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PURIFICATION AND PROPERTIES OF ONE COMPONENT OF ACID PHOSPHATASE PRODUCED BY *ASPERGILLUS NIGER*

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Summary

One component, the *i* form, of acid phosphatase (orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2) produced by *Aspergillus niger* was purified from the mycelial extract. The purified enzyme was homogenous on Sephadex G-200 gel filtration, disc electrophoresis and heat inactivation. The purified enzyme was studied and the following results were obtained:

1. The enzyme catalyzed the hydrolysis of a wide variety of phosphomonoesters, but not that of bis(*p*-nitrophenyl)phosphate, adenosine 3′,5′-cyclic monophosphate, fructose 1,6-diphosphate, adenosine 5′-diphosphate or adenosine 5′-triphosphate.

2. Fluoride, orthophosphate, arsenate, borate, molybdate and (+)-tartrate acted as inhibitors. This enzyme was inactivated by *N*-bromosuccinimide and 2-hydroxy-5-nitrobenzyl bromide, and was not affected by *p*-chloromercuribenzoate, *N*-acetylimidazole, *p*-diazobenzenesulfonic acid and tetranitromethane. From these results, tryptophan was estimated to play an important role in the enzyme activity.

3. The apparent molecular weight was 310 000 by Sephadex G-200 gel filtration. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate suggested that the molecular weight of the subunit was approximately 89 000.

4. The purified enzyme contained 29% carbohydrate consisting of glucosamine, mannose and galactose. The amino acid composition of this enzyme was not specific compared with other known acid phosphatases.

Introduction

Acid phosphatase (orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2) is widely distributed in nature and often occurs in multiple forms in mammals, plants and in microorganisms. However, the functional

and structural relations of these multiple forms are obscure, except that sialic acid attachment to the enzyme molecule is known to contribute to the multiple forms of acid phosphatase in human prostate [1,2] and in pig liver [3].

In our preliminary experiment, it was found that a strain of *Aspergillus niger* produces three forms of acid phosphatase (*e*, *i* and *b*) which differ in electrophoretic mobility and molecular weight. The *e* form is abundant in the extracellular phosphatase which also contains minor amounts of forms *i* and *b*, while the intracellular phosphatase contains mainly form *i* and no form *e* [4]. Recently, we observed that form *i* was converted to a different form of phosphatase having the same electrophoretic mobility as form *e* when incubated with the cell extract which had been prepared from the same mold by crushing and centrifugation (unpublished data). These findings provide us a suitable system for studying the relationship between excretion and the multiple forms of the enzyme.

This paper describes the purification of form *i*, one component of the multiple form acid phosphatase produced by *Asp. niger*, and some characteristics including molecular weight, amino acid and sugar compositions of the purified enzyme.

Materials and Methods

Organism and culture methods

Aspergillus niger U20-2-5, an adenine-requiring strain maintained in this laboratory [5], was employed.

The medium for first seed culture contained the following in 1 l of 0.1 M citrate buffer (pH 4.6): potato starch, 150 g; peptone, 7 g; adenine, 0.75 mmol; KH_2PO_4, 2 g; $CaCl_2 \cdot 2H_2O$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g. In the medium for second seed and main cultures, potato starch in the first seed culture medium was replaced by 50 g of glucose.

First seed culture was inoculated with conidia from an agar slant, and after 40 h 10% of culture broth was transferred to the second seed culture. These cultivations were carried out in 500-ml Erlenmeyer flasks (working volume 100 ml) on a rotary shaker (180 rev./min, radius 4 cm) at 30° C. Main cultures were carried out in 100-l jar fermenters (Marubishi Co.) of 60-l working volume at 30° C for 24 h, inoculating 5% of 24-h second seed culture broth, agitating at a speed of 100 rev./min and aerating at a rate of 1.0 vols. air/vol. medium per min.

Assay of acid phosphatase activity

The acid phosphatase activity was measured by a spectrophotometric method using *p*-nitrophenyl phosphate as substrate [6]. The release of 1 μmol of *p*-nitrophenol per min was defined as one unit of activity.

The amount of inorganic phosphate released from phosphate esters was measured by the method of Fiske and SubbaRow [7].

