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Purification of a Nucleoside Diphosphate Kinase from Pea Seed and Phosphorylation of the Enzyme with Adenosine (^{32}P)Triphosphate

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A nucleoside diphosphate kinase has been extracted and purified in good yield from dried pea seed flour. The procedure is simple, involving water extraction, precipitation and fractionation with acetone, followed by three or four successive chromatographic separations on DEAE-Sephadex. The molecular weight of the enzyme as determined by ultracentrifugation was 70 000. During a short incubation with (^{32}P)ATP, the enzyme incorporated about 3 phosphoryl groups per enzyme molecule, indicating an intermediate phosphorylation of the enzyme during its action. When the ^{32}P-labelled enzyme was hydrolyzed in alkali, 1-(^{32}P)phosphohistidine, 3 (^{32}P)phosphohistidine, and N^{ε}-(^{32}P)phospholysine were released in small amounts. The dominating radioactive degradation products in the hydrolysate were some peptide components, apparently identical with the main degradation products from the ^{32}P-labelled bovine liver enzyme (*J. Biol. Chem.* **243** (1968) 3947).

Nucleoside diphosphate kinase (EC 2.7.4.6) is an enzyme catalyzing a transphosphorylation reaction between a nucleoside triphosphate and a nucleoside diphosphate. In several cases, the enzyme has been shown to be phosphorylated by its substrate (^{32}P)ATP.[1–7] Different labelled products have been obtained from the ^{32}P-labelled enzyme after alkaline hydrolysis, depending on the source of the enzyme. Hydrolysis of the enzyme from baker's yeast yielded predominantly 1-(^{32}P)phosphohistidine, suggesting that this phosphoamino acid plays an important part in the mechanism of action of the enzyme. Small amounts of 3-(^{32}P)phosphohistidine and N^{ε}-(^{32}P)-phospholysine were also found.[6] However, with mammalian enzymes, the principal hydrolytic products were phosphopeptides. In addition, small amounts of the three labelled phosphoamino acids mentioned were isolated.[4,8] The phosphopeptides have been hydrolyzed further and shown to contain 1-(^{32}P)phosphohistidine.[9]

These findings suggest, that in the nucleoside diphosphate kinase preparations studied so far, the phosphorylation occurs essentially at Nitrogen 1 of a histidine imidazole side chain during enzyme action.

The aim of the present study was to find a source rich in nucleoside diphosphate kinase, from which it could easily be isolated for further studies of the enzyme. Pea seeds were found to contain considerable amounts of nucleoside diphosphate kinase. The enzyme has been purified, and some of its properties have been studied. It has been shown to incorporate phosphoryl groups after a brief incubation with (^{32}P)ATP, indicating an intermediate phosphorylation of the enzyme. The phosphorylated enzyme has been hydrolyzed in alkali, and the hydrolytic products have been studied.

MATERIALS

ATP, dGDP, lactate dehydrogenase (EC 1.1.1.27) (type II), pyruvate kinase (EC 2.7.1.40) (type II), NADH, and phosphoenolpyruvate were purchased from Sigma. Sephadex and DEAE-Sephadex were products of Pharmacia. (^{32}P)ATP, labelled at the γ-P, was prepared according to Engström.[10] The specific radioactivity of the (^{32}P)ATP ranged from 0.21 to 1.00×10^6 counts $\times$ min^{-1} $\times$ nmol^{-1}. 1-Phosphohistidine and 3-phosphohistidine were prepared as described by DeLuca *et al.*[11] and isolated as previously described.[12] N^{ε}-Phospholysine was synthetized from lysine and phosphorus oxychloride.[13] Flour of dried pea seed (*Pisum sativum*) was obtained from Mälardalens Lantmannaförbund, Sweden. Nucleoside diphosphate kinase from bovine liver [4] was a kind gift from Dr. O. Wålinder, Uppsala, Sweden.

The molarity of the buffers containing triethanolamine-acetic acid is given with respect to added triethanolamine.

METHODS

Radioactivity was measured as described previously.[8] Protein concentration was measured by either the biuret method,[14] a micro-Kjeldahl method, or by measuring the ultraviolet absorbance at 280 nm. The absorbance of a solution containing 1 mg/ml of purified protein was found to be 1.41 (1 cm light path). The protein concentration was determined by the Kjeldahl method, assuming the nitrogen content of the protein to be 16 %. Acid-labile phosphate was estimated as described recently.[15] Ultrafiltration and dialysis were performed by methods described earlier.[8] Phosphate incorporating activity was measured as described.[8] Ultracentrifugation studies were performed in a Spinco Model E ultracentrifuge with interference optics, using a double sector cell equipped with sapphire windows. Molecular weight experiments were performed at 20 410 rpm at 20°, according to Yphantis' "high speed method".[16] The protein concentration was 0.7 mg/ml.

