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Purification of Chicken Pancreas Nuclease by Substrate Elution from Phosphorylated Cellulose*

James Eley

ABSTRACT: A nuclease from chicken pancreas adsorbed on phosphorylated cellulose can be specifically eluted by the substrate, ribonucleic acid, and is purified 60–70-fold by this single step. Ribonucleic acid which has been thoroughly hydrolyzed with KOH or with chicken pancreas nuclease will not elute the enzyme. Deoxyribonucleic acid, a poor substrate of the enzyme, and ribonucleic acid which has been thoroughly digested with bovine pancreatic ribonuclease will also elute the enzyme, but the elution is much less sharp than that obtained with ribonucleic acid. The ribonucleic acid can be removed from the phosphorylated cellulose eluent by readsorption of the enzyme onto fresh phosphorylated cellulose and subsequent salt elution. The negatively charged ribonucleic acid is not retained by the cation exchanger.

Whenever a protein interacts specifically and reversibly with another substance the reaction offers a possible means of purifying that protein. Lerman found that certain *p*-azophenol groups covalently bound to cellulose inhibited tyrosinase activity (Lerman, 1953). Such *p*-azophenol celluloses specifically retained tyrosinase while the bulk of the protein in a crude preparation passed through in the wash volume. The tyrosinase could subsequently be eluted by a change in pH of the elution buffer or by sodium benzoate, a strong competitive inhibitor of tyrosinase. A similar procedure has been used more recently for the purification of flavin-requiring enzymes (Arsenis and McCormick, 1964, 1966). These methods, to be useful for purification purposes, require that the substance interacting with the protein (substrate or inhibitor) be irreversibly bound to a stable insoluble medium.

Another approach to the technique is that used by Pogell (1962, 1966) for the purification of fructose-1,6-diphosphatase. Here the phosphatase, adsorbed on CM-cellulose, was specifically eluted from the adsorbent by the substrate fructose 1,6-diphosphate. A considerable purification of the enzyme, much in excess of that obtained by conventional purification techniques, was obtained by this procedure.

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Because of the evident ease and rapidity of the substrate elution technique it was decided to attempt the purification of nucleases by this method. Chicken pancreas RNase was chosen as a model enzyme (Eley and Roth, 1966a,b). This report describes the purification of this nuclease using P-cellulose as adsorbent and the substrate, yeast RNA, as eluting agent. This technique resulted in a considerable purification of the enzyme.

Materials and Methods

Chemicals and Reagents. Yeast RNA (sodium nucleate) obtained from Schwartz Bioresearch Inc. was used throughout this work. For those RNase assays which were done to determine specific activities, RNA which was cleaned as follows, was used.

A 5% solution of the RNA in 0.1 M NaCl was mixed with two volumes of 95% cold ethanol. The precipitate was collected by centrifugation and dissolved in the original volume of 0.1 M NaCl. This procedure was repeated a total of three times. The RNA, after the third precipitation and collection, was lyophilized and stored as a dried powder. RNA treated in this manner gave blank values in the nuclease assay which were about two-thirds the values obtained with nontreated RNA (0.200 *vs.* 0.300).

Salmon testes DNA and crystalline bovine pancreatic RNase were obtained from Worthington Biochemical Corp. The 5'-ribonucleotides and yeast RNA, prepared by the method of Crestfield, Smith, and Allen, were obtained from Sigma Chemical Co. The P-cellulose (P 11) was a Whatman product. Bacteriophage R 17 were purified by the method of Paranchych and Graham (1962) and the RNA was extracted by the phenol procedure (Gierer and Schramm, 1956).

Nuclease Assay. Assays were carried out in 3-ml clear plastic centrifuge tubes. To each tube was added 0.3 ml of a buffer (pH 7.5) containing 0.3 M Tris-HCl and 0.003 M $MgCl_2$. Enzyme and enough water were then added to bring the volume to 0.5 ml. At zero time 0.2 ml (2 mg) of RNA was added and the samples were incubated at 37°. After 30 min 0.7 ml of ice-cold 10% $HClO_4$ was added and the samples were put in ice-water. After 10–20 min they were centrifuged for 10 min at 2800 rpm in the type 269 rotor of an International Model PR-2 centrifuge. The supernatants were poured off and an aliquot (0.05 ml) was diluted 60-fold (to 3.0 ml) with water. The absorbance at 260 mμ was read using a Beckman Model DU spectrophotometer. A unit of enzyme is defined as that amount of enzyme which gives an increase in the 260-mμ absorbance of 1.000 under these assay conditions. Figure 1 shows the linearity of the enzyme assay when the cleaned RNA (see preceding) is the substrate.

