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PREPARATION AND SOME PROPERTIES OF HUMAN PANCREATIC AMYLASE INCLUDING A COMPARISON WITH HUMAN PAROTID AMYLASE

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SUMMARY

A method for the preparation of α-amylase (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) from human pancreatic juice is described. Procedures utilizing $(NH_4)_2SO_4$ fractionation, ion-exchange chromatography and gel filtration resulted in an amylase preparation of high purity (2085 maltose units per mg) and good yield (59%). Electrophoresis on polyacrylamide disc gels yielded characteristic isoenzyme patterns with up to six bands on anionic gels.

Comparisons with crystalline human parotid amylase indicated that the two principal human amylases are closely related enzymes but also exhibit organ specific variation. The two enzymes yielded the same amino acid composition and similar but not identical peptide maps. Each appears to consist of a single polypeptide chain. At neutral pH both amylases exhibited the same action pattern. Differences were noted in molecular weight, gel filtration profile, carbohydrate content and the accessibility of reactive sulfhydryl groups. A both high and low temperature extremes pancreatic amylase was more labile than the parotid enzyme. A less compact configuration is indicated for pancreatic amylase.

A relationship is postulated between the number of sulfhydryl groups in the amylase molecule and the mode of action. Amylases possessing 1 sulfhydryl group, namely those from human pancreatic juice, human parotid saliva and rat parotid saliva, exhibited the same type of action pattern whereas porcine pancreatic amylase, with 2 sulfhydryls, was different.

INTRODUCTION

As far as is known, in man as in a number of other mammals, the enzyme α-amylase (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) is secreted primarily by the salivary glands and the pancreas. The gross similarity between the amylases derived from these two sources caused earlier investigators to conclude that the human amylases were identical[1,2]. More recent studies on human and other mammalian

amylases, however, utilizing more sensitive biochemical techniques, have revealed differences not only between the amylases of different species but also between organs within a species and even within the amylases produced by a particular organ[3–6]. It is understandable that few direct investigations of pancreatic amylase from the human subject have been attempted. Although Bernfeld *et al.*[2] worked with crystalline human pancreatic amylase, the majority of later comparative studies have been carried out either on impure biological fluids and extracts[7–10] or in non-human species[3,4].

The goal of the present work has been the preparation of amylase from human pancreatic juice in highly purified form in order that certain of its properties might be defined and compared with those of equally pure human parotid amylase.

MATERIALS AND METHODS

Enzyme samples

The freeze-dried powder of human pancreatic juice, collected from one individual[11] was used as the starting material.

For analytical procedures involving human pancreatic amylase, the sample consisted of the enzyme purified as described under Enzyme Purification or where specified that obtained by the glycogen–enzyme complexing method of Schramm and Loyter[12], followed by filtration on P-10 Bio-Gel. By either method of preparation the enzyme sample was free of non-amylolytic components as based on the criteria of polyacrylamide disc gels.

Human parotid amylase, both the crystalline preparation and samples of the A and B isoenzyme families, were prepared by the method of Kauffman *et al.*[5]. Rat parotid amylase was purified by the procedure of Loyter and Schramm[13], and porcine pancreatic amylase was obtained from Worthington Biochemical Corporation.

Column chromatography

The columns were packed as prescribed by the manufacturers of the materials. All chromatography was carried out at 4 °C. Protein concentrations were calculated from the absorbance at 280 nm, using an extinction coefficient of $E_{1\,cm}^{1\,\%}$ equal to 16 in the case of mixed protein solutions, and 23.3 in the case of the more highly purified amylase fractions[14].

Storage of enzyme samples

Purified sample stock was stored either frozen or as the lyophilized powder, at −20 °C, or under toluene at 4 °C. Any turbidity following storage was removed by centrifugation in a Beckman L-2 ultracentrifuge.

Polyacrylamide disc gel electrophoresis

Electrophoresis was performed under alkaline conditions using the system of Davis[15], and under acidic conditions by the method of Reisfeld *et al.*[16] as modified by Neidle and Waelsch[17]. To test for impurities anionic gels were run until the tracking dye, 1 mM bromophenol blue, reached the end of the tube. For wider isoenzyme separation, electrophoresis was allowed to proceed for a longer time, normally 2.5 h at 3 mA per gel.

The gels were stained overnight with a 1% solution of amido black in 7% acetic acid, and destained electrophoretically.

Starch slide zymograms

Protein bands exhibiting amylolytic activity were located by placing the polyacrylamide gels on starch-coated slides, using the procedure of Keller and Allan[11] except that the composition of the I_2 solution was 1% I_2 in 0.5 M KI.

Specific enzymatic activity

Amylase activity was determined by the dinitrosalicylic acid procedure of Fischer and Stein[19], except that the assays were carried out at 30 °C and the buffer consisted of 20 mM sodium phosphate, 10 mM NaCl (pH 6.9).

Specific amylase activity was defined as the ratio of absorbance at 540 nm to enzyme concentration, calculated from the absorbance at 280 nm using the extinction coefficient $E^{1\%}_{1\,cm} = 23.3$ (ref. 14).

For the experiments testing the effects of increased temperatures, pancreatic and parotid amylase samples were incubated at 45, 50, and 55 °C. At intervals up to 90 min, aliquots were withdrawn and their activity measured at 30 °C as described above. The buffer was changed to 20 mM sodium glycerophosphate, 10 mM NaCl (pH 6.9) since this appeared to enhance amylase activity at temperatures above 30 °C.

