AFFINITY CHROMATOGRAPHY OF TRYPSIN INHIBITORS ON TRYPSINOGEN-AGAROSE EXCHANGERS

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1. Introduction

Dlouhá and Keil [1, 2] have reported that two trypsin inhibitors complex with trypsinogen as well as with trypsin. We have prepared trypsinogen-agarose and trypsin-agarose columns and have compared the chromatographic behaviour of crude peanut trypsin inhibitor (PTI) and Kunitz soybean trypsin inhibitor (SBTI) on these columns. Only the PTI was bound to the trypsinogen-agrose columns while both PTI and SBTI were bound to the trypsin-agarose columns. The PTI could be eluted from the trypsinogen-agarose columns under milder conditions than from the trypsin-agarose columns.

2. Materials and methods

The trypsin-agarose and the trypsinogen-agarose were prepared by cyanogen bromide activation [3–5] of the agarose (Sepharose 4B-200) (Sigma) followed by the coupling of the beads to bovine trypsin (Sigma) or bovine trypsinogen (Worthington). The trypsin activity was determined by the method of Erlanger, Kokowsky and Cohen [6] with *N*-benzoyl-DL-arginine *p*-nitroanilide (Schwarz/Mann) as the substrate. Commercial SBTI (Worthington) was used without further purification. The PTI was prepared in our laboratory by a method which will be reported at a later time.

3. Results

The chromatographic behavior of the PTI on a trypsinogen-agarose column, on a trypsin-agarose column and on an unmodified agarose column at room temperature are illustrated in fig. 1. The chromatography of the PTI on a trypsinogen-agarose column (fig. 1A) in a pH 8.6 tris-HCl buffer system resulted in the elution of a large amount of slightly retarded material with no trypsin inhibitor activity. One peak of inhibitor activity was eluted after about five column volumes of buffer were passed through the column. The application of a step gradient of 0.01 M HCl resulted in the elution of another peak of trypsin inhibitor activity at the break-through volume. The application of a second step gradient of 0.1 M HCl did not elute any additional material or inhibitor activity. The behavior of the PTI activity on a trypsin-agarose column (fig. 1B) was quite different. The inhibitor was more tightly bound, 0.01 M HCl was required for the elution of the first inhibitor peak and 0.1 M HCl was required for the elution of the second inhibitor peak. When the PTI was chromatographed on agarose (fig. 1C) the inhibitor activity was not retarded by the column.

The chromatographic behavior of the SBTI on trypsinogen-agarose and trypsin-agarose columns is illustrated in fig. 2. The SBTI was not bound to the trypsinogen-agarose column to any great extent (fig. 2A). Ninety-four percent of the inhibitor activity

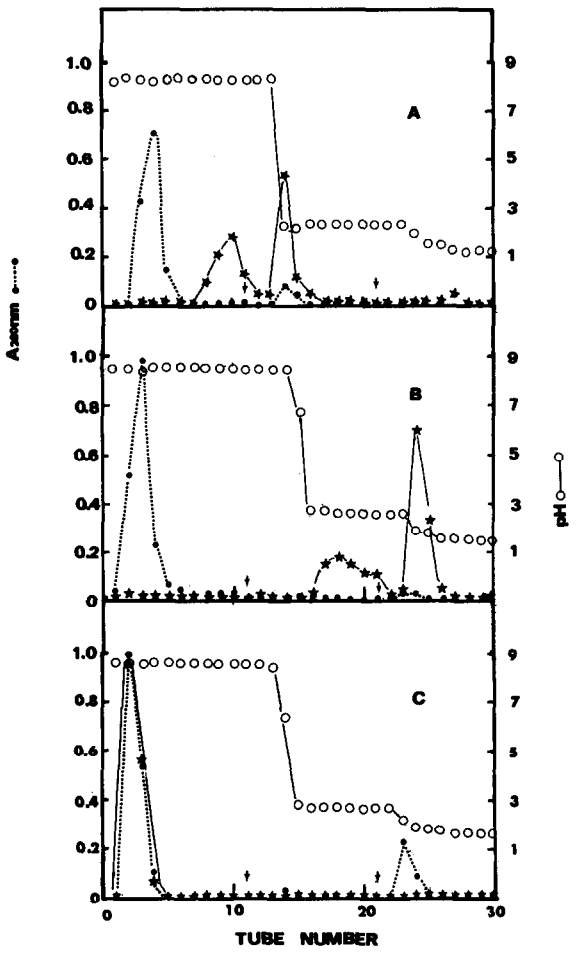

Fig. 1. Affinity chromatography of crude peanut trypsin inhibitor: (A) on a column (0.9 × 13 cm) of trypsinogen-agarose, (B) on a column (0.9 × 9 cm) of trypsin-agarose, and (C) on a column (0.9 × 9 cm) of agarose. The peanut inhibitor was dissolved in 0.4 ml of pH 8.6, 0.05 M tris-HCl buffer with 40 mg sucrose and applied to the column. The column was eluted with 37 ml of buffer, then 37 ml of 0.01 M HCl and then 37 ml of 0.1 M HCl. The point of application of each step gradient is marked with an arrow. Fractions of 60 drops (3.7 ml) were taken. The absorbance at 280 nm (at 1 cm path length), pH, and trypsin inhibitor activity were measured for each fraction. See text for details.