Preparation of P-cellulose. The P-cellulose was washed with 1 M NaOH then 1 M HCl. This wash sequence was repeated twice. Each wash was for 30 min with constant agitation. After the second 1 M HCl wash, the P-cellulose was suspended in water and the pH was adjusted to 7.5 by the addition of Tris. After mixing overnight the pH was readjusted to 7.5 and the cellulose was then washed three times with 0.01 M Tris-HCl (pH 7.5). It was stored at 4° until used for column chromatography. In some cases NaN_3 (0.02%) was added as a growth retardant. P-cellulose which had been used for chromatography was reprocessed as described above and used again without any evident change in its chromatographic properties.

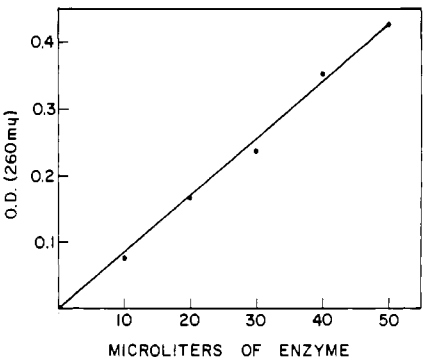

FIGURE 1: Linearity of RNase assay using cleaned Schwartz yeast RNA as substrate. See Materials and Methods for procedure. The plotted values have been corrected for the blank value obtained when no enzyme was in the assay (blank value 0.200).

Preparation of Crude Enzyme. Chicken pancreases, obtained from a local slaughterhouse, were trimmed of fat and extraneous tissue and stored at −40° until processed as follows.

Pancreas tissue was mixed with 0.1 M Tris-HCl (pH 7.5) and homogenized with a Ten-Broeck homogenizer in a cold room (1 g/10 ml). The homogenate was centrifuged for 60 min at 28,000 rpm in the type 30 rotor of a Beckman Model L ultracentrifuge. The supernatant (enzyme fraction I, Table I) was brought to 50% saturation with $(NH_4)_2SO_4$ by adding solid salt to the solution at 0°. The resulting precipitate was removed by centrifugation for 15 min at 10,000 rpm in the type SS-34 rotor of a Sorvall RC2 centrifuge. The supernatant was brought to 90% saturation with $(NH_4)_2SO_4$ and the precipitate was collected in the SS-34 rotor as above. The pellet was dissolved in 0.01 M Tris-HCl (pH 7.5) and diluted with buffer to a volume one-fourth that of the original homogenate volume. This protein solution was then dialyzed 12–24 hr against 0.01 M Tris-HCl (pH 7.5) using 2 l. of buffer/25 ml of protein. This dialyzed solution (enzyme fraction II) was the crude enzyme which was used for all P-cellulose chromatography. It was stored at −40° until applied to the columns. This method of preparation is based on that of Eley and Roth (1966a).

P-cellulose Column Chromatography. The cleaned P-cellulose was allowed to settle by gravity in the column. After excess buffer had run through, the crude enzyme solution (enzyme fraction II) was pipetted onto the cellulose and rinsed in with two to three volumes of 0.01 M Tris-HCl (pH 7.5). Stepwise elution was carried out as indicated in each figure. Fractions of 4.5 ml were collected except for Figure 2 where the fractions were 9 ml. The absorbance of the fractions at 280 mμ was determined with the DU spectrophotom-

TABLE I: Summary of Nuclease Purification.[a]

Fraction	Protein (mg/ml)	Enzyme (units/ml)	Sp Act. (units[b]/ mg of protein)	Purifcn
I (high-speed supernatant)	6.0	15.3	2.6	1
II (ammonium sulfate)	3.4	35	10	4
III (column eluent)	0.01	7.2	720	280

[a] For details, see Materials and Methods. [b] One unit of enzyme activity is that amount of enzyme which results in an increase of the 260-mμ absorbance of 1.000 under the assay conditions described in Materials and Methods.

eter. Aliquots of 50 μl were used for nuclease assays.

Removal of RNA from the Enzyme. Those fractions in the RNA front containing the maximum enzyme activity were combined (enzyme fraction III) and applied to fresh P-cellulose. The enzyme was rinsed in with a small volume of 0.01 M Tris-HCl (pH 7.5) and the column was eluted with 0.1 M NaCl–0.025 M Tris-HCl (pH 7.5) followed by 0.4 M NaCl–0.05 M Tris-HCl (pH 7.5). Those fractions containing the enzyme were combined and stored at −40°.

