Purification of a 34 kDa ribonucleoprotein (p34) from spinach chloroplasts as an effective phosphate acceptor for casein kinase II

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Abstract: A 34 kDa ribonucleoprotein (p34) was purified to homogeneity from a 1.0 M KCl extract of spinach chloroplasts as an effective phosphate acceptor for casein kinase II (CK-II) by means of heparin-agarose, DEAE-cellulose, Sephacryl S300, dsDNA-cellulose and Mono Q column chromatography. Biochemical analysis of purified p34 show that the native form of p34 is a monomeric protein and p34 is phosphorylated specifically by CK-II in the chloroplasts.

Key words: Protein purification; chloroplast ribonucleoprotein; casein kinase II (CK-II); spinach.

Introduction. Casein kinase II (CK-II) is a cAMP- and Ca\(^{2+}\)-independent serine/threonine protein kinase, which is distributed widely among eukaryotic cells. This kinase has been shown to play important roles in a variety of processes, such as regulation of cell proliferation, gene expression and DNA replication, because the kinase specifically modifies DNA-binding proteins [DNA topoisomerase,\(^{2)}\) DNA ligase\(^{3)}\) and transcriptional factors (Sp1\(^{5)}\) and serum response factor.\(^{6)}\)

In plant cells, CK-II has been purified and characterized biochemically from tobacco,\(^{7)}\) maize,\(^{8)}\) pea,\(^{9)}\) broccoli\(^{10)}\) and liverwort.\(^{11)}\) Plant CK-II is suggested to function as a kinase which regulate gene expression and cell proliferation. However, the precise biological roles of the kinase in plant cells remain unknown. For elucidation of biological roles of plant CK-II, to determine its native substrates is important.

Recently, it has been reported that (i) CK-II exists in spinach chloroplasts; and (ii) a 34 kDa ribonucleoprotein (RNP), which are thought to be involved in mRNA processing and/or stabilization in chloroplasts, functions as an effective phosphate acceptor for CK-II.\(^{12)}\)\(^{13)}\) In order to clarify the biological significance of the phosphorylation of p34 by CK-II in chloroplasts, the biochemical studies, such as determination of phosphorylating domain for CK-II in p34 and analysis of effects of the phosphorylation on the activity of the RNP, should be done. It is necessary for these analytical studies to purify p34 with high purity and yield from spinach chloroplasts.

The present study describes a method for purification of p34 from the crude extract of spinach chloroplasts and some biochemical properties of the protein.

Materials and methods. Chemicals. [\(\gamma\)\(^{32}\)P]ATP was obtained from Amersham Corp. (Arlington Heights, IL, U.S.A.); phenylmethylsulfonyl fluoride (PMSF), p-aminobenzamidine from Sigma Chemicals Corp. (St. Louis, MO, U.S.A.); trypsin inhibitor (soybean) from wako Pure Chemical Ind. (Osaka, Japan); and dsDNA-cellulose, Sephacryl S300, Mono Q HR 5/5 and Superdex 75 pg from Pharmacia Fine Chemicals (Uppsala, Sweden).

Crude extract of p34 from chloroplasts. Chloroplasts were isolated from leaves of spinach by the method described by Ohyama et al.\(^{14)}\) To extract p34, chloroplasts (about 1 kg wet weight) were homogenized with a glass homogenizer in 3,000 ml of Buffer A [50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10 mM 2-mercaptoethanol (2-ME), 0.1 mM Mg\(^{2+}\), 0.1 mM PMSF and 10% glycerol] containing 1.0 M KCl, 5 mg/ml of trypsin inhibitor and 0.5 mM p-aminobenzamidine and then sonicated for 30 sec in an ice bath. After centrifugation (33,000 \(\times\) g for 30 min), the supernatant was dialyzed against Buffer A containing 0.2 M KCl. The dialysate was used as a crude p34 extract for further purification.

Purification of CK-II from spinach chloroplasts. Two forms (\(\alpha\)-monomeric and oligomeric forms) of CK-II were
purified separately from a crude p34 extract of spinach chloroplasts by means of successive heparin-agarose column chromatography and gel filtration on Superdex 200 pg (HPLC), as reported previously. The oligomeric form of CK-II in the Superdex 200 pg fraction was further purified by Mono Q column chromatography and used as a purified CK-II in this study.

