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PURIFICATION AND CHARACTERIZATION OF PROTEIN KINASE C FROM A HIGHER PLANT, *Brassica campestris* L.

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SUMMARY: Protein kinase C (PKC) was partially purified from *Brassica campestris* L., by successive chromatographies on DEAE-cellulose membrane, hydroxyapatite and phenyl-5PW columns. The purified preparation showed typical characteristics of the conventional type of mammalian PKC that responds to Ca^{2+}, phosphatidylserine, and diacylglycerol or the tumor-promoting phorbol ester, phorbol 12-myristate 13-acetate. The plant PKC activity was apparently associated with a 75-kDa polypeptide that was recognized by an antibody against the catalytic domain of rat PKC. Substrate specificity of the plant PKC was similar to that of the rat PKC. A synthetic peptide corresponding to residues 4-14 of myelin basic protein, which is a selective substrate for the mammalian PKC, was phosphorylated efficiently by the plant PKC. These results indicate the existence of a PKC equivalent in higher plant cells.

Protein kinase C (PKC) plays crucial roles in signal transduction coupling with hydrolysis of phosphoinositides and other membrane phospholipids (1). PKC was first found in mammalian brain tissues and has been shown to be distributed widely in the animal kingdom. The mammalian PKC is revealed to be a family of proteins that have structures related to one another. Initially, the conventional PKC group (with the α-, βI-, βII-, and γ-subspecies) was isolated, and diacylglycerol and tumor-promoting phorbol esters are shown to activate enzymes of this group in the presence of membrane phospholipids and Ca^{2+}. The second is the novel PKC group (with the δ-, ε-, η-, and θ-subspecies) that is activated by diacylglycerol and phorbol ester even in the absence of Ca^{2+}.

Intracellular signaling pathway through hydrolysis of phosphoinositides has been studied not only in animals but also in plants, and most of the components of the mammalian signal

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Abbreviations: PKC, protein kinase C; PS, phosphatidylserine; DO, diolein; PMA, phorbol 12-myristate 13-acetate; MBP, myelin basic protein.

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transduction system have been found to have structural and functional equivalents in plant cells (2,3). Protein kinase activities resembling the mammalian PKC have been isolated in lower eukaryotes such as baker's yeast (4-6) and slime molds (7-9). Attempts have also been made to investigate PKC activity in higher plants, and PKC-related kinase activity has been reported in various higher plants (10-17). However, the plant protein kinase fractions in most of these studies were still contaminated with other well-known Ca^{2+}-dependent protein kinases (18,19). The protein kinase activity detected in oat plasma membrane is reported to be dependent on both Ca^{2+} and lipid, although its biochemical and immunochemical properties are distinct from those of mammalian PKC (15). In addition, the molecular mass estimated for the PKC-like activity varied among the reports (12,14,15,17). Thus, it is not clear whether the protein kinase activity reported in higher plants derives from a gene homologous to the mammalian PKC. On the other hand, the gene encoding PKC homologue itself has not yet been isolated in higher plants, although a number of studies have been carried out (2,18). Drøbak discussed that PKC may not have a direct equivalent in higher plant cells and other protein kinases carry out functions equivalent to those of PKC in mammalian cells (2).

In this study, PKC in plant cells has been identified and partially purified, using *Brassica campestris* L. as an enzyme source. Here we report the presence of a plant PKC that resembles the conventional type of mammalian PKC.

MATERIALS AND METHODS

Plant growth. Seeds of *Brassica campestris* L. were planted in vermiculite. After one-week growth in the dark at 25°C, individual seedlings were inserted through the hole of a rubber stopper and secured with a foam rubber plug. Plants were grown hydroponically with roots in half-strength Ohtuka solution (Ohtuka chemicals Co. Ltd.) sparged continuously with air for three weeks at 25°C. The incubator was programmed for a 12-hr light/12-hr dark cycle at 25°C.