Amylase action pattern

The enzymatic action pattern was determined by measuring the decrease in blue color and the increase in reducing groups at given intervals of digestion as described by Robyt and French[20].

Following 10 min equilibration at 40 °C, enzyme was added (12 ng/ml in 20 mM glycerophosphate, 10 mM NaCl buffer, pH 6.9). Incubation was carried out at 40 °C with mechanical stirring. At zero time, and at given intervals up to 90 min, 5.0-ml aliquots were withdrawn and treated as summarized in Fig. 1.

The loss in blue value (absorbance at 615 nm) was expressed as a percentage of the color at zero time. Ferricyanide reduction (absorbance at 420 nm) was converted to μg of maltose from a maltose standard curve.

Molecular weight

The sodium dodecyl sulfate polyacrylamide gel electrophoresis procedure of Weber and Osborn[21] was used as modified by Keller *et al.*[6] for parotid amylase analysis. The gels were stained for 4 h with 0.25% Coomassie brilliant blue in 50% methanol–glacial acetic acid (91:9, by vol.) and destained electrophoretically with methanol–acetic acid–water (5.0:7.5:87.5, by vol.).

Amino acid analysis

Aliquots of purified amylase each containing 0.3 mg protein were analyzed. One sample was oxidized for cysteic acid determination according to the method of Hirs[22] Acid hydrolysis was performed at 108 °C in evacuated tubes for 16, 40 and 72 h The samples were analyzed in a Beckman Autoanalyzer, Model 120 C.

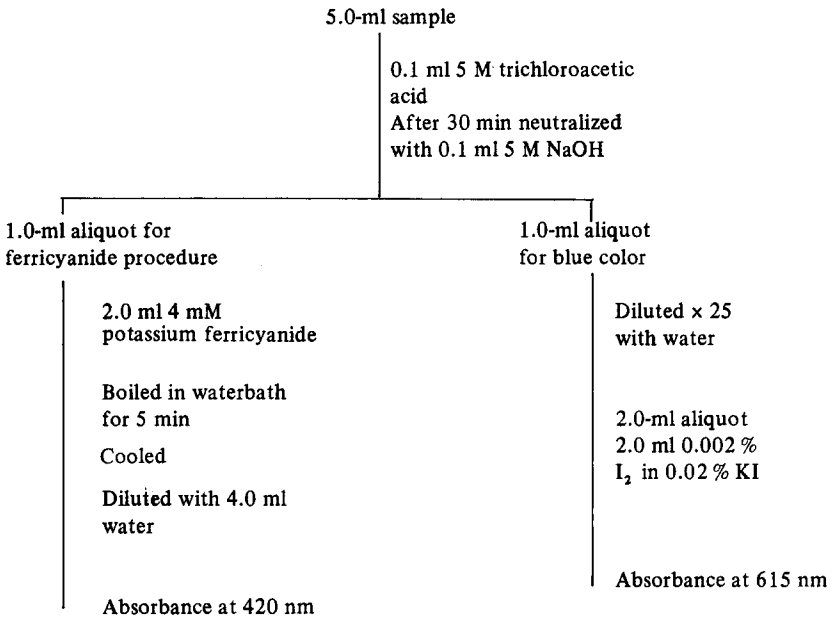

Fig. 1. Outline of iodimetric and ferricyanide reduction procedures for measuring amylase activity in action pattern studies.

The tryptophan content was analyzed from a sample of 0.4 mg protein, in 0.1 M NaOH, according to the spectrophotometric method of Bencze and Schmidt[23].

Sulfhydryl groups

Sulfhydryl groups were determined by the procedure of Schramm[24]. The assay mixture, 40 mM Tris buffer (pH 7.9) containing 1.5 mg amylase (28 nmoles, based on a mol. wt of 54 000) was incubated for 10 min at 25 °C with 10 mM EDTA and 1% sodium dodecyl sulfate. Dithiobisnitrobenzoic acid obtained from Sigma and dissolved in 40 mM Tris (pH 7.9) was added to a concentration of 0.2 mM, and after 5 min the absorbance at 412 nm noted. A control mixture containing neither sodium dodecyl sulfate nor EDTA was tested.

Carbohydrate analysis

Neutral sugar was determined on a sample of 1.6 mg amylase by the phenol–H_2SO_4 method of Hirs[25] using glucose as a standard. The proportion of covalently-linked carbohydrate was established by boiling a sample of amylase (2.1 mg) for 5 min in 0.33 M potassium phosphate buffer (pH 6.0) and subtracting the carbohydrate released by denaturation from the total.

Peptide mapping

Peptic digests of human pancreatic amylase and human parotid amylase (B family) were prepared according to Kauffman *et al.*[5] and subjected to high-voltage electrophoresis followed by descending paper chromatography. Separation of the acidic and basic peptides was achieved by electrophoresis at pH 6.5 for 35 min at

