STUDIES ON STARCH-DEGRADING ENZYMES

PART XIV*. THE MULTIPLE FORMS OF PORCINE, PANCREATIC ALPHA-AMYLASE

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ABSTRACT

Crystalline, porcine, pancreatic alpha-amylase has been fractionated into four distinct fractions by ion-exchange chromatography on DEAE-cellulose. Each fraction hydrolyses amylose in a manner identical to that of the parent enzyme, *i.e.*, at optimal pH, the reaction pattern corresponds to multiple attack, whereas in the presence of glycerol, or at high pH, it changes to multichain attack. Ultracentrifugation and gel exclusion-chromatography showed that the molecular weights of the fractions are similar to one another and to the parent enzyme, suggesting that the fractions are isoenzymes. However, determination of the amino-acid content of the multiple forms failed to reveal any reason for their different migratory rates through DEAE-cellulose. It is suggested that the multiple forms are artefacts, arising during the isolation of the enzyme.

INTRODUCTION

The alpha-amylase (α-1,4 glucan 4-glucanohydrolase, E.C. 3.2.1.1) isolated from porcine pancreas has been shown[1] to be quite distinct from other plant, fungal, and salivary alpha-amylases, in that it exhibits a pronounced degree of multiple attack when amylose is the substrate. In this attack, the enzyme severs a bond randomly, and then hydrolyses several other bonds in the immediate vicinity of the first point of cleavage, with the production of small oligomers, predominantly maltose and maltotriose. Consequently, these products are present in appreciable quantities from the start of the hydrolysis, whereas such small oligomers can be detected only at advanced stages of the hydrolysis when amylose is subjected to the action of other alpha-amylases. However, Robyt and French[1] also found that, by using a high pH for the hydrolysis, the action pattern changed to multichain, *i.e.*, the non-random component in the hydrolysis was suppressed. Subsequently, we confirmed the findings of Robyt and French, and extended them by showing that 40% aqueous glycerol would also effect the change from multiple to multichain attack[2]. In view of the repeated observation[3–6] that there are multiple forms of porcine, pancreatic alpha-amylase,

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we tentatively suggested that these isoenzymes exhibited slightly different action patterns, and that one of them was inhibited by the use of high pH, and in the presence of glycerol. We have now carried out a separation of these multiple forms, and have examined the action pattern of each. In addition, the amino-acid contents and molecular weights of the fractions have been measured.

EXPERIMENTAL

Enzymes. — Crystalline, porcine, pancreatic alpha-amylase was obtained from the Sigma Chemical Company and the Worthington Biochemical Corporation.

Substrates. — Linear amylose was leached from potato-starch granules[7].

Fractionation of alpha-amylase on DEAE-cellulose. — Whatman DEAE 52 ion-exchange cellulose, with an ion capacity of 1.0 mequiv./g was used for the separation of the fractions. After equilibration of the DEAE-cellulose with the eluting buffer (10mM Tris-HCl, pH 8.5; 3mM with respect to calcium chloride), it was packed into a glass column (30 × 1.5 cm). Enzyme (5 mg) was dissolved in the eluting buffer and applied to the top of the column. Fractions (0.6 ml) were collected, and constantly monitored at 254 nm, using an L.K.B. "Uvicord" flow photometer and fraction collector, with a photocell drop-counter.

Measurement of alpha-amylase activity. — The unit of activity used in this work is the iodine-colour dextrin unit, *i. e.*, the amount of enzyme necessary to decrease the iodine stain of a standard amylopectin β-limit-dextrin by one-third in 100 min. Details of the experimental procedure have been given previously[8].

Action of porcine, pancreatic alpha-amylase on amylose. — The type of degradative mechanism operative may be obtained from the graph of reducing power of the digest as a function of iodine stain[1,2]. The former parameter was obtained by means of the alkaline ferricyanide technique[9]; the latter was measured in the standard manner[2].

