Biochimica et Biophysica Acta, 341 (1974) 305–317
© Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

BBA 67167

ISOLATION OF SALIVARY RIBONUCLEASE, DEOXYRIBONUCLEASE AND AMYLASE FROM THE PAROTID GLAND OF THE RAT, *RATTUS NORVEGICUS ALBINUS*

WILLIAM D. BALL

Department of Zoology and The Center for Research in Oral Biology, University of Washington, Seattle, Wash. 98105 (U.S.A.)

(Received October 29th, 1973)

SUMMARY

The salivary enzymes amylase, deoxyribonuclease and ribonuclease have been purified from a crude homogenate of parotid glands from the rat, *Rattus norvegicus albinus*. Initial separation of the three enzymes was effected by chromatography on DEAE-Sephadex, with elution of amylase and DNAase by a NaCl gradient. Amylase was further purified by chromatography on Sephadex G-100, exploiting its tendency to bind to the dextran gel and to elute as if it were a much smaller molecule. When analyzed by anionic disc gel electrophoresis at pH 8.3, one major and two minor amylase species were detected in the preparation. The RNAase and DNAase fractions were each separated on Sephadex G-100 and then on ion-exchange resins. RNAase was purified on a Bio-Rex 70 column, and showed three distinct peaks when eluted with 0.2 M phosphate buffer (pH 6.15). The two major peaks showed identical, single bands when analyzed by cationic disc electrophoresis at pH 4.5. DNAase was prepared by SE-Sephadex chromatography, using an acetate gradient at pH 4.7, and eluted as a single peak at 0.35 M sodium acetate buffer. The DNAase preparation showed a major and a minor band with DNAase activity, when subjected to anionic disc gel electrophoresis at pH 8.3.

INTRODUCTION

The salivary glands of rodents produce and secrete digestive enzymes with functional capacities similar to those produced by the pancreas. The parotid gland secretes α-amylase [1, 2] (EC 3.2.1.1), a deoxyribonuclease [2–5] and a ribonuclease [6], the submaxillary gland produces trypsin- and chymotrypsin-like proteases [7–9].

Previous studies concerning the accumulation of these enzymatic activities during embryonic development of the rat, *Rattus norvegicus albinus*, had raised the question of the significance of their presence in the embryonic and neonatal salivary glands [6, 12] (Ball, W. D., unpublished). It became apparent that their effective quantitative documentation would require the preparation of the purified adult enzymes to provide reference standards for the enzyme activities detected in crude

tissue extracts. This paper reports the isolation of amylase, DNAase and RNAase from a crude homogenate of adult rat parotid glands.

Amylase has been previously purified from both the pancreas and the parotid gland of the rat [10–12], and the predominant molecular species found in the parotid gland has been shown to be specifically different from the amylase produced in the pancreas [12]. DNAase and RNAase had not previously been purified. The preparations of DNAase, RNAase, and amylase which are described here, have been purified 31-fold, 2000-fold and 8-fold, respectively, and have no contaminants detectable by disc gel electrophoresis.

MATERIALS AND METHODS

Experimental animals

The animals used in this study are from a colony of Sprague–Dawley rats maintained in this laboratory since 1968, and which originally were purchased from Rush Laboratories, Beaverton, Oregon.

Preparation of crude extract

Parotid glands were dissected from 30 etherized adult male rats which had been starved for 24 h, and were collected on ice. After carefully trimming away connective tissue, fat, and other contaminants, the glands were minced and placed in 30 ml of a buffer solution which contained 0.025 M KCl, 5 mM $MgCl_2$, 8.5% sucrose, and 0.05 M Tris–HCl buffer (pH 8.0). The mixture was homogenized in a Sorvall Omnimixer at the highest speed setting, for 4 times at 1 min each. A preliminary centrifugation was performed for 5 min at 500 $\times$ g, to remove intact cells and tissue fragments, and the resulting supernatant was centrifuged for 90 min at 100 000 $\times$ g in a Spinco Type 40 rotor.

Determination of enzyme activities

An inhibitor of protease activity, phenylmethylsulfonyl fluoride [14], was included in some solutions described below [4, 5], and the isopropanol is the vehicle in which the inhibitor is dissolved. Bovine serum albumin (Pentex, Fraction V Powder) is included in the buffers used to dilute RNAase and DNAase, since these enzymes have been found to lose activity at high dilutions in the absence of other protein (Ball, W. D., unpublished).