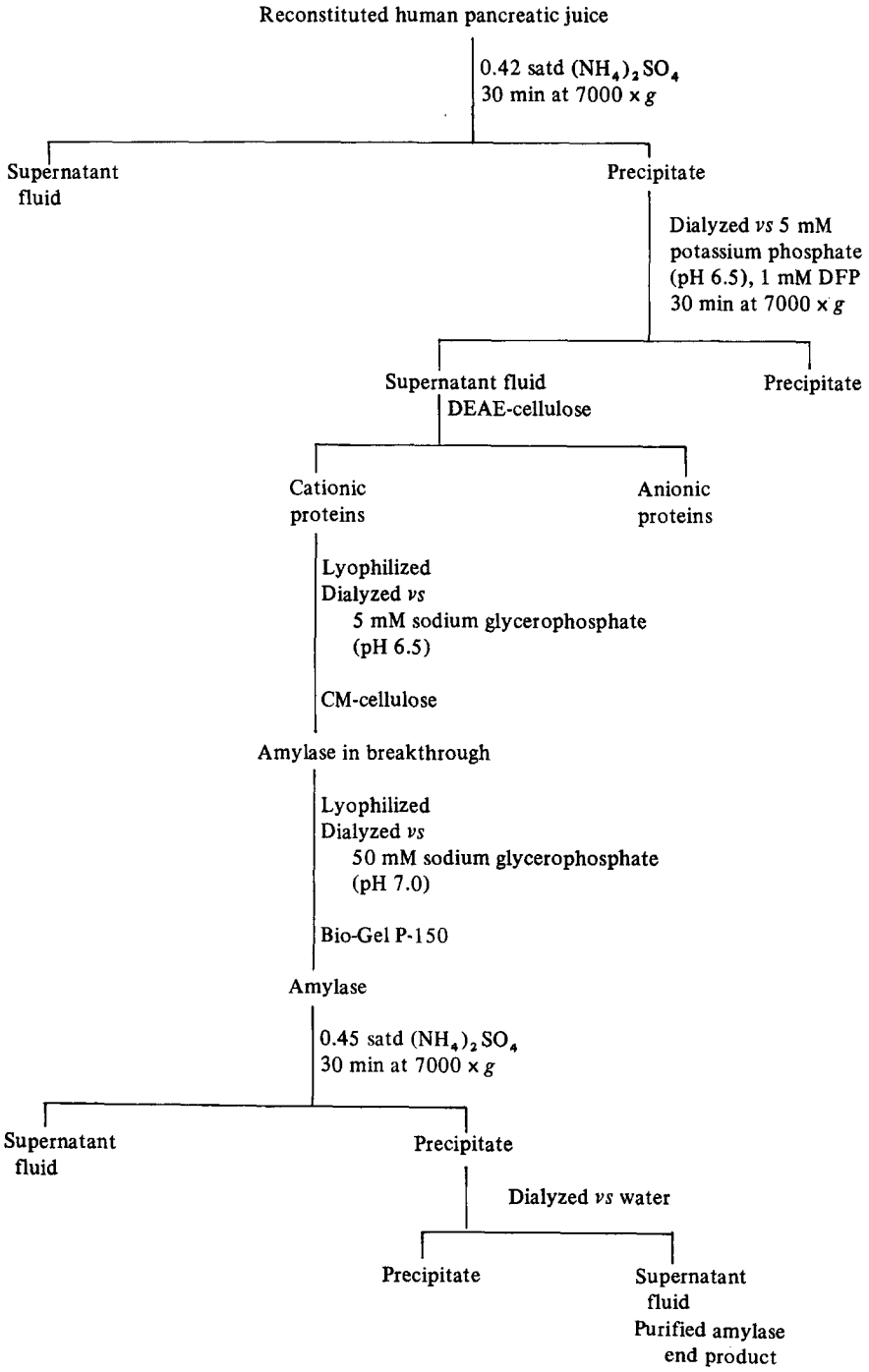

Fig. 2. Outline of method of preparation of human pancreatic amylase. Details of the procedure are presented in Table I.

50 V/cm. The neutral area was subjected to further electrophoresis at pH 1.9 for 45 min at 50 V/cm. The acidic, basic and neutral peptides were separated in a second direction by paper chromatography, using pyridine–butanol–acetic acid–water (10:15:3:12, by vol.). Following drying, the chromatograms were stained with 1% ninhydrin–0.66% cadmium acetate (100:15, by vol.) and the stain developed at 50 °C.

ENZYME PURIFICATION, EXPERIMENTAL RESULTS AND DISCUSSION

Preparation of human pancreatic amylase

Several methods of purification of α-amylase from human pancreatic juice were attempted. These were based on various properties of the enzyme, namely, molecular size, charge and the specific binding of enzyme to substrate. The most productive procedure both in terms of yield and final purity consisted of a combination of $(NH_4)_2SO_4$ precipitation, ion-exchange chromatography and gel filtration. This method is outlined in Fig. 2. Details of the chromatography are given in Table I.

TABLE I
CHROMATOGRAPHIC PROTOCOL FOR PURIFICATION OF HUMAN PANCREATIC AMYLASE

Chromatography step	*Column dimensions (cm)*	*Buffer*	*Flow rate (ml/h)*	*Sample volume (ml)*
DEAE-cellulose	1.8 × 65	5 mM potassium phosphate (pH 6.5) 0.1 mM DFP	17.0	17.5*
CM-cellulose	35.5 × 0.9	5 mM sodium glycerophosphate (pH 6.5), 0.1 mM DFP	12.0	10.2
Bio-Gel P-150	2.5 × 90	50 mM sodium glycerophosphate (pH 7.0)	12.5	9.6

* DFP added to sample to 1.0 mM concentration.

Precaution was taken throughout to prevent proteolysis during preparation either through the addition of ε-amino caproic acid or DFP as indicated.

Step I. $(NH_4)_2SO_4$ precipitation. The procedure was carried out on ice and the pH maintained at 7.0. Lyophilized human pancreatic juice was reconstituted with 5 mM potassium phosphate buffer (pH 6.5) 0.15 M in ε-amino caproic acid. $(NH_4)_2SO_4$ was added gradually with vigorous stirring to 0.42 saturation and the mixture equilibrated for 30 min.

