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PURIFICATION AND CHARACTERIZATION OF HOMOGENEOUS SUNFLOWER SEED ACID PHOSPHATASE

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Key Word Index—*Helianthus annuus*; Compositae; sunflower; nonspecific phosphomonoesterase acid optimum; acid phosphatase; purification; substrate specificity; amino acid composition; inhibition.

Abstract—Acid phosphatase (EC 3.1.3.2) from sunflower seed was purified 1800-fold to homogeneity using both conventional and affinity chromatographic methods. The purified enzyme was a mixture of two enzyme forms distinguishable by polyacrylamide gel electrophoresis (PAGE). Gel exclusion chromatography, which did not distinguish between the two forms, gave an apparent M_r of 103 000. Preparative PAGE permitted the separation of the two forms, and SDS-PAGE showed that they contained equivalent peptide subunits of apparent M_r 56 000 and 52 000. Amino acid analysis indicated that both enzyme forms have similar amino acid compositions. Data on substrate specificity and pH dependence is presented. The kinetic constants for hydrolysis of *p*-nitrophenyl phosphate as catalysed by sunflower seed acid phosphatase were independent of pH in the range 3–5. The enzyme was competitively inhibited by inorganic phosphate and non-competitively inhibited by phosphomycin.

INTRODUCTION

Acid phosphatases (orthophosphoric monoester phosphohydrolases; EC 3.1.3.2) are enzymes that catalyse the hydrolysis of a variety of phosphate esters and appear to exhibit pH optima below 6. The enzymes are widely found in nature [1, 2] and are usually rather nonspecific. Acid phosphatases are apparently ubiquitous in nature, having already been identified in numerous organisms and tissues. In general, the acid phosphatases occur in very small quantities, are unstable in dilute solution, and are subject to surface denaturation in the pure state. These properties, and their tendency to occur in multiple forms, often make the isolation of acid phosphatases difficult. Studies on pure acid phosphatases have so far focused mainly on those of clinical significance from animal sources [2–4].

Perhaps because of the difficulty of obtaining the pure enzymes, the functions of the nonspecific acid phosphatases are poorly defined. Among possible functions of acid phosphatases might be the role of providing inorganic phosphate for metabolic excretory and some secretory purposes. Acid phosphatases may have a role in the mammalian reproductive process [5]. A possible role of human prostatic acid phosphatase may be in the dephosphorylation of esters to liberate fructose, which serves as an energy source for spermatozoa, or in the formation of choline from choline phosphate. In plants, acid phosphatase activity in seeds often increases greatly during germination [6], indicating a possible role in phosphate mobilization.

Previous work in this laboratory on wheat germ phosphatases has resulted in useful information on the chemical mechanism of the enzyme [7, 8]. However, because of the hexaploid nature of wheat and its frequent commercial genetic manipulation, it appeared possible that a plant such as sunflower might be a more reliable source of plant phosphatase. It has been shown that the acid phosphatase isoenzymes in sunflower seed are specified by a single gene which has at least four codominant alleles S, B, I and F [9, 10]. Based on the behaviour of the crude enzyme, it was concluded that the enzyme possessed an M_r of 95 000 (estimated by gel chromatography), while based on genetic data, it was hypothesized that the enzyme was a dimer. In addition, the individual isoenzymes were reportedly under genetic control, and PAGE with activity staining could be used to reveal the isoenzyme forms. Thus, in addition to the general goal of carefully studying some properties of a plant acid phosphatase, it seemed important to ascertain, based on studies with homogeneous enzyme, if this protein is indeed a dimer. We describe here the isolation and characterization of SSAP,† a pure acid phosphatase from sunflower seed.