Ribonuclease activity was measured by a modification of the assay method of Beard and Razzell [14], which was developed as a microassay for small quantities of RNAase in embryonic tissues (Ball, W. D. and Rutter, W. J., unpublished). An aliquot containing 10 μl of the enzyme source in a buffer solution (0.1 M Na_2SO_4, 0.055 M Tris-HCl buffer (pH 8.0), 1 mg/ml albumin, 0.5 mM phenylmethylsulfonyl fluoride, 2.5% isopropanol) was added to 50 μl of 1 mg/ml RNA in 8 mM ammonium acetate, 8 mM potassium phosphate, and 9 mM Tris-HCl buffer (pH 7.8). The mixture was incubated at 37 °C, and the reaction was stopped with 50 μl of cold acid–ethanol (76% ethanol in HCl). After standing 10 min in ice, the tubes were centrifuged at 15 000 $\times$ g for 5 min, the supernatant was diluted 0.2 in water, and the absorbance was measured at 260 nm on a Zeiss PMQ II spectrophotometer. In some assays, polycytidylic acid (Miles Laboratories) was used as the substrate at 0.28 mg/ml. The reactions were

stopped by the addition of 50 μl of 16% $HClO_4$, and the absorbance was measured at 280 nm.

Amylase activity was determined by a micro-modification of the Bernfield method [16]. To prepare substrate solution, 1% soluble starch (Baker potato starch) was dissolved in amylase assay buffer (0.03 M KH_2PO_4, 0.02 M NaCl, 0.002% Trition-X-100, 1 mM phenylmethylsulfonyl fluoride, 5% isopropanol (pH 6.9)) by gently heating the mixture to boiling, and filtering it through Whatman No. 2 filter paper.

Dinitrosalicylate reagent was prepared by dissolving 1.0 g of 3, 5-dinitrosalicylic acid (K. and K.) in 20 ml of 2 M NaOH and 50 ml of water. To this was added 30 g sodium potassium tartrate (Rochelle salt), and the mixture was brought to 100 ml and tightly capped to protect it from CO_2. The enzyme source was diluted in amylase assay buffer, a 10-μl aliquot was mixed with 10 μl of substrate solution in a 400-μl polyethylene centrifuge tube (Beckman–Spinco Microfuge tube), and the mixture was incubated at 37 °C. The reaction was stopped by adding 20 μl of dinitrosalicylate reagent, mixing, and chilling the tube in ice. The tube was then heated 5 min in a boiling water bath, chilled, and mixed with 200 μl water. Color was allowed to develop for at least 30 min, and the absorbance was measured at 540 nm. Activity units are expressed in reference to a maltose standard curve, as μmoles maltose released per min per mg protein, at 37 °C.

DNAase activity was measured by a modification of the method of Kunitz [17]. The enzyme source was diluted in a solution containing 1 mg/ml albumin, 1 mM phenylmethylsulfonyl fluoride, 5% isopropanol, 0.01 M Tris–HCl buffer (pH 7.4). The DNA substrate solution contained 0.05 mg/ml DNA (Calbiochem, salmon sperm), 5 mM $MgCl_2$ and 0.05 M sodium *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonate buffer (pH 7.7). Commercial preparations of bovine pancreatic DNAase (Worthington, DSV) were assayed with the same procedure, except that the buffer was 0.05 M sodium morpholinopropane sulfonate (Calbiochem) pH 7.0. A 10-μl aliquot of enzyme solution was added to 0.99 ml of substrate, mixed by inversion, and the enzyme activity was measured at 22 °C by the increase in absorbance at 260 nm. Activity units are expressed as A/min per mg protein.

Total protein content was measured with a micromodification of the Folin–Lowry technique [4, 18], and expressed in terms of a bovine serum albumin standard.

Column chromatography

Samples were brought to 10% sucrose and applied to the column beds under an overlying buffer layer. The column effluents were monitored by their absorbance at 280 nm, using an LKB 8300 Uvicord II absorption spectrophotometer. The concentrations of salt gradients were determined by measuring the conductivity, using a Radiometer Type CDM2e conductivity meter with a CDC114 conductivity cell. Fractions were collected with an LKB 7000 Ultrorac Fraction collector. Enzyme peaks from the column effluents were concentrated under N_2 prior to the next chromatographic step, by using Amicon No. 202, No. 52 or MC-8 pressure–filtration cells with UM-2 membranes. The samples were then equilibrated with the appropriate elution buffers by pressure–dialysis using the same equipment. All chromatography steps were done at 4 °C.

DEAE-Sephadex chromatography was used for the initial fractionation of the

crude supernatant. DEAE-Sephadex, A-50, was equilibrated with 0.09 M Tris–HCl buffer (pH 8.0), packed in a column 2.5 cm × 40 cm (volume = 196 ml), and washed with 1 l of the buffer under a 20-cm pressure head. 27 ml of the crude, 100 000 × *g* supernatant was applied, and the column was eluted with the Tris buffer at 20 ml/h. Proteins which bound to the gel under these ionic conditions were eluted with a linear salt gradient, between 0 and 0.10 M NaCl.

Each of the enzyme peaks obtained from the DEAE-Sephadex column was further purified on Sephadex G-100. The gel was equilibrated with 0.09 M Tris–HCl (pH 8.0) and packed in a 2.5 cm × 45 cm column (volume = 220 ml). Samples of 7.5 ml RNAase, and 15 ml for DNAase and amylase were eluted at 15–25 ml/h with a pressure head of 15–20 cm.