Step II. Chromatography on DEAE-cellulose. This procedure, while serving as a general means of purification, also specifically eliminated any residual trypsinogen from the preparation. The breakthrough peak consisted of the non-adsorbed cationic proteins, amylase, lipase and proelastase. A major advantage of the present method is the early removal of both trypsinogen and chymotrypsinogen, thus minimizing the possibility of proteolytic degradation in the course of the subsequent steps.

Step III. Chromatography on CM-cellulose. Separation of amylase from pro-

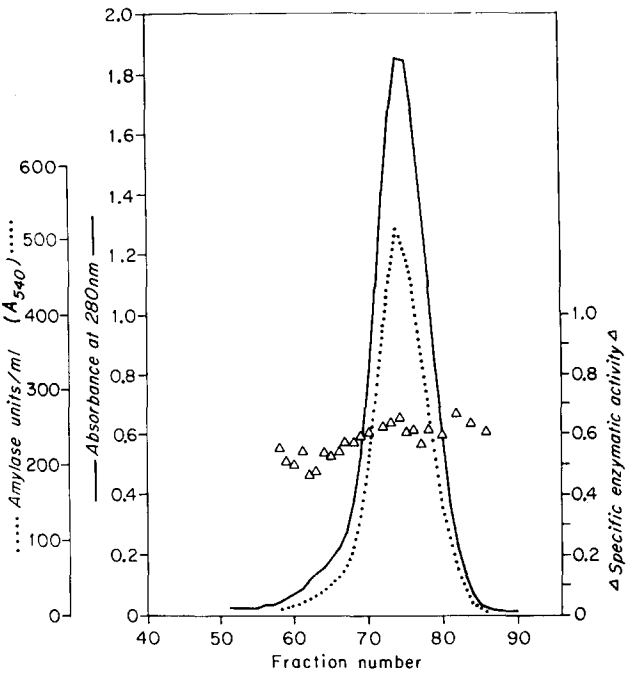

Fig. 3. Gel filtration of human pancreatic amylase on Bio-Gel P-150 (100–200 mesh). The column was equilibrated and eluted with 50 mM sodium glycerophosphate (pH 7.0). 34.8 mg protein were applied and 4.2-ml fractions collected at a flow rate of 12.5 ml/h. The absorbance at 280 nm (———), amylase units (· · · · · ·) expressed as units $A_{540\ nm}$ per ml, and specific amylase activity (Δ) expressed as $A_{540\ nm}$ units per μg amylase were measured.

elastase and lipase was achieved by this step with amylase being eluted in the breakthrough peak fractions.

Step IV. Gel filtration on Bio-Gel P-150. For analytical purposes, as well as additional purification, the amylase sample obtained from the CM-cellulose column

TABLE II

AMYLASE YIELDS AT DIFFERENT STAGES OF PURIFICATION

Sample	*Protein (mg)*	*Specific activity**	*Maltose (units/mg)*	*Total maltose*	*Recovery*
Whole human Pancreatic Juice	520	0.059	198	102 960	100
$(NH_4)_2SO_4$					
(a) Whole euglobulin	248	0.098	329	81 592	79
(b) Euglobulin supernatant	168	0.131	440	73 920	70
Dialyzed DEAE B.T.**	90	0.247	829	74 610	72
CM-cellulose B.T.**	37	0.566	1900	70 300	68
P-150 Bio-Gel	32	0.590	1981	62 600	61
Second $(NH_4)_2SO_4$ (purified amylase end product)	29	0.621	2085	60 465	59

* Absorbance at 540 nm in the dinitrosalicylic acid assay procedure per μg protein.
** B.T., breakthrough peak.

was applied to a Bio-Gel P-150 column. As shown in Fig. 3, the single protein peak corresponded closely, although not completely, with amylase activity, indicating some heterogeneity of the product. However, all fractions were included in the pooling of the material.

Step V. Additional $(NH_4)_2SO_4$ treatment. A second $(NH_4)_2SO_4$ precipitation was used as the final purification step.

The enzyme yields and progressive enrichment in activity at various stages of the preparative process are presented in Table II. In converting the observed absorbance at 280 nm to quantity of protein, the change from using an extinction coefficient of 16.0 to a factor of 23.3 was made following chromatography on CM-cellulose, since from this stage on the sample consisted of amylase essentially free of other proteins. However, the progressive increase in specific activities following subsequent fractionation on Bio-Gel P-150 and the final $(NH_4)_2SO_4$ precipitation indicate that these procedures further enhanced the purity of the enzyme.

Molecular properties of human pancreatic amylase

The high degree of purity of human pancreatic amylase prepared by the above method was established by means of polyacrylamide disc gel electrophoresis in conjunction with starch slide zymograms, as well as by the determination of specific enzymatic activity as shown in Table II. Comparisons of a number of properties were made with the corresponding analytical data for crystalline human parotid amylase. Since the latter enzyme can be fractionated on columns of Bio-Gel P-150 into two isoenzyme families[5], designated A and B, these two forms of parotid amylase were considered as separate entities.

Electrophoretic patterns

Polyacrylamide disc gel electrophoresis of freshly purified pancreatic amylase revealed the presence of isoenzymes. Under anionic conditions up to six protein bands could be seen on freshly stained gels containing 390 μg protein and run 180 min. The corresponding zymograms showed that all protein zones possessed amylolytic activity. By this criterion, therefore, the sample was free of non-amylolytic impurities.