Determination of protein and sugars

Protein and sugar concentrations were determined by the Lowry [8] and the phenol-sulfate [9] methods using bovine serum albumin and glucose as standards, respectively.

Polyacrylamide gel electrophoresis

Standard 7% acrylamide gel (pH 8.0) [10] was used for disc electrophoresis. Electrophoresis was carried out at a constant current of 1.5 mA per tube (0.6 × 7 cm) at 4°C. Protein bands were detected by Amido Black staining. Sugar bands were detected by periodic acid-Schiff staining [11]. And the activity band of acid phosphatase was detected by the diazonium method using Fast Blue and α-naphthyl phosphate [12].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis in 7.5% acrylamide gel was carried out according to the method of Shapiro et al. [13]. Electrophoresis was carried out in the presence of 0.1% sodium dodecyl sulfate at room temperature at 8 mA per tube (0.6 × 7 cm), with samples denatured at 35°C for 15 h in 1% sodium dodecyl sulfate and 0.2% dithiothreitol. The gels were stained with 0.25% Coomassie Brilliant Blue.

Carbohydrate composition

The neutral sugars in the purified acid phosphatase form *i* were determined by the procedure of Misaki et al. [14]. After hydrolysis of 1.36 mg of the purified enzyme in 0.5 M H_2SO_4 at 100°C for 9.5 h, the hydrolyzate was passed through a small column of Dowex 50 x4 (H^+ form), coupled to a column of Dowex 1 x8 ($HCOO^-$ form). The unadsorbed neutral sugars eluted with water were hydrogenated with sodium borohydride, and then acetylated by heating with pyridine/acetic anhydride (1 : 1 mixture) at 100°C for 2 h. The acetylated alditol derivatives were analyzed by gas-liquid chromatography (applied to a Shimadzu GC-6AM gas chromatogram) using a column (200 cm) of 3% ECNSS-M on Gas Chrom Q at 180°C. The materials adsorbed on both columns were used for amino acid analysis.

Composition of amino acid and hexosamine

Composition of the amino acid in the *i* form of the purified acid phosphatase were determined by the method of Moore [15]. The materials adsorbed on Dowex 50 x4 and Dowex 1 x8 were eluted with 3 M HCl and 3 M HCOOH, respectively. These eluted samples were hydrolyzed with 4 M methane sulfonic acid containing 0.2% of indolethylamine at 110°C for 24 h, and hydrolysate was applied to a Hitachi KLA-3B amino acid analyzer. Tryptophan was determined spectrophotometrically [16]. The determination of sulfhydryl groups in the enzyme was performed by the colorimetric procedure of Ellman [17], after reduction with sodium borohydride in 0.1 M phosphate buffer (pH 7.9) containing 8 M urea.

The quantitative determination of hexosamine in the enzyme was made by the method of Elson and Morgan [18], after the hydrolysis with 4 M HCl at 100°C for 14 h.

Purification of acid phosphatase form i

Step 1. Preparation of curde enzyme. A 24-h culture broth was chilled in ice and all subsequent operations were performed at 0—5°C. To separate mycelia, the culture broth (120 l from two batches in 100-l jar fermenters) was filtered through a cotton cloth. The mycelia, washed with water, were suspended in 0.02 M citrate buffer (pH 4.6), and crushed in a Dyno-Mill (Type KDL, W.A.

Bachofen, Switzerland) using a 0.6-l glass container and 0.75-mm diameter glass beads in a continuous flow operation with a mean retention time of 2 min. After centrifugation (15 000 × *g*, 10 min) of the crushed mycelial homogenate, the supernatant was used as crude enzyme solution.

Step 2. Acetone fractionation. The crude enzyme solution (20 l) was fractionated with cold acetone (−15° C). The protein was fractionated between 30 and 60% acetone. The precipitate collected by centrifugation (9000 × *g*, 10 min) was dissolved in an appropriate amount of 0.01 M citrate buffer (pH 3.0).