Determination of the molecular weight of porcine, pancreatic alpha-amylase by gel-permeation chromatography. — The molecular sizes of (*a*) the parent alpha-amylase and (*b*) the fractions obtained after passage through DEAE-cellulose were obtained by using "Biogel P-60" (column size, 30 × 1.5 cm). The void volume of the column was obtained by the use of Blue Dextran 2,000, and the column was calibrated by using serum albumin, ovalbumin, and trypsin inhibitor.

Measurement of the sedimentation coefficient. — For the parent amylose, sedimentation coefficients were measured in 10mM Tris-HCl buffer (pH 8.5) containing 3mM calcium chloride, at a protein concentration of 2 mg/ml, as previously described[10]. However, the isoenzyme fractions obtained from the DEAE-cellulose column were too dilute for use with the Schlieren optical system of the ultracentrifuge. Recourse was therefore made to the technique of Bordman *et al.*[11] In this method, the protein sedimented from solution during ultracentrifugation is trapped in filter paper at the bottom of the cell, and the sedimentation coefficient obtained from the relation

$$S = [-1.75 \log(1 - \Delta c/c_0\lambda)]/\Sigma\bar{\omega}^2 \Delta t,$$

where Δc is the change in concentration of the protein solution resulting from ultracentrifugation, c_0 is the initial protein concentration, $\bar{\omega}$ is the mean speed of rotation, and Δt is the time (min) of ultracentrifugation. The parameter λ is obtained from

$$\lambda = r_P^2/(r_P^2 - r_m^2),$$

where r_m and r_P are the distances from the axis of rotation to the meniscus of the solution and to the surface of the filter paper, respectively. The value of $\bar{\omega}$ must be corrected for the periods of acceleration, and also deceleration, in the manner prescribed[11]. When this technique is applied to an enzyme, the concentration term $\Delta c/c_0$ may be converted into activity units, *i.e.* $\Delta a/a_0$.

Three thicknesses of Whatman No. 1 filter paper were cut to fit into the base of the ultracentrifuge cell. The cell was then filled with a dilute solution of alpha-amylase, the activity of which was known. After ultracentrifugation at 50,740 r.p.m. for 45 min, the solution was mixed by gentle rolling of the cell, and withdrawn by means of a syringe, care being taken not to touch the surface of the filter paper during the process. The activity of this solution was then measured. To obtain the factor λ, a photograph of the cell contents was taken during deceleration (usually at a speed of 4,000 r.p.m.), and the distances r_m and r_P were subsequently measured by using a travelling microscope.

Amino acid analysis. — The technique used for analysis of amino acids was that of Speckman *et al.*[12], as modified by Patterson and Benson[13]. The apparatus used in this work was the "Technicon" automatic amino acid analyser.

Samples (2 mg) were hydrolysed in sealed Pyrex tubes at 105° for 24 h, using constant-boiling (6M) hydrochloric acid (6 ml). After removal of the acid, samples were dissolved in sodium citrate buffer (67mM; pH 2.2) containing norleucine and L-1-amino-2-guanidinopropionic acid hydrochloride as internal standards (the concentration of each was 0.2 μmole per ml of buffer).

RESULTS AND DISCUSSION

Ion-exchange chromatography

The result of passing porcine, pancreatic alpha-amylase (Sigma Chemical Company) through DEAE-cellulose is shown in Fig. 1*a*; the corresponding results for the Worthington enzyme appear in Fig. 1*b*. The former preparation yields four distinct patterns, whereas the latter shows one major component, and insignificantly small amounts of three other components. The significance of this quite profound difference in elution pattern will be dealt with in more detail later.

Rowe *et al.*[5] found only two major peaks on submitting their porcine, pancreatic alpha-amylase to ion-exchange chromatography on DEAE-cellulose. However, their experimental procedure of combining the eluant into 4-ml aliquots prior to measuring absorbance probably disguised the fact that four distinct protein-fractions were present. Using slightly different conditions, Cozzone *et al.*[6] obtained three fractions, but the elution pattern for their third fraction is rather broad and suggests

the presence of two overlapping components (see Fig. 1 of Ref. 6). Thus, our present results are not inconsistent with published data.