Under cationic conditions equivalent amounts of the same preparations showed two protein bands, both having amylolytic activity. A third slower band was present only in trace amounts. Thus, by cationic electrophoresis also, the preparation appears to be free of non-amylolytic components.

The precautions taken to prevent proteolysis and the fact that the isoenzyme patterns were consistent irrespective of the method of preparation (Keller, P. J. and Stiefel, D. J., unpublished) support the view that the multiple forms are not artifactual products of the purification process.

Although the total number of bands is similar in preparations of human pancreatic and human parotid amylase, their respective distribution and electrophoretic mobilities are different. These results are in accord with earlier observations by Allan *et al.*[18]. Electrophoretic differences were also reported by Norby[7]; Kamaryt and Laxova[8], Berk *et al.*[9] and by Goetz *et al.*[10].

Molecular properties

Analytical data on the human amylases are summarized in Table III.

(1) Molecular weight. In a series of electrophoretic analyses in the presence of sodium dodecyl sulfate and β-mercaptoethanol, human pancreatic amylase yielded one discrete band and human parotid amylase yielded two bands corresponding to Families A and B (ref. 6). Although the difference in mobility between parotid B and pancreatic amylase was slight, separate zones could be distinguished on gels containing a mixture of the two proteins. Mean values of their molecular weights, calculated from seven separate determinations, were 57 000 ± 1800 for human parotid amylase B and 54 000 ± 1000 for human pancreatic amylase. These values are within experimental error of the molecular weight of 55 200 ± 1500, established by Mutzbauer and Schultz[26] for crystalline human salivary amylase.

TABLE III

COMPARISON OF ANALYTICAL DATA FOR HUMAN PANCREATIC AND HUMAN PAROTID AMYLASES

Data	*Pancreatic*	*Parotid*	
		Family A	*Family B*
Molecular weight	54 000	62 000	56 000
Carbohydrate (neutral)	1 mole/mole	8 moles/mole	<1 mole/mole
Sulfhydryls			
+EDTA, sodium dodecyl sulfate	1.0/mole	1.0/mole	1.0/mole
−EDTA, sodium dodecyl sulfate	0.4/mole	0.0/mole	0.0/mole

Human pancreatic amylase appears to exist as a single polypeptide chain. This observation is in agreement with those of other investigators who have found single chains for amylases from various sources, namely, human parotid[6], rabbit parotid and pancreas[3], rat parotid and pancreas[4] and pig pancreas[27].

Parotid amylase Family A, as analyzed in these experiments and under similar conditions by Keller *et al.*[6] exhibited a distinctly higher molecular weight of 61 900 ± 1900, a reflection in part at least of the significantly higher levels of covalently bound carbohydrate present in Family A of parotid. All of the human amylases studied are retarded on Bio-Gel columns well beyond the elution volumes expected for molecules of their size[18].

(2) Amino acid composition. The amino acid analytical data for pancreatic amylase are presented in Table IV, and compared with those obtained previously under similar conditions for the parotid isoenzymes[5]. It is evident that amylase secreted by the human pancreas is very similar in composition to that secreted by the parotid gland.

(3) Sulfhydryl groups. The sulfhydryl content for human pancreatic amylase was equivalent to 1 mole of cysteine per mole of enzyme, the same value as reported by Kauffman *et al.*[5] for the human parotid amylases. Analysis of purified rat parotid amylase also yielded 1 mole of sulfhydryl per mole of enzyme. A difference, however, was noted in the sulfhydryl reactivity in the absence of EDTA and sodium dodecyl sulfate. Of all amylases tested to date, only the human pancreatic enzyme reacted

TABLE IV

COMPARISON OF AMINO ACID COMPOSITION OF HUMAN PANCREATIC AND HUMAN PAROTID AMYLASE

Amino acid	*Residues per 1000 residues*		
	*Pancreatic**	*Parotid***	
		Family A	*Family B*
Lysine	45	45	46
Histidine	22	22	23
Arginine	56	58	59
Aspartic acid	148	145	155
Threonine	41	42	41
Serine	62	67	64
Glutamic Acid	69	69	68
Proline	50	51	53
Glycine	101	98	100
Alanine	54	49	48
Half-cystine	15	18	18
Valine	69	73	73
Methionine	17	18	21
Isoleucine	54	56	55
Leucine	52	49	49
Tyrosine	41	44	41
Phenylalanine	49	53	53
Tryptophan***	34	36	32

* Prepared by the glycogen–enzyme complexing method[12] followed by filtration on P-10 Bio-Gel.

** From Kauffman *et al.*[5].

*** Determined by the method of Bencze and Schmidt[23].

with dithiobisnitrobenzoic acid without prior denaturation, yielding 40% of total reactivity under these conditions. The human pancreatic enzyme, therefore, appears to have a less compact configuration than the parotid enzyme, at least in the vicinity of the sulfhydryl group.

(4) Carbohydrate analysis. A determination of the neutral sugar content of pancreatic amylase indicated the presence of 33 nmoles of carbohydrate per 28 nmoles of enzyme, of which 26 nmoles remained protein-bound after heat denaturation. Hence, covalently-bound carbohydrate was present in a 1 mole per mole ratio, based on a glucose standard curve.

In contrast to human parotid amylase which is fractionated on columns of Bio-Gel into two families, with the A family comprising glycosidated isoenzymes and the B family consisting of nonglycosidated isoenzymes[6], no separation into isoenzyme families was observed for human pancreatic amylase. The latter enzyme emerged as a single peak from gel filtration columns and presented only one band on electrophoretic gels run in the presence of sodium dodecyl sulfate. These observations suggest that the carbohydrate moiety may be uniformly distributed among the isoenzymes of pancreatic amylase and that selective glycosidation is not the basis of their existence. The total carbohydrate content is very low, however, and precise knowledge of its nature and distribution must await isolation of the individual isoenzymes.