RESULTS

Although the acid phosphatase is not present in large amounts in sunflower seed, the use of affinity chromatography steps made it possible to purify the enzyme. Table 1 summarizes the purification procedure that was developed. After an 1822-fold purification, the enzyme was obtained in a 14% yield and had a specific activity of 113 μmol/min/mg, when assayed with *p*-nitrophenyl phosphate at pH 5. Acetone precipitation appeared to be unavoidable in order to remove lipid from the extract, despite the low recovery in this step. When a 35–50%

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†Abbreviations: SSAP, sunflower seed acid phosphatase as isolated following the Sephacryl S-200 step of the purification procedure; SSAPI and SSAPII, two forms of SSAP obtained upon subsequent preparative gel electrophoresis. Unless otherwise indicated, SSAP was used for kinetic studies.

Table 1. Purification of sunflower seed acid phosphatase

Step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification
Initial extract	6770	127000	7830	0.062	(100)	(1)
50% Acetone precipitation	1770	41000	3600	0.088	46	1.4
Con-A Sepharose	2135	662	2210	3.4	28	55
Affi-Gel Blue Sepharose	1100	154	1620	10.7	21	172
DEAE-Sepharose	6.3	24	1010	29.8	13	480
Sephacryl S-200	15.7	9.6	1080	113.0	14	1820

ammonium sulphate fractionation was done with the initial buffer extract, the precipitation of protein was difficult, apparently because of the extremely high lipid content. Moreover, gel exclusion chromatography on a Sephacryl S-200 column, calibrated using standard proteins, showed that sunflower seed acid phosphatase had an unexpectedly high M_r, larger than catalase, whose estimated M_r is 210000. This was not the case following introduction of the acetone precipitation step.

Analytical PAGE of the enzyme solution that was obtained following the Sephacryl S-200 Sepharose column run at pH 5 showed two distinct bands on both activity and Coomassie staining. However, exactly corresponding bands were obtained with each staining method, consistent with the conclusion that both proteins were acid phosphatases. Since attempts to use ion exchange chromatography failed to separate the two bands that were apparent on PAGE, we performed preparative PAGE at 4° in order to separate two enzyme forms SSAPI and SSAPII (see Experimental). Enzyme activity assays and protein determinations were done spectrophotometrically for each gel extract. For each fraction containing acid phosphatase activity, analytical PAGE was done to check the separation of the proteins. Figure 1 shows that preparative PAGE separated the two forms of enzyme, while denaturing PAGE also shows the purity of these proteins (Fig. 2). Fractions containing only one protein also showed a single band on SDS-PAGE.

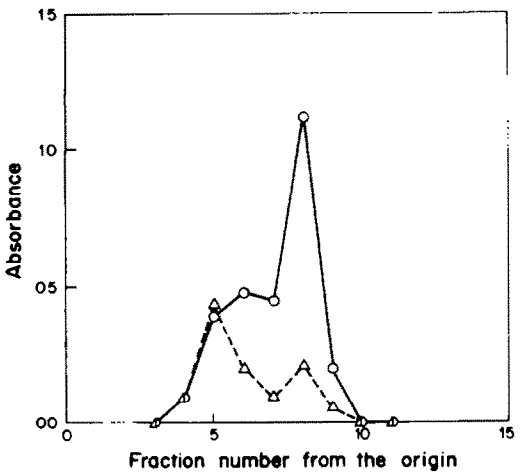


Fig. 1. Separation of sunflower seed acid phosphatase into forms SSAPI and SSAPII by preparative PAGE as described in the Experimental, using protein obtained after Sephacryl S-200 gel chromatography. The gel was cut into 2 mm slices and extracted with buffer (0.05 M NaOAc, pH 5 containing 0.15 M NaCl). Each fraction is numbered from the origin (+). Absorbance at 280 nm was obtained by subtracting the absorbance value of fractions which contained only gel extract. Each fraction was assayed by measuring E_{280} nm (△——△) and enzyme activity (○——○).

