AFFINITY CHROMATOGRAPHY OF CHICKEN PEPSIN USING PEPSIN INHIBITOR FROM *Ascaris Lumbricoides* AS AFFINANT*

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Chicken pepsin was purified on a column of Sepharose 4B with covalently bound pepsin inhibitor isolated from the body walls of the roundworm *Ascaris lumbricoides*. In this single operation the specific activity of chicken pepsin increases about 8-times. In the reverse process, chicken pepsin was covalently coupled to Sepharose 4B and its inhibitor was chromatographed.

During the past few years affinity chromatography has become the method of choice for the preparation of enzymes and their inhibitors. Three different types of affinity chromatography have been used for proteinases of the carboxyl type. Sepharose 4B has been used as a solid support in all cases whereas poly-L-lysine[1], pepstatin[2], and amino-caproyl-L-phenylalanyl-D-phenylalanine[3] were the affinants. We have made use of the free thiol group in the molecule of chicken pepsin for its isolation on a mercurial Sepharose column in the preceding study[4]. In this paper we report on the use of the interaction between chicken pepsin and the naturally occurring pepsin inhibitor from *Ascaris lumbricoides* for affinity chromatography of either chicken pepsin or its inhibitor.

EXPERIMENTAL

Material. Crude pepsin from chicken forestomachs was isolated by the method of Bohak[5]. Sepharose 4B, SE-Sephadex C-25, and Sephadex G-75 were products of Pharmacia, Sweden.

Enzyme assays. The inhibitory activity was assayed as follows. The inhibitor solution (50 µl) was treated with 50 µl of the enzyme solution. After 5 min of interaction at pH 3·0, the proteolytic activity of the mixture was determined as described earlier[4]. The quantity of inhibitor which inhibits 1 µg of pepsin under the experimental conditions given was taken for 1 unit.

Preparation of Ascaris lumbricoides *pepsin inhibitor.* The inhibitor was isolated from the body walls of the roundworm *Ascaric lumbricoides*, var. *suis*, by the method of Peanasky and Abu-Erreish.[6] The original method was slightly modified: we replaced SE-Cellex by SE-Sephadex and the active fraction was subsequently chromatographed on Sephadex G-75 in 0·1M-Tris-HCl buffer at pH 3·8. We obtained two well separated peaks. Both fractions were concentrated and dialyzed by pressure dialysis and stored at −20°C. A solution (A_{280nm} 1·250) of the fraction emerging

in the lower elution volume, which was essentially homogeneous on disc electrophoresis at pH 8·3, was used in all our experiments.

Ascaris inhibitor Sepharose column: Sepharose 4B, activated by cyanogen bromide[7], was washed exhaustedly with cold 0·1M-$NaHCO_3$, pH 9, and then with cold 0·01M phosphate buffer, pH 6·8. The suspension (10 ml) was treated dropwise with 20 ml of inhibitor solution (at pH 6·8). The mixture was stirred overnight at 4°C, packed into a column, and washed with the phosphate buffer, pH 6·8, till the effluent was free of inhibitory activity (tested with chicken pepsin). Since the pepsin-pepsin inhibitor complex is formed in acid media, the column was subsequently equilibrated with acetic acid at pH 3. One ml of Sepharose with attached inhibitor was capable of binding about 2 mg of chicken pepsin.

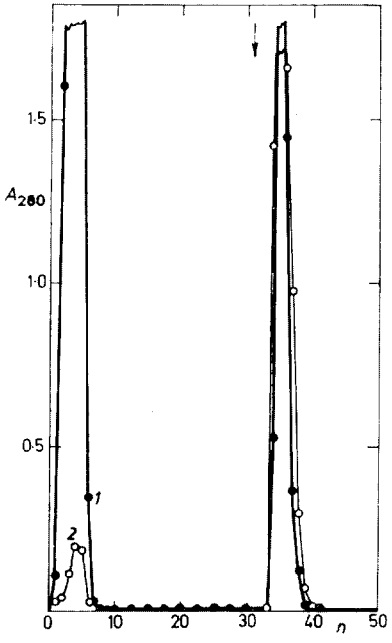

FIG. 1

Affinity Chromatography of Chicken Pepsin on Sepharose 4B with Covalently Attached Pepsin Inhibitor from *Ascaris lumbricoides*

50 mg of crude chicken pepsin was applied onto a 1×6 cm column. The flow rate was 60 ml/h. *1* absorbance at 280 nm; *2* proteolytic activity; the addition of 0·01M phosphate buffer at pH 6·8 is marked by an arrow; *n* tube number.

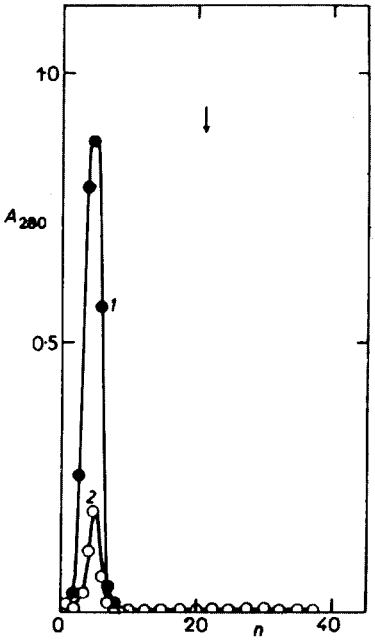

FIG. 2

Affinity Chromatography of Chicken Pepsin on Unmodified Sepharose 4B

2 mg of crude chicken pepsin was applied onto a 1×6 cm column. The flow rate was 60 ml/h. *1* absorbance at 280 nm; *2* proteolytic activity; the addition of 0·01M phosphate buffer at pH 6·8 is marked by an arrow; *n* tube number.