Chemicals. [γ-^{32}P]ATP was obtained from ICN. Phosphatidylserine (PS) and diolein (DO) were purchased from Serdary Research Laboratories. Phorbol 12-myristate 13-acetate (PMA) was a product of LC Services. Myelin basic protein (MBP), protamine sulfate, and soybean trypsin inhibitor were obtained from Sigma. Leupeptin, pepstatin, and chymostatin were purchased from Peptide Institute (Osaka). (*p*-Amidinophenyl)methanesulfonyl fluoride hydrochloride was purchased from Wako (Osaka). Prestained SDS-PAGE standards (phosphorylase B, 106 kDa; bovine serum albumin, 80.0 kDa; ovalbumin, 49.5 kDa; carbonic anhydrase, 32.5 kDa; soybean trypsin inhibitor, 27.5 kDa; lysozyme, 18.5 kDa) were obtained from Bio-Rad. The peptide substrates, αPKC peptide (corresponding to residues 15-31 of αPKC, substituting Ser for Ala^{25}; DVANRFARKGSLRQKNV) (20) and MBP_{4-14} (residues 4-14 of MBP; QKRPSQRSKYL) (21) were synthesized with an automated peptide synthesizer (Applied Biosystems, model 430A). Calf thymus H1 histone and rat brain αPKC were prepared as described (22). An anti-rat γPKC kinase domain serum was prepared as described (23). All other chemicals were of analytical grade.

Enzyme assay. Protein kinase activity was assayed by measuring the incorporation of ^{32}Pi from [γ-^{32}P]ATP into the phosphate acceptor as described (20,23). The reaction mixture (50 μl) contained 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 μM [γ-^{32}P]ATP (3,000

cpm/pmol), 200 μg/ml H1 histone, enzyme, and other chemicals such as 80 μg/ml PS, 8 μg/ml DO, 16.2 nM PMA, and various concentrations of $CaCl_2$ as indicated in each experiment. After incubation for 10 min at 30°C, aliquots of the mixture (40 μl) were withdrawn and spotted onto phosphocellulose strips (P81, Whatman). The strips were washed for 5 min five times by immersion in 75 mM phosphoric acid (10 ml per sample), and the radioactivity remaining on the strips was quantified by Cerenkov counting using a scintillation spectrometer. Where indicated, phosphate acceptors such as MBP (200 μg/ml), protamine sulfate (400 μg/ml), αPKC peptide (200 μM), and MBP_{4-14} (40 μM) were employed instead of H1 histone.

Immunoblot analysis. Immunoblot analysis was performed with an antibody against the protein kinase domain of rat γPKC as described previously (23). Samples were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore) by using a semi-dry blotter (Sartorius). The membrane was soaked in T-TBS (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween 20) containing 1% skim milk for 1 hr at room temperature, and then incubated with the first antibody 100-fold diluted in the T-TBS/skim milk solution for 16 hr at 4°C. After washing with T-TBS, the membrane was incubated with alkaline phosphatase-conjugated anti-rabbit IgG (Cappel) for 1 hr at room temperature, and the immune complex was detected by using 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt as substrate.

RESULTS

Purification of plant PKC. All procedures for chromatography were performed on an FPLC system (Pharmacia LKB). All manipulations were carried out at 0-4°C. Whole seedlings (50 g wet weight) were suspended in 100 ml of 20 mM Tris-HCl (pH 7.8) containing 10 mM EGTA, 2 mM EDTA, 10 mM 2-mercaptoethanol, 50 μM (*p*-Amidinophenyl)methane-sulfonyl fluoride hydrochloride, 0.1 mg/ml leupeptin, 0.5 mg/ml pepstatin, 0.5 mg/ml chymostatin, and 0.5 mg/ml soybean trypsin inhibitor. The cell suspension was homogenized for 3 min by using a Cycle blender (Oster Corporation, USA). The homogenate was filtered through cotton gauze, and was homogenized further by a Teflon-glass homogenizer (Omega Electric, Tokyo). The resulting homogenate was centrifuged for 60 min at 120,000 x g. Ammonium sulfate was added to the supernatant up to 50% saturation. After centrifugation for 20 min at 10,000 x g, the precipitate was recovered and dissolved in 10 ml of buffer A (20 mM Tris-HCl (pH 7.8), 0.5 mM EGTA, 0.5 mM EDTA and 20 mM 2-mercaptoethanol). The solution was dialyzed against Buffer A for 3 hr at 4°C, and then diluted with buffer A till the electric conductivity of the solution reached to 2.0 mMho/cm. The diluted sample was applied onto a DEAE-MemSep 1000 membrane (Millipore) that had been equilibrated with buffer A, at a flow rate of 1 ml/min. After washing the membrane with 30 ml of buffer A, protein was eluted with a linear gradient of NaCl (0-0.4M) in 80 ml of buffer A. Fractions (2 ml each) were collected and assayed for protein kinase activity using H1 histone as substrate. Two peaks of protein kinase activity appeared as shown in Fig. 1 (fractions 5-8 and 9-14). Both fractions required Ca^{2+} for their enzymatic activity, and the protein kinase activity in the latter peak stimulated further by the presence of PS and DO comparing with that in the former peak.