The ribonuclease peak from the Sephadex G-100 column was chromatographed on Bio-Rex 70, 100–200 mesh. The resin was prepared as described by Hirs et al. [19] and equilibrated with 0.2 M potassium phosphate buffer (pH 6.15). A 0.9 cm × 14.5 cm column was packed with the resin, and the 1-ml sample was eluted at 5 ml/h.

The deoxyribonuclease peak from the Sephadex G-100 separation was further purified on SE-Sephadex, C-50, using the method of Price et al. [20]. The gel was equilibrated with 0.2 M sodium acetate buffer (pH 4.7), and packed in a 2.5 cm × 40 cm column (volume = 196 ml). The 5-ml sample was initially eluted with the equilibrating buffer, at 30 ml/h with a 20-cm pressure head, followed by a gradient of 0.20–0.57 M sodium acetate buffer (pH 4.7). An additional purification was done on DEAE-Sephadex, using a 0.9 cm × 28 cm column (volume = 17.6 ml) and a 1-ml sample eluted at 5 ml/h.

Polyacrylamide disc gel electrophoresis

Purified enzyme preparations were analyzed using the cationic procedure of Reisfield et al. [21] with 15% gels, and the anionic method of Davis [22] with 7.5% gels.

All gels were polymerized with $(NH_4)_2S_2O_8$ at 0.7 mg/ml. Samples were made up in 0.1 ml of large pore gel buffer containing 10% sucrose, and loaded to the top of the gel under an overlying layer of tray buffer.

Electrophoresis was performed at 4 °C, using 6-mm gel tubes and a current of 5.8 mA/gel. Electrophoretic mobility is expressed as R_F = mobility of protein band/mobility of marker dye, and the marker dyes used were bromphenol blue (Eastman) in the anionic system [22], and bismark brown R (Baker), at 31 μg/gel, in the cationic system.

Gels to be stained for protein were rimmed in 7.5% acetic acid and cut at the dye front with a razor blade. After a 30-min soak in methanol–acetic acid–water (5:1:5, by vol.), the gels were stained overnight in a solution of 0.25% Coomassie Brilliant Blue [23] (Sigma, Brilliant Blue R) in this mixture. Gels were destained in 5% methanol, 7.5% acetic acid (v/v) using a Canalco transverse destainer, and stored in 7.5% acetic acid.

Gels to be assayed for enzymatic activity were rimmed in the appropriate assay buffer. RNAase activity was directly assayed in the gels using the procedure of Wilson [24]. DNAase activity was detected by incubating the gels on a DNA–agar plate and staining with methyl green [4]. Amylase activity was localized by a brief incubation of the gels on starch–agar plates containing 1% Noble Agar (Difco), 0.5% soluble starch

(prepared as above), 0.02 M KH_2PO_4 and 0.01 M NaCl. The unhydrolyzed starch was stained by irrigating the plate with a solution of 3.5 mM I_2, 0.25 M KI.

RESULTS

DEAE-Sephadex chromatography of the 100 000 × g crude supernatant

With the equilibrating buffer ($I = 0.05$), ribonuclease activity eluted at the leading edge of the first major protein peak (Fig. 1). The fractions in Region 1 of the

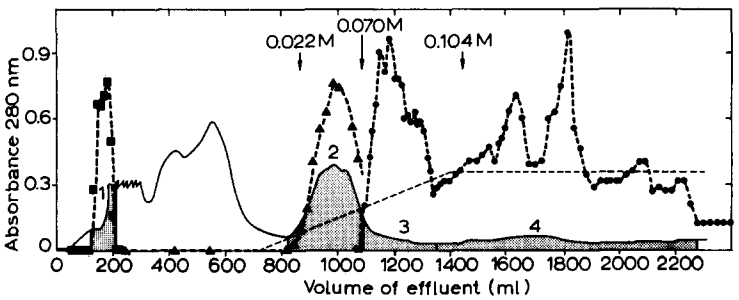

Fig. 1. Chromatography on DEAE-Sephadex, A-50, of the crude 100 000 × *g* supernatant of a rat parotid gland homogenate. The solid line is a trace of absorbance at 280 nm. The lightly-dashed line represents the concentration of NaCl in the starting buffer of 0.09 M Tris–HCl buffer (pH 8.0, $I = 0.05$), increasing from 0 to 0.10 M. Shaded regions of the absorbance trace are the fractions pooled for the isolation of each enzyme; 1, RNAase; 2, amylase; 3, DNAase Batch I; 4, DNAase Batch II. Filled characters represent enzyme activity; squares, RNAase; triangle, amylase; circles, DNAase.

absorbance trace were pooled, and showed a 29-fold purification (Table I). DNAase and amylase were eluted with a salt gradient between 0 and 0.10 M NaCl in the Tris buffer. Amylase eluted at 0.02 M NaCl ($I = 0.07$) as a protein peak which was coincident with amylase activity (Fig. 1). The fractions in Region 2 were pooled, and showed a purification of 5.4 with 82% recovery (Table II).