(5) Peptide mapping. Figs 4, 5 and 6 indicate an overall similarity in the

peptide patterns of human pancreatic and human parotid amylase. The similarity in pattern is most marked in the basic peptides (Fig. 4). All 22 basic paptides from pancreatic amylase have qualitative counterparts on the parotid amylase map. Variations are of a minor quantitative nature. Peptide B-2, strongly evident in the parotid enzyme but only weakly present in the pancreatic, tended to vary in intensity on other maps of parotid B amylase.

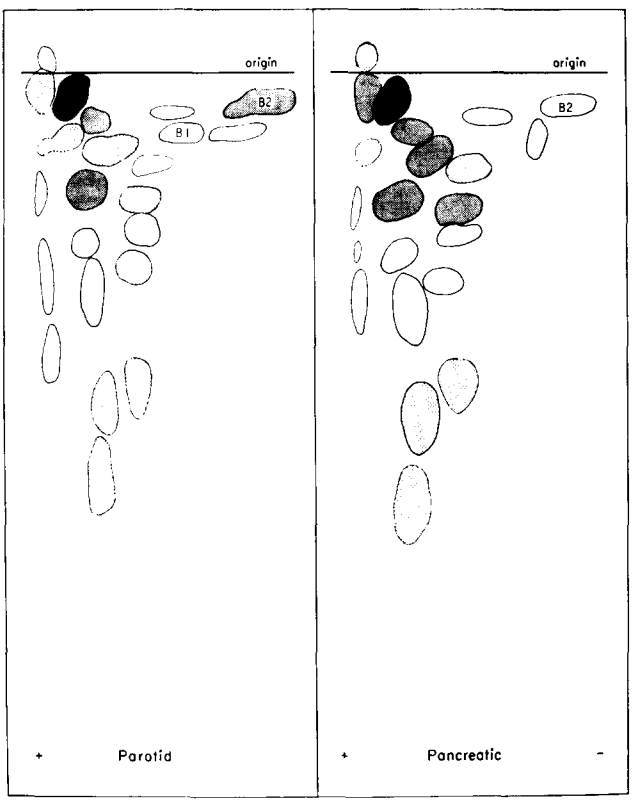

Fig. 4. Maps of the basic peptides yielded by peptic digests of human pancreatic and parotid amylase. Electrophoresis, at pH 6.5 was in the horizontal direction; chromatography was in the vertical direction from the origin as shown. The peptides were graded in the order of increasing staining intensity from 1 to 4 (1, blank; 2, lightly dotted; 3 heavily dotted; 4, blackened).

A comparison of the acidic patterns (Fig. 5) also reveals considerable similarities. However, noticeable differences both of a quantitative and qualitative nature, are apparent in the peptides remaining close to the chromatographic origin. In contrast to the acidic and basic peptides, the pattern of neutral peptides (Fig. 6) shows considerably greater differences between the two enzymes. Although the total number of peptides is again approximately the same, only the areas most mobile on chromatography have an obvious correspondence. A significant difference in amino acid sequence is therefore indicated.

Enzymatic properties

Specific enzymatic activity. A comparison of the amylase preparations presented in Table V showed human parotid amylase to be the most active with a specific activity of 0.932. The corresponding maximum activity for purified human pancreatic amylase was 0.621 or 67% that of the parotid. The present pancreatic amylase sample, when assayed under the conditions used by Fischer *et al.*[28] had an activity comparable

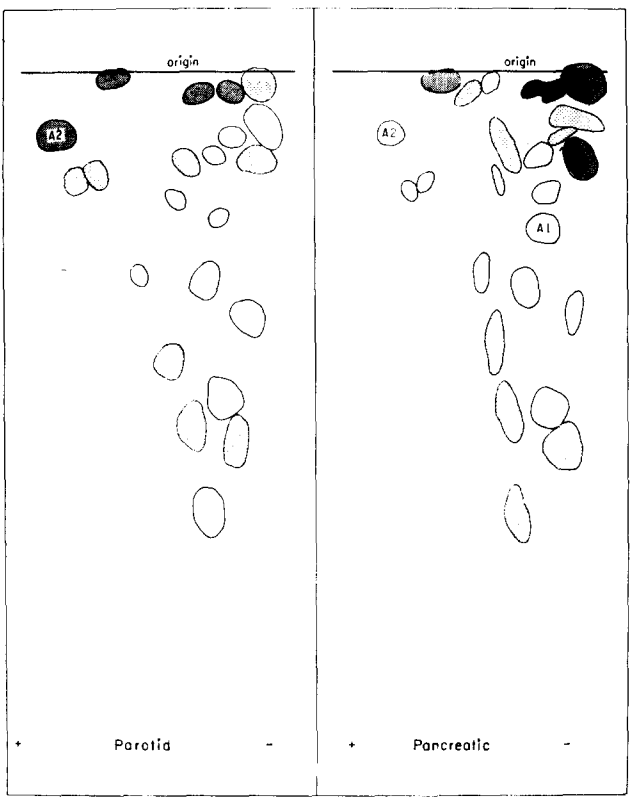

Fig. 5. Maps of the acidic peptides yielded by peptic digests of human pancreatic and parotid amylase. Electrophoresis, at pH 6.5, was in the horizontal direction; chromatography was in the vertical direction from the origin as shown. The peptides were graded in the order of increasing staining intensity from 1 to 4 (1 blank; 2, lightly dotted; 3, heavily dotted; 4, blackened).

to that obtained by the latter investigators for crystalline human pancreatic amylase.