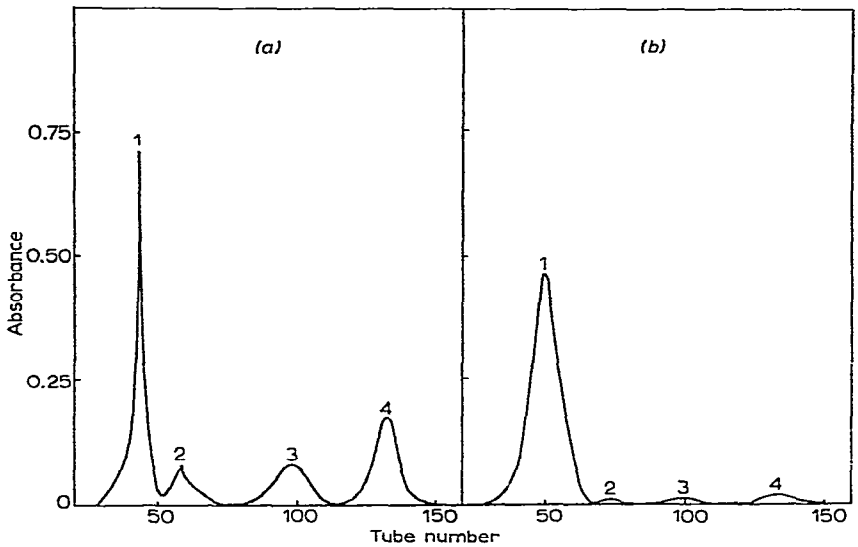

Fig. 1. The fractionation of porcine, pancreatic alpha-amylase on a DEAE-cellulose column; (*a*) alpha-amylase obtained from the Sigma Chemical Company, (*b*) alpha-amylase obtained from the Worthington Biochemical Corporation.

To ensure that the four fractions were not artefacts arising as a result of ion-exchange chromatography on DEAE-cellulose, each fraction was submitted to re-chromatography. The elution patterns are shown in Fig. 2. Each fraction is seen to emerge as a single peak, indicating that the chromatographic process *per se* does not cause the formation of artefacts within the protein.

The action pattern of the parent alpha-amylase, and of its multiple forms

To differentiate between multiple and multichain attack, it is most convenient to measure the reducing power–iodine stain relation, using amylose as substrate[1,2]. This relation is shown in Fig. 3 for the parent enzyme, and its four multiple forms, both in the presence and absence of 40% glycerol, the incubation being carried out at pH 5.5. In the absence of glycerol, the action pattern for the parent alpha-amylase and the four fractions is multiple attack (the difference between the curve for the parent sample and that for the fractions is experimental error). However, in the presence of 40% glycerol, the action pattern becomes random for all the samples. A similar effect was noted on carrying out the digestion in an aqueous medium at pH 10.5, in agreement with earlier results[1]. Thus, our suggestion[2] that the change in action pattern may be due to one of the multiple forms having slightly different properties from the others must be discarded. We have recently postulated that there

