE-cellulose chromatography after dialysis: about 80% of the activity loaded did not bind to the column at low salt (70 mM KCl), whereas the remaining 20% was retained (results not shown). This procedure is a well-established approach to the separation of casein kinases I and II (the enzymes'

nomenclature has originated from the actual sequence of elution from DEAE-cellulose). Although DEAE-cellulose chromatography resulted in a good separation of the two activities, it was not very practical for a large-scale procedure, because it required prior dialysis of large volumes. We have observed that the DEAE-cellulose flow-through (casein kinase I) adsorbed to phenyl-Sepharose at moderate salt concentrations (500 mM), whereas the DEAE-cellulose-adsorbing fraction (casein kinase II-like) required the addition of at least 1 M $(NH_4)_2SO_4$; accordingly, hydrophobic-interaction chromatography on phenyl-Sepharose can be a useful alternative of separating casein kinase I from other activities without the need for dialysis. Thus our final preparative procedure included direct loading of the phosphocellulose eluate on to phenyl-Sepharose. The phenyl-Sepharose eluate was concentrated and depleted of ethylene glycol by chromatography on CM-Sephacel. The CM-Sephacel fraction was loaded on CKI-7–Sepharose and eluted as an essentially pure protein by ethylene glycol. The summary of the purification procedure is given in Table 1, and the polypeptide composition of sequential fractions revealed by silver staining is shown in Figure 1. The final fraction consisted of at least 95% of a protein doublet migrating at 36–37 kDa. Some minor contaminant of ~ 24 kDa, not exceeding 5% of protein, was visible in certain preparations; it may correspond to a degradation product. The relative intensities of the two bands varied slightly during purification; in particular, the lower band appeared to be more abundant in the phenyl-Sepharose and CM-Sephacel pools, whereas quite visible enrichment of the upper band took place during the final affinity-step. At this step, about 10% of the activity loaded was recovered in the flow-through fraction, which was enriched in the lower band. Additional minor differences may be caused by different representation of the bands in peak fractions; however, no separation of casein kinase I activity into discernible peaks or detectable variation of band intensity within a peak was observed with any of the chromatographic techniques used. The relative intensities of the two bands did not change upon storage at −20 °C up to several months, and were not affected by treatment with calf alkaline phosphatase (results not shown).

Identification of the catalytic subunit

The enzymic activity could be renatured after SDS/PAGE, and the enzyme assay in situ was performed in a gel which included partially dephosphorylated casein added before polymerization. Two closely migrating bands of casein-phosphorylating activity were observed at 36–38 kDa (Figure 2a), corresponding to the predominant protein components visible after silver staining in the purified fraction. The enzyme fraction used for this ex-
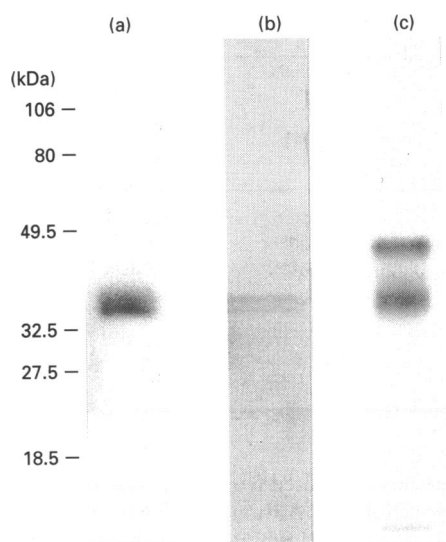
Figure 2   Identification of the catalytic and immunoreactive subunit of casein kinase I

(a) Activity gel. Autoradiogram of an activity gel with separated and renatured 0.1 nmol/min casein kinase I of phenyl-Sepharose pool. (b) Western blot. Immunoblot of 2 μg protein of the CM-Sephacel pool containing ∼ 0.28 μg of broccoli casein kinase I, probed with a 1:50 dilution of immune antiserum raised against bovine casein kinase I. (c) Autophosphorylation. Autoradiogram of an SDS/PAGE gel separating phosphorylation products of a reaction mixture containing 0.5 nmol/min of casein kinase I of the CKI-7-Sepharose pool incubated for 10 min without any exogenous substrate. The positions of prestained molecular-mass markers are given in kDa.

Figure 3   Identification of autophosphorylated amino acids in casein kinase I

Autoradiogram of hydrolysis products of phosphorylated casein kinase I (CKI-7-Sepharose pool) separated by thin-layer electrophoresis at pH 3.5 as described in the Experimental section. Abbreviations: P-Ser, phosphoserine; P-Thr, phosphothreonine; P-Tyr, phosphotyrosine.

periment, the phenyl-Sepharose pool, showed a stronger lower band of the purified doublet upon silver staining, which indicates that the relative intensities of the two activity bands match those of the silver-stained bands at this particular stage of purification (compare Figure 2a and Figure 1, lane 2).

