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PURIFICATION AND PROPERTIES OF SUCROSE-6-PHOSPHATASE FROM *PISUM SATIVUM* SHOOTS

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Key Word Index—*Pisum sativum*, Leguminosae, pea, purification, sucrose phosphatase, properties, MW, inhibition

Abstract—Sucrose-6-phosphatase from pea shoots, which was purified to homogeneity, consists of two similar subunits each with an MW of about 55 000 The pH optimum was at 6 8, the K_m for sucrose-6-phosphate was 250 μM and the K_m for magnesium was 175 μM The enzyme was specific for sucrose-6-phosphate and was not inhibited by sucrose except at very high concentrations

INTRODUCTION

In higher plants the biosynthesis of sucrose [1] is considered to be catalysed by the sequential action of sucrose phosphate synthetase (UDP-glucose D-fructose-6-phosphate-2-glucosyl transferase, EC 2 4 1 14) and sucrose phosphatase (sucrose phosphohydrolase, EC 3 1 3 24) *De novo* sucrose synthesis takes place in photosynthetic cells from triose-phosphate exported from the chloroplast, sucrose is then transported to other parts of the plant Sucrose phosphatase has been partially purified from sugar cane and carrot root tissue [2, 3], and in both cases was shown to be inhibited by sucrose at physiological concentrations (i.e 70% inhibition by 50 mM sucrose) As part of a study of the regulation of photosynthesis by assimilate partitioning in plants this enzyme was purified to homogeneity for the first time and an attempt was made to repeat the previously observed inhibition of the enzyme by sucrose

RESULTS AND DISCUSSION

Enzyme purification

Table 1 shows the purification profile of pea shoot sucrose phosphatase The initial mincing of the frozen pea shoots and subsequent thawing produced a very concentrated extract at an early stage and reduced the volumes of liquid being handled This method did not appear to reduce the final yield A final purification of 715 fold was achieved This was largely due to the use of the fast protein liquid chromatography (FPLC) step The enzyme obtained from this step was considered homogeneous as judged by polyacrylamide gel electrophoresis and ultracentrifugation

Properties

Enzyme activity was rapidly lost if the enzyme was stored without special precautions A range of media were compared to determine the optimum storage conditions The enzyme was most stable when stored in buffer (10 mM HEPES, pH 7) with 50% glycerol and 1 mM dithiothreitol added at −20°

The pH optimum of sucrose phosphate hydrolysis was fairly sharp at pH 6 7 which is the same as that obtained for the sugar cane enzyme [3] The enzyme was specific for sucrose-6-phosphate as its substrate and did not catalyse the hydrolysis of 10 mM solutions of glucose-6-phosphate, ribose-5-phosphate, 3-phosphoglycerate or fructose-1,6-diphosphate in standard assays A trace of activity was observed with *p*-nitrophenyl phosphate (2 5% of the rate with sucrose phosphate)

Using the procedure of Lineweaver and Burk [4], a K_m for sucrose-6-phosphate of 250 μM was obtained This is the same as that demonstrated by Hawker [3] for the sugar cane enzyme

Sucrose-6-phosphatase requires magnesium for activity Removal of all traces of magnesium from the enzyme was difficult Dialysis alone was not sufficient and residual enzyme activity (20%) remained To remove all bound magnesium it was necessary to treat the enzyme with 100 mM EDTA and then dialyse Under these conditions it was possible to eliminate all enzyme activity, which could be restored completely by adding magnesium Lineweaver–Burk plots were then used to calcu-

Table 1 Purification of sucrose-6-phosphatase

Step	Procedure	Volume (ml)	Total activity (nkat)	Total protein (mg)	Specific activity (nkat/mg)	Purification	Yield
A	Crude Brei	270	805	4185	0 18		
B	Post G25	70	1025	1084	0 93	5 ×	127%
C	DE52 fractions	20	250	119	2 08	11 ×	31%
D	FPLC fractions				130	722 ×	

late the K_m for magnesium at three concentrations The K_m for magnesium was 175 μM

Estimation of MW

Gel electrophoresis under dissociating conditions [5] indicated a MW of *ca* 55 000 (± 5000), while gel filtration [6] gave an estimate of 115 000 (± 5000) Ultracentrifugation was performed using the Yphantis equilibrium method [7] In three separate runs using the same sample, an MW of 120 000 (± 10 000) was calculated It appears therefore that sucrose phosphatase consists of two similarly sized sub-units of 55 000 MW and exists in its native form as a dimer of *ca* 120 000 MW