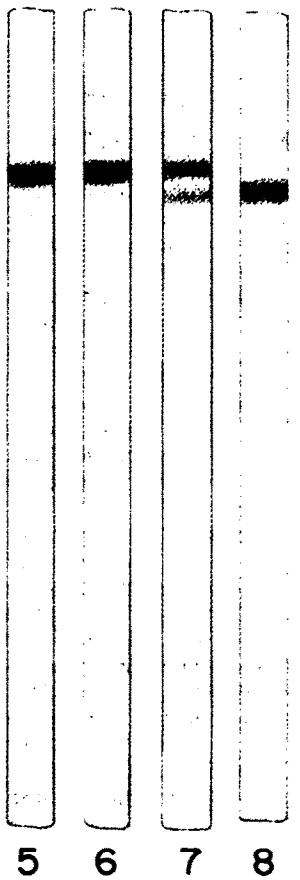


Fig. 2. Analytical disc gel electrophoresis of the sunflower seed acid phosphatase forms obtained by preparative gel electrophoresis. Electrophoresis was performed at pH 5 as described in the Experimental. Gels were stained with Coomassie blue and are numbered corresponding to the fraction numbers shown in Fig. 1. The gel for fraction 7 shows a mixed enzyme composition.

SDS-PAGE of each enzyme in the denatured and reduced states showed slightly different M_rs for SSAPI compared to that for SSAPII (Fig 3). The relative mobility for each protein was calculated by dividing the migration in cm from the origin (−) by the distance the dye migrated in cm [11, 12]. A least-squares program was used to fit these data to a protein standard curve and gave apparent M_rs of 56 000 for SSAPI and 52 000 for SSAPII. The M_r determined by gel exclusion chromatography was 103 000 daltons (composite value for both enzyme forms).

Because both enzymes appear to be glycoproteins (they both bind to the ConA-Sepharose column, for example), the difference in M_rs between the two enzyme forms may simply be due to the carbohydrate portion. The amino acid composition (Table 2) is consistent with this possibility. In the amino acid composition calculation, the number of residues in each subunit was calculated using a residue minimization program [13]. Only the tyrosine results do not agree within the limits of experimental error.

Acid phosphatase from sunflower seed has a broad specificity toward orthophosphoric monoesters. The enzyme hydrolysed a series of substrates tested by inorganic phosphate assays as shown in Table 3. Michaelis constants for *p*-nitrophenyl phosphate measured using

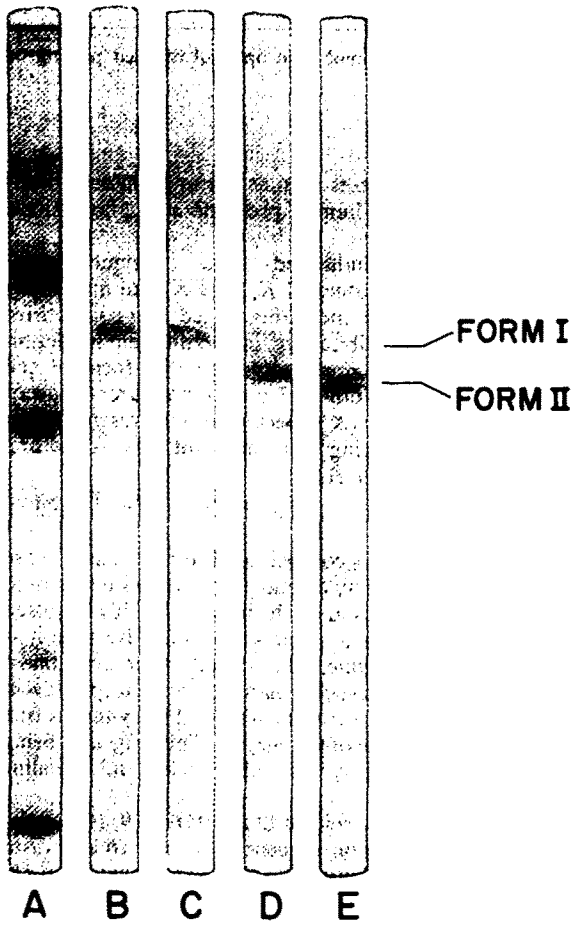


Fig. 3. SDS-PAGE of the sunflower seed acid phosphatase forms. Lane A shows standard proteins. Lanes B–E are samples from fraction 5, 6, 7 and 8, respectively, as indicated for Fig. 1.