TABLE I

PURIFICATION OF RIBONUCLEASE

Activity units are expressed as the μg quantity of a standard RNAase (EC 2.7.7.16, bovine pancreatic ribonuclease A, Worthington, RASE) which will effect the same hydrolysis.

	Total protein (mg)	Units of enzyme activity	Units/mg protein	Purification	Recovery (%)
100 000 × *g* crude supernatant	880	44.2	0.051	1	100
DEAE-Sephadex peak	21.8	32.3	1.48	29.0	73.0
Sephadex G-100 peak	2.4	23.3	9.79	192	52.7
Bio-Rex 70 Peak I	0.181	0.64	3.89	76	1.4*
Bio-Rex 70 Peak II	0.057	6.10	107	2090	13.8*
Bio-Rex 70 Peak III	0.047	4.13	87.9	1730	9.3*

* Total recovery in Bio-Rex 70 peaks was 24.5%.

TABLE II

PURIFICATION OF AMYLASE

	Total protein (mg)	Units of enzyme activity	Units/mg protein	Purification	Recovery (%)
100 000 × *g* crude supernatant	880	202 000	230	1	100
DEAE-Sephadex peak	133	166 000	1250	5.4	82
Sephadex G-100 peak	92.7	165 000	1780	7.7	82

DNAase activity eluted at 0.07 M NaCl ($I = 0.12$), and appeared at the trailing edge of the amylase peak. In this separation, the DNAase eluted anomalously, in that following an initial peak, the remainder of the activity was spread over a large number of fractions, at constant ionic strength. Preliminary experiments had shown the DNAase to elute from DEAE-Sephadex as a single, sharp peak at 0.07 M NaCl. The two different batches containing DNAase activity (shaded areas 3 and 4 in Fig. 1) behaved identically in the remaining purification steps. Rechromatography of the combined batches after the last purification step (Fig. 7) demonstrates that the elution pattern seen in Fig. 1 is a technical artifact, and that the enzyme is homogenous in its binding to DEAE-Sephadex.

Purification of RNAase

Sephadex G-100 chromatography. The DEAE-Sephadex fractions which contained ribonuclease activity (see Fig. 1) were concentrated to 7.5 ml and further resolved on Sephadex G-100. RNAase activity eluted well behind the first peak of excluded molecules, and ahead of the low molecular weight components (Fig. 2). The fractions in the shaded portion of the graph were pooled and concentrated, and the RNAase activity showed a 192-fold purification over the crude supernatant (Table I).

Bio-Rex 70 chromatography. The Sephadex G-100 concentrate was loaded to a Bio-Rex 70 column and eluted at 5 ml/h with 0.2 M potassium phosphate buffer (pH 6.15). RNAase activity was eluted in three distinct peaks, the first and smallest peak

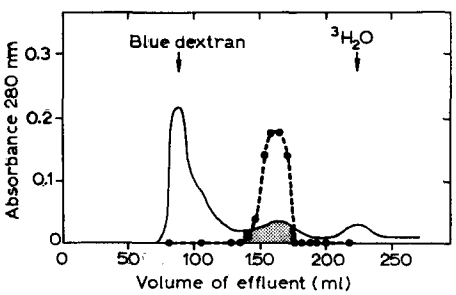

Fig. 2. Sephadex G-100 chromatography of the RNAase peak eluted from the DEAE-Sephadex column (see Fig. 1). The solid line is a trace of absorbance at 280 nm. Points represent RNAase activity, and the shaded portion of the trace defines the fractions containing RNAase activity which were collected for further purification. The vertical arrows mark the effluent volumes at which blue dextran and ^3H_2O were eluted from the column.

being associated with the trailing edge of an absorbance peak (Fig. 3). The two major peaks of RNAase activity showed no detectable absorbance, consistent with the low total amount of protein (Table I) and were obtained with approx. a 2000-fold purification (Table I). No additional RNAase activity eluted with phosphate buffer at pH increasing to 8.0.

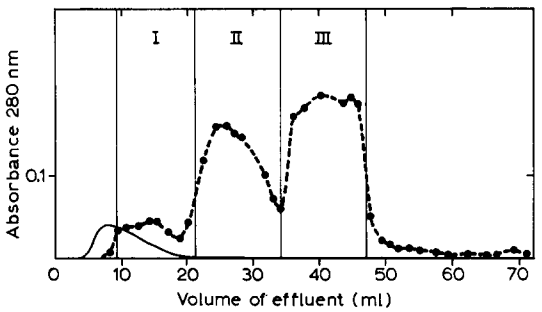

Fig. 3. Bio-Rex 70 chromatography of the RNAase peak eluted from the Sephadex G-100 column (see Fig. 2). The solid line is a trace of absorbance at 280 nm. Points represent RNAase activity, and the three peaks of RNAase activity were each separately pooled as indicated by the vertical lines.

