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EVIDENCE THAT PSEUDORENIN ACTIVITY IN BOVINE SPLEEN IS DUE TO CATHEPSIN D

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Abstract—Pseudorenin and cathepsin D activity from bovine spleen were found to behave identically on DEAE-cellulose, Sephadex G-100, and concanavalin A-agarose chromatography. The molecular weight of pseudorenin and of cathepsin D was estimated to be 50,000. The binding of the enzymatic activity to concanavalin A-agarose and elution with α-methyl-D-mannoside indicates that pseudorenin (cathepsin D) is a glycoprotein. It is suggested that pseudorenin activity in the spleen is due to cathepsin D.

Pseudorenin is an enzyme which, like renin, is capable of producing angiotensin I from tetradecapeptide renin substrate and purified hog renin substrate. Pseudorenin also resembles renin in that it is inhibited by the pentapeptide, pepstatin [1]. Pseudorenin differs from renin in that it is chromatographically distinct from renin, has its optimum enzymatic activity at a lower pH value, and is unable to produce angiotensin I from substrate occurring naturally in plasma [2].

Pseudorenin activity has been shown by Skeggs *et al.* [2] to be widely distributed in rat tissues, with the kidney containing relatively low amounts of activity in comparison to the spleen and thymus. In addition, pseudorenin activity has been found in our laboratory in bovine spleen, kidney, thymus, adrenal medulla, and adrenal cortex [3, 4].

During the course of our studies of this enzyme, we found that partially purified extracts of bovine spleen which contained pseudorenin activity also possessed cathepsin D activity. Since cathepsin D from bovine anterior pituitary had previously been shown capable of hydrolyzing an artificial octapeptide substrate of renin [5], it appeared possible that bovine spleen pseudorenin and cathepsin D were the same enzyme. To test this possibility, the simultaneous purification of pseudorenin and cathepsin D was undertaken.

MATERIALS AND METHODS

Materials. Tetradecapeptide renin substrate (TDP) and (^{125}I)angiotensin I were obtained from Schwarz/Mann, Orangeburg, New York, and New England Nuclear, Boston, MA, respectively. Angiotensin I for the radioimmunoassay was obtained from the National Institute for Biological Standards, Holly Hill, London. Hemoglobin (Type II), blue dextran, cytochrome *c*, ribonuclease, chymotrypsinogen A, ovalbumin, bovine albumin (Fraction IV), and α-methyl-D-mannoside were all obtained from Sigma Chemical Company, St Louis, MO. Glycosylex A was obtained from Miles Laboratories, Elkhart, IN.

Purification of bovine spleen pseudorenin and cathepsin D activity. Ten grams of frozen bovine spleen were thawed at 4° overnight and then homogenized with 50 ml of 5 mM EDTA, pH 7.0. The homogenate was centrifuged for 15 min at 10,000 rpm, the supernatant decanted, and the residue rehomogenized with another 50 ml of 5 mM EDTA, pH 7.0. This second homogenate was centrifuged for 15 min at 10,000 rpm. The residue was discarded and the supernatant combined with the supernatant obtained from the first homogenization. Ammonium sulfate was added to the combined supernatants until the resulting mixture was 1.3 M. This mixture was centrifuged for 15 min at 10,000 rpm. Ammonium sulfate was added to the resulting supernatant until a concentration of 2.5 M was obtained. The precipitate obtained from centrifugation for 15 min at 10,000 rpm was reconstituted in 12 ml of H_2O and dialyzed for 20 hr against 5 mM sodium phosphate buffer, pH 7.5. The insoluble residue was removed by centrifugation at 12,000 rpm for 30 min to provide 14 ml of a solution containing both pseudorenin and cathepsin D activity.

This crude enzyme solution was applied to a DEAE-cellulose column (1.5 × 28 cm) which had been equilibrated with 5 mM sodium phosphate buffer, pH 7.5. The column was washed with 5 mM sodium phosphate buffer, pH 7.5, until 180 ml of eluant had been collected, at which time elution with 0.2 M NaCl in starting buffer was begun. The elution of protein from the column was followed by measuring the absorbance of the collected fractions at 280 nm. Fractions of 5 ml were collected and assayed for pseudorenin and cathepsin D activity, as described below. The fractions containing the enzymatic activity which eluted between 25 and 55 ml were combined and concentrated using a Millipore immersible molecular separator.

The concentrated enzyme solution obtained from the DEAE-cellulose column was next applied to a Sephadex G-100 column (1.5 × 58 cm) equilibrated with 5 mM sodium phosphate buffer, pH 7.5. Fractions of 5 ml were collected and assayed for enzymatic activity. The active fractions eluting between 25 and 45 ml were pooled, concentrated, and dialyzed against

10 mM sodium phosphate buffer, pH 7.0, 0.15 M NaCl.