Table 2. Amino acid composition of sunflower seed acid phosphatase

Amino acid	Number of residues per subunit: Enzyme form I	form II
Asp	64	64
Thr	35	33
Ser	39	44
Glu	46	50
Pro	35	32
Gly	42	42
Ala	35	36
Val	38	35
Met	8	9
Ile	23	26
Leu	34	34
Tyr	27	18
Phe	21	19
His	17	16
Lys	23	25
Arg	17	19
Residues per subunit	504	501
Subunit M_r =	55 100	54 100

Input data are for the 24 hr hydrolysis reaction. The presence of cysteine was also apparent, but quantitative measurements of cysteine (as cysteic acid) were not done. Similarly, tryptophan was not determined.

SSAPI and II were 2.9 and 9.8×10^{-5} M, respectively, calculated by the Cleland HYPER program [14] to fit the experimental data to the Michaelis–Menten equation. Since the estimated error is 10%, these values differ by a small but experimentally significant amount.

Table 4 shows the dependence of the enzyme kinetic constants K_m and V on pH. Treatment of the data following Dixon and Webb [15] shows the importance of the ionization of a group at pH 5.2 which probably represents the ionization of substrate.

Inorganic phosphate, which is one of the products of the phosphatase reaction, competitively inhibited sunflower seed acid phosphatase. Non-linear least squares fitting to a hyperbolic equation, using the maximum velocity value obtained in the absence of inhibitor, was used to estimate inhibition constants. For inorganic phosphate at pH 5 we found $K_i = 2.4 \times 10^{-4}$ M. Phosphomycin (*cis*-1,2-epoxypropyl phosphonic acid) inhibited sunflower seed acid phosphatase noncompetitively, giving K_{is} and K_{ii} values 1.3×10^{-3} M and 2.8×10^{-4} M, respectively, where K_{is} and K_{ii} are expressed as follows:

$$v = \frac{V[So]}{K_m\left(1+\frac{[I]}{K_{is}}\right)+[So]\left(1+\frac{[I]}{K_{ii}}\right)}$$

L-(+)-Tartrate, which is competitive inhibitor of several acid phosphatases from animal sources [16, 17], did not inhibit this enzyme.

DISCUSSION

In the present experiments, SDS-PAGE of the isoenzymes separated by preparative PAGE showed single,

Table 3. Substrate specificity of sunflower seed acid phosphatase

Substrate*	Specific activity†
p-Nitrophenyl phosphate	75
D-Glucose-6-phosphate	28
Phosphoenolpyruvate	52
α-Glycerophosphate	35
β-Glycerophosphate	47
O-Phosphoryl choline	<1
O-Phosphoryl ethanolamine	24
O-Phosphoserine	16
Phosphocreatine	<1
3',5'-AMP	0
2'-AMP	0
3'-AMP	37
5'-AMP	12
ATP	0
NADP	28
Pyrophosphate	91
DNA‡	0
Phenyl phosphate	57
Diphenyl phosphate	4
α-Naphthyl phosphate	23
β-Naphthyl phosphate	35
O-Carboxyphenyl phosphate§	16
Thymolphthalein monophosphate	3.3
Phenolphthalein monophosphate	13

*The concentration of each substrate was 10 mM if not otherwise specified. To each substrate solution, 50 μl of dilute enzyme solution was added and enzymic activity was determined.

†Specific activity in μmol of inorganic phosphate released per mg protein per min at 25°, based on 10 min reaction times.

‡1 mg in incubation mixture.

§5 mM in incubation mixture.