The specific activity of the Batch III preparation against both native RNA and polycytidylic acid substrates is given in Table IV, and compared with the values obtained for a commercial preparation of bovine pancreatic RNAase. The parotid ribonuclease has lower specific activity against both substrates. It shares with the bovine enzyme a distinct preference for poly(C) as a substrate, but the preference is quantitatively less, with a poly(C)/RNA activity ratio of 3.4 compared to 8 for the pancreatic ribonuclease. Since the low total amount of protein made its quantitation uncertain, the specific activity calculations are only approximate estimates.

Purification of amylase by Sephadex G-100 chromatography

Amylase preparation from the mouse submaxillary gland [25], the rat pancreas [11] and the human parotid (Keller, P., personal communication), are known to be retarded on Sephadex gels. Preliminary experiments had shown (Ball, W. D., unpublished) that the rat parotid amylase eluted more slowly than cytochrome c (M_r = 12 700), although it has a molecular weight of 56 000 [12]. When the amylase peak (Region 2, Fig. 1) was chromatographed on Sephadex G-100, all of the amylase activity was eluted just ahead of the low molecular weight components (Fig. 4). This step showed an additional purification of 1.4, and an overall purification of 7.7, with 82% recovery of the initial amylase activity (Table II).

Purified rat parotid amylase has previously been obtained at specific activities of 3000 [26], 2500 [27] and 2780 [12] units/mg protein, where units are equal to mg maltose per 3 min at 30 °C. Expressed in these units, the preparation described here had a specific activity of 1920 when protein was estimated by the Folin–Lowry method and 2530 with protein estimated on the basis of an $A^{1\%}_{280\ nm}$ = 23.3, the value reported by Hsui et al. [28] for human salivary amylase. Since it appeared that the amylase preparation might have lost activity in storage, a second preparation was made using

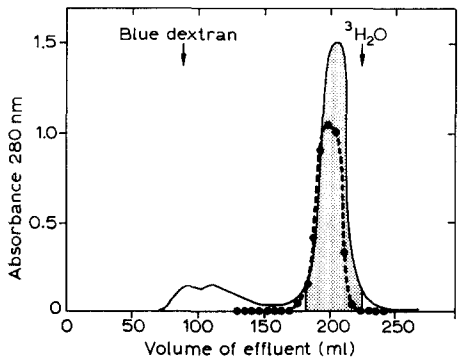

Fig. 4. Sephadex G-100 chromatography of the amylase peak eluted from the DEAE-Sephadex column (see Fig. 1). The solid line is a trace of absorbance at 280 nm, and the points represent amylase activity. The vertical arrows mark the effluent volumes at which blue dextran and ^3H_2O were eluted from the column.

the same procedure and the specific activity of this preparation was 2310 based on Folin–Lowry protein, and 3040 based on 280-nm absorbance measurement.

Purification of DNAase by Sephadex G-100 and SE-Sephadex chromatography

The pooled fractions from the DEAE-Sephadex column were chromatographed on Sephadex G-100 (Fig. 5). The DNAase activity is associated with a major peak of absorbance just behind the void peak of excluded molecules ($M_r \leqq 100\,000$). The large peak which appears later in the fractionation is a contaminating fraction of amylase.

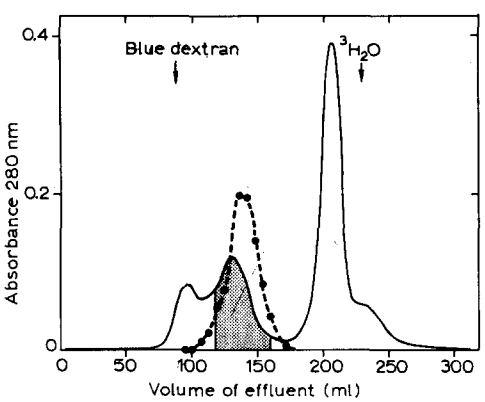

Fig. 5. Sephadex G-100 chromatography of the DNAase peak (Batch I) eluted from the DEAE-Sephadex column (see Fig. 1). The solid line is a trace of absorbance at 280 nm and the points represent DNAase activity. The fractions in the shaded portion of the trace were collected for further purification. The vertical arrows mark the effluent volumes at which blue dextran and ^3H_2O were eluted from the column.

The Sephadex G-100 isolation provided a further 4-fold purification of the enzyme (Table III). The DNAase recovered from the Sephadex G-100 column was further resolved on a SE-Sephadex column (Fig. 6). A minor peak of absorbance eluted at 0.2 M sodium acetate buffer (pH 4.7) and DNAase appeared at 0.35 M sodium

TABLE III

PURIFICATION OF DEOXYRIBONUCLEASE

	Total protein (mg)	Units of enzyme activity	Units/mg protein	Purification	Recovery (%)
100 000 × *g* crude supernatant	880	103	0.117	1	100
DEAE-Sephadex peak*	167	49.5	0.296	2.5	48.0
Sephadex G-100 peak*	36.8	43.5	1.18	10.1	42.2
SE-Sephadex peak*	5.4	19.7	3.64	31.1	19.2

* The quantities given are the sums of the data for the Batch I and Batch II preparations which were separately purified (see text and Fig. 9).