Assay of enzyme activity. Nucleoside diphosphate kinase activity was measured by the coupled pyruvate kinase lactate dehydrogenase method of Mourad and Parks.[17] The nucleoside diphosphate used was dGDP. The reaction mixture (1 ml) contained 1.5 μg of pyruvate kinase and 7.5 μg of lactate dehydrogenase. Corrections were made for the adenosine triphosphatase background activity, when present. One unit of nucleoside diphosphate kinase activity is defined as described previously.[6]

Investigation of an alkaline hydrolysate of ^{32}P-labelled enzyme. Isolation of phosphoamino acids. Purified protein, 0.6 – 5.0 mg in 0.2 – 0.4 ml of 0.01 M triethanolamine-acetic acid buffer, pH 7.4, was incubated with 0.03 – 0.17 μmol of (^{32}P)ATP, and hydrolyzed for 3 h in 3 M KOH at 100° in a sealed tube.[8] The acid-labile phosphoamino acids in the hydrolysate were isolated by chromatography on Dowex 1 × 8, and identified by paper electrophoresis and paper chromatography.[8]

Paper electrophoresis. Nucleoside diphosphate kinase from pea seed and bovine liver were incubated with (^{32}P)ATP and hydrolyzed in KOH as described.[4] The hydrolysates were examined by paper electrophoresis at pH 8.25, followed by autoradiography.[4]

RESULTS

Purification of the enzyme. Purification of the enzyme, including centrifugation, was carried out at about 5°. The purification and the results of a typical preparation are shown in Table 1.

Table 1. Purification of nucleoside diphosphate kinase from pea seed. For this preparation, 12 kg of dried pea seed flour were used. The protein content in operations 1 to 4 was estimated by the biuret method, and in the chromatographic steps by measuring the ultraviolet absorbance at 280 nm.

Purification step	Total protein *g*	Total activity Units	Specific activity Units/mg protein	Purification factor	Recovery %
1. Extraction	1 560	1 200 000	0.8	1	100
2. pH 5 precipitation (supernatant)	358	910 000	2.5	3	76
3. Acetone precipitation (50 %)	110	523 000	4.8	6	44
4. Acetone fractionation (40 – 55 %)	14	395 000	27	34	33
5. DEAE-Sephadex, chromatography I	0.88	337 000	380	480	28
6. DEAE-Sephadex, chromatography II	0.32	293 000	920	1 200	24
7. DEAE-Sephadex, chromatography III	0.20	285 000	1 400	1 800	24
8. DEAE-Sephadex, chromatography IV	0.15	270 000	1 800	2 300	23

Extraction. To 48 l of distilled water were added 12 kg of flour from dried pea seed with slow stirring. The pH of the mixture was adjusted to pH 7.4 by the addition of 1 M triethanolamine (about 700 ml). The mixture was stirred slowly for about 15 min.

pH 5 precipitation. The mixture was then adjusted to pH 5 by addition of 4 M acetic acid (approximately 600 ml). The precipitate, together with the insoluble debris from the extraction step, were separated by centrifugation 60 000 – 150 000 $g \times$ min. The supernatant, approximately 36 l, was collected.

Acetone precipitation. An equal volume (36 l) of acetone was added to the supernatant, stirring carefully. The solution was left for at least 15 min. It was then centrifuged 120 000 $g \times$ min. The precipitate was suspended, using a Turmix blender, in 6 l of 0.05 M triethanolamine-acetic acid, pH 7.4, containing 1 mM EDTA. Insoluble debris was separated by centrifugation 150 000 $g \times$ min. Solid magnesium acetate, 200 g/l, was dissolved in the extract.

Acetone fractionation. Acetone was then added to give a final concentration of 40 % (v/v) – the percentage is based on the volume (5.8 l) of the solution before the addition of the magnesium acetate. After a minimum of 15 min,

the mixture was centrifuged 150 000 $g \times$ min. To the supernatant (9.3 l), acetone was added to give a final concentration of 55 % (v/v). The mixture was stood for at least 15 min and then centrifuged 60 000 $g \times$ min. The pellet was dissolved in 400 ml of 0.01 M triethanolamine-acetic acid buffer, pH 7.4, containing 1 mM EDTA, and carefully dialyzed against the same buffer without EDTA. The dialyzed material was centrifuged 150 000 $g \times$ min, and the pellet was extracted with 100 ml of 0.01 M triethanolamine-acetic acid buffer, pH 7.4. The extract was added to the dialyzed solution.

Chromatography on DEAE-Sephadex. The material was applied to a column (7.0 × 54.6 cm) of DEAE-Sephadex, equilibrated with 0.01 M triethanolamine-acetic acid buffer, pH 7.4. The column was first eluted with 1.5 l of the same buffer (0.1 M). The eluent showed no enzyme activity. During this treatment, the column shrunk considerably. The enzyme activity was eluted as described in the legend of Fig. 1.

