Immunochemical Relationship between α -Amylases of Rat Liver, Serum, Pancreas and Parotid Gland

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1. Rabbit antisera to purified rat parotid α -amylase were prepared. 2. The relationships between rat parotid-gland, pancreatic, serum and liver amylase were investigated by using the antisera in immunodiffusion, immunoelectrophoresis and immunoinhibition experiments. 3. Serum and liver amylase were identical, and very similar to parotid-gland amylase; pancreatic amylase was, however, quite distinct, and showed only some of the determinants present on parotid-gland amylase. 4. The data strengthen the suggestion that the liver is the main source of serum amylase.

McGeachin and his co-workers (McGeachin & Reynolds, 1961; McGeachin *et al.*, 1961, 1966) have extensively used antisera against pig pancreatic and human salivary α -amylases (EC 3.2.1.1) in an attempt to study relationships between amylases of crude tissue extracts in a variety of animal species. They concluded from the relative lack of inhibition of liver amylase by such antisera that the liver enzyme is immunologically and structurally distinct from pancreatic, salivary and, by implication, serum amylases (McGeachin, 1968). Mordoh *et al.* (1968) found a difference in K_m for glycogen between amylases of rat liver and serum, and therefore similarly proposed that liver amylase differs from serum amylase: however, their experiments were done with crude mixtures in which the K_m values may be altered by other components.

Other studies involving cellulose acetate electrophoresis of partially purified amylase preparations (Hammerton & Messer, 1971) suggest that rat liver and serum amylase are identical, which would be consistent with the fact that the perfused rat liver secretes amylase into the perfusion medium (Arnold & Rutter, 1963). Further, the intracellular distribution of liver amylase resembles that of a secretory protein (Hammerton & Messer, 1973).

In the present work the relationship between rat tissue amylases in solubilized extracts has been investigated, by using rabbit antisera to purified rat parotid-gland amylase and to a crude rat parotid-gland extract.

Methods

Amylase assay

Amylase activity was measured by the method of Bernfeld (1955), but with 3% starch in 0.1M-

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Tris-maleate (pH 6.9)–20mM-NaCl as substrate. Incubations were for 10min. One unit of amylase activity is defined as 1.0 μ mol of maltose equivalent liberated per min at 37°C.

Purification of rat parotid-gland amylase

Parotid-gland amylase was purified from homogenates (in 5mM-Tris-HCl, pH 7.4; 1:5, w/v) of the parotid glands of 20 male Wistar rats. The method consisted of gel filtration of a supernatant of this homogenate (100000g centrifugation for 60min) on Sephadex G-100 and then on Bio-Gel P-150, with 5mM-Tris-HCl, pH 7.4, as eluent, essentially as described by Frati & Caputo (1970), but followed by DEAE-Sephadex chromatography at pH 8.0 as described by Ball (1974). This additional step was done to ensure removal of any traces of pancreatic-type amylases that might be present in the parotid gland; these have isoelectric points higher than 8.7 (Sanders & Rutter, 1972) and therefore should not be bound by the DEAE-Sephadex column.

Amylase solutions were concentrated by ultrafiltration through Amicon PM-10 membranes (Amicon Corp., Lexington, Mass., U.S.A.).

Preparation of antisera

Antisera were prepared in New Zealand White rabbits by repeated intramuscular injections, each of 1 mg of protein in 0.25 ml of a 1:1 mixture of complete Freund's adjuvant and 0.9% NaCl. Antiserum A was derived from a rabbit which received injections of crude extract of rat parotid gland (100000g supernatant) on days 0, 17, 33 and 46, and antisera B and C were derived from rabbits which received purified parotid-gland amylase. Rabbit B was injected on days 0, 17 and 33 and rabbit C on days 0, 17, 33, 46 and 55.

Partial purification of serum, liver and pancreatic amylases

Because serum and liver contain only relatively small amounts of amylase, the enzyme extracts were concentrated to workable concentrations (at least 30 units/ml) by partial purification. Rat serum amylase was partially purified by gel filtration on Sephadex G-100, with a recovery of 83%.