Chicken pepsin Sepharose column. Fifty ml of cyanogen bromide activated and washed Sepharose 4B was equilibrated in 0·01M phosphate buffer at pH 6·8. Purified chicken pepsin (200 mg) was dissolved in 15 ml of the same buffer and the solution was added dropwise to the Sepharose suspension. The mixture was slowly stirred for additional 24 h at 4°C. It was packed into a column and excess unattached pepsin was washed out with the same buffer. About 3 mg of chicken pepsin had been coupled to 1 ml of Sepharose.

RESULTS AND DISCUSSION

Pepsin inhibitor from *Ascaris lumbricoides* inhibits, in addition to hog pepsin, also hog gastricsin[8], human pepsin[8] and the tissue proteinase cathepsin *E* (ref.[9]). After we had found that it inhibited also the enzymatic activity of chicken pepsin, we used it as a specific affinant, attached to Sepharose, for the isolation of chicken pepsin.

Ascaris pepsin inhibitor Sepharose column. In preliminary experiments, a suspension of Ascaris pepsin inhibitor Sepharose (4 ml) was diluted by 5 ml of acetic acid at pH 3 and mixed with a solution of 4 mg of crude chicken pepsin in 1 ml of acetic acid, pH 3. Residual pepsin activity of the supernatant was determined at intervals. As shown in Table I, the inactivation of pepsin is almost instantaneous and a marked activity drop, compared to a control, can be observed already after 1 min. The affinity chromatography of chicken pepsin on an Ascaris pepsin inhibitor Sepharose column is shown in Fig. 1. After the emergence of the first peak, attached chicken pepsin was displaced from the complex by 0·01M phosphate buffer at pH 6·8. The yield was 90% of enzymatic units originally applied. The specific activity increased about 8-times in this single operation. This purified pepsin gave on disc electrophoresis (at pH 8·3) 2 main zones and 3 minor zones of lower mobility. Obviously we again obtained a mixture of all forms of chicken pepsin. When the fraction of chicken pepsin obtained by chromatography on the mercurial Sepharose column[4] was chromatographed on the Ascaris pepsin inhibitor Sepharose column, its activity increased by approximately 15%; hence, affinity chromatography on Ascaris pepsin inhibitor Sepharose is slightly

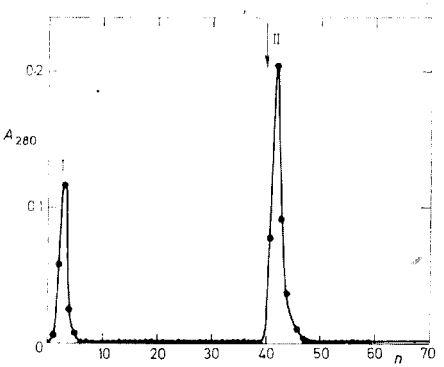

FIG. 3

Affinity Chromatography of Pepsin Inhibitor from *Ascaris lumbricoides* on Sepharose 4B with Covalently Attached Chicken Pepsin

The inhibitor (230 units) was applied to a 1.6 cm column. Peak II represents 85% of the original inhibitory activity. Flow rate 60 ml/h. ●——● absorbance at 280 nm; the addition of 0·01M phosphate buffer at pH 6·8 is marked by an arrow; *n* tube number.

more specific. Chromatography on Ascaris pepsin inhibitor Sepharose may find wide application in the isolation of proteinases of the carboxyl type which are inhibited by this inhibitor. Thus, *e.g.* we found in preliminary experiments in this laboratory that both hog pepsin and hog gastricsin form a complex with Ascaris pepsin inhibitor coupled to Sepharose and can be displaced from this complex in active state by an acetate buffer containing NaCl. Under different conditions, cathepsin E, prepared from rabbit bone marrow can be attached also to Ascaris pepsin inhibitor Sepharose and displaced later[10].

A control experiment was run with pure, unmodified Sepharose. As shown in Fig. 2, the entire peptic activity emerges in the first peak from the column at pH 3·0.

TABLE I

Adsorption of Chicken Pepsin to Sepharose 4B with Covalently Attached Pepsin Inhibitor from *Ascaris lumbricoides*

Time of reaction of components min	Residual peptic activity	
	A_{280nm}	%
0	0·500	100
1	0·080	16
10	0·030	6
20	0·015	3

Chromatography of Ascaris pepsin inhibitor on Sepharose with attached chicken pepsin. Chicken pepsin, unlike hog pepsin, is stable over a relatively wide pH range, 1–8 (ref.[5]). It can be therefore coupled directly to Sepharose at pH 6·8 without the danger of a total inactivation of the enzyme. The chicken pepsin Sepharose column was treated with acetic acid (pH 3). The solution of purified inhibitor adjusted at the same pH, was slowly applied onto the column. After the removal of free uncoupled inhibitor by exhaustive washing of the column with acetic acid (pH 3), the inhibitor attached was displaced from the complex by 0·01M phosphate buffer at pH 6·8. The course of the chromatography is shown in Fig. 3. The yield of the operation in terms of inhibitory activity was 85% of the material applied.

We assume that affinity chromatography on Sepharose with covalently attached chicken pepsin may serve as an effective method in the search and isolation of naturally occurring inhibitors of other pepsin-like proteinases.

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