Table 4. Kinetic constants for the hydrolysis of *p*-nitrophenyl phosphate catalysed by sunflower seed acid phosphatase

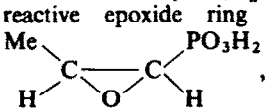
Buffer	pH	K_m (mM) ± s.d.	V^* ± s.d.
Glycine–HCl	3.6	0.83 ± 0.2	32 ± 3.5
Acetate	3.6	0.40 ± 0.02	22.0 ± 0.4
	3.8	0.11 ± 0.02	26.6 ± 1.1
	4.0	0.17 ± 0.01	32.2 ± 0.6
	4.2	0.14 ± 0.03	36.1 ± 1.7
	4.5	0.14 ± 0.02	45.1 ± 1.5
	4.8	0.13 ± 0.04	50.1 ± 1.0
	5.0	0.08 ± 0.02	48.5 ± 3.9
	5.2	0.13 ± 0.01	57.5 ± 1.4
	5.5	0.17 ± 0.02	57.7 ± 1.6
Cacodylate	5.5	0.23 ± 0.04	39.9 ± 2.1
	5.8	1.13 ± 0.16	44.8 ± 3.0
	6.2	1.12 ± 0.03	27.1 ± 0.4
	6.5	1.69 ± 0.2	22.1 ± 1.2
	6.8	1.64 ± 0.26	16.8 ± 1.2
	7.0	2.40 ± 0.18	14.1 ± 0.4
	7.3	3.70 ± 0.36	13.1 ± 0.6
Barbital	7.3	1.63 ± 0.12	30.5 ± 0.7
	7.6	1.28 ± 0.10	18.7 ± 0.4
	7.8	1.20 ± 0.07	13.9 ± 0.1
	8.2	1.04 ± 0.12	5.3 ± 0.1
	8.5	1.34 ± 0.18	3.3 ± 0.1
	8.8	1.17 ± 0.1	1.8 ± 0.03

*V is expressed as μmol *p*-nitrophenol released per mg of protein per min.

distinct bands for each isoenzyme. Comparison of the M_r determined by gel chromatography (native state) with that SDS-PAGE (denatured and reduced state) indicates that the two enzyme forms SSAPI and SSAPII, in addition to differing slightly from one another in M_r, are themselves dimers. The amino acid compositions of the two forms are similar. Within the limits of the present measurements, they may be identical (with the possible exception of the tyrosine data). If they do have the same amino acid composition, and furthermore, are the same protein, then the differences in M_r and electrophoretic mobility could perhaps be explained in terms of varied carbohydrate contents. Immunological and/or peptide mapping studies may ultimately be necessary to clarify the suggestion, based on genetic studies, that the proteins are distinct gene products [9, 10].

In the pH dependence study, cacodylate buffer, which was used in the pH range 5.5–7.3, shows some degree of interference in measuring steady state kinetic constants. Both V and K_m are slightly different in this buffer than at similar pH values in acetate or barbital. Still, the maximum velocity clearly shows a decrease in the pH range above 7–7.5 which would be consistent with the involvement of a histidine residue, as known to be the case for other acid phosphatases such as wheat germ acid phosphatases [7, 8] and human prostatic acid phosphatase [18].

Phosphomycin inhibited the enzyme non-competitively. The values of K_{is} and K_{ii} are marginally different in this case, indicating mixed non-competitive inhibition, i.e. the inhibitors act in two different modes on the enzyme, one competing for the free form of the enzyme (K_{is}) and the other affecting the apparent concentration of the enzyme (K_{ii}). Because of the presence of the reactive epoxide ring as part of its structure,



, it appears possible that phosphomycin may act as a covalent inhibitor. This may prove to be a general property of acid phosphatases which bind phosphate derivatives and which have suitably disposed nucleophilic residues at their active sites. Because it is likely that this enzyme, like several other acid phosphatases [18], acts in such a manner, then phosphomycin may act to inhibit the enzyme irreversibly by means of a covalent modification of the enzyme. This reagent is being tested with a variety of other acid and alkaline phosphatases.

Earlier work by Torres and Diedenhofen [9, 10] involving the rehybridization of genetically controlled forms suggested the possibility that subunit aggregation might occur in a reversible manner. This raises the possibility that subunit affinity chromatography could be used in the purification and study of this enzyme, as has been done with mammalian aryl sulphatase A [19]. The present purification procedure and enzyme characterization

should facilitate these and other structural and biophysical investigations.