The above dialyzed material was then applied to a Glycosylex A column (agarose-concanavalin A, 1.0 × 16 cm), equilibrated with 10 mM sodium phosphate buffer, pH 7.0, 0.15 M NaCl. The column was washed with this same buffer until the elution of protein was no longer detectable. After 60 ml of eluant had been collected, elution was begun with 0.3 M α-methyl-D-mannoside in starting buffer. Five ml fractions were collected and assayed as before for pseudorenin and cathepsin D activity.

Molecular weight determination. Proteins of known molecular weight were chromatographed on the same Sephadex G-100 column used in the gel filtration step described above. Proteins used included: cytochrome *c* (12,900), ribonuclease (13,899), chymotrypsinogen A (25,000), ovalbumin (45,000), and bovine albumin (69,000). Blue dextran was used to calculate the void volume (V_0) of the column. The elution volume (V_e) of each protein was determined and this value, along with the value of the void volume (V_0) and total bed volume (V_t), were substituted into the following formula to generate each protein's K_{av} value.

$$K_{av} = V_e - V_0/V_t - V_0$$

A semilogarithmic plot of K_{av} versus the molecular weight gave a standard curve from which the molecular weight of pseudorenin (cathepsin D) was calculated.

Assay of pseudorenin activity. The fractions obtained from the above chromatographic columns were assayed for pseudorenin activity in the following manner. Fifty μl of a 60-fold dilution of each fraction were incubated at 37° in a final volume of 100 μl containing 50 mM sodium citrate buffer, pH 5.0 and 1000 pmoles/ml of TDP. After 30 min the enzymatic reaction was stopped by placing the incubation mixtures on ice and diluting them with 0.8 ml of 0.1 M Tris–acetate buffer, pH 7.4. The angiotensin I produced was assayed by radioimmunoassay as described below. Each fraction was assayed in duplicate. The enzymatic activity was expressed as μg antiotensin I generated/hr/ml. Pseudorenin activity of the pooled, concentrated fractions was measured by these same procedures except that 500–1000-fold dilutions of the enzyme were used. Specific activities of the concentrated fractions were expressed as μg angiotensin I generated/hr/mg of protein and were derived from protein concentration measurements obtained by the method of Lowry *et al.* [6].

Radioimmunoassay of angiotensin I. A modification of the method described by Haber *et al.* [7] was used in this study. Samples (50 μl) of the incubation mixtures to be assayed were mixed with 50 μl of [^{125}I]angiotensin I (4500 cpm) in Tris–acetate buffer, pH 7.4, containing bovine serum albumin (5.0 mg/ml). Rabbit antiserum, 100 μl of a 1:10,000 dilution in 0.1 M Tris–acetate buffer, pH 7.4, was added and the mixture allowed to equilibrate at 4° for 24 hr. The antiserum was prepared as described by Haber *et al.* [7]. A charcoal suspension (0.8 ml) containing 3.9 g/l charcoal and 0.39 g/l dextran in 0.1 M Tris–acetate buffer, pH 7.4, was then added. The mixtures were thoroughly mixed and then centrifuged at 7000 rpm for 10 min. The supernatant was decanted, mixed with 4.5 ml of scintillation fluid (Aquasol) and counted in a Packard Tri-Carb liquid scintillation counter. Known amounts of angiotensin I varying from 25 to 1600 pg were treated in a similar manner to produce a standard curve.

Assay of cathepsin D activity. A modification of the method of Anson [8] was used. Aliquots (0.1 ml) of each fraction were mixed with 0.1 ml H_2O, 0.1 ml of a freshly prepared solution of 2% hemoglobin in H_2O, and 0.2 ml of 0.2 M sodium citrate buffer, pH 3.5. The samples were incubated at 37° for 30 min, after which time they were placed on ice and treated