Other Procedures. Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. All protein solutions containing Tris were dialyzed before assaying for protein since Tris interfered with the assay. Neither hydrolyzed nor unhydrolyzed RNA had any effect on the protein assay.

Results

Elution with Substrates. Preliminary experiments indicated that the chicken pancreas nuclease was retained by the cation exchanger, P-cellulose. A NaCl concentration of 0.2 M or greater, buffered with 0.025 M Tris-HCl (pH 7.5), was necessary to elute the enzyme.

Figure 2 shows the elution pattern of the P-cellulose when RNA was the eluting agent. A small amount of enzyme, usually 8–12% of that applied, was eluted with the protein front. If a larger amount of enzyme elutes with this nonadsorbed protein, then the column is too small for the sample applied. When the protein front containing the enzyme activity was reapplied to a second column of the same size, 8–12% of the reapplied enzyme still eluted with the protein front. The protein in this front appears to interfere with adsorption of the enzyme since a column of this size (2.5 × 20 cm) will retain over 1000 units of the purified enzyme.

The first eluting agent, 0.05 M NaCl (A), brought off no enzyme but did elute some protein (shoulder of protein front). Neither protein nor enzyme was detectable when eluting with Tris-HCl–$MgCl_2$ (pH 7.5) (step B). This elution step was used to remove the NaCl from the column thereby minimizing any nonspecific salt elution which might occur during step C. Elution with RNA, step C, gave a sharp peak of activity which came off with the RNA front. The absorbance of the RNA at 280 mμ masked the absorbance of any protein present. Columns such as this routinely resulted in a 60–70-fold purification of the enzyme

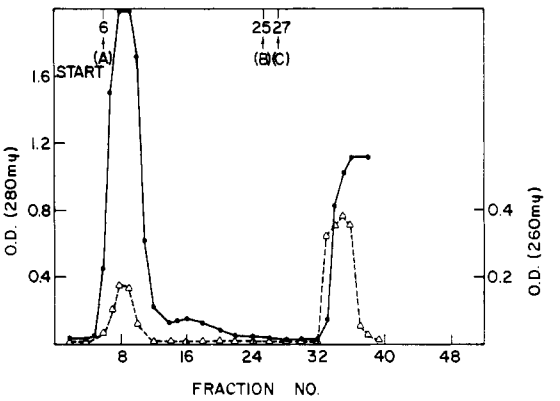

FIGURE 2: Elution of enzyme by RNA. Protein (19 mg) (fraction II, 522 enzyme units) was applied (column size 2.5 × 20 cm). Stepwise elution was carried out with 0.025 M Tris-HCl (pH 7.5)–0.001 M $MgCl_2$ containing (A) 0.05 M NaCl, (B) no addition, and (C) RNA (100 μg/ml). Fractions of 9 ml were collected. $OD_{280\,m\mu}$ of fractions (●—●); $OD_{260\,m\mu}$ of nuclease assays (△----△).

activity (also see Table I). Recoveries ranged from 50 to 80%. Results identical with those of Figure 2 were obtained when the high molecular weight ribonucleates, bacteriophage R 17 RNA or Crestfield, Smith, and Allen yeast RNA, were used as eluting agents in place of Schwartz yeast RNA.

An elution pattern identical with that of Figure 2 was also found when 0.002 M EDTA was used in all the elution steps in place of 0.001 M $MgCl_2$. Since EDTA completely inhibits (Eley and Roth, 1966a) this nuclease (reversibly), the RNA need not be hydrolyzed in order for it to bring about the elution of the enzyme.

Figure 3 shows the elution pattern when DNA was used in step C instead of RNA. There was a slow constant elution of the enzyme. If the DNA was replaced with RNA, step D, a sharp peak of activity came out with the RNA front. These results agree with previous work indicating DNA to be a poor substrate (Eley and Roth, 1966b).

A bovine pancreatic RNase hydrolysate of RNA also eluted the enzyme, but as was the case with DNA the enzyme came off slowly.

Nonsubstrates Do Not Elute Enzyme. Neither a mixture of the 5′-ribonucleotides, 25 μg/ml of each of AMP, UMP, CMP, and GMP, nor a mixture of the 3′- and 2′-ribonucleotides, a KOH hydrolysate of RNA (100 μg/ml), eluted the enzyme from the P-cellulose.

RNA which had been thoroughly digested with the chicken pancreas nuclease would also not elute the enzyme (Figure 4). The enzyme remained active on the column and could subsequently be eluted with nondigested RNA (step D).