The two peaks of protein kinase activity (fractions 5-14) were pooled and directly applied onto a hydroxyapatite KB column (Koken (Tokyo), 0.78 x 10 cm) previously equilibrated with

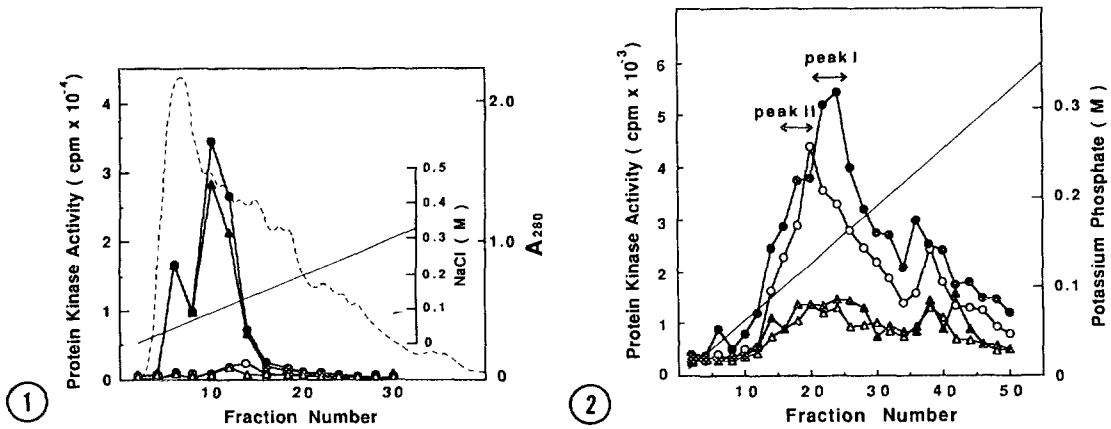

Figure 1. DEAE-MemSep membrane chromatography of PKC from *Brassica campestris* L. Protein kinase activity of each fraction was assayed with H1 histone as substrate as described in MATERIALS AND METHODS. (●——●), in the presence of PS, DO, and 10 μM $CaCl_2$; (O——O), in the presence of PS, DO, and 0.5 mM EGTA; (▲——▲), in the presence of 10 μM $CaCl_2$ without PS and DO; (Δ——Δ), in the presence of 0.5 mM EGTA without PS and DO; (----------), absorbance at 280 nm; (————), NaCl concentration.

Figure 2. Hydroxyapatite column chromatography of PKC from *Brassica campestris* L. Protein kinase activity of each fraction was assayed with H1 histone as substrate as described in MATERIALS AND METHODS. (●——●), in the presence of PS, DO, and 10 μM $CaCl_2$; (O——O), in the presence of PS, DO, and 0.5 mM EGTA; (▲——▲), in the presence of 10 μM $CaCl_2$ without PS and DO; (Δ——Δ), in the presence of 0.5 mM EGTA without PS and DO; (————), potassium phosphate concentration.

buffer B (20 mM potassium phosphate (pH 7.5), 0.5 mM EGTA, 0.5 mM EDTA, 10% glycerol, and 10 mM 2-mercaptoethanol) at a flow rate of 0.4 ml/min. After washing with 50 ml of buffer B, enzymes were eluted with a 50-ml linear concentration gradient (20 to 320 mM) of potassium phosphate in buffer B. Fractions (1 ml each) were collected and assayed for protein kinase activity as shown in Fig. 2. The protein kinase activity recovered from the hydroxy-apatite column was stimulated efficiently by PS and DO, comparing with that of DEAE-MemSep fractions, suggesting that most of the Ca^{2+}-dependent and lipid-independent kinases were removed by the hydroxyapatite column chromatography. Among the active fractions, the main peak (peak I, fractions 21-25) was stimulated by Ca^{2+} in the presence of PS and DO, whereas another peak (peak II, fractions 16-20) eluted in front of peak I was stimulated by PS and DO even in the absence of Ca^{2+}. These results suggest that the hydroxyapatite column fraction contains at least two distinct enzymes. The properties of the protein kinase activities in peak I and peak II are similar to those of the conventional and novel types of mammalian PKC, respectively.