Step 3. Batch treatment on CM-cellulose. One fourth of the enzyme solution (40 ml) obtained in Step 2 was diluted to 2 l in 0.01 M citrate buffer (pH 3.0). CM-cellulose (150 g dry wt, Serva, W. Germany), activated and equilibrated with 0.01 M citrate buffer (pH 3.0), was added to this diluted enzyme solution and gently agitated for 30 min. The resulting CM-cellulose was packed in a column (8.6 × 50 cm) and after washing with 0.01 M citrate buffer (pH 3.0) the adsorbed material was eluted with the same buffer containing 0.1 M NaCl. The active fractions were collected and the enzyme was precipitated by adding acetone to a final concentration of 60%. The precipitate collected by centrifugation was dissolved in a small volume of 0.01 M citrate buffer (pH 4.6).

Step 4. Batch treatment on DEAE-cellulose. The enzyme solution from Step 3 was diluted with 0.01 M Tris · HCl buffer (pH 7.2) and subjected to batch treatment on DEAE-cellulose (Serva, W. Germany) by the same procedure as that used in Step 3 except with 0.01 M Tris · HCl buffer (pH 7.2) containing 0.15 M NaCl as elution buffer. Form *b* was not contained in this eluted fraction. The active fractions were collected and the enzyme was precipitated with acetone as described above. The precipitate was dissolved in a small volume of 0.02 M citrate buffer (pH 4.6).

Step 5. Gel filtration through Sephadex G-200. The enzyme solution (10 ml) from Step 4 was passed through a column (2.64 × 45 cm) of Sephadex G-200 equilibrated with 0.02 M citrate buffer (pH 4.6). Elution was performed with the same buffer at a flow rate of 20 ml per h, and the active fractions were recovered from 120 to 170 ml of eluate.

Step 6. Column chromatography on DEAE-cellulose. The active eluate (50 ml) from Step 5 was concentrated and desalted by Amicon ultrafiltration apparatus with PM-30 filter. The enzyme solution, adjusted to pH 7.2 with 0.01 M Tris, was applied to a column (2.0 × 22 cm) of DEAE-cellulose equilibrated with 0.01 M Tris · HCl buffer (pH 7.2). After washing the column with 100 ml of the equilibrating buffer, elution was carried out with 800 ml of the buffer having a linear gradient of NaCl concentration from 0 to 0.5 M at a flow rate of 50 ml per h. The enzyme activity was recovered from 220 to 310 ml of eluate at about 0.1 M NaCl concentration.

Step 7. Column chromatography on CM-cellulose. The eluate (90 ml) was ultrafiltered by the same procedure as in Step 6. The enzyme solution, adjusted to pH 3.0 with 0.01 M citrate, was applied to a column (1.9 × 26 cm) of CM-cellulose equilibrated with 0.01 M citrate buffer (pH 3.0). After washing the column with 100 ml of the equilibrating buffer, the enzyme was eluted with 800 ml of the buffer having a linear gradient of NaCl concentration from 0 to 0.5 M at a flow rate of 35 ml per h. The enzyme activity was eluted at 0.06 M NaCl.

Step 8. Gel filtration through Sephadex G-200. The active fractions (50 ml) from Step 7 were concentrated to 4 ml by ultrafiltration as described above, and passed through a column (2.64 × 95 cm) of Sephadex G-200 equilibrated with 0.02 M citrate buffer (pH 4.6). Elution was performed with the same buffer at a flow rate of 25 ml per hour, and the activity was recovered from 240 to 280 ml of eluate.

Step 9. Isoelectric focussing. The technique described by Svensson [19] was used. The active eluate (40 ml) from Step 8 was concentrated and desalted by ultrafiltration as described above. The enzyme solution was subjected to column electrophoresis (110 ml) with carrier ampholyte (pH gradient between 4 and 6, LKB, Sweden). The applied potential was 400 V for the first 20 h, then increased gradually at a rate of 70 V per hour. After the potential had reached 900 V, electrophoresis was continued at this voltage for another 20 h. The activity was recovered from 42 to 48 ml of eluate.

Step 10. Gel filtration through Sephadex G-200. The active eluate (6 ml) from Step 9 was desalted and concentrated to 4 ml by ultrafiltration as stated above, and passed through a column (2.64 × 95 cm) of Sephadex G-200 equilibrated with 0.02 M citrate buffer (pH 4.6). Elution was performed with the same buffer at a flow rate of 15 ml per h, and the activity was recovered from 240 to 265 ml of eluate.