Effects of inhibitors

Addition of 250 mM sucrose to standard assays with varying substrate concentrations produced an increase in K_m and a slight decrease in V_{max}, indicating that sucrose is probably a competitive inhibitor of the enzyme The effects on the enzyme of several other dissacharides were investigated These were melibiose, trehalose, cellobiose and maltose, of these maltose gave the highest degree of inhibition of 27% at 100 mM, this compared with 9% inhibition by sucrose at the same concentration

Sucrose-6-phosphatase from sugar cane and carrot root is reported to be inhibited by sucrose at physiological concentrations (K_i = 10 mM) and it has been suggested that this effect is responsible for the regulation of sucrose biosynthesis *in vivo* [2] However, we were unable to demonstrate inhibition of the enzyme from pea leaves by sucrose except at very high concentrations, 35% inhibition at 500 mM The role of sucrose-6-phosphatase as a control point in sucrose synthesis must therefore be questioned, at least in pea

EXPERIMENTAL

Materials Pea plants (*Pisium sativum* var Alaska) were grown in compost in a greenhouse with supplementary heating and lighting in the winter Whole shoots were harvested 2–3 weeks after planting

Chemicals Sucrose-6-phosphate was synthesized using the method of ref [8] Sephadex was obtained from Pharmacia, ion exchange cellulose DE52 from Whatman, and thioglycerol (3-mercapto-1,2-propanediol) from Aldrich All other chemicals were purchased from B D H

Enzyme purification A Homogenization Young pea shoots (500 g) were frozen overnight at −20° and then minced The homogenate was allowed to thaw and the slurry was filtered through two layers of muslin The filtrate was brought to pH 6 8, made 10 mM in thioglycerol, and then clarified by centrifugation at 20 000 *g* for 20 min *B* $(NH_4)_2SO_4$ *fractionation* The protein fraction precipitating from the supernatant between 33% and 45% with $(NH_4)_2SO_4$ was redissolved in a small vol of buffer (20 mM HEPES, pH 6 8, 1 mM thioglycerol) and desalted on a column of Sephadex G25 (11 × 4 5 cm) *C DE52 Chromatography* The protein was applied to a column of Whatman DE52 ion exchange cellulose equilibrated in buffer (20 mM HEPES pH 6 8, 1 mM thioglycerol) and the column was washed with buffer The enzyme was eluted by a 100 ml + 100 ml gradient of 0 to 0 5 M NaCl in the same buffer 10 ml fractions were collected and the column eluate was monitored at 280 nm Sucrose-6-phosphatase activity was detected in fractions eluting between 60 and 110 ml of the gradient *D Fast protein liquid chromatography (FPLC)* Final purification was achieved by FPLC on a QAE ion exchange column (Q-column, Pharmacia) The desalted post DE52 eluate (5 ml) in 2 mM Tris–HCl, pH 7, was applied and the column was eluted with a linear gradient of 0–0 5 M KCl in the same buffer at a flow rate of 1 ml/min The enzyme was eluted in fractions 15 and 16

Protein determinations For crude samples the biuret method [9] was used Where less protein was available either the direct spectrophotometric method [10] or, for more accurate measurements, the method of ref [11] was used

Enzyme activity was measured using a discontinuous assay to follow phosphate release from sucrose-6-phosphate The assay mixture contained, in 0 1 ml, 2 5 μmol 2-(*N*-morpholino)ethanesulphonic acid (MES), pH 6 8, 1 μmol MgCl, and 0 1 μmol sucrose-6-phosphate P_i was estimated, after stopping the reaction with 15% trichloroacetic acid, using the assay of ref [12] An enzyme unit was defined as the amount of enzyme required to hydrolyse one μmol of sucrose-6-phosphate per min at 30°

Polyacrylamide gel electrophoresis was carried out in 7 5% polyacrylamide gels at pH 7 5 Gels were run at 4 mA/tube until the tracking dye reached the bottom of the tube The gels were stained overnight in 0 01% Coomassie blue R250 in 27% *iso*-PrOH–10% HOAc and then destained in 10% HOAc–10% EtOH

SDS gel electrophoresis The method of ref [5] was used Protein samples were prepared by heating at 90° for 15 min with 1% SDS and 10 mM thioglycerol Protein bands were visualized with Coomassie blue

Fast protein liquid chromatography (FPLC) was carried out using equipment supplied by Pharmacia Chromatography was carried out on a QAE ion-exchange column (Q-column) using the conditions detailed in the purification procedure

Ultracentrifuge studies These were carried out on Beckman model E analytical ultracentrifuge equipped with interference optics The rotor speed was 17 900 rpm and the temp 15 7°

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