Immunological cross-reactivity

After an overnight binding reaction with a 1:50 dilution of the rabbit serum raised against bovine casein kinase I, a cross-reacting double band of 37–38 kDa was detected in a highly enriched fraction of broccoli casein kinase I (Figure 2b). At the same dilution, the antibody did not cross-react with highly purified preparations of broccoli casein kinase II and Ca^{2+}-dependent protein kinase containing about 0.2–0.5 μg of the kinase protein in overstained Western-blot or dot-blot assays (L. J. Klimczak, unpublished work).

Autophosphorylation

When the phosphorylation reaction was performed with the pure enzyme fraction without the addition of exogenous protein substrates, and radioactively labelled protein products were analysed, a double band of 36–37 kDa became labelled at the start of the reaction and several slower-migrating bands appeared during its progress (Figure 2c shows an intermediate state of the reaction). Although most of these bands were weak, and their labelling intensity did not increase with time, an intense wide band of 42 kDa accumulated during the reaction and became the predominant labelled band at its later stages. The appearance of this radiolabelled band was accompanied by a decrease in the intensity of the silver-stained 36–37 kDa doublet and the appearance of a wide silver-stained band of 42 kDa (results not
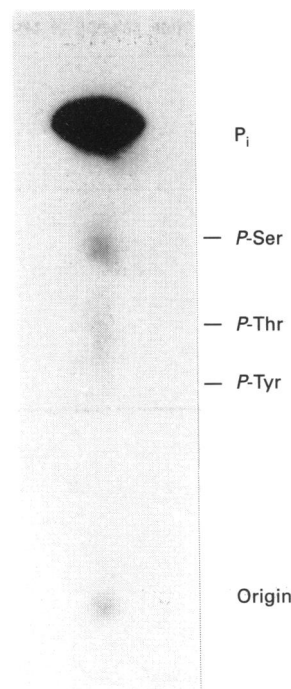
shown). These results resemble very closely the kinetics of autophosphorylation of the bovine enzyme (Dahmus, 1981a). They correspond to the conversion of the casein kinase I proteins into slower-migrating phosphorylated forms with the wide 42 kDa band as the final product, which is most likely an incompletely resolved doublet. When this product was isolated from a gel, it was determined that up to 4 molecules of phosphate could be incorporated into broccoli casein kinase I by auto-phosphorylation, similarly to the 2–4 molecules incorporated into the bovine enzyme (Dahmus, 1981a). Hydrolysis of the autophosphorylated sample and analysis of phosphoamino acids by thin-layer electrophoresis revealed that serine and threonine were the targets of phosphorylation, with the respective ratio of about 3–4:1 (Figure 3).

Determination of native molecular mass

Gel filtration of the phenyl-Sepharose fraction performed together with standard calibration proteins revealed a Stokes radius of 2.76 nm (Figure 4a), and glycerol-gradient sedimentation revealed a sedimentation coefficient, $s_{20,w}$, of 3.0 S (Figure 4b). The native molecular mass of 34.2 kDa was calculated from the equation of Siegel and Monty (1966). It corresponds to the molecular mass obtained by SDS/PAGE and indicates that the enzyme is composed of a single subunit. The frictional ratio calculated for casein kinase I from the native parameters was 1.288, and corresponded to an axial ratio of 5.5 for a prolate ellipsoid. The mass difference observed by SDS/PAGE between the two isolated isoforms was beyond the resolution capacity of the native-molecular-mass determination methods, since only single symmetrical peaks were observed (results not shown).
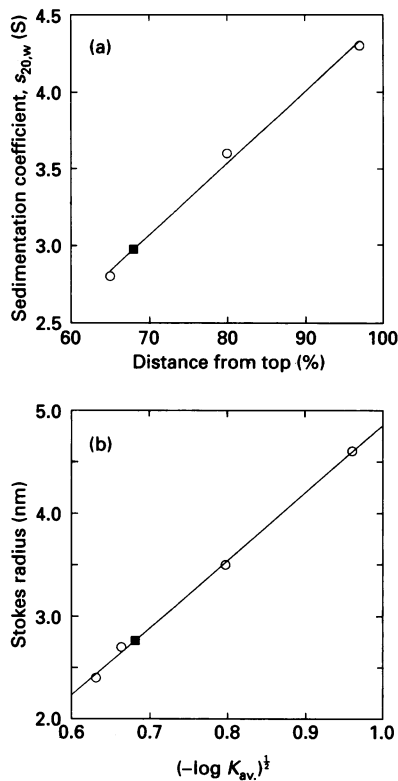
Figure 4   Determination of native molecular mass of casein kinase I

(a) Glycerol-gradient sedimentation. Sedimentation-coefficient standards, shown as ○, were: BSA ($s_{20,w}$ = 4.3 S); ovalbumin (3.6 S); carbonic anhydrase (2.8 S). The position of the casein kinase I peak is indicated by ■. (b) Gel filtration. Stokes-radius standards, shown as ○, were: rabbit muscle aldolase (4.6 nm), BSA (3.5 nm), ovalbumin (2.7 nm) and carbonic anhydrase (2.4 nm). The position of the casein kinase I peak is indicated by ■.