Results

Homogeneity of purified acid phosphatase form i

The purification process is summarized in Table I. In the last purification procedure, molecular sieve chromatography on Sephadex G-200, the elution pattern of the activity, as well as of protein and of carbohydrate, was symmetrical. The specific activities of the enzyme eluted from 240 to 265 ml were constant: 265 units per mg protein and 910 units per mg carbohydrate. Protein

TABLE I

SUMMARY OF PURIFICATION OF ACID PHOSPHATASE FORM *i*

Purification step	Total activity (units)	Total protein (mg)	Total sugar (mg)	Specific activity		Yield (%)
				(units/mg protein)	(units/mg sugar)	
1 Crude extract	18 400	55 400	63 600	0.332	0.290	100
2 Acetone fractionation (30—60%)	15 000	9 350	6 840	1.60	2.19	81.5
3 CM-cellulose (batch)	11 500	1 880	544	6.12	21.1	62.5
4 DEAE-cellulose (batch)	8 540	1 040	300	8.21	28.5	46.4
5 Sephadex G-200	8 180	696	242	11.7	33.8	44.5
6 DEAE-cellulose (linear)	6 090	180	41.8	33.8	146	33.1
7 CM-cellulose (linear)	4 820	68	21.0	70.9	229	26.2
8 Sephadex G-200	4 400	35.2	10.4	125	423	23.9
9 Isoelectric fractionation	3 300	12.5	3.67	264	899	17.9
10 Sephadex G-200	2 130	8.03	2.34	265	910	11.6

of the purified enzyme amounted to 8.03 mg from 120 l of culture broth, and purification of the cell extract reached about 800-fold in specific activity on a protein basis and about 3100-fold on a carbohydrate basis, with a recovery of 11.6% of activity. In all subsequent experiments, this enzyme solution was used.

To test the homogeneity of the purified enzyme, polyacrylamide disc electrophoresis was performed. The quantity of enzyme used for protein, carbohydrate and activity staining were 57, 57 and 4 μg, respectively. The electrophoeretogram obtained showed single bands of protein, carbohydrate and enzyme activity having the same mobility were detected.

Properties of purified acid phosphatase form i

The acid phosphatase form *i* (3 mg enzyme per ml of 0.05 M citrate buffer, pH 4.6) can be stored for at least three months without loss of activity at −15°C.

The optimum pH for hydrolysis of *p*-nitrophenyl phosphate was 4.0 in 0.1 M citrate buffer system. The K_m value obtained at pH 4.0 and 40°C for *p*-nitrophenyl phosphate was 1.0 mM. Effect of pH on the enzyme stability was tested at pH ranging from 2 to 8 by using 0.05 M glycine/HCl buffer (pH 2—3), 0.05 M citrate buffer (pH 3—6) or 0.05 M Tris/maleate buffer (pH 6—8). After treatment at 50°C for 3 h, pH was adjusted to 4 for activity measurement. Loss of activity was not observed after treatment at pH between 5 and 6.

To determine the isoelectric point of the enzyme, dialyzed enzyme (30 μg) was subjected to column electrophoresis with carrier ampholyte, pH gradient

TABLE II

SUBSTRATE SPECIFICITY OF ACID PHOSPHATASE FORM *i*

The enzyme (0.1 μg) was incubated with each substrate at 10 mM for 40 min at 40°C in 0.1 M acetate buffer (pH 4.0). The reaction was terminated by the addition of trichloroacetic acid at a final concentration of 10%. The amount of released inorganic phosphate was measured by the method of Fiske and SubbaRow. All values were expressed as the percentage of the amount of inorganic phosphate released from *p*-nitrophenyl phosphate.

Substrate	Relative activity (%)
p-Nitrophenylphosphate	100
α-Naphthylphosphate	86
α-Glycerophosphate	38
β-Glycerophosphate	72
Bis(*p*-nitrophenyl)phosphate	2
Adenosine 5′-monophosphate	105
Adenosine 5′-diphosphate	14
Adenosine 5′-triphosphate	0
Adenosine 3′,5′-cyclic monophosphate	0
Cytidine 5′-monophosphate	97
Guanosine 5′-monophosphate	33
Glucose 1-phosphate	45
Fructose 6-phosphate	32
Fructose 1,6-diphosphate	4
Ribose 5-phosphate	36
6-phosphogluconate	17
Phospho*enol*pyruvate	40

between 4 and 6. Electrophoresis was carried out by the same procedure as described in Materials and Methods. The isoelectric point of the enzyme was thus found to be pH 4.6.