Stability at low temperatures. Activity determinations following storage at 4 °C or at −20 °C revealed that parotid amylase was extremely stable over periods of several months. In contrast, pancreatic amylase, although essentially stable for 7–9 days, lost up to 17% of its activity after longer storage.

Effect of increases in temperature. In contrast to the relative stability of the amylases at low temperatures, marked losses in activity were noted when the temperature was increased beyond 45 °C, as shown in Fig. 7. However, in each case, the

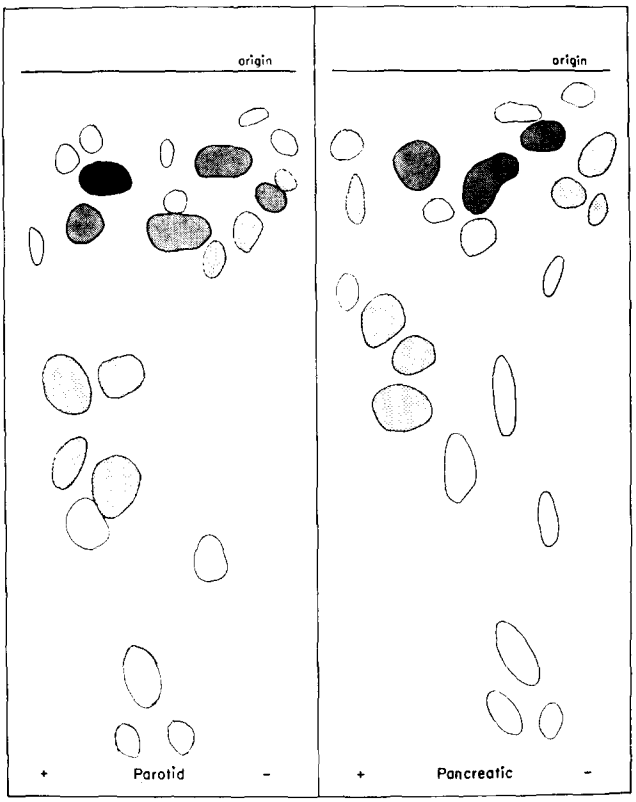

Fig. 6. Maps of the neutral peptides yielded by peptic digests of human pancreatic and parotid amylase. Electrophoresis was in the horizontal direction and carried out at pH 1.9 on the neutral area from the pH 6.5 run; chromatography was in the vertical direction from the origin as shown. The peptides were graded in the order of increasing staining intensity from 1 to 4 (1, blank; 2, lightly dotted; 3, heavily dotted; 4, blackened).

TABLE V

SPECIFIC ENZYMATIC ACTIVITY OF VARIOUS AMYLASE PREPARATIONS

Sample	*Specific activity**	*Maltose (units/mg)*
Human parotid amylase	0.932	2800
Human pancreatic amylase		
(1) Present preparation	0.621	2085
(2) Glycogen-complexing method	0.499	1484
(3) Glycogen-complexing and chromatography on P-10 Bio-Gel	0.417	1253
(4) Fischer preparation**	0.332	1000
(5) Present preparation under Fischer conditions**	0.327	982
Porcine pancreatic amylase	0.333	1000

* Absorbance at 540 nm per ng amylase.
** Assayed at 20 °C and 3.5 mM Cl^-.

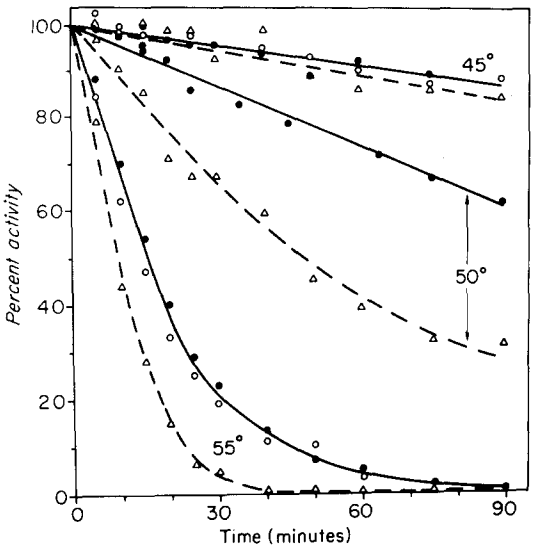

Fig. 7. Effect of increases in temperature on amylase activity. After time periods of incubation at elevated temperatures as indicated, amylase was assayed by means of the dinitrosalicylic acid procedure at 30 °C. Samples consisted of (Δ) human pancreatic amylase; (○) human parotid amylase, Family A; (●) human parotid amylase, Family B.

decline in activity was less marked for parotid amylase, indicating that at the higher temperatures, as at the low extremes, this enzyme was relatively more stable than pancreatic amylase.

Amylase action pattern. α-Amylases have been reported to hydrolyze starch in a multiple attack manner, that is, through the cleavage of several bonds during the life time of a particular enzyme-substrate complex. Robyt and French[20] reported that the degree of multiple attack differed for amylases prepared from different sources, *e.g.* human saliva and porcine pancreas. To determine whether the differences observed by Robyt and French[20] were due to species or organ differences, a similar study was made with the purified preparations of human parotid and human pancreatic amylase. Rat parotid and porcine pancreatic amylases were included in the study.