TABLE IV

SPECIFIC ACTIVITY OF RIBONUCLEASE

Specific activities are expressed as change in absorbance/min per mg protein, under the assay conditions described in Materials and Methods.

	Specific activity		Ratio poly (C)/RNA
Substrate	RNA	Poly(C)	
Bovine pancreatic ribonuclease	$6.0 \cdot 10^5$	$4.8 \cdot 10^6$	8.0
Parotid ribonuclease	$8.1 \cdot 10^4$	$2.7 \cdot 10^5$	3.3

acetate buffer. The apparently homogeneous peak of DNAase activity was obtained with 3-fold purification from this step, an overall purification of 31-fold, and a recovery of 19% (Table III). The specific activity of the preparation was 360 *A* units/min per mg protein. Bovine pancreatic DNAase (see Materials and Methods) showed a similar specific activity of 350 *A* units/min per mg protein.

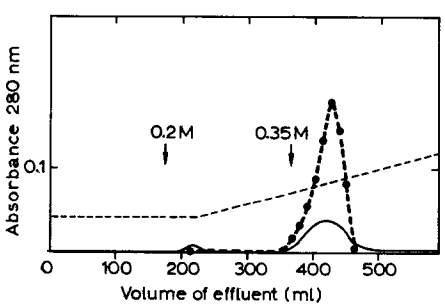

Fig. 6. SE-Sephadex chromatography of the DNAase peak from the Sephadex G-100 column. The solid line is a trace of absorbance at 280 nm and the points represent DNAase activity. The dashed line represents the concentration of sodium acetate buffer (pH 4.7).

Disc gel electrophoresis of the purified enzyme preparations

Ribonuclease was analyzed by disc gel electrophoresis at pH 4.5 [21]. Gels were stained with Coomassie brilliant blue for visualization of protein bands, and

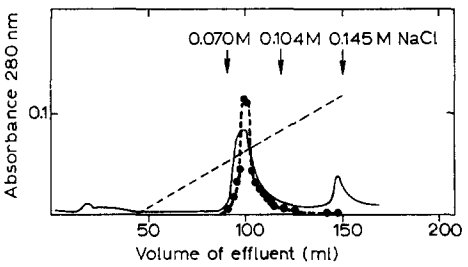

Fig. 7. Rechromatography of the purified DNAase combined Batch I and Batch II preparations on DEAE-Sephadex. The solid line is a trace of absorbance at 280 nm. The lightly-dashed line represents the concentration of NaCl in the starting buffer of 0.09 M Tris–HCl buffer (pH 8.0, I = 0.05), increasing from 0 to 0.145 M. Points represent DNAase activity.

RNAase activity was detected as described in Materials and Methods. Peaks II and III migrated as single protein bands, both with $R_F = 0.38$ (Fig. 8, Gels 3 and 5). Identical gels which had been incubated and stained for RNA showed clear bands with the same mobility (Fig. 8, gels 4 and 6). The Peak I preparation showed four bands when stained for total protein, at $R_F = 0.20$, 0.26, 0.30 and 0.35 (Gel 1). In a replicate

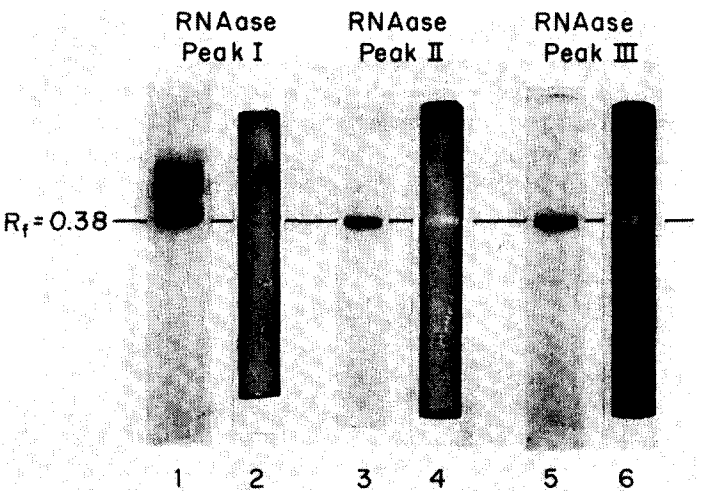

Fig. 8. Cationic disc gel electrophoresis of the purified ribonuclease preparations. Gels 1, 3 and 5 were stained for total protein, and Gels 2, 4 and 6 were analyzed using the RNAase activity detection technique. The direction of migration toward the cathode is to the bottom of the figure.

gel which was assayed for RNAase activity, a band was present at $R_F = 0.36$, and a very faint band at $R_F = 0.27$ (Gel 2).