Removal of RNA. RNA was removed from the RNA enzyme eluent by adsorption of the enzyme onto fresh P-cellulose followed by salt elution (Figure 5). No enzyme was detectable in the region where RNA was eluted and little or no RNA came off with the enzyme. Of the enzyme applied to this column, 100% of the activity was recovered in the peak fractions. The 280 mμ/260 mμ absorbance ratio of the eluted enzyme was 1.5. Other columns have given enzyme solutions with 280/260 ratios ranging from 1.1 to 1.7 with recoveries of enzyme activity varying from 70 to 100%. This step can also be used to concentrate the purified enzyme provided a large quantity is applied to a relatively small column. A twofold concentration of the activity was obtained in the column described in Figure 5. As this column indicates the P-cellulose has a much greater capacity for the purified enzyme than it has for the enzyme in the crude preparation (compare with Figure 2).

Sample Purification. Table I summarizes the results of a typical purification. The P-cellulose step resulted in a 70-fold purification of the enzyme activity to give an over-all purification of 280-fold. Of the activity put on the column 54% was recovered in the enzyme peak which eluted with the RNA front.

Four separate complete purifications using the same batch of pancreases were performed and in each case results almost identical with those given in Table I were obtained. Pancreases obtained on different dates have given enzyme which had a two- to threefold greater specific activity than that shown in Table I but in all cases the purification obtained by the P-cellulose step was similar to that given in the table (60–70-fold).

Enzyme Storage. The enzyme, either in the RNA solution or freed of RNA by P-cellulose chromatography, can be stored at −40° for up to 6 months without any significant loss in activity.

Discussion

The procedure described for the purification of the chicken pancreas nuclease results in an enzyme of about twofold greater purity than has been obtained previously using conventional methods of enzyme purification (Eley and Roth, 1966a). The advantages of this method are its rapidity and the relatively high yield of purified enzyme, routinely greater than 50%. The major disadvantage is the low capacity of the P-cellulose for the crude enzyme. This is not a drawback if only enzyme activity is wanted, but it is a definite drawback to the preparation of quantities of protein for physical characterization.

The technique of substrate elution is presently being investigated as a means of purification of other nucleases and of other enzymes involved in nucleic acid metabolism, particularly the RNA polymerases. The method also has potential use as a rapid means of as-

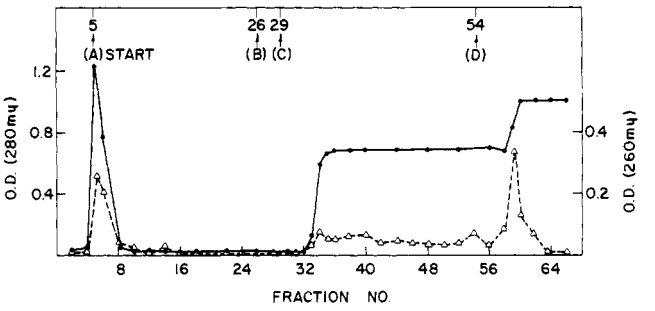

FIGURE 3: Elution of enzyme by DNA followed by RNA. Enzyme fraction II (6.3 mg; 174 units) was applied (column size 2 × 7 cm). Stepwise elution was carried out with 0.025 M Tris-HCl (pH 7.5)–0.001 M $MgCl_2$ containing (A) 0.05 M NaCl, (B) no addition, (C) DNA (100 μg/ml), and (D) RNA (100 (μg/ml). $OD_{280\,m\mu}$ of fractions (●—●); $OD_{260\,m\mu}$ of nuclease assays (△----△).

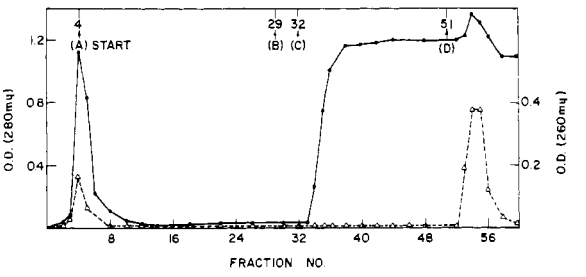

FIGURE 4: Elution by a chicken pancreas nuclease hydrolysate of RNA. Enzyme fraction II (6.3 mg) was applied to the column (2 × 7 cm). Stepwise elution was carried out with 0.025 M Tris-HCl (pH 7.5)–0.001 M $MgCl_2$ containing (A) 0.05 M NaCl, (B) no addition, (C) nuclease RNA hydrolysate (100 μg/ml), and (D) RNA (100 μg/ml). RNA (10 mg) was hydrolyzed at 37° for 3 hr with purified nuclease (8 units of a phosphorylated cellulose eluent) in 2.2 ml containing 200 μmoles of Tris-HCl (pH 7.5) and 2 μmoles of $MgCl_2$. $OD_{280\,m\mu}$ of fractions (●—●); $OD_{260\,m\mu}$ of nuclease assays (△----△).