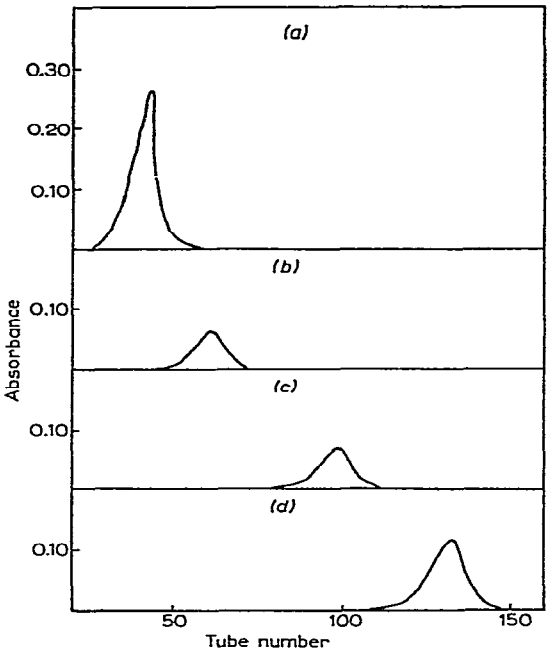

Fig. 2. Fractions re-chromatographed on DEAE-cellulose; (*a*) fraction 1, (*b*) fraction 2, (*c*) fraction 3, and (*d*) fraction 4.

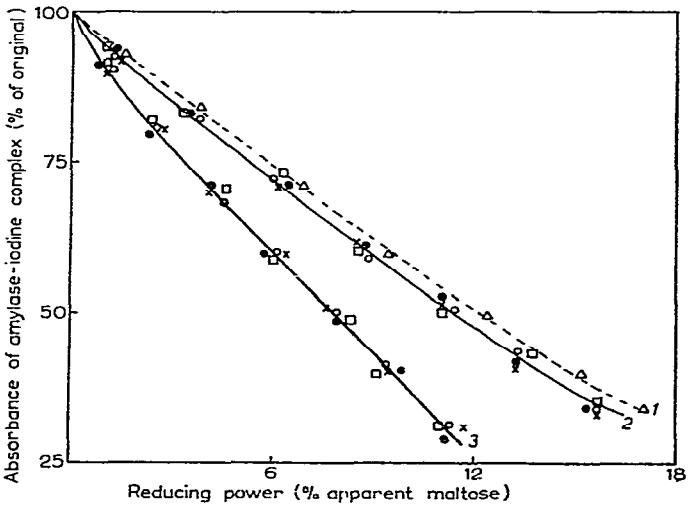

Fig. 3. The absorbance of the iodine complex as a function of the reducing power developed on incubating amylose with porcine, pancreatic alpha-amylase, and its multiple forms; (*1*) the parent enzyme, at pH 5.5, (*2*) the fractions, at pH 5.5, and (*3*) the fractions, at pH 5.5 in the presence of 40% glycerol. Fraction 1 (×); fraction 2 (●); fraction 3 (○); fraction 4 (□).

TABLE I

MOLECULAR PARAMETERS FOR PORCINE, PANCREATIC ALPHA-AMYLASE, AND ITS FRACTIONS

	Gel-permeation chromatography	*Sedimentation*[a]					
	Mol.wt. $\times 10^{-3}$	λ	$\Delta a/a_0$	$10^{11}\Sigma\bar{\omega}^2\Delta t$	$S \times 10^{13}$ (*sec*)	*Mol. wt.* $\times 10^3$	*Mean Mol.wt.* $\times 10^{-3}$
Parent enzyme	48.4	3.67	0.314	1.42	4.7	47.1	47.8
Fraction 1	47.9	3.44	0.288	1.42	4.7	47.1	47.5
Fraction 2	50.6	3.40	0.318	1.42	5.3	53.1	51.9
Fraction 3	47.6	3.72	0.330	1.42	5.0	50.1	48.9
Fraction 4	47.9	3.58	0.325	1.42	5.1	51.1	49.5

[a]By the method of Bordman *et al*[11].

are two substrate-binding sites in porcine, pancreatic alpha-amylase, and that one of them is inhibited at high pH and in the presence of glycerol[14].