A number of phosphoric acid esters were used to test the substrate specificity (Table II). Acid phosphatase form *i* was a typical orthophosphoric-monoester phosphohydrolase, scarcely active toward bis(*p*-nitrophenyl)phosphate, adenosine 3′,5′-cyclic monophosphate, di-, triphosphate esters or fructose 1,6-diphosphate, and highly active toward *p*-nitrophenylphosphate, α-naphthyl phosphate, β-glycerophosphate, adenosine 5′-monophosphate and cytidine 5′-monophosphate.

The effects of various inhibitors on the activity are shown in Table III.

TABLE III

EFFECT OF VARIOUS INHIBITORS ON THE ACTIVITY OF ACID PHOSPHATASE FORM *i*

The activity was determined by incubation of the enzyme for 20 min at 40°C in the presence of 4 mM *p*-nitrophenylphosphate and various inhibitors in a total volume of 2.5 ml of 0.1 M citrate buffer (pH 4.0). In the case of ethylenediaminetetraacetate, *p*-chloromercuribenzoate, diisofluoropropyl phosphate, *N*-bromosuccinimide and 2-hydroxy-5-nitrobenzyl bromide, the enzyme was incubated with each compound in 0.05 M acetate buffer (pH 4.0). In the case of *p*-diazobenzene sulfonic acid and tetranitromethane, incubation was performed in 0.05 M Tris · HCl buffer (pH 8.0), and in the case of *N*-acetyl imidazol, in 0.05 M borate buffer (pH 7.5). After incubation of 1.5 ml enzyme with each compound for 30 min at 25°C, 1.0 ml of 10 mM *p*-nitrophenylphosphate in 0.2 M acetate buffer (pH 4.0) was added and residual activity was determined by incubation for 20 min at 40°C. The activity was expressed as the percentage of that observed without inhibitor. Enzyme concentration was 0.2 μg per ml.

Inhibitor	Concentration (mM)	Relative activity (%)
NaF	80	0
	8	12
H_3PO_4	80	9
	8	75
H_3BO_3	80	73
	8	96
$Na_2MoO_4 \cdot 2\ H_2O$	80	0
	8	56
KH_2AsO_4	80	9
	8	67
$FeSO_4 \cdot 7\ H_2O$	8	90
$FeCl_3 \cdot 6\ H_2O$	8	45
(—)-Tartarate	80	92
(+)-Tartarate	80	10
	8	66
Sodium dodecyl sulfate	8	4
Ethylenediaminetetraacetate	1	100
p-Chloromercuribenzoate	0.1	92
Diisofluoropropylphosphate	1	100
N-Bromosuccinimide	0.01	0
2-Hydroxy-5-nitrobenzylbromide	1	0
N-Acetylimidazole	1	100
p-Diazobenzenesulfonic acid	1	105
Tetranitromethane	1	102
None	—	100

Fluoride, orthophosphate, molybdate, arsenate, sodium dodecyl sulfate and (+)-tartarate inhibited the enzyme reaction at 8 mM, but (−)-tartarate did not affect the activity. When *p*-nitrophenyl phosphate was used as the substrate, fluoride, orthophosphate and arsenate were competitive inhibitors, the K_i values being 0.45 mM, 18 mM and 4.8 mM, respectively. The enzyme activity was not affected by the presence of the following metal ions at 8 mM; Na^+, K^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Cu^{2+} and Zn^{2+}, but Fe^{3+} at 8 mM inhibited the activity to 45%. Ethylenediaminetetraacetate, *p*-chloromercuribenzoate, diisofluoropropyl phosphate, *N*-acetyl imidazol, *p*-diazobenzene sulfonic acid and tetranitromethane did not affect the enzyme activity. However, *N*-bromosuccinimide (0.01 mM) and 2-hydroxy-5-nitrobenzyl bromide (1 mM) completely inhibited the enzyme reaction.

Molecular weight of acid phosphatase form i

Molecular weight of the enzyme was estimated by gel filtration on Sephadex G-200 [20]. Standard proteins (Boehringer-Mannheim, W. Germany) used were ferritin, bovine liver catalase, rabbit muscle aldolase, bovine serum albumin and egg albumin. Estimated molecular weight of the acid phosphatase form *i* was $3.1 \cdot 10^5$.