With a highly polymerized substrate such as Superlose measurement of the decline in amylose iodine blue color gives an indication of the size of the polysaccharide remaining after enzymatic action, whereas the ferricyanide-reduction procedure measures the increase in total number of reducing groups, expressed as maltose molecules. Plots of these values resulted in characteristic action patterns as presented in Fig. 8.

At neutral pH, in spite of their different specific activities, human parotid and human pancreatic amylases yielded identical action patterns. The curves were independent of enzyme concentration. Furthermore, although both human parotid and human pancreatic amylase exhibited twice as much activity in sodium glycerophosphate as in sodium phosphate buffer of the same concentration, chloride content and pH, the mode of action remained the same in both buffer systems.

Under varying pH conditions the response of the two human amylases differed

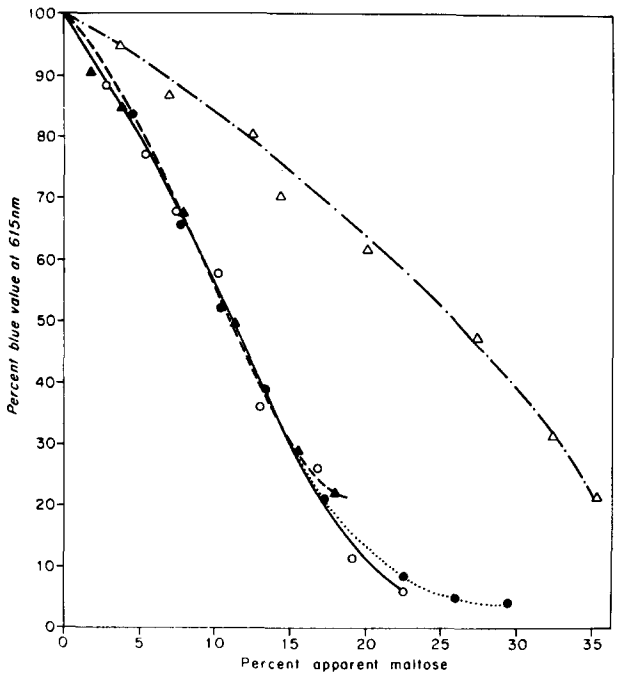

Fig. 8. Amylolysis by amylases from various sources; comparison of action pattern curves. The following amylase samples were tested: (△) porcine pancreatic, (▲) human pancreatic, (○) rat parotid, (●) human parotid (Family B). All enzyme concentrations were 12 ng/ml in 20 mM sodium glycerophosphate, 10 mM NaCl buffer (pH 6.9). Substrate: 0.1% Superlose. The human pancreatic amylase was prepared by the glycogen-binding method[12] followed by filtration on Bio-Gel P-10.

according to whether the change was made toward the acid or the alkaline side. At alkaline pH (9.0) the pancreatic enzyme remained relatively more active and retained a higher degree of multiple attack than the parotid enzyme, while at acid pH levels (5.5) the converse held true. These observations appear consistent with the physiological conditions under which the enzymes function.

Whereas the curve of rat parotid amylase was similar to that of the human enzymes, the action of porcine pancreatic amylase yielded more reducing value per corresponding drop in blue color, consistent with a greater degree of multiple attack. In addition to being consistent with the results reported by Robyt and French[20] for crystalline amylases from pig pancreas and mixed human saliva, the present findings indicate that the variations in action pattern are essentially species rather than organ differences.

It is interesting to speculate on the relationship of the free sulfhydryl groups and the activity of the amylase molecule. Those amylases having one free sulfhydryl group, namely the two human enzymes and that of rat parotid, appear to have a very similar mode of action. On the other hand, porcine pancreatic amylase with two sulfhydryl groups (ref. 24 and Cozzone, P., personal communication) exhibits an action pattern indicative of a higher degree of multiple attack. Loyter and Schramm[29] have demonstrated the presence of two binding sites in the porcine pancreatic

amylase molecule. The latter report and the present experiments are consistent with the hypothesis that the sulfhydryl groups are part of the binding site or sites of the enzyme (Cozzone, P., personal communication). On the assumption that the binding sites cooperate to bind a single polymeric substrate molecule such as Superlose, the presence of an additional sulfhydryl group in porcine pancreatic amylase may well account for the increased degree of multiple attack, and hence the type of action pattern, observed for this enzyme.

The relationship of enzyme structure to function is a very close one, and even small differences in composition or sequence may have an effect on tertiary structure and consequently on catalytic action. Such appears to be the case for the amylases of human pancreatic and parotid origin.

Evidence for a less compact configuration of the pancreatic amylase molecule compared to the parotid has been provided by several observations: the relatively greater accessibility of the sulfhydryl group in undenatured human pancreatic amylase, the greater instability of pancreatic amylase toward heat denaturation, observed in the present study as well as by Berk *et al.*[9], and its more marked lability at low temperatures. Configurational differences have also been postulated by Aw and Hobbs[30] to be the reason for the greater inhibition of human pancreatic amylase by a given antiserum compared to human parotid.

The human enzymes thus follow the pattern of organ specificity observed with amylases in other animals, namely those of Malacinski and Rutter[3] for rabbit, Sanders and Rutter[4] for rat, and Sick and Nielsen[31] for salivary and pancreatic amylases of the mouse.

The present comparative study was performed on mixtures of isoenzymes. Determination of the physicochemical and possibly functional differences between the respective molecular forms will require their isolation in quantities not presently available.

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