To characterize the conventional type of plant PKC, fractions 21-25 (peak I) were pooled and subjected to further purification. Solid ammonium sulfate was added to the pooled fraction to a final concentration of 0.8 M, and the solution was applied on a TSK phenyl-5PW column

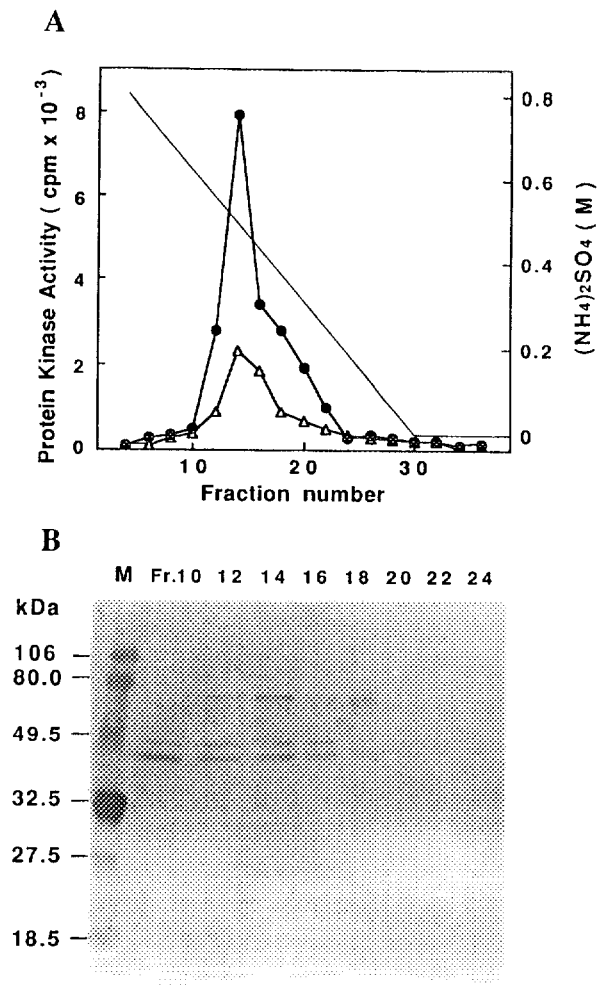

Figure 3. TSK phenyl-5PW column chromatography of PKC from *Brassica campestris* L. **(A)** Protein kinase activity. Each fraction was assayed with H1 histone as substrate as described in MATERIALS AND METHODS. (●——●), in the presence of PS, DO, and 10 μM $CaCl_2$; (Δ——Δ), in the presence of 0.5 mM EGTA without PS and DO; (————), ammonium sulfate concentration. **(B)** Immunoblot analysis. Fractions (20 μl each) from the column were analyzed by immunoblotting as described in MATERIALS AND METHODS. M, Prestained marker proteins (Bio-Rad).

(TOSOH (Tokyo); 2.15 x 15 cm) equilibrated with 0.8 M ammonium sulfate in buffer A. After washing the column with 50 ml of buffer A containing 0.8 M ammonium sulfate, PKC was eluted by application of a 90-ml decreasing linear concentration gradient of ammonium sulfate (0.8-0 M) in buffer A at a flow rate of 3 ml/min. Fractions (3 ml each) were collected, and assayed for the enzyme activity as shown in Fig. 3A. There appeared a single peak of PKC activity that is stimulated by Ca^{2+}, PS, and DO. Immunoreactive proteins in each fraction were analyzed by immunoblotting with an antibody against the kinase domain of rat γPKC (Fig. 3B). Three major polypeptide bands (75 kDa, 49 kDa, and 40 kDa) were detected by this antibody.

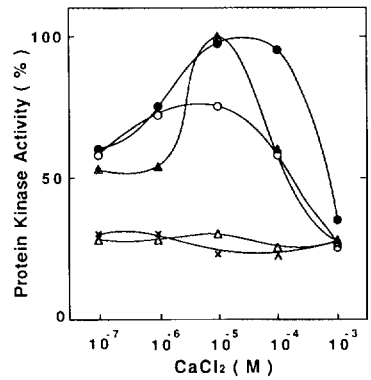

Figure 4. Kinetic properties of the plant PKC. PKC purified from *Brassica campestris* L. was assayed with H1 histone as substrate in the presence or absence of PS, DO, and PMA at various concentrations of $CaCl_2$ as described in MATERIALS AND METHODS. The results were normalized to the maximum activity. (●——●), in the presence of PS and DO; (▲——▲), in the presence of PS and PMA; (○——○), in the presence of PS alone; (x——x), in the presence of DO alone; (△——△), in the presence of PMA alone.