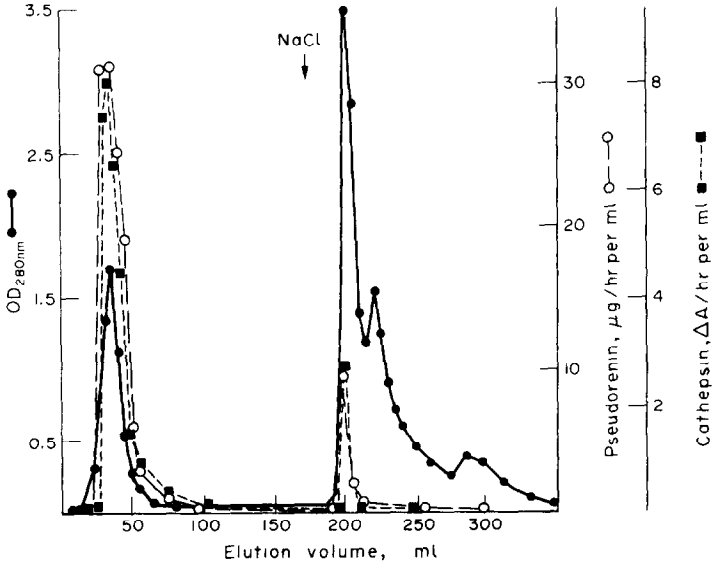

Fig. 1. DEAE-cellulose column chromatography of 2.5 M ammonium sulfate fraction from bovine spleen extract. Elution was with 5 mM sodium phosphate buffer, pH 7.5. At an elution volume of 180 ml, NaCl (0.2 M) was added to the eluting buffer. Optical density at 280 nm ●——●, pseudorenin activity O----O, and cathepsin D activity ■----■.

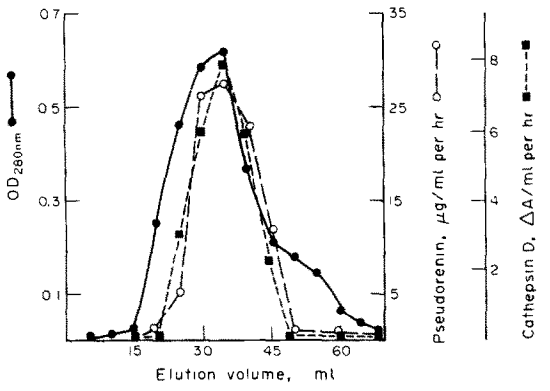

Fig. 2. Sephadex G-100 column chromatography of DEAE-cellulose purified pseudorenin (cathepsin D) activity. Elution was carried out in 5 mM sodium phosphate buffer, pH 7.5. Optical density at 280 nm ●——●, pseudorenin activity O----O, and cathepsin D activity ■----■.

with 0.1 ml of 25% trichloroacetic acid. The samples were centrifuged for 10 min at 7000 rpm and the optical density of the supernatants determined at 280 nm. Controls were treated the same as the samples except that the enzyme was added after the incubation period. Cathepsin D activity was expressed as the difference in absorbance at 280 nM (ΔA) between sample and control/hr/ml.

Cathepsin D activity of the concentrated fractions was measured in a manner similar to that described above except that 0.01 ml of enzyme and 0.19 ml of H_2O were used instead. Specific activity of these fractions was expressed as ΔA/hr/mg protein.

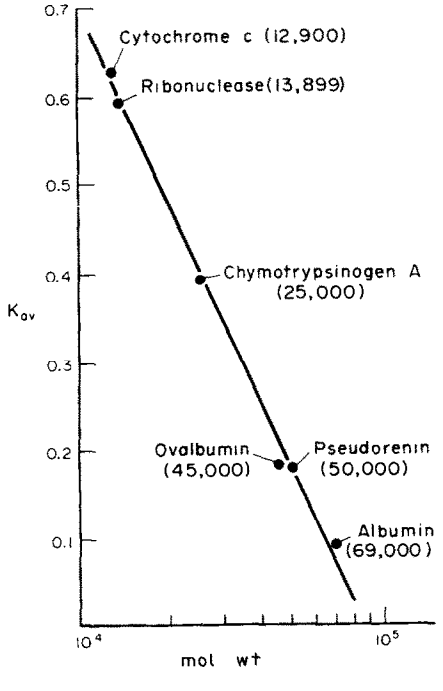

Fig. 3. Molecular weight estimation of pseudorenin (cathepsin D) by chromatography on a Sephadex G-100 column equilibrated with 5 mM sodium phosphate buffer, pH 7.5.

RESULTS

The chromatographic pattern of the 2.5 M ammonium sulfate fraction on DEAE-cellulose is shown in Fig. 1. Approximately 80 per cent of the pseudorenin and cathepsin D activity appeared in the non-retained fractions. The remainder of the enzymatic activity was eluted from the column with 0.2 M NaCl. This type of behavior on DEAE-cellulose has been observed previously during the purification of cathepsin D from porcine spleen [9]. The specific activity of the non-retained pseudorenin and cathepsin D activity was increased 10-fold in both cases. This material was subsequently placed on a Sephadex G-100 column. As depicted in Fig. 2, both pseudorenin and cathepsin D were eluted together with an elution volume of 35 ml. When compared to the elution position of proteins of known molecular weight, this elution volume corresponds to a molecular weight of 50,000 (Fig. 3). Previous studies have found the molecular weight of cathepsin D to range from 42,000 [10] to 58,000 [11, 12]. Thus our results would appear to be in agreement with these values. After gel chromatography on Sephadex G-100 the enzymatically active fractions were concentrated and placed on a concanavalin A-agarose affinity column. As shown in Fig. 4, both pseudorenin and cathepsin D activity were retained on the column and subsequently eluted when the column was washed with 0.3 M α-methyl-D-mannoside. The binding of glycoproteins with α-D-mannopyranosyl or α-D-glucopyranosyl end residues to concanavalin A-agarose columns is well known [13]. Thus it would appear that pseudorenin and cathepsin D are glycoprotein in nature. The binding of pseudorenin to concanavalin A does not appear to involve the active site since concanavalin A does not inhibit the ability of pseudorenin to produce angiotensin I from TDP (unpublished results).