EXPERIMENTAL

Materials. Concanavalin A, *p*-nitrophenyl phosphate (*p*-NPP) and other phosphoester compounds were purchased from Sigma. Sephacryl S-200, standard proteins for gel exclusion chromatography, DEAE-Sepharose were obtained from Pharmacia, while Affi-Gel Blue Sepharose was from Bio-Rad. Reagents used for electrophoresis were purchased from LKB. Commercial, hulled sunflower seed was obtained from Agway, Inc., Box 169, Grandin, ND 58036. Dialysis tubing were purchased from Spectrum Medical Industries, Inc. Diaflo Ultrafiltration membranes were obtained from Amicon.

Assay of enzyme activity. Acid phosphatase activities were determined spectrophotometrically as the rate of formation of *p*-nitrophenol caused by the enzyme catalysed hydrolysis of *p*-NPP. Enzyme soln (25–50 μl) was added to 2 ml of 5 mM *p*-NPP in 0.05 M NaOAc, 0.15 M NaCl, pH 5. After 2–10 min of incubation at 25°, the reaction was stopped by addition of 0.4 ml of 1.25 M NaOH and the A was read at 400 nm. The molar extinction of *p*-nitrophenolate ion at 400 nm at pH values greater than 9.0 is 1.8×10^4/M/cm [20]. One unit of enzyme activity corresponds to the hydrolysis of 1 μmol of *p*-NPP per min at 25°. Sp. act. is defined as the number of enzyme units present per mg of protein. Protein concns were determined colorimetrically by the procedure described in ref. [21] using BSA as a standard. During chromatographic steps in protein purification, where only the relative levels were desired, the protein content was estimated by measuring the A of the solns at 280 nm.

The method of ref. [22] was employed for the analysis of Pi liberated by the enzymatic cleavage of phosphate esters by quantitation of the reduced form of phosphomolybdic acid at pH 4, where hydrolysis of labile phosphate esters is presumably at a minimum. This product has a molar extinction coefficient of 4.2×10^3/M/cm at 700 nm. In a typical analysis of reaction, 1 ml of 0.05 M NaOAc, 0.1 M NaCl, pH 5 buffer containing 10 mM substrate was incubated with 20–50 μl of diluted enzyme soln for 10 min. The enzymatic reaction was quenched by addition of 2 ml of 3% ammonium molybdate in 0.2 M NaOAc, pH 4 buffer. After addition of 0.2 ml of 1% ascorbic acid in 0.2 M NaOAc, pH 4 buffer as a reducing agent, the colour was developed for 30 min.

The pH dependence of the enzyme was determined at 25° using *p*-NPP at concns ranging from 0.2 to 5 times K_m. Eight to ten substrate concns were used at each pH in 0.05 M glycine–HCl (pH 3.6), cacodylate (pH 5.5–7.3), and 0.05 M barbital (pH 7.3–8.8) buffer containing 0.1 M NaCl. Triplicate determinations were done at each pH and the Michaelis constants (K_m and V) were calculated using the Cleland HYPER program [14].

Polyacrylamide gel electrophoresis to check the purity of the enzyme prepared was performed at neutral pH or low pH. The stock solns for the preparation of gels are those of ref. [23] for the pH 7 runs, and the method of ref. [24] was employed for low pH runs. The 7.5% gels were formed to a height of 10 cm in 0.55 × 12.5 cm glass tubes. Samples were layered on the stacking gel surface in 20–50 μl containing 30–40% sucrose (w/v) and small vol. of 0.05% methyl green (low pH) or bromophenol blue (neutral pH) as a tracking dye. When the protein soln was diluted, maximally up to 200 μl of sample was located on the gel. PAGE was carried out at 4° using a current of 3 mA per each tube gel until the tracking dye was near the bottom of gel. It typically took about 4.5 hr. Following electrophoresis, gels were removed from tubes and placed in incubating tubes containing 0.1 M NaOAc, pH 5 buffer at room temp.