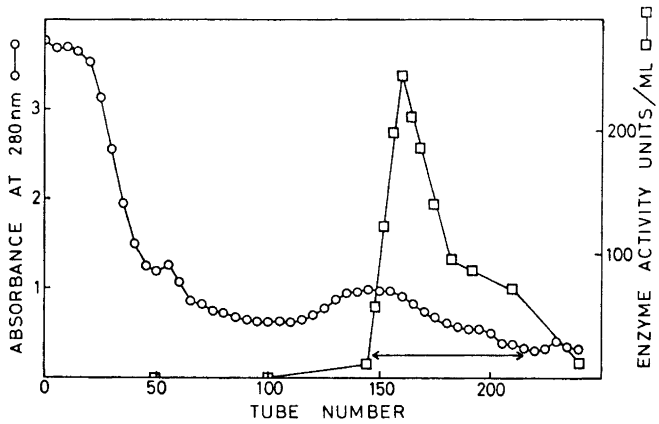

Fig. 1. Chromatography of nucleoside diphosphate kinase from pea seed on a DEAE-Sephadex column. The column (7.0 × 54.6 cm) was eluted with a linear gradient (total volume 8 l) formed from 0.1 M triethanolamine-acetic acid buffer, pH 7.4, and 0.4 M sodium acetate in the same buffer. 25 ml fractions were collected, and those with enzyme activity were pooled as indicated in the figure.

The fractions containing the main enzyme activity were pooled (Fig. 1), concentrated by ultrafiltration, and dialyzed carefully against 0.01 M triethanolamine-acetic acid buffer, pH 7.4.

This material (280 ml) was applied to a column (3.1 × 33.1 cm) of DEAE-Sephadex equilibrated with dialyzing buffer. The column was then eluted as described for the preceding chromatographic step (see legend Fig. 2), using 200 ml of 0.1 M buffer and a 4 l linear gradient. The inactive material was separated from the active material, which was pooled as indicated in Fig. 2A, and then carefully dialyzed against the 0.01 M buffer.

The chromatography was repeated once or twice (Fig. 2B and C).

The portion eluted with the 0.1 M buffer showed no nucleoside diphosphate kinase activity. The active material was pooled as indicated in Fig. 2.

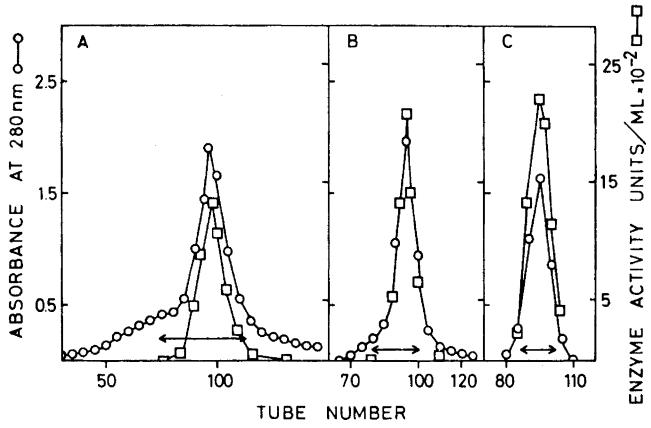

Fig. 2. Three successive rechromatographies of nucleoside diphosphate kinase from pea seed on a DEAE-Sephadex column. The columns (3.1 × 33.1 cm) were eluted with a linear gradient (total volume 4 l), formed from 0.1 M triethanolamine-acetic acid buffer, pH 7.4, and 0.4 M sodium acetate in the same buffer. 15 ml fractions were collected. Fractions containing enzyme activity were pooled as indicated in the figure.

Investigations of the purified enzyme

Chromatography on Sephadex G-200. The active material from the fourth DEAE-Sephadex chromatography (190 ml) was ultrafiltered, and the resulting small volume (9 ml) was applied to a column (3.1 × 32 cm) of Sephadex G-200, equilibrated with 0.05 M triethanolamine-acetic acid buffer, pH 7.4, containing 1 M sodium chloride. The column was eluted with the same buffer. The enzyme appeared as a single peak (Fig. 3) with a fairly constant ratio of enzyme activity to protein concentration over the peak region. The enzyme was eluted after about 0.6 column volume.

Ultracentrifugation. The molecular weight of the enzyme was found to be 70 000. The partial specific volume of the enzyme was assumed to be 0.75 cm^3/g.

Incorporation of (^{32}P)phosphate from (^{32}P)ATP. The purified enzyme was shown to incorporate ^{32}P from (^{32}P)ATP. Within experimental error, all of the radioactivity incorporated into the enzyme was acid-labile. 3.1 mol of phosphate were bound per mol of enzyme. Another preparation showed a value of 2.6. Varying the ATP concentration from 5 μM to 86 μM in the incubation mixture did not produce any detectable difference in phosphate incorporation.