For the preparation of partially purified liver amylase, a microsomal fraction was obtained by the method of de Duve *et al.* (1955) from 67 g of livers of unstarved rats. To solubilize the enzyme, the microsomal fraction was suspended in 5 mM-Tris-HCl, pH 7.4, containing 0.1% Triton X-100, sonicated, and then dialysed for 24 h against 10 mM-Tris-HCl, pH 7.4, containing 10 mM-CaCl₂; this was done to permit the release of amylase from the microsomal fractions and the digestion of glycogen to which much of such released amylase would initially be adsorbed (Mordoh *et al.*, 1968; M. Messer & R. T. Dean, unpublished work). The dialysed solution was centrifuged at 100 000g for 60 min and the amylase of the supernatant was partially purified by fractional precipitation with 66% ethanol as described by Hammerton & Messer (1971). A final recovery of 36% of the total liver amylase, or 70% of the microsomal amylase, was obtained.

Rat pancreatic amylase was similarly partially purified from a 100 000g supernatant of rat pancreas homogenized in 5 mM-Tris-HCl, pH 7.4 (1:5), with 80% recovery.

Immunochemical techniques

Immunoinhibition and immunoprecipitation studies were done by mixing 100 μ l of amylase solution (adjusted to 7 units/ml by dilution with 1% normal rabbit serum in 10 mM-Tris-HCl, pH 7.4) with 100 μ l of 0.1 M-Tris-maleate, pH 6.9, and 100 μ l of antiserum B (which had been dialysed against 0.9% NaCl). The antiserum was used in various dilutions (with 0.9% NaCl) up to a maximum of 800 times. After incubation for various times at room temperature (25°C), 100 μ l of the mixture was assayed for amylase activity.

For immunoprecipitation, the same mixtures were then stored at 4°C for 20 h and centrifuged (15 000g for 10 min); the supernatants were assayed for amylase activity.

The amylolytic activity of the antiserum was also estimated and subtracted from that of the rat amylase solutions; this correction was only of significance in experiments with rat pancreatic amylase for which large amounts of antiserum were required. A control experiment showed that the antiserum had no effect on the amylase activity of normal rabbit serum. In

further control experiments with normal rabbit serum in place of antiserum, no immunoinhibition of any amylase was observed.

Double immunodiffusion analysis by the method of Ouchterlony (1967) was performed by using 50 μ l capacity wells in 1% agarose in either 20 mM-potassium phosphate buffer, pH 7.0, containing 0.9% NaCl, or Tris-barbitone buffer, pH 8.6 (Tris, 150 mM; barbitone, 50 mM). After 16–20 h of diffusion the plates were washed, dried and stained with 0.1% Coomassie Brilliant Blue (Barrett, 1973) as described by Dean (1974). This staining provides much greater sensitivity than is obtained by viewing unstained precipitin lines under dark-ground illumination. Immunoelectrophoresis was performed at pH 8.2 as described previously (Dean, 1974).

'Rocket' immunoelectrophoresis (electrophoresis of amylase into agarose gels containing antiserum) was performed as described by Laurell (1966) by using Tris-barbitone buffer, pH 8.6, and an electrophoresis time of 16 h. The amount of antiserum required to precipitate a known amount of amylase was calculated from the areas under the 'rockets', which were measured by weight by using paper cut-outs.

Results

Purity and characteristics of the parotid-gland amylase used as antigen

The final freeze-dried product (56 mg) had $E_{280}^{1\%} = 21.5$ and, on the basis of this value, a specific activity of 3420 units/mg of protein. At 30°C it liberated 2630 mg of maltose-water/3 min per mg of protein; this compares with values ranging from 2500 to 3000 reported for purified rat parotid-gland amylase by other workers (see Ball, 1974).