Protein gels were stained with freshly made 0.2% Coomassie brilliant blue R-250 in 40% MeOH–20% HOAc for 1–3 hr. Then, the gel was transferred to a Bio-Rad Model 172A Diffusion Destainer containing 25% EtOH–8% HOAc for 1 day. The enzyme activity was located with α-naphthyl phosphate and Fast Red violet LB salt 1 mg/ml each, in 0.1 M NaOAc, pH 5 buffer. Developed gels were stored in 7.5% HOAc.

Preparative PAGE was carried out similarly to analytical PAGE at low pH. The 7.5% gels were made to a height of 9.5 cm in 1.5 × 12 cm glass tubes. Samples were layered on the stacking gel surface in a vol. of 2–3 ml containing 2–3 mg of protein. Electrophoresis was performed in 0.037 M β-alanine–HOAc, pH 4.4, using a current of 5 mA/cm^2 gel in the beginning, and after 1 hr, the current applied was increased gradually up to 12.5 mA/cm^2 gel as a final reading. It took *ca* 7 hr for tracking dye to move to the bottom of gel, and running was continued for an additional 2 hr for better separation. After electrophoresis, gels were sliced with a 2–3 mm thickness and each gel was homogenized in 0.05 M NaOAc, pH 5 buffer containing 0.15 M NaCl. Then, the gel extract was centrifuged 10 min at 17400 *g* and enzyme activity assays and protein determination were done for each supernatant.

SDS-PAGE was carried out according to the method of ref. [25]. Protein (15–20 μg) was denatured and reduced by heating at 110° with 2% SDS and 5% 2-mercaptoethanol for several min and placed on the 10% slab gel made with a thickness of 1.5 mm. PAGE was carried out in the presence of 0.1% SDS at 5° with a current of 20 mA per one gel plate for 1.5 hr and the current was increased to 30 mA. Protein staining and destaining was the same as that used in native gels. Standard proteins were prepared in an identical manner to the enzyme soln and were simultaneously run on the same gel plate. The standards used to make a plot of log M_r *versus* mobility of protein band were: phosphorylase B (M_r 92500), bovine serum albumin (M_r 68000), ovalbumin (M_r 43000), α-chymotrypsinogen (M_r 25700) and β-lactoglobulin (M_r 18400).

M_r *determination.* A 2.6 × 90 cm column of Sephacryl S-200 equilibrated with 0.05 M Tris–HCl, pH 7 buffer containing 0.15 M NaCl was used to estimate the M_r of the enzyme. Blue dextran (M_r 2000000), aldolase (M_r 158000), bovine serum albumin (M_r 67000), chymotrypsinogen A (M_r 25000) and ribonuclease A (M_r 13700) were used as standards to calibrate the column.

Amino acid composition. Salt-free proteins were prepared by dialysing eluents from PAGE and passing them through a 1.5 × 5 mm column of Sephadex 6-25 equilibrated with 1% HOAc. The effluent was monitored by A at 280 nm and the protein-containing solns from the column were lyophilized. Constant boiling 6 M HCl was added to 0.15–0.3 mg of lyophilized, salt-free SSAPI and II, respectively. Each sample was divided into three portions and placed in amino acid analysis tubes. For the 24 and 48 hr hydrolysis samples, the amino acid analysis tube containing protein soln was degassed using a vacuum pump and sealed. (Tubes were sealed without prior degassing for the 24 hr hydrolysis sample under oxidizing conditions.) Then tubes were placed in a heating block at 110° for 24 and 48 hr, respectively. After cooling to room temp., each sample was evaporated to dryness *in vacuo* in a desiccator containing P_2O_5 and NaOH. Analyses were performed on a Durrum 500 amino acid analyser.

Purification of sunflower seed acid phosphatase. Initial extraction of enzyme was done at 4°. Some of the chromatographic steps were done at room temp. Sp. act. was measured at each step of the purification.