Isolation of 1-(^{32}P)phosphohistidine, 3-(^{32}P)phosphohistidine, and N^e-(^{32}P)phospholysine from an alkaline hydrolysate of ^{32}P-labelled enzyme. The three phosphoamino acids accounted for 2.1 – 5.4 % of the total acid-labile radioactivity of the hydrolysate. In three different preparations 1-(^{32}P)phosphohistidine accounted for 0.7 %, 1.6 %, and 3.2 %, 3-(^{32}P)phosphohistidine for 2.2 %, 0.2 %, and 1.2 %, and N^e-(^{32}P)phospholysine for 0.6 %, 0.3 %, and 1.0 %. The identities of the phosphoamino acids were established as described previously.[8]

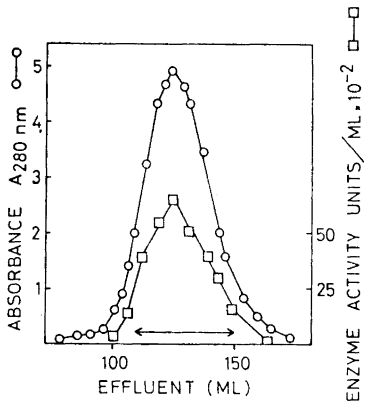

Fig. 3. Chromatography of nucleoside diphosphate kinase from pea seed on a Sephadex G-200 column. The column (3.1 × 32 cm) was eluted with 0.05 M triethanolamine-acetic buffer, pH 7.4, containing 1 M sodium chloride. Fractions of 2.8 ml were collected. They were pooled as indicated.

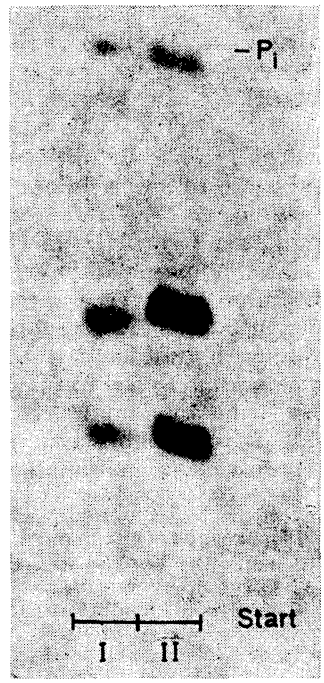

Fig. 4. Radioautogram of an electrophoretic run at pH 8.25 of an alkaline hydrolysate of ^{32}P-labelled nucleoside diphosphate kinase from pea seed and bovine liver. Whatman No. 3 paper, electrophoresis time 2 h, voltage 20 V/cm. X-Ray film (Ilford, Ilfex) exposed for 24 h. Nucleoside diphosphate kinase from bovine liver (I) and pea seed (II). The amounts applied corresponded to about 2 μg of each protein.

Electrophoresis of an alkaline hydrolysate of ^{32}P-labelled enzyme. Paper electrophoresis at pH 8.25, followed by radioautography, revealed that the main alkaline degradation products of the ^{32}P-labelled enzyme were (^{32}P)-orthophosphate and some slower migrating material, presumably phosphopeptides. The radioautogram was apparently identical with that from the ^{32}P-labelled bovine liver enzyme (Fig. 4).

DISCUSSION

The aim of the present study was to find a source rich in nucleoside diphosphate kinase, from which the enzyme could easily be isolated.

Pea seed was found to fulfil these requirements. The enzyme has thus been obtained in a good yield, and its chromatographic behavior indicates that it is

fairly pure. The molecular weight of the pea seed enzyme (70 000) is somewhat lower than that (100 000) of the other nucleoside diphosphate kinases studied so far.[2,5,8,18]

The molar incorporation of phosphate (2.6 – 3.1) from ATP was of the same order as that of the bovine liver and baker's yeast enzymes.[6,8] Since the incorporation was obtained after short incubation with ATP, it was assumed to be an intermediate phosphorylation, similar to that for the bovine liver enzyme.[19]

On alkaline hydrolysis of the ^{32}P-labelled enzymes from pea seed, bovine liver,[4,8] and human red blood cells,[4] small amounts of the aforementioned phosphoamino acids were obtained. When released from the ^{32}P-labelled pea seed enzyme, their relative proportions varied for as yet unknown reasons. The dominating radioactive degradation products from the pea seed enzyme seemed according to electrophoresis to be identical with the 1-phosphohistidine peptides [9] from an alkaline hydrolysate of the (^{32}P)ATP incubated mammalian enzymes.[4] It is therefore assumed that the active site of the pea seed enzyme is similar to that of mammalian nucleoside diphosphate kinases.

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