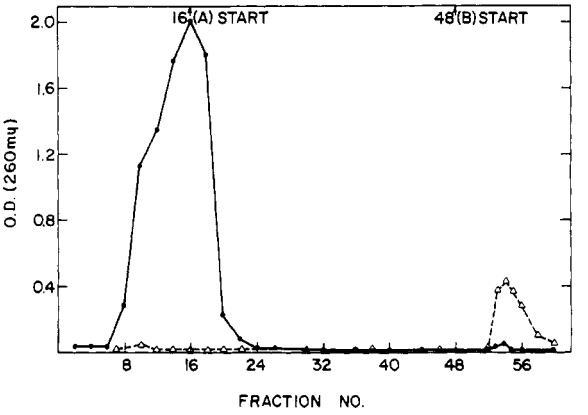

FIGURE 5: Removal of RNA. Fractions of the RNA front containing enzyme from columns such as that shown in Figure 2 were combined and put on a fresh phosphorylated cellulose column (943 units, column size 2.0 × 15 cm). Stepwise elution was carried out with (A) 150 ml of 0.1 M NaCl–0.025 M Tris-HCl (pH 7.5) and (B) 50 ml of 0.4 M NaCl–0.05 M Tris-HCl (pH 7.5). $OD_{260\,m\mu}$ of fractions (●—●); $OD_{260\,m\mu}$ of nuclease assays (△----△).

sessing relative substrate specificities of different nucleases. For example, the bovine RNase hydrolysate of RNA eluted the chicken nuclease because the specificities of the two enzymes differ.

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Characterization of the Cyanogen Bromide Peptides from the α1 Chain of Chick Skin Collagen*

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ABSTRACT: The α1 chain of chick skin collagen was cleaved at the methionyl residues with cyanogen bromide and the resulting peptides were separated and characterized. Ten peptides of varying molecular weight ranging from 242 to 25,000 were obtained. The sum of the molecular weights of these peptides as determined by gel filtration was 91,567, and that determined by amino acid analysis was 90,721, values which are in good agreement with the known molecular weight of the α1 chain.

The sum of the amino acid compositions also agrees with the composition of the whole α1 chain. These data indicate that the ten peptides account for the entire amino acid sequence represented in the original α1 chain. The finding of only ten unique peptides from a chain containing nine methionyl residues indicates that the two α1 chains of chick skin collagen are identical or very similar in their amino acid sequence. These peptides are similar and clearly homologous to the cyanogen bromide peptides previously isolated from the α1 chain of rat skin collagen. They are identical in every respect examined with the cyanogen bromide peptides from the α1 chain of chick bone collagen except for the degree of hydroxylation of lysine. This result suggests that chick bone and skin collagens are derived from the same structural genes and, therefore, that the different properties of the two tissues cannot be explained on this basis.

The amino acid sequence of the collagen molecule, a rigid three-chain rod-shaped structure (3000 Å long and 13 Å wide), has been difficult to establish because of its great size. It has been possible to separate and isolate the three chains by gentle heat denaturation and chromatography on CM-cellulose which, in the case of most vertebrate collagens, provides two α1 chains identical in amino acid composition and one α2 chain with a different amino acid composition and chromatographic behavior. Both α1 and α2 have a molecular weight of about 95,000. CNBr cleavage of the seven methionyl bonds of the α1 chain of rat skin collagen has produced eight peptides of different sizes, ranging from 1,400 to 25,000 in molecular weight. These have been separated, purified, and characterized as to their molecular weight and amino acid composition (Butler *et al.*, 1967) and the amino acid sequence has been determined for two of the smaller ones (Kang *et al.*, 1967; Bornstein, 1967). In addition, by a combination of chemical analyses and localization of one of the larger peptides by electron microscopy, Piez *et al.* (1969) have succeeded in tentatively ordering all cyanogen bromide peptides along the length of the α1 chain of rat skin collagen.

In order to obtain a more general picture of the primary structure of collagen, it was deemed essential to study a collagen of another class, which can be purified as well as that from mammals, can be readily solubilized, and is stable under usual laboratory conditions. We report here the isolation and characterization of the CNBr peptides from the α1 chain of chick skin collagen. The recent study on chick bone collagen (Miller *et al.*, 1969) provides an opportunity

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† Senior Investigator of the Arthritis Foundation.