Step 1. Sunflower seed kernel was ground in a stainless steel Waring blender. Cold 0.1 M NaOAc at pH 4.5 buffer containing 0.1 M NaCl and 1 mM EDTA was added to the ground seed,

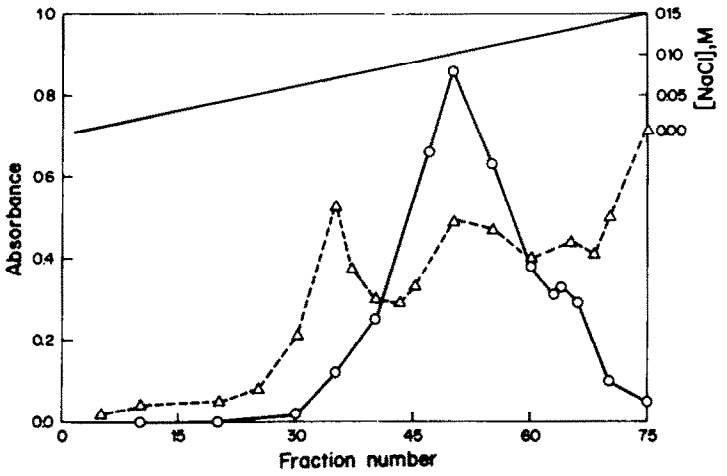


Fig. 4. Elution of sunflower seed acid phosphatase from the DEAE-Sepharose column. Enzymic activity (○—○) and protein (E_{280}, △—△) were monitored. A linear gradient composed of 0.01 M Tris–HCl, pH 7 and of the same buffer containing 0.15 M NaCl was applied. Fractions 46–60 were pooled and condensed for chromatography on Sephacryl S-200.

using a vol. of 1.5–2 ml per g of seed. After several hr, the resulting slurry was centrifuged 15 min at 11 000 *g* and the supernatant was filtered through cheesecloth. The seed paste in tube was re-extracted for better recovery and buffer-extracted soln was dialysed against deionized H_2O with four changes.

Step 2. To a dialysed protein soln, an equivalent vol. of cold Me_2CO (−10°) was slowly added with gentle, continuous stirring and the resulting buffer-extracted-Me_2CO soln was kept at −10° for 3–4 hr. Then, the Me_2CO soln was centrifuged for 15 min at 11 000 *g*, and the pellet was dissolved in ice-cold 0.05 M NaOAc at pH 6 and centrifuged 10 min at 10 000 *g*.

Step 3. A 3.6 × 10 cm Concanavalin A-Sepharose (ConA-Sepharose) column was freshly regenerated with 0.05 M NaOAc buffer, pH 6, containing 1 mM each of Mg^{2+}, Mn^{2+} and Ca^{2+} ions and it was then equilibrated with 0.5 M NaOAc buffer, pH 6. The supernatant from step 2 was applied. The column was washed with the same buffer, and then with the buffer containing 0.4 M NaCl until the *A* at 280 nm was zero in each case. Batch elution of enzyme was done using the same buffer containing 0.4 M NaCl and 0.4 M mannose. The eluant was dialysed against 0.05 M NaOAc, pH 5.

Step 4. Dialysed enzyme soln from step 3 was loaded on an Affi-gel Blue Sepharose column equilibrated with 0.05 M NaOAc buffer, pH 6. The column was washed with the same buffer until the *A* at 280 nm was zero, and then the enzyme was gradually eluted using an NaCl concn gradient with the final NaCl concn of 1 M. The resulting soln was dialysed against 0.01 M Tris–HCl buffer, pH 7.

Step 5. Enzyme soln from step 4 was applied on a DEAE-Sepharose column equilibrated with 0.01 M Tris–HCl buffer, pH 7. After extensive washing of the column with the same buffer, a gradual increase of NaCl concn up to 0.15 M caused the proteins to be eluted as shown in Fig. 4. Fractions 46–60 were pooled and condensed by Diaflow dialysis using a PM-10 membrane.

Step 6. The enzyme soln was applied to a 1.5 × 90 cm column of Sephacryl S-200 equilibrated with 0.01 M NaOAc buffer, pH 6, containing 0.1 M NaCl and eluted with the same buffer at a flow rate of 10 ml/hr. The chromatographic profile showed a major activity band and coincident protein band. These fractions were pooled and concd by Diaflow dialysis using PM-10 membrane and stored at 4°.

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