**SDS-PAGE and autoradiography.** Polypeptides phosphorylated by CK-II were detected by SDS-PAGE followed by autoradiography, after incubation (30 min at 25°C) of the partially purified or purified p34 fractions with the purified CK-II and 20 μM [γ-32P]ATP (500 cpm/pmol) in the presence of 3 mM Mn2+ and 10 μg/ml poly-Lys.

**Results and discussion.** To remove CK-II, the crude p34 extract (about 1,200 mg protein) was passed through a column of heparin-agarose in Buffer A containing 0.2 M KCl. After dialysis against Buffer A containing 0.15 M KCl, the heparin-agarose eluant (about 1,050 mg protein) was applied on a column (5 x 30 cm) of DEAE-cellulose. Elution was carried out in a step-wise manner with Buffer A containing 0.15 M, 0.6 M and 1.0 M KCl, successively (Fig. 1A). p34 was detected as one of the phosphorylated polypeptides when the 0.6 M KCl fraction was incubated with purified CK-II in the presence of 20 μM [γ-32P]ATP (500 cpm/pmol), 3 mM Mn2+ and 10 μg/ml poly-Lys (lane 6, Fig. 1B). After concentration by vacuum dialysis, p34 in the 0.6 M KCl fraction (about 250 mg protein) was further purified by gel filtration on Sephacryl S3000 (1.6 x 100 cm) in the presence of 0.6 M KCl. p34 was eluted between apparent molecular sizes of 25 kDa and 45 kDa, as estimated from the marker proteins tyroglobulin (669 kDa), γ-globulin (150 kDa), ovalbumin (45 kDa) and trypsin inhibitor (22 kDa) (Fig. 2).

Because p34 has a binding activity to nucleic acids, the protein in the Sephacryl S3000 fraction (about 60 mg protein) was purified by dsDNA-cellulose column (2.6 x 6 cm) chromatography after dialysis against Buffer B [50 mM Tris-HCl (pH 7.4), 0.2 mM EDTA, 10 mM 2-ME, 0.1 mM PMSF and 10% glycerol] containing 0.15 M KCl. Elution was carried out step-wise with Buffer B containing 0.15 M KCl then 0.7 M KCl (Fig. 3A). p34 was detected in the 0.7 M KCl eluted fraction (Figs. 3B, C). This step using the dsDNA-cellulose was an effective method for purification of p34, because more than 95% of the contaminated proteins eluted by 0.15 M KCl.

Finally, p34 in the dsDNA-cellulose fraction (about 2 mg protein) was purified by Mono Q column chromatography (HPLC) after dialysis against Buffer B containing 0.2 M KCl. The elution was carried out with a linear gradient between 0.2 M and 0.5 M KCl. As shown in Fig 4A, at least three distinct protein peaks (P-I, P-II and P-III) were obtained. The molecular size of the main polypeptide in the P-I, P-II and P-III fractions was estimated as 25 kDa (designated p25), 28 kDa (designated p28) and 34 kDa, respectively. It has been shown that p25 and p28 are degradation products derived from p34 during protein purification: (i) the protein ratio of P-III (p34) to P-I (p25) and P-II (p28), in Mono Q column chromatography (Fig. 3A), significantly decreases when the heparin-agarose p34 eluant is incubated for a long time (more than 5 hrs at 25°C).
in the absence of protease inhibitors (PMSF, p-aminobenzamidine and trypsin inhibitor); and (ii) the N-terminal 11 residues (A-V-L-E-G-E-S-D-P-E-G) of p28 (P-II fraction) are identical with the corresponding sequences of p34.\(^\text{12}\) p34 in the P-III fraction was phosphorylated by CK-II (lane 7, Fig. 4C) and pre-incubation of p34 with calf thymus dsDNA (10 \(\mu\)g/ml) resulted in great stimulation of its phosphorylation (lane 8, Fig. 4C). Moreover, p34 in the P-III fraction was a homogeneous phosphorylating polypeptide (lane 6, Fig. 5). Therefore, this fraction was used as purified p34 for the biochemical studies.

The native molecular weight of purified p34 was determined by gel filtration on Superdex 75pg column (HPLC) in Buffer B containing 0.6 M KCl. The size was found to be approximately 33 kDa as estimated from the elution positions after HPLC of BSA (67 kDa), ovalbumin (45 kDa), chymotrypsinogen (25 kDa) and cytochrome C (12.4 kDa) (Fig. 6). This value was corresponding to that
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approximately 34 kDa) determined by SDS-PAGE. These results show that the native form of p34 is a monomeric protein in the chloroplasts.