The molecular size of the parent enzyme and its fractions

The results of the gel-permeation chromatography for the calibrating substances, the parent alpha-amylase, and the four fractions are shown in Fig. 4, in which V_e/V_o is graphed as a function of the logarithmic molecular weight. With the exception of

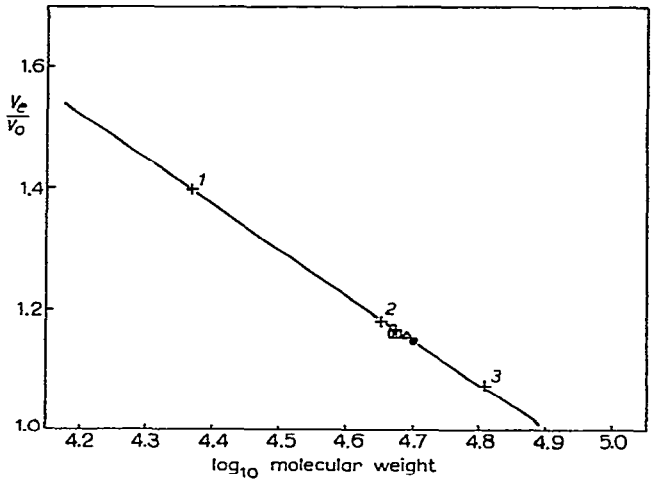

Fig. 4. Dependence of the elution volume on the molecular weight for the parent alpha-amylase, its four multiple forms, and calibrating proteins. Calibrants are denoted by (+) and are (*1*) trypsin inhibitor, (*2*) ovalbumin, and (*3*) serum albumin. The alpha-amylases are, the parent (△), fraction 1 (×), fraction 2 (●), fraction 3 (○), and fraction 4 (□).

fraction 2, each of the protein fractions yielded a value of V_e/V_o, which was virtually identical with that of the parent enzyme. The molecular weight obtained for fraction 2 was some 4% higher, as shown in Table I.

Ultracentrifugal examination of the parent enzyme, using the Schlieren optical system, showed that the protein material was homogeneous with respect to frictional properties. At an enzyme concentration of 0.2%, the measured value of the sedimentation coefficient (S) was 4.7×10^{-13} sec. The sedimentation coefficients, and the parameters from which they were calculated, for solutions a hundredfold more dilute than the above, using the method of Bordman *et al.*[11], are shown in Table I. For the parent enzyme, the S-value obtained in this manner is 4.8×10^{-13} sec, in excellent agreement with the figure resulting from the conventional technique. Thus, the method of Bordman *et al.*[11] gives the necessary degree of accuracy for measuring sedimentation coefficients.

The sedimentation coefficients of the fractions are scattered over a range of 10%, but, in agreement with the results of gel-permeation chromatography, the highest

S-value (and hence the highest molecular weight) is again associated with fraction 2. Molecular weights were calculated from the Svedberg relation

$$M = RTS/(1-\overline{V}\rho)D,$$

where $\overline{V}$, the partial specific volume of the protein, is taken[15] as 0.70 ml/g, and D, the diffusion coefficient, has a value[15] of 8.1×10^{-7}. The resultant values of M are shown in Table I.

The agreement between the molecular weights determined by gel-permeation chromatography and those derived from sedimentation measurements is fairly good. For the parent enzyme, the mean molecular weight derived from our measurements is 47,800, which should be compared with the value of 47,300 $\pm$2,300 obtained by Danielsson[15]. With the exception of the enzyme isolated from *B. stearothermophilus*, the molecular weights of alpha-amylases from most sources appear to lie in this region[16]. The fact that the molecular weights of the four fractions are approximately the same as that of the parent enzyme shows that the results of column chromatography on DEAE-cellulose cannot be explained by a disaggregation process. Rather, they must represent the presence, in the parent enzyme, of four molecular species differing in charge but not molecular size. The amino acid compositions of the parent enzyme, and its four fractions, were therefore determined.

Amino acid composition

The minimum contents of amino acids for the parent alpha-amylase and the fractions are shown in Table II. These results were obtained by converting the experimentally available parameter, *i.e.* the weight fraction of each amino acid, into its mole fraction and then dividing the figure obtained by that for histidine.