When examined by polyacrylamide-disc-gel electrophoresis (Davis, 1964) by using 7% gels and a discontinuous Tris-glycine system (Clarke, 1964), one major and four minor bands were seen after staining one-half of the gel, cut lengthwise, with Coomassie Brilliant Blue (Plate 1). Each of these bands had amylase activity, as shown by incubation of the other half of the gel on starch-agarose (Ball, 1974). Multiple electrophoretic forms of rat and human parotid-gland amylases have been investigated in detail previously (Kauffman *et al.*, 1970; Sanders & Rutter, 1972; Ball, 1974). The mobility of the purified amylase on sodium dodecyl sulphate-polyacrylamide-gel electrophoresis as described by Neville (1971) was intermediate between those of bovine serum albumin and hen ovalbumin, which is consistent with a molecular weight of 56 000 determined by Sanders & Rutter (1972). No subunits with smaller molecular size were observed in this system, run either in the presence or in the absence of

EXPLANATION OF PLATE I

Polyacrylamide-gel electrophoreses of purified parotid-gland amylase

The sample protein loadings were: (a) 100 μ g; (b) 200 μ g. Migration was from top to bottom and gels were stained for protein as described in the Methods section. Four bands are visible at the lower loading (a), and faint traces of a fifth (just above the group of four) at the higher loading (b). All five bands contained active amylase.

EXPLANATION OF PLATE 2

Representative Ouchterlony gel-diffusion test of the specificity of antiserum B

The central well contained antiserum B; peripheral wells 1–3 contained dilutions of a crude extract of rat parotid gland, and well 4 contained purified parotid-gland amylase. The precipitin lines on a replicate plate were shown to contain amylase by the method described in the text.

EXPLANATION OF PLATE 3

Ouchterlony gel-diffusion analysis of tissue amylases

(a) The sample wells contained: L, concentrated liver amylase; Pr, purified parotid-gland amylase; S, concentrated serum amylase; A, antiserum B. (b) The sample wells contained: Pr, purified parotid-gland amylase; Pn, partially purified pancreatic amylase; A, antiserum B. Fusion of the pancreatic and parotid-gland lines was clear on the original plate. Sequential observations suggested that the sharp minor precipitin line between antiserum and parotid-gland amylase in (b) was due to band splitting, and this was confirmed by assaying the precipitates as described in the text.

10mm-dithiothreitol. Thus by the usual criteria of protein chemistry the parotid-gland amylase was pure.

The purity of the parotid-gland amylase was also tested by immunoelectrophoresis against the polyspecific antiserum A, which was found to precipitate at least five proteins in the crude parotid-gland extract. In conventional immunoelectrophoresis, a single precipitin line was observed in a position towards the anode corresponding to that of amylase, when the purified enzyme was the antigen.

'Rocket' immunoelectrophoresis, although originally designed for quantitative determination of antigens (Laurell, 1966), is highly suitable for the assessment of purity of antigens and specificity of antisera; the technique ensures that antigen and antibody reach equivalence, and that all molecules of any antigen to which there are an excess of antibodies are finally precipitated in a 'rocket'. Thus a wide range of antigen/antibody ratios can be tested much more easily in this system than in those involving diffusion of the interacting components, and, further, contaminating proteins present in low concentrations are more likely to be detected if antibodies to them are present. Indeed, simultaneous quantitative determination of a number of antigens has been reported (Laurell, 1972). We have therefore used 'rocket' immunoelectrophoresis against 20% antiserum A for final assessment of the parotid-gland amylase preparation; only one precipitin line was observed. To demonstrate that this precipitin line was due to amylase, it was cut out, suspended in 2ml of 20mm-potassium phosphate buffer, pH 7.0, in 0.9% NaCl, and dispersed by sonication. A control contained gel cut from within the 'rocket'. The suspensions were assayed for amylase activity; the precipitin line was found to contain 1.1% of the applied activity (40units) whereas the control contained no activity. As shown below, immunoprecipitated amylase is strongly inhibited, and this accounts for the apparent low recovery of activity. No other precipitin lines were observed during experiments with antiserum A at lower concentrations. Thus, even by these highly sensitive immunochemical criteria, the parotid-gland amylase preparation contained only amylase, and none of the other antigens originally present in the parotid gland.