To examine the kinase specificity for phosphorylation of p34 in vitro, the purified p34 was incubated separately with four different protein kinases [monomeric CK-II from spinach chloroplasts, histone kinase II (HK-II) and histone kinase III (HK-III) from liverwort, and cAMP-dependent

Fig. 4. Separation of three distinct fractions (P-I, P-II and P-III) from the dsDNA-cellulose fraction by Mono Q column chromatography. (A) p34 in the DNA-cellulose fraction (approximately 2 mg protein) was further purified by Mono Q column chromatography (HPLC). Elution was carried out with a linear gradient between 0.2 M and 0.5 M KCl, and 1.0 ml fractions were collected. Absorbance at 280 nm (—). (B) To determine the main polypeptide in the indicated Mono Q fractions (P-I, P-II and P-III), each fraction (10 μl each) was analyzed by SDS-PAGE and polypeptides on the gel were stained with Coomassie Brilliant Blue R-250. Lane 1, Pre-Mono Q fraction (DNA-cellulose fraction); lane 2, P-I; lane 3, P-II; and lane 4, P-III. (C) To detect p34, aliquots (20 μl) of these three fractions (P-I, P-II and P-III) were incubated separately for 30 min at 25°C with oligomeric CK-II (0.1 μg) and 20 mM [γ-32P]ATP (500 cpm/pmol) in the presence (lanes 2, 4, 6 and 8) or absence (lanes 1, 3, 5 and 7) of calf thymus dsDNA (10 μg/ml) and 3 mM Mn2+. Lanes 1 and 2, Pre-Mono Q fraction; lanes 3 and 4, P-I; lanes 5 and 6, P-II; and lanes 7 and 8, P-III.

Fig. 5. Homogeneity of the purified p34. To analyze the homogeneity of p34 in the crude preparation (lane 1), the heparin-agarose fraction (lane 2), the DEAE-cellulose fraction (lane 3), the Sephacryl S300 fraction (lane 4), the dsDNA-cellulose fraction (lane 5) and the Mono Q fraction (lane 6), aliquots (20 μl) of these fractions were incubated separately for 30 min at 25°C with oligomeric CK-II (0.1 μg) and 20 μM [γ-32P]ATP (500 cpm/pmol). The reaction mixtures were analyzed by SDS-PAGE and autoradiography.

Fig. 6. Molecular weight of p34 determined by gel filtration on Superdex 75 pg (HPLC). To determine the molecular weight of p34, the Mono Q fraction was applied on a column of Superdex 75 pg which had been equilibrated with Buffer B containing 0.6 M KCl. Bovine serum albumin (BSA, 67 kDa), ovalbumin (45 kDa), chymotrypsinogen (25 kDa) and cytochrome C (12.4 kDa) were used as marker proteins.
protein kinase (A-kinase) from Ehrlich ascites tumor (EAT) cells. Phosphorylation of p34 was detected when monomeric CK-II was incubated with the protein and 20 μM [γ-32P]ATP (500 cpm/pmol) in the presence of 3 mM Mn2+, whereas no phosphorylation of p34 by other kinases was detected (Fig. 7). In addition, oligomeric CK-II from spinach chloroplasts also phosphorylated p34 in vitro (Figs. 1–5). These results suggest that both monomeric and oligomeric forms of CK-II are kinases responsible for phosphorylation of p34.

As shown in Fig. 4C, dsDNA stimulated CK-II catalyzed phosphorylation of p34. RNAs also have similar stimulating effects on the phosphorylation. It seems that chloroplast RNPs, such as p34 and 28 kDa RNP from spinach, bind to mRNAs and are involved in post-transcriptional steps of light-induced chloroplast gene expression. Therefore, the stimulating effect of nucleic acids on the phosphorylation of p34 by CK-II may have an important role in the chloroplast gene expression. Further biochemical studies of purified p34 will be required to understand the biological significance of specific phosphorylation of the protein by CK-II.

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Fig. 7. The protein kinase-specificity for p34 phosphorylation in vitro. To examine the protein kinase-specificity of p34, monomeric CK-II (lanes 1 and 5), HK-II kinase (lanes 2 and 6), HK-III kinase (lanes 3 and 7) and cAMP-dependent kinase (A-kinase) (lanes 4 and 8) were separately incubated with 3 mM Mn2+ and 20 μM [γ-32P]ATP (500 cpm/pmol) in the presence (lanes 5 through 8) and absence (lanes 1 through 4) of purified p34.

References