The amylase preparation was analyzed at pH 8.3 with a total protein load of 25 μg/gel. When stained with coomassie blue, three protein bands were visible; a major band at $R_F = 0.41$, and minor bands at $R_F = 0.37$ and 0.44 (Fig. 9, Gel 1).

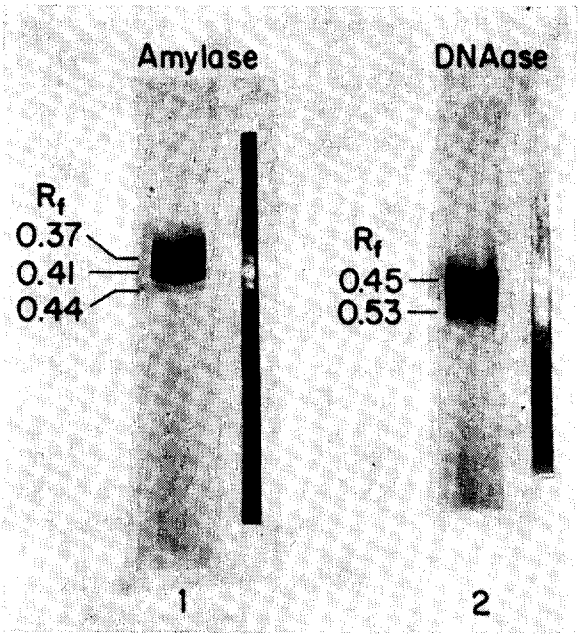

Fig. 9. Anionic disc gel electrophoresis of the purified amylase and deoxyribonuclease preparations. The mobility of the protein bands is indicated on the gels stained with Coomassie blue. The accompanying pictures show the enzyme activities detected on substrate–agar plates. The direction of migration toward the anode is to the bottom of the figure.

The accompanying photograph shows a starch plate on which an identical gel had been briefly incubated, and reveals one major and two minor bands of amylase activity in the same region as the bands of protein.

A similar analysis of the DNAase preparation with a total protein load of 17 μg, revealed a major protein band at $R_F = 0.45$ and a single minor band at $R_F = 0.53$, and showed a major and a minor band of enzyme activity in the same positions (Fig. 9, Gel 2).

DISCUSSION

The enzyme preparations obtained from the parotid gland appear to be fairly pure, based on the absence of contaminants which are detectable on disc gel electrophoresis. Ribonuclease was resolved by Bio-Rex 70 chromatography as three peaks of activity (Fig. 3). When these were separately analyzed by disc gel electrophoresis, single, identical protein bands were obtained for Peaks II and III, on gels which had received a total protein load of 2.5 μg (Fig. 8). Control experiments using bovine pancreas RNAase have shown that bands containing 0.2 μg of protein are easily detectable with this staining procedure (Ball, W. D., unpublished). Therefore, it is likely that no single contaminant is present at 8% or more of the quantity of ribonuclease in both Peaks II and III. The RNAase activity of Peak I is much less (Table I), and the presence of multiple protein bands which lack RNAase activity indicates that this preparation is not pure (Fig. 8, Gel 1). The small total amount of RNAase

obtained in these preparations did not permit more extensive electrophoretic analysis, or the use of higher protein loads.

Parotid gland amylase has been previously purified by several procedures [10–12]; the method used here is essentially that of Sanders and Rutter [12]. The Sephadex G-100 step is useful in that the amylase elutes from the column as if it were a relatively small molecule, and thus is easily separated from most cellular protein.

The SE-Sephadex separation of DNAase was used by Price et al. [20], to resolve two forms of the bovine enzyme, which eluted at what appeared to be 0.23 and 0.27 M sodium acetate buffer (pH 4.7). The parotid gland DNAase was eluted at 0.35 M and shows no indication of multiple peaks (Fig. 6). In addition to the separation of two forms of bovine pancreatic DNAase on SE-Sephadex, chromatography of this enzyme on phosphocellulose has revealed 4 distinct isozymes which vary both in their amino acid composition and in their carbohydrate side chains [29]. The rat parotid DNAase is homogeneous on the basis of SE-Sephadex chromatography, but when analyzed by disc gel electrophoresis showed a minor band with DNAase activity (Fig. 9). The same two enzymatically active protein bands are detected in electrophoretic separations of crude parotid homogenates (Ball, W. D., unpublished). The nature of the difference between these two forms is not known.

The above data demonstrate that no proteins lacking amylase and DNAase activity are evident on electrophoretic analysis of the purified amylase and DNAase preparations. On the assumption (discussed above) that 0.2 μg of protein is detectable as a protein band, no single protein contaminant which migrates to the anode at pH 8.3, could be present in quantity greater than 1.2% of the DNAase, or 0.8% of the amylase preparation.

The relationship between the parotid gland enzymes and those of the pancreas is of obvious interest. The parotid amylase has been shown to be similar but not identical to pancreatic amylase [12]. The pancreas of the rat, unlike the bovine pancreas, contains no secretory DNAase [3, 4].