TABLE II

MINIMUM CONTENTS OF AMINO ACID RESIDUES (WHOLE NUMBERS) FOR PORCINE, PANCREATIC ALPHA-AMYLASE AND ITS FRACTIONS

Amino acid	*Parent enzyme*	*Fraction 1*	*Fraction 2*	*Fraction 3*	*Fraction 4*
Asp	7	7	9	7	7
Thr	3	3	3	2	3
Ser	4	4	4	4	3
Glu	4	6	5	4	4
Pro	2	3	3	2	2
Gly	6	7	7	6	6
Ala	4	4	5	4	4
Val	4	5	5	4	4
Met	1	1	1	1	1
Ile	2	3	3	2	2
Leu	3	3	3	3	3
Tyr	2	2	2	2	2
Phe	2	2	3	3	2
Lys	2	2	2	2	2
His	1	1	1	1	1
Arg	3	3	3	3	3

The composition of the parent enzyme is somewhat different to that first reported[17] for porcine, pancreatic alpha-amylase, but is in good agreement with recent studies[6,18]. In particular, we find only *ca.* 65% of the phenylalanine found by Caldwell *et al.*[17], but some 35% more arginine.

The amino acid compositions of the fractions are similar to one another, and to that of the parent protein. There is no evidence of an increase in content of acidic amino acids in going from fraction 1 to fraction 4. Even accepting that the slight variations in the amino acid contents of the various fractions are real, rather than merely reflecting experimental error and the inaccuracies caused by rounding-off to the nearest whole number of amino acids, the only differences between the parent and fractions 3 and 4 are to be found in neutral residues. We conclude, therefore, that the measured contents of amino acids for the four fractions do not provide an explanation of why the parent enzyme can be fractionated on DEAE-cellulose.

CONCLUSIONS

The multiple-form properties of porcine, pancreatic alpha-amylase can be summarized as follows:

(*1*) Whilst some dubiety exists about the exact number of multiple forms present in the parent enzyme, there is no doubt that passing that material through DEAE-cellulose does lead to fractionation. However, the relative amounts of the various fractions are grossly dependent on the isolation procedure, as may be seen by comparing Figs. 1*a* and 1*b*.

(*2*) The molecular weights of the fractions are similar to one another, and to that of the parent enzyme. This observation is sufficient to exclude the possibility that the enzyme dissociates into monomer units during passage through DEAE-cellulose. Thus, the separation on that material must represent charge differences.

(*3*) The two fractions obtained by Rowe *et al.*[5] were identical in terms of thermal and pH stability, and in their dependence of V_{max} and K_m on pH. In the present work, we have shown that, at optimal pH, the action pattern of the parent enzyme and the four fractions is that of multiple attack; at optimal pH in the presence of glycerol (40%), or at pH 10.6, this mechanism changes, in all cases, to random attack. If isoenzymes were, in fact, the explanation for the fractionation on DEAE-cellulose, one might reasonably expect to detect some minor differences in the action of these fractions on amylose.

(*4*) The amino acid analysis of the four fractions fails to reveal any gradation in properties which would result in fractionation being obtained on DEAE-cellulose.

We believe that these four points, taken together, are completely inconsistent with the suggestion[5] that the multiple forms of porcine, pancreatic alpha-amylase are isoenzymes, under genetic control. The experimental observations are, however, easily explained on the basis of the enzyme's undergoing slight molecular modification during the commercial isolation procedure. It should be noted that ~20% of all the amino acid residues present are accounted for by aspartic and glutamic acids. In the

parent enzyme, these residues may be present either as the free acids or as the amides. We suggest, therefore, that certain isolation procedures may remove small numbers of amide side-groups. The resultant difference in charge would enable the various molecular species to be separated on DEAE-cellulose. The relative abundance of the fractions would then vary from preparation to preparation (as is observed experimentally), depending on the details of the isolation procedure. Also, the fractions would have the same molecular weight, and, because the structural differences are comparatively trivial, would behave towards their substrate in an identical manner.

It is our conclusion, therefore, that the multiple forms of porcine, pancreatic alpha-amylase are artefacts, arising during the commercial isolation of the enzyme.

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