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BOVINE PEPSINOGEN AND PEPSIN

IV. A NEW METHOD OF PURIFICATION OF THE PEPSIN

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SUMMARY

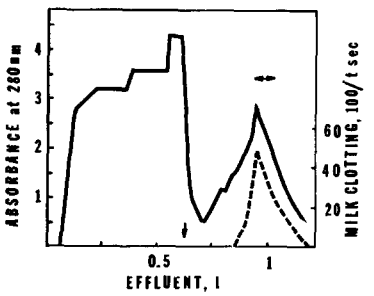
A new purification procedure for bovine pepsin (EC 3.4.4.1), based on affinity chromatography, is more rapid, and gives a higher yield and activity than previous procedures. It may be used for purification of other pepsins and of pepsinogen. Pepsinogen is extracted from gastric mucosa, fractionated with ammonium sulfate, and activated to pepsin. The pepsin is purified by chromatography on poly-L-lysine Sepharose 4B, CM-cellulose and Sephadex G-100. The final product is almost homogeneous.

Conventional methods for purifying pepsinogens and pepsins are laborious and give low yields in spite of the high content of zymogens in the stomach mucosa. Affinity chromatography as developed by AXÉN *et al.*[1] and PORATH *et al.*[2] has been successful in purifying many enzymes including chymotrypsin[3] and wheat proteases[4]. The present report describes the successful purification of bovine pepsin on Sepharose 4B (ref. 2) coupled to poly-L-lysine. The use of polylysine is based on the observation of KATCHALSKI *et al.*[5] that it is a pepsin inhibitor.

The procedure for the activation of Sepharose 4B by CNBr was similar to that described by CUATRECASAS *et al.*[3]. Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N.J.) was washed with water on a coarse fritted glass funnel under suction. 50 g of the washed gel were suspended in 37.5 ml water and 62.5 ml of a CNBr (Eastman) solution containing 80 mg/ml were added to the stirred mixture at 25°. The pH was immediately adjusted to 11 by the addition of 2.5 M NaOH; the mixture was maintained at pH 11 ± 0.3 for 8–10 min. The activated gel was immediately transferred to a glass funnel and washed under suction with 20 vol. each of cold water and cold 0.1 M $NaHCO_3$. The washed gel was transferred to a screw-top bottle and suspended in 50 ml of cold 0.1 M $NaHCO_3$. 50 ml of a poly-L-lysine solution (5 mg/ml) in 0.1 M $NaHCO_3$ were added and the mixture gently shaken by inversion at 4° for 24 h. (The poly-L-lysine, mol. wt. 50 000–100 000, was purchased from Pierce Chemical Co., Rockford, Ill.) The gel was poured into a column (1.6 cm × 55 cm) and washed with 750 ml of 0.1 M $NaHCO_3$ and 750 ml of 0.5 M NaCl adjusted to pH 9.2. After washing, the column was equilibrated at 4° with 0.15 M NaCl in 0.01 M sodium acetate buffer (pH 5.2).

Pepsin was detected in chromatographic peaks by milk clotting activity. Specific activity (expressed as μg equivalents of Worthington crystalline porcine pepsin per $A_{280\,nm}$ unit of bovine pepsin) was determined with hemoglobin ass ubstrate. Both methods were described by CHOW AND KASSELL[6].

The mucosa of the fourth stomach, which was removed from freshly slaughtered cows, washed and frozen, was supplied by Pelfreez Biologicals, Rogers, Ark. The pepsinogen was extracted and fractionated with ammonium sulfate[6]. The zymogen was activated at pH 2 by the same method used for purified pepsinogen[7]. A large amount of insoluble material, formed on activation, was removed by centrifugation without loss of pepsin. The supernatant solution containing the enzyme was dialyzed first against 0.01 M sodium acetate (pH 5.2) and then against 0.15 M NaCl in the same buffer; any precipitate formed was centrifuged off. At this stage the specific activity was 61 μg of pepsin per $A_{280\,nm}$ unit. The solution, 560 ml containing 175 mg of pepsin, was placed on the poly-L-lysine Sepharose 4B column, which was eluted