Table 2   Enzymic properties of casein kinase I

Conditions	Activity (%)
Standard assay	100
KCl added (mM):	
50	103
100	50
200	26
400	22
EGTA added (2 mM)	100
Heparin added (μg/ml):	
0.5	78
2	53
Polylysine added (200 μg/ml)	33
CKI-7 added (μg/ml):	
10 μM	63
50 μM	33
Partially dephosphorylated casein replaced by:	
Histone IIIS (0.25 mg/ml)	1
α-Casein (0.25 mg/ml)	105
β-Casein (0.25 mg/ml)	131
Phosvitin (0.25 mg/ml)	98
Unlabelled ATP added (1 mM)*	2
Unlabelled GTP added (1 mM)*	100

*To determine the degree of competition, only incorporation of radiolabelled ATP was measured in this experiment.

Enzymic properties

As shown in Table 2, broccoli casein kinase I phosphorylated preferentially several characteristic acidic proteins, but, in contrast with casein kinase II, was not stimulated by polylysine. It utilized only ATP as a phosphate donor, and no competition for ATP was observed with a 100-fold excess of GTP. The effects of univalent cations and some inhibitors are also summarized in Table 2. The IC_{50} value for CKI-7 was 21 μM. The K_m value for ATP was 5.1 μM at 0.25 mg/ml partially dephosphorylated casein, and the K_m value for partially dephosphorylated casein was 0.12 mg/ml at 50 μM ATP. The $V_{max.}$ from both determinations was 2100 nmol/min per mg.

DISCUSSION

The comparison of recently obtained casein kinase I sequences from vertebrates (Rowles et al., 1991) and yeast (Robinson et al., 1992; Wang et al., 1992) reveals quite significant differences in the predicted protein sizes: 37–39 kDa for the bovine enzyme and ~ 62 kDa for the yeast enzyme. The latter also contains a putative C-terminal prenylation site (Cys-Cys), which reflects its membrane localization, as opposed to the cytosolic casein kinase I from animal tissue (Wang et al., 1992). These substantial differences contrast with a high degree of conservation of casein kinase I sequences in the area of homology (about 54 % identity in a 300-amino-acid region, and 74 % identity in a 80-amino-acid fragment of the catalytic domain) and raise the question of possible divergent evolution of the biological function of casein kinase I, similar to that of Ras proteins (Wigler, 1990).

In light of the differences between yeast and animal casein kinases I, useful insights should be obtained from the study of these kinases in other groups of organisms, in particular plants. It recently became apparent that some plant protein kinases possess unique features distinct from those of their animal counterparts (see, e.g., Putnam-Evans et al., 1990). Therefore, rigorous biochemical and molecular-genetic studies of plant casein kinase I could further the understanding of the degree of structural and functional similarities among the members of this enzyme group.

Although several enzymes with the properties of casein kinase I have been described in plants (for full listing see Tuazon and Traugh, 1991), most of them were relatively crude preparations in which proteins in a wide molecular-mass range of 20–60 kDa were identified. The most advanced purification protocol to date was published by Rychlik and Zagórski (1980), describing the purification from wheat germ of a 20 kDa protein with a specific activity of 215 nmol/min per mg. Although the authors did not explicitly address its relationship to casein kinase I, the pI of 9.2 suggested that the preparation contained in fact casein kinase I. Since the specific activity of that enzyme was 7–10 times less than the values found for casein kinase I (Ahmad et al., 1984; Rowles et al., 1991; the present work), it appears that that protein was either a poorly active degradation product or it was an enriched contaminant in a preparation which was only partially pure. Indeed, an abundant contaminant of about 20 kDa can be seen in this study in partially pure fractions (Figure 1, lane 3). The work of Dobrowolska et al. (1987) described in maize seedlings a casein kinase I preparation with a specific activity of 69 nmol/min per mg and systematically classified its properties as matching those of casein kinase I. Some differences observed therein were probably due to a low degree of purity, e.g. the absence of sensitivity to heparin (cf. Ahmad et al., 1984).