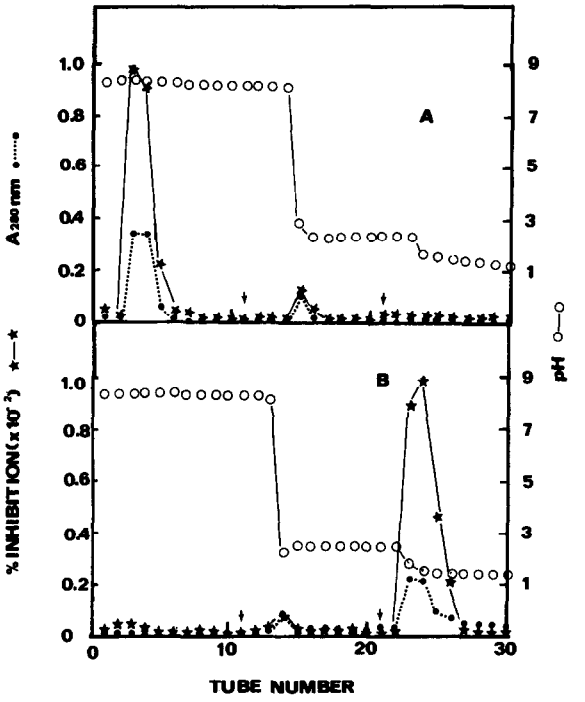

Fig. 2. Affinity chromatography of Kunitz soybean inhibitor: (A) on a column (0.9 × 13 cm) of trypsinogen-agarose, and (B) on a column (0.9 × 9 cm) of trypsin-agarose. The conditions were identical to those given in fig. 1.

came through only slightly retarded, 6% eluted at the breakthrough volume for the 0.01 M HCl, and less than 0.5% was eluted with 0.1 M HCl. As was expected, the SBTI was tightly bound to the trypsin-agarose columns (fig. 2B) and 0.1 M HCl was required to elute the inhibitor activity. The results of chromatography of PTI and SBTI on chymotrypsinogen-agarose columns indicated that neither inhibitor was bound significantly under similar conditions.

4. Discussion

Thus we have found that PTI can be chromatographed on trypsinogen-agarose columns. As the SBTI did not appear to bind to the trypsinogen-agarose, the binding of plant protease inhibitors to trypsinogen-agarose shows some species specificity. The lack of any significant binding of the SBTI to the trypsinogen-agarose column indicates that there was little active trypsin on the trypsinogen-agarose column and that the PTI must bind to the trypsinogen of the zymogen-agarose since the inhibitor does not bind to agarose. PTI is eluted under milder conditions from the trypsinogen-agarose columns than

from the trypsin-agarose columns and thus the affinity of PTI for the trypsin-agarose is greater than the affinity for trypsinogen-agarose. Dlouhá and Keil [1] reported similar results for the interaction of basic pancreatic trypsin inhibitor with free trypsin and trypsinogen. Although the binding for the PTI differs on the exchangers, the high selectivity of each exchanger for the PTI is quite apparent. This high selectivity can be accounted for by postulating that the binding site for the PTI is the active site of the trypsin or the precursor of the active site in the catalytically inactive zymogen. The difference in the affinity of binding of the PTI could then be ascribed to the differences in the geometry of this site in the zymogen and in the active enzyme.

Trypsinogen-agarose columns should prove to be useful in the isolation of those protease inhibitors which bind to trypsinogen such as PTI, basic pancreatic trypsin inhibitor, and the cow colostrum inhibitor [2]. Hixson and Laskowski [7] have reported that some of the plant protease inhibitors were extensively modified by the insolubilized trypsin when isolated by affinity chromatography on insolubilized trypsin by Hochstrasser et al. [8–11]. In light of the present work it would seem quite possible that some of these plant inhibitors could be isolated in an unmodified form by using affinity chromatography on the insolubilized trypsinogen instead of the insolubilized trypsin.

One would also expect that, in general, zymogen exchangers could be useful for substrate isolations whenever the site specificity of the enzyme was present in the zymogen while the catalytic activity was not.

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