The results from the copurification of pseudorenin and cathepsin D activity are summarized in Table 1. The purification procedures resulted in a 42-fold increase in the specific activity of pseudorenin while an 84-fold increase in specific activity of cathepsin D was obtained. The overall recovery of pseudorenin and cathepsin D activity was 5 per cent and 10 per cent respectively.

The difference in recovery and specific activity observed in the concanavalin A-agarose chromatographic step is probably due to the difference in time at which the assays were carried out since subsequent purifications where the two assays were performed simultaneously did not show a difference. It is clear that the two enzymatic activities elute in parallel on concanavalin A-agarose as shown in Fig. 4.

DISCUSSION

Since the initial description of pseudorenin [2], very little additional work has been carried out to determine what physiological significance, if any, this enzyme has. Studies in our laboratory have shown pseudorenin to be similar to such acid proteases as pepsin, cathepsin D, and renin since the enzyme is inhibited by the pentapeptide, pepstatin [1]. In addition, we have found that purified bovine spleen pseudorenin can act on homologous serum substrate at

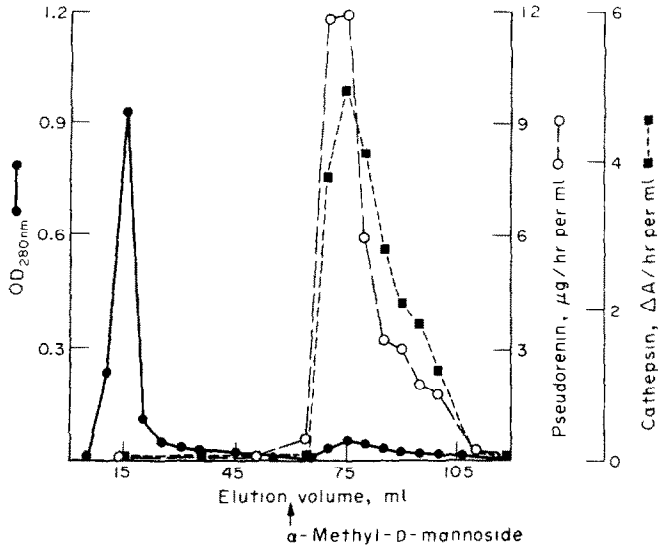

Fig. 4. Glycosylex A column chromatography of Sephadex fraction of pseudorenin-cathepsin D activity. The initial elution was carried out with 10 mM sodium phosphate in 0.15 M NaCl, pH 8.0. At an elution volume of 60 ml the eluting solvent was changed to 0.3 M α-methyl-D-mannoside. Optical density at 280 nm ●——●, pseudorenin activity O----O, cathepsin D activity ■----■.

Table 1. Purification of bovine spleen pseudorenin and cathepsin D activity

Fraction	Total protein (mg)	Pseudorenin activity: Specific activity (μg/mg/hr)	Pseudorenin activity: Recovery (%)	Pseudorenin activity: Purification	Cathepsin A activity: Specific activity (ΔA/mg/hr)	Cathepsin A activity: Recovery (%)	Cathepsin A activity: Purification
Extract	509	7.4	100	1	2.45	100	1
Ammonium sulfate precipitation	187	9.9	49	1.3	3.02	45	1.2
DEAE-cellulose	11	72	21	9.7	25.0	22	10.0
Sephadex G-100	4.7	80	10	10.8	35.5	13	14.1
Glycosylex A	0.6	310	5	42	205	9.9	83.5

pH 5.5 to produce angiotensin I [4]. Rat plasma pseudorenin, however, is not regulated in the same way as plasma renin [14].

Recent studies on the renin activity present in dog brain have shown it to be due to cathepsin D [15]. The present study shows that pseudorenin and cathepsin D behave identically on DEAE-cellulose, Sephadex G-100, and concanavalin A-agarose chromatography. Since the separations which we employed utilize differences in chemical and/or physical properties (anionic charge, molecular weight, and affinity for a lectin, respectively), it would appear that the pseudorenin activity found in bovine spleen is also due to cathepsin D. Thus the present study is another indication that studies on extrarenal "renin" must be interpreted with caution in order to eliminate the possible influence of cathepsin D.

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