The parotid ribonuclease appears similar to the bovine pancreatic RNAase in its heat-stability and its preference for poly(C) as a substrate. Whether any of the three RNAase species obtained here are identical to that of the rat pancreas is not known. The rat pancreatic ribonuclease (EC 2.7.7.16) has been purified [30–32] and its amino acid sequence has been reported [33]. Preliminary experiments (Ball, W. D., unpublished) indicate that the rat pancreas and parotid RNAase behave identically on disc gel electrophoresis at pH 4.5, and both have higher activity toward a polycytidylic acid substrate than toward native RNA. The parotid enzymes will be further characterized in the course of studies now in progress, which focus on their production during embryonic and neonatal development.

ACKNOWLEDGMENTS

This work was supported by U.S. Public Health Service Research Grant HD-04281 to the author, and by U.S.P.H.S. Grant DE-02600 to the Center for Research in Oral Biology, University of Washington. The author would like to thank Kathleen J. Smith, H. Stephen Hart and Norma J. Nelson for their capable technical assistance.

REFERENCES

1 Schramm, M. (1964) in Salivary Glands and their Secretions (Sreebny, L. M. and Meyer, J., eds), pp. 315–323, Pergamon Press, Oxford
2 Redman, R. S. (1971) Dev. Biol. 25, 248–279
3 Sreebny, L. M., Ruark, G. W. and Tamarin, A. (1967) Arch. Oral Biol. 12, 777–781
4 Ball, W. D. and Rutter, W. J. (1971) J. Exp. Zool. 176, 1–14
5 Ball, W. D. (1972) J. Exp. Zool. 178, 331–342
6 Robinovitch, M. R., Sreebny, L. M. and Smuckler, E. A. (1968) J. Biol. Chem. 243, 3441–3446
7 Sreebny, L. M., Meyer, J., Bachem, E. and Weinman, J. P. (1955) Growth 19, 57–73
8 Riekkinen, P. J. and Hopsu, V. K. (1965) Ann. Med. Exp. Fenn. 43, 6–14
9 Riekkinen, P. J. and Hopsu-Havu, V. K. (1966) Acta Chem. Scand. 20, 2169–2178
10 Loyter, A. and Schramm, M. (1962) Biochim. Biophys. Acta 65, 200–206
11 Vandermeers, A. and Christophe, J. (1968) Biochim. Biophys. Acta 154, 110–129
12 Sanders, T. G. and Rutter, W. J. (1972) Biochemistry 11, 130–136
13 Laskowski, Sr, M. (1961) in The Enzymes, 2nd edn. (Boyer, P. B., Lardy, H. A. and Myrback, K., eds), Vol. 5, Part B, pp. 123–147, Academic Press, New York
14 Gold, A. M. and Fahrney, D. (1964) Biochemistry 3, 783–791
15 Beard, J. R. and Razzell, W. E. (1964) J. Biol. Chem. 239, 4186–4193
16 Bernfield, P. (1955) in Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds), Vol. 9, pp. 149–158, Academic Press, New York
17 Kunitz, M. (1950) J. Gen. Physiol. 33, 349–362
18 Rutter, W. J. (1967) in Methods in Developmental Biology (Wilt, F. H. and Wessells, N. K., eds), pp. 671–684, Crowell, New York
19 Hirs, C. H. W., Moore, S. and Stein, W. H. (1953) J. Biol. Chem. 200, 493–506
20 Price, P. A., Liu, T., Stein, W. H. and Moore, S. (1969) J. Biol. Chem. 244, 917–923
21 Reisfield, R. A., Lewis, U. J. and Williams, D. E. (1962) Nature 195, 281–283
22 Davis, B. J. (1964) Ann. N.Y. Acad. Sci. 121, 404–427
23 Meyer, T. S. and Lamberts, B. L. (1965) Biochim. Biophys. Acta 107, 144–145
24 Wilson, C. W. (1969) Anal. Biochem. 31, 506–511
25 Frati, L. and Caputo, A. (1970) J. Chromatogr. 48, 547–550
26 Schramm, M. and Danon, D. (1961) Biochim. Biophys. Acta 50, 102–112
27 Loyter, A. and Schramm, M. (1962) Biochim. Biophys. Acta 65, 200–206
28 Hsiu, J., Fischer, E. H. and Stein, E. A. (1964) Biochemistry 3, 61–66
29 Salnikow, J., Moore, S. and Stein, W. H. (1970) J. Biol. Chem. 245, 5685–5690
30 Beintema, J. J. and Gruber, M. (1965) Bull. Soc. Chim. Biol. 47, 2307–2310
31 Cozzone, A. and Marchis-Mouren, G. (1966) Biochim. Biophys. Acta 128, 396–399
32 Kemp, J. D., Walther, B. T. and Rutter, W. J. (1972) J. Biol. Chem. 247, 3941–3952
33 Beintema, J. J. and Gruber, M. (1967) Biochim. Biophys. Acta 147, 612–614