To obtain information on the subunits of the enzyme, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out according to the method described in Materials and Methods. Standard proteins used were bovine serum albumin, egg albumin, bovine pancreas chymotrypsinogen and horse heart cytochrome *c*. The electrophoretogram obtained showed a single protein band corresponding to a molecular weight of approximately 89 000.

Composition of carbohydrate in acid phosphatase form i

The presence of carbohydrate in the enzyme molecule was detected by the phenol-sulfate reaction, periodic acid-Schiff staining after disc gel electrophoresis, and precipitation with concanavalin A. The neutral sugar content of the enzyme was 31% (w/w), as measured by the phenol-sulfate method after sufficient dialysis of the sample. Identification and quantitative determination of neutral sugars in the purified enzyme was performed. The acetylated alditol derivatives of neutral sugars were analyzed quantitatively by gas-liquid chromatography. As shown in Fig. 1, major (T_x = 2.1) and minor (T_x = 2.4) peaks were identified as mannose and galactose derivatives, respectively, in the molar proportion 89 : 11 (Table IV).

Amino acid composition of acid phosphatase form i

Amino acid composition of the enzyme sample was determined by the method described in Materials and Methods, and the result is shown in Table IV. The amino acid composition is not so different from that of other acid phosphatases [21—23]. The relative number of residues of amino acid, hexosamine and neutral sugars in the enzyme molecule against total residues are shown in the first column. From these values, total number of residues in the enzyme were calculated based on a molecular weight of 89 000. The result of amino acid analysis indicated that acid phosphatase form *i* contained at least 2.41% glucos-

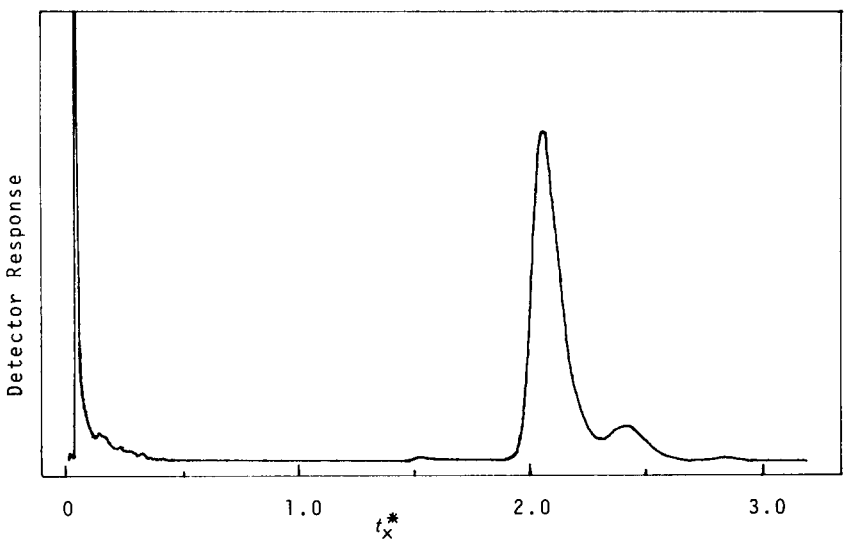

Fig. 1. Gas chromatogram of the neutral sugars of acid phosphatase form *i*, as the acetylated alditol. Neutral sugars were separated as the acetylated alditol on a column of 3% ECNSS-M. The column and detector temperatures were 180°C and 240°C, respectively. The flow rate of carrier nitrogen gas was maintained at 40 ml per minute. t_x *: the retention time of D-xylitol was defined as 1.0.