Specificity of antisera B and C

The specificity of antiserum B (obtained from a rabbit which had been immunized with purified parotid-gland amylase) was carefully tested by Ouchterlony double immunodiffusion (Plate 2) and immunoelectrophoresis by using a wide range of ratios of antiserum/antigen. In double immunodiffusion, the antiserum was tested against serial dilutions of highly concentrated samples of rat serum and of solubilized extracts of rat parotid gland and

liver, as well as dilutions of purified parotid-gland amylase. In 'rocket' immunoelectrophoresis the antigens, in the same ranges of concentrations, were run into gels containing up to 20% antiserum. In conventional immunoelectrophoresis the antiserum was tested only against crude parotid-gland and liver extracts. In all cases, either a single precipitin line or no line was observed. The 'rocket' precipitin produced between purified parotid-gland amylase and antiserum B and the immunodiffusion precipitin between a parotid-gland extract and antiserum B were shown to be due to amylase, by the method described above. Fusions (observed in immunodiffusion; see under 'Immunochemical relationship between rat α -amylases') between precipitin lines from samples of serum, liver and pancreas with those due to parotid-gland amylase showed that these precipitates were also due to amylase. For 'rocket' immunoelectrophoresis of concentrated liver extracts, a heavy protein streak, presumably due to lipoproteins, may have obscured the presence of some precipitin lines; nevertheless, no other precipitin lines were obtained with samples largely freed of lipoprotein by ethanol precipitation (see the Methods section) or in conventional immunoelectrophoresis of the crude concentrated extracts. Thus there was no evidence that antiserum B reacted with any components of the tissues under study other than amylase. The production of a monospecific antiserum to amylase is also consistent with the evidence already presented for the purity of the parotid-gland amylase preparation.

The specificity of antiserum C was not extensively assessed; however, no evidence was obtained for precipitation of components other than amylase by this antiserum.

Immunochemical relationship between rat α -amylases

Immunodiffusion and 'rocket' immunoelectrophoresis. Relationships between antigens can be investigated with monospecific or polyspecific antisera, provided the precipitin lines due to the components of interest can be identified. It has already been shown that antiserum B reacts only with amylase, and that the only component in the parotid-gland amylase preparation reacting with any of the antisera is amylase. Criteria for identifying amylase precipitin lines (by line fusions and by activity measurements) were thus available, so all three antisera were used to study the relationships between the tissue amylases.

Agarose-gel double diffusion at pH 8.6 or 7.0 of purified parotid-gland and partially purified serum and liver amylase preparations gave a single continuous precipitin line (reaction of identity) against antisera A, B or C (Plate 3). A precipitin line was also seen with pancreatic amylase but

Fig. 1. Inhibition of α -amylases of rat serum (a), liver (b), parotid gland (c) and pancreas (d) by rabbit antiserum prepared against purified rat parotid-gland amylase

Assays for amylase were made on mixtures containing various concentrations of antiserum B and a fixed concentration of amylase at pH 6.9 (see the Methods section). ●, Amylase activity after 2h incubation at room temperature; ○, amylase activity of the supernatant after centrifugation after precipitation for 20h at 4°C.

only at higher concentrations of antigen (500 units/ml), a reaction of partial identity being obtained between this line and that due to parotid-gland amylase; a spur without deviation was observed on the parotid-gland amylase line (Plate 3).

When parotid-gland, serum or liver amylases were allowed to electrophorese into agarose gels containing antiserum B (0.1%) or antiserum C (0.5%), single rocket-shaped precipitin lines were seen in each case. When the antigen wells were placed close together, these lines fused and no spurs were seen. Evidence has already been presented that these lines were due to amylase. No precipitin lines could be detected

with pancreatic amylase under these conditions, but 'rockets' were seen with this amylase when electrophoresed into 20% antiserum B in the reverse (cathodal) direction. This is due to the high isoelectric points of pancreatic amylase, which at 8.77 and 8.95 (Sanders & Rutter, 1972) are above the pH of the electrophoresis buffer.

From the areas of the 'rockets' the ratios of antiserum B to amylase in the precipitin lines were calculated to be 0.38, 0.34, 0.32 and 10.0 μ l per amylase unit for parotid-gland, serum, liver and pancreatic amylase respectively. With antiserum C the ratios were 1.7, 1.7 and 1.6 μ l per unit (ratio for

pancreatic amylase not determined with this antiserum).