In the present work, we describe the purification of plant casein kinase I to near homogeneity. Although broccoli is in our

experience one of the better sources of plant protein kinases, the enzyme is less abundant than in certain animal tissues (Rowles et al., 1991), since the specific activity in the crude extract is 3–10 times lower. As a consequence, the final purification factor is about 10 times higher than the ~ 6000-fold enrichment required for the purification of the bovine enzyme (Rowles et al., 1991). In addition, the enzyme is initially more diluted, since at least several kg of starting material are required for a successful purification. We overcame the problems of low abundance and the resulting loss of activity by decreasing the number of chromatographic steps and by using a very efficient and selective step of affinity chromatography on the immobilized casein kinase I inhibitor, CKI-7. Similar purification strategies, in particular the use of related approaches reviewed by Ferrari and Thomas (1991) and Jenö and Thomas (1991), could be applied for purification of many other plant protein kinases which so far had to be studied predominantly in a quite crude form.

The properties of casein kinase I from broccoli are very similar to those of the enzymes purified from animal sources. It displays their characteristic chromatographic properties, such as the elution from phosphocellulose at 500 mM KCl, and no adsorption to DEAE-cellulose at 70 mM KCl. The latter is a reflection of the very basic character of casein kinase I (pI 9.0–9.6, reviewed by Tuazon and Traugh, 1991). The broccoli enzyme showed several characteristic enzymic features of animal casein kinase I, in particular those distinct from casein kinase II: utilization of ATP alone as a phosphate donor and no stimulation of casein phosphorylation by polylysine. The K_m value for ATP is similar to those reported for other casein kinase I preparations (usually 7–25 μM; Tuazon and Traugh, 1991). So is the IC_{50} value for CKI-7 (9.5 μM for casein kinase I from bovine testis; Chijiwa et al., 1989). However, the plant enzyme is not stimulated by univalent cations (Table 2).

Broccoli casein kinase I is composed of a single subunit of 36–37 kDa. The purification of a protein doublet is not surprising, since band microheterogeneity was observed in many other protein kinase preparations, including those of animal casein kinase I (Ahmad et al., 1984). In particular, those protein kinases which are encoded by multiple genes can be separated into closely related isoforms. In the case of a higher degree of heterogeneity, such isoforms can be separated chromatographically (e.g. protein kinase C isoenzymes; Huang and Huang, 1991). When the isoforms are very closely related to each other, the separation can only be achieved by techniques of higher resolution, such as SDS/PAGE (e.g. plant Ca²⁺-dependent protein kinase; Putnam-Evans et al., 1990) or isoelectric focusing (microheterogeneity within individual protein kinase C isoenzymes; Huang and Huang, 1991). Since casein kinase I is encoded by multiple genes in animal and yeast systems (Rowles et al., 1991; Robinson et al., 1992), it is likely that this is also the case in plants and that the two bands are products of different genes. Since the bands are not inter-converted by phosphatase treatment, it is rather unlikely that the microheterogeneity would result from differential phosphorylation *in vivo*. However, in spite of the use of protease inhibitors during isolation, it cannot still be excluded that the two proteins may be products of proteolytic cleavage close to the terminus. This matter can be fully resolved when isolation of plant casein kinase I genes will allow generation of sequence-specific antibodies. These tools will also facilitate intracellular localization studies.

The protein doublet purified from broccoli showed immunological cross-reaction with the antibodies raised against bovine casein kinase I. The antibody concentration used for binding to the blot was higher than the 1:1000 dilution required to recognize the bovine enzyme (M. Dahmus, personal communication), but no cross-reaction was observed with other plant protein kinases at the same conditions. The anti-(bovine casein kinase I) antibody was also characterized previously, and no cross-reaction was observed with calf casein kinase II and cyclic-AMP-dependent protein kinase (Dahmus, 1981b). Therefore, it can be concluded that the plant casein kinase I is related to the animal enzyme, but significant divergence of their sequences must have taken place. This is in agreement with the previously mentioned sequence comparisons of the bovine and yeast enzymes, which reveal a homology of more than 70 % only within a region of 80 amino acids. The quantification of the relatedness of the plant enzyme, as well as questions about the number of distinct forms of casein kinase I in plants, can be addressed more closely when the corresponding gene sequence(s) are available.

Although the importance of casein kinase I for the control of some essential cellular function was demonstrated recently by genetic studies in yeast (Robinson et al., 1992), no precise physiological function or possible mode of regulation is known for this multifunctional protein kinase. The elucidation of these unknowns will require a stronger focus on the *in vivo* substrates of various casein kinase I forms from different biological systems. The availability of a purified plant casein kinase I opens the opportunities of such studies in plant systems.

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