TABLE IV

COMPOSITION OF ACID PHOSPHATASE FORM *i*

Composition	Mol % *	Residues per molecule **	Nearest integral number of residues
Amino acids			
Lysine	1.84	13.0	13
Histidine	0.94	6.6	7
Arginine	1.80	12.7	13
Aspartic acid	9.98	70.5	71
Threonine	4.64	32.8	33
Serine	6.09	43.0	43
Glutamic acid	5.95	42.0	42
Proline	4.70	33.2	33
Glycine	7.72	54.6	55
Alanine	6.02	42.6	43
Valine	3.44	24.3	24
Methionine	1.04	7.3	7
Isoleucine	2.68	19.0	19
Leucine	4.64	32.8	33
Tyrosine	3.74	26.4	26
Phenylalanine	3.07	21.7	22
Tryptophan ***	1.25	8.9	9
Cysteine ****	1.46	10.3	10
Glucosamine ****	4.88	34.5	35
Neutral sugars			
Mannose	21.42	151.4	151
Galactose	2.68	18.9	19
Total	99.98	706.9	708

* Molar ratio of each component relative to total.

** Calculated based on the molecular weight of the enzyme, 89 000.

*** Determined spectrophotometrically.

**** Determined colorimetrically.

amine; the value obtained by the method of Elson and Morgan was 4.88%. In a preliminary experiment, a sample of lysozyme and glucosamine was analyzed by the same method. The recovery of glucosamine was 55%, by which the glucosamine content obtained by amino acid analyzer, 2.41%, was corrected to 4.38%. This shows the value given by the Elson and Morgan method to be reliable.

Discussion

The isolated acid phosphatase form *i* had a substrate specificity typical of acid phosphatases [22,24—27]: it was active toward various phosphomonoesters shown in Table II, but not toward bis(*p*-nitrophenyl) phosphate, adenosine 3′,5′-cyclic monophosphate, fructose 1,6-diphosphate, adenosine 5′-diphosphate and adenosine 5′-triphosphate. As with typical acid phosphatases, the enzyme activity was inhibited by orthophosphate, fluoride, borate, arsenate, molybdate and (+)-tartarate [22,24—27]. In spite of these similarities, the enzyme was sensitive to *N*-bromosuccinimide and 2-hydroxy-5-nitrobenzyl bromide but resistant to *p*-chloromercuribenzoate, *N*-acetyl imidazol, *p*-diazobenzenesulfonic acid and tetranitromethane (Table III). These findings suggest that a tryptophan residue in the enzyme molecule plays an important role in its activity. A metal ion in violet colored acid phosphatase of sweet potato [22], a cysteine residue in bovine brain acid phosphatase [25] and tyrosine and tryptophan residues in human prostate acid phosphatase [24] are essential groups for activity. The present enzyme is resistant to *p*-chloromercuribenzoate and ethylenediaminetetraacetate up to 1 mM and 0.1 mM respectively, indicating cysteine and metal ion(s) do not participate in the activity in this enzyme.

The *i* form of the enzyme from *Asp. niger* is a glycoprotein, as evidenced by phenol-sulfate reaction, periodic acid-Schiff staining after acrylamide gel electrophoresis and by precipitation with concanavalin A. Some acid phosphatases were reported to be glycoproteins containing glucosamine, sialic acid, fucose, mannose, galactose and glucose (human prostate [21]), or glucosamine and mannose (*Neurospora crassa* [23]). As shown in Table IV, the carbohydrate moiety of acid phosphate form *i* contained somewhat different sugars as mentioned above, glucosamine, mannose and galactose. Concanavalin A precipitates specifically with carbohydrate containing α-D-mannose, α-D-glucose, β-D-fructose or α-D-arabinose residues at the nonreducing end [28], suggesting that some nonreducing ends of the carbohydrate chain of this enzyme are α-D-mannose.

Generally, in glycoproteins with asparagine-linked carbohydrate units, the carbohydrate unit combines with asparagine through two molecules of *N*-acetyl glucosamine [29]. This acid phosphatase form *i* contained 35 molecules of *N*-acetyl glucosamine and 170 molecules of neutral sugars (Table IV), so if we assumed that this enzyme has asparagine-linked homogeneous carbohydrate units, it must have 17 carbohydrate moieties consisting of 12 molecules of carbohydrate.

The molecular weights of polymer enzyme and its subunit obtained by Sephadex G-200 molecular shieve chromatography and SDS acrylamide gel electrophoresis were 310 000 and 89 000, respectively. According to Odds

and Hierholzer [30], estimation methods employed in these experiments are not so accurate for glycoproteins, so these values may include error that makes the number of enzyme subunits calculated not an integral number.

To study the relationship between excretion and multiform enzyme, purification of form *e* is in progress.

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