Immunoinhibition and immunoprecipitation. To determine the inhibition of amylase activity by antiserum, a suitable amount of each amylase preparation was mixed with various concentrations of antiserum B, and the remaining activity was determined after 2h. (Control experiments for various times of incubation showed that inhibition was maximal at 2h.) The inhibition curves for serum and liver amylases were identical (Fig. 1), and both were similar to the curve for parotid-gland amylase but very different from that for pancreatic amylase; 50% inhibition was produced by 0.46, 0.46, 0.56 and 39 μ l of antiserum per amylase unit respectively. Further, serum, liver and parotid-gland amylases could each be completely inhibited by a suitable amount of antiserum, whereas pancreatic amylase could not.

The mixtures of amylase and antiserum were further examined by assaying the amylase activity in the supernatant after centrifugation after precipitation over 20h at 4°C. For serum, liver and parotid-gland amylase there was only a small further loss of activity (Fig. 1), but with pancreatic amylase there was considerable loss. Evidently the immunoprecipitate formed with pancreatic amylase retained significant amylase activity, consistent with the failure to achieve complete inhibition.

Discussion

Our results with immunodiffusion, 'rocket' immunoelectrophoresis and immunoinhibition demonstrate that rat liver and serum amylases are identical and that both are very similar to parotid-gland amylase. Immunoinhibition has been shown to be a very sensitive technique for differentiating between human amylases (Aw & Hobbs, 1968) and between several bacterial amylases (Matsuzaki *et al.*, 1974). The minor difference in immunoinhibition characteristics between serum and liver amylases on the one hand and parotid-gland amylase on the other may therefore reflect a genuine minor structural difference; previous electrophoretic observations on these amylases (Hammerton & Messer, 1971) would be consistent with this.

All three amylases differ greatly from pancreatic amylase, a distinction which was seen most clearly in the immunoinhibition studies. In the reaction of partial identity between pancreatic and parotid-gland amylases observed in immunodiffusion, the spur was present on the parotid precipitin line; it may therefore be deduced that pancreatic amylase lacks some antigenic determinants present in parotid-gland amylase. Sanders & Rutter (1972), using rabbit antiserum to rat pancreatic amylase, have previously presented limited evidence of partial identity between

rat pancreatic and parotid-gland amylase, as well as more detailed studies on differences in their amino acid compositions and isoelectric points.

In contrast with our results with liver amylase, McGeachin & Reynolds (1961) and McGeachin *et al.* (1966) observed relatively little inhibition of rat liver amylase by rabbit antisera to pig pancreatic or human salivary amylases or by rooster or goat antisera to pig pancreatic amylase. However, as pointed out by Arnold & Rutter (1963), this evidence (unlike that in the present work) is based on studies with crude liver extracts in which a major part of the amylase is associated with glycogen and microsomal fractions. As discussed in detail by Cinader (1967), substrate (in this case glycogen) interferes with the inhibitory effect of subsequently added antibody. Further, the microsomal membrane may provide a greater barrier to antibodies than it does to some components of the starch substrate. To a lesser extent the problem of relative accessibility of the amylases to substrate and antibody may have affected the results of McGeachin *et al.* (1961, 1966) on other tissues; nevertheless, their data (McGeachin & Reynolds, 1961) imply the existence of a clear difference between rat pancreatic and parotid-gland amylases.

Our results support previous suggestions that pancreatic and parotid-gland amylases are the products of different genes (Sick & Nielsen, 1964; Malacinski & Rutter, 1969; Sanders & Rutter, 1972), but refute the possibility that liver amylase might be due to the activity of a third locus.

Our evidence on the immunochemical identity of liver and serum amylases and their difference from pancreatic amylase strengthens previous suggestions that the liver is the main source of serum amylase (see Hammerton & Messer, 1971), and, further, eliminates the possibility of the pancreas being such a source.

The availability of a monospecific antiserum to liver amylase may facilitate experiments on the intracellular localization and physiological function of this enzyme.

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