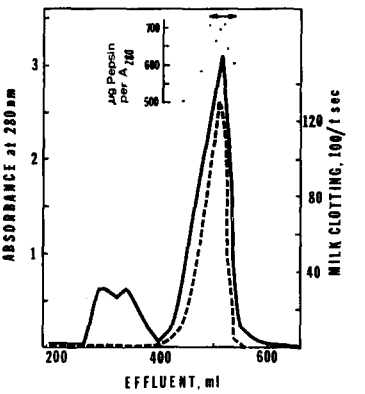


Fig. 1. Elution profile of bovine pepsin from poly-L-lysine Sepharose 4B. Column 1.6 cm × 55 cm; sample 175 mg in 560 ml; fractions 4.5 ml; flow rate 22 ml/h. Starting buffer, 300 ml of 0.15 M NaCl in 0.01 M sodium acetate buffer (pH 5.2). Gradient buffer, 300 ml of 1 M NaCl in the same buffer. The buffers were in paired interconnected open bottles to form a linear gradient, started at the vertical arrow. The fractions pooled are indicated by the horizontal arrow. ———, $A_{280\,nm}$; — — —, milk clotting activity equivalent to 0.1 ml of undiluted solution.

Fig. 2. Gel filtration of partially purified pepsin on Sephadex G-100. Column 3.2 cm × 100 cm; sample 136 mg in 25 ml; fractions 3.5 ml; flow rate, 20 ml/h; buffer, 0.01 M sodium acetate (pH 5.2). The fractions pooled are indicated by the horizontal arrow. ———, $A_{280\,nm}$; — — —, milk clotting activity equivalent to 0.1 ml of undiluted solution; ●, specific activity.

with a linear gradient of 0.15 M to 1 M NaCl in the acetate buffer (Fig. 1). The portion of the peak having the highest specific activity was pooled (average 421 μg pepsin per $A_{280\,nm}$ unit). The recovery was 73% on the basis of proteolytic activity.

The pooled solution was dialyzed against 0.01 M sodium acetate (pH 5.2). To remove contaminating basic peptides, the solution was adjusted to pH 3.5 with 0.5 M acetic acid and was passed through a CM-cellulose column (3 cm × 22 cm) equilibrated with 0.01 M sodium citrate (pH 3.5). The fraction collector tubes contained 0.4 ml of 0.4 M sodium acetate buffer (pH 6.0); the solution was at the low pH for less than 1 h. The enzyme was concentrated and passed through a Sephadex G-100 column (3.2 cm × 100 cm) equilibrated with 0.01 M sodium acetate (pH 5.2) (Fig. 2).

The portion of the peak with specific activity above 600 μg of pepsin per $A_{280\ nm}$ unit was pooled; 89% of the activity was recovered. The overall purification from the crude active pepsin was 10 fold, with a yield of 63%. A second passage through the same Sephadex column gave no further purification; a single peak appeared with constant specific activity across the peak (645 μg pepsin per $A_{280\ nm}$ unit).

The amino acid composition of the enzyme purified by this procedure was in agreement with that found by LANG AND KASSELL[8]. Since bovine pepsin does not contain lysine, the absence of this amino acid is a test for homogeneity. The lysine content was less than 0.2 residue per mole.

Porcine pepsin behaves similarly on the poly-L-lysine column. This procedure can therefore be used for the purification of other pepsins. Pepsinogen (at pH 6.5) was bound as strongly as pepsin at pH 5.2; thus this method can also be used to purify pepsinogen.

The elution pattern of the crude pepsin solution on the poly-L-lysine resin (Fig. 1) appears to result from a combination of affinity chromatography and ion exchange. A high degree of purification in one step is typical of affinity chromatography, but retention of some inactive material on the column after elution of the pepsin shows that ion exchange is also occurring.

This method of purification has several advantages over our previous procedure[7]. The activity is 10–20% higher than bovine pepsin prepared from pepsinogen by the older method (*cf.* ref. 8), probably because less denaturation occurs in this much shorter time of preparation. The yield is considerably higher. The column has been used many times without loss of efficiency.

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