The intensity of 75-kDa band was in accordance with the PKC activity, while other polypeptides did not show such an accordance (Fig. 3A and B). These small immunoreactive polypeptides were also observed in some purified PKC preparations (23), and supposed to be degradation products of PKC. Immunoblot analyses using other antibodies against the conserved regions of mammalian PKC gave similar results (data not shown). Since the conventional type of mammalian PKC has molecular mass of 77-78 kDa (1), the results above suggest that the 75-kDa protein is most likely the conventional type of plant PKC. Finally, fractions 12-16 were pooled and used for characterization of the enzyme.

Enzymatic properties of plant PKC. Effects of PS, DO, and PMA on the purified plant PKC were examined using H1 histone as a phosphate acceptor at various concentrations of Ca^{2+} (Fig. 4). The characteristics of the plant PKC were very similar to those of mammalian conventional PKC. The enzyme required PS, DO, and Ca^{2+} to exhibit full enzymatic activity, and PMA replaced the effect of DO. Substrate specificity of the plant PKC was also studied comparing with rat αPKC (Table 1). No significant difference was found between the two enzymes when MBP, H1 histone, αPKC peptide, and MBP_{4-14} were used as substrates, but protamine sulfate was a poor substrate for the plant PKC. It is worth noting that the plant PKC efficiently phosphorylated MBP_{4-14} which has been demonstrated to be a synthetic substrate specific to PKC among various mammalian protein kinases (21).

DISCUSSION

In this study, we characterized a plant PKC partially purified from *Brassica campestris* L. Separation of the plant PKC from other protein kinases such as Ca^{2+}-dependent kinases was crucial for the identification and characterization of this enzyme. This was performed by using

Table 1. Substrate specificity of the plant PKC

Substrate	Activity (%)	
	Plant PKC	Rat αPKC
MBP	100	100
H1 histone	97	86
Protamine sulfate	47	129
αPKC peptide	154	174
MBP_{4-14}	89	83

PKC activity was assayed with each substrate in the presence of PS, DO, and 10 μM $CaCl_2$ as described in MATERIALS AND METHODS. Activity is expressed as a percentage of that obtained with MBP for each enzyme.

hydroxyapatite column chromatography that is usually used for separation of mammalian PKC isozymes (20,22). The Ca^{2+}-dependent kinase activity was recovered in the flow-through fraction of the chromatography. Hydroxyapatite column chromatography was also effective to remove ribulose-1,5-bisphosphate carboxylase/oxygenase that is abundantly present in plant leaves. Two distinct PKC activities (peak I and II) were found in the hydroxyapatite column chromatography (Fig. 2) and seemed to be similar to the conventional and novel types of mammalian PKC, respectively. The peak I fraction was further purified in this study. An immunoreactive polypeptide of 75 kDa was detected in the final preparation by immunoblotting with an antibody against the catalytic domain of rat PKC (Fig. 3B). The activity found in the presence of 0.5 mM EGTA without PS and DO (Fig. 3A) is higher than that of mammalian PKC. The purified enzyme recognized MBP_{4-14} (Table 1), which has been used as a selective substrate for mammalian PKCs, and was activated by diacylglycerol and phorbol ester in the presence of phospholipid and Ca^{2+} (Fig. 4). Thus, it was concluded that the purified enzyme has the characteristics of the conventional type of mammalian PKC. As to the putative novel type of PKC (peak II) found in the hydroxyapatite column chromatography, we were unable to detect its activity in the factions of the following TSK-phenyl 5PW column chromatography. The peak II enzyme seems to be unstable during the purification procedure, however, further studies are necessary to purify and characterize this type of PKC.

It is now evident that some of the cellular signaling pathways in higher plants are mediated by protein phosphorylation and dephosphorylation as in animal cells (2,3). For example, the activity of nitrate reductase in spinach leaf is modulated by reversible phosphorylation in a phytochrome-mediated fashion (24-26). Since the light-mediated stimulation of nitrate reductase in maize can be mimicked by phorbol ester, the involvement of PKC-type enzyme is suggested in this pathway (15). PKC is also implicated in the stomatal opening in response to white light (27). Protein kinases such as conventional and novel type of PKCs we have shown here may be involved in these signaling pathways. However, the structural and genetical features of the plant PKC are presently unknown. Further enzymological and molecular

biological studies may clarify the physiological role of PKC in cellular signal transduction of higher plants.

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