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AFFINITY CHROMATOGRAPHY OF PROTEASES ON HYDROXYALKYL METHACRYLATE GELS WITH COVALENTLY ATTACHED INHIBITORS

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SUMMARY

The efficient isolation of trypsin and chymotrypsin from a crude pancreatic extract was achieved by affinity chromatography on specific adsorbents prepared by coupling of both naturally occurring protease inhibitors and also synthetic low-molecular-weight protease inhibitors to hydroxyalkyl methacrylate gels. Specific sorbents prepared with synthetic inhibitors are stable and are suitable for the isolation of chymotrypsin and trypsin even on a large scale.

INTRODUCTION

The last few years have witnessed great progress in the simple isolation of numerous biologically active substances, especially enzymes, owing to the introduction of affinity chromatography. This method makes use of the property of these substances to form stable, specific and reversible complexes such as, *e.g.*, complexes of enzymes with their inhibitors, substrates or effectors, antibodies with antigens and lectins with polysaccharides. The efficient isolation of trypsin and chymotrypsin from a crude pancreatic extract on specific adsorbents prepared by the coupling of protease inhibitors to hydroxyalkyl methacrylate gels may serve as an example.

The gels used were developed and prepared by Čoupek *et al.*[1] at the Institute of Macromolecular Chemistry of the Czechoslovak Academy of Sciences and their structure is shown in Fig. 1. The copolymerization of hydroxyalkyl methacrylate with alkylene dimethacrylates gives rise to heavily crosslinked microparticles of a xerogel, which subsequently aggregate and yield macroporous structures of spheroids. Because of this structure, the gels have some chemical properties in common with the most commonly used support, agarose. Thus, *e.g.*, the hydroxyl groups of the gel can be activated with cyanogen bromide[2], in a similar manner to the hydroxyl groups of agarose. Amino acids, peptides and proteins can be bound to the activated gels through their amino groups. At the same time, however, the gels resemble, because of their macroreticular structure, inorganic supports. They do not change in volume with

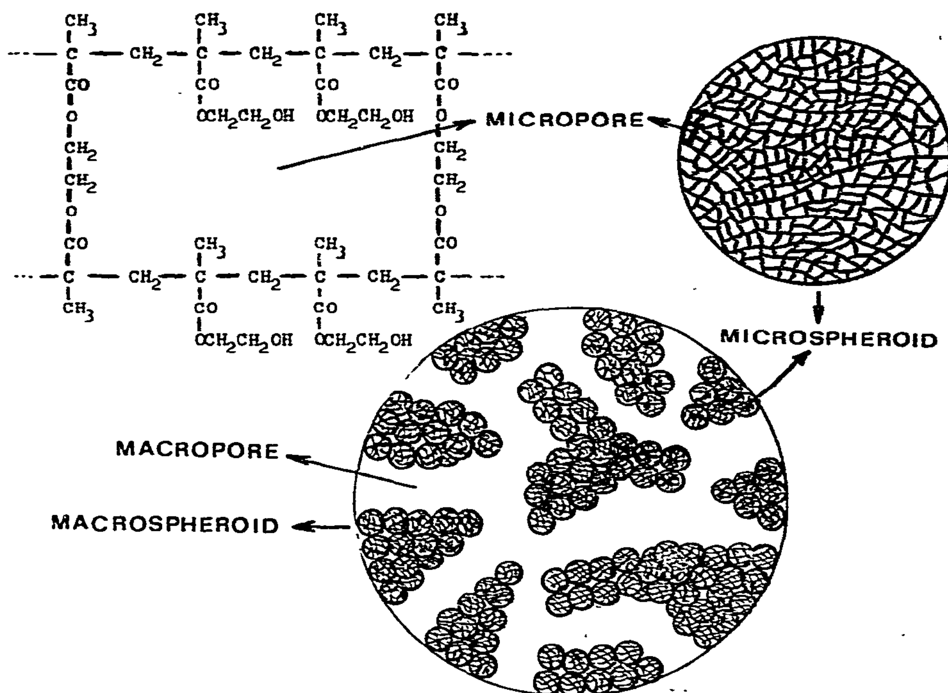

Fig. 1. Structure of hydroxyalkyl methacrylate gels (Spheron).

changes in pH or after the addition of organic solvents, and they are not attacked by microorganisms and show excellent flow properties because of their rigidity. These properties permit the use of the gels in large-scale operations.

EXPERIMENTAL

Materials

The hydroxyalkyl methacrylate gels (Spheron 300) of particle size 100–200 μm were prepared by the method described earlier[1], as well as Spheron with attached hexamethylenediamine (NH_2-Spheron)[2,3] and N-benzyloxycarbonylglycyl-D-phenylalanine-NH_2-Spheron (Z-Gly-D-Phe-NH_2-Spheron)[4]. Aminobenzamidine was attached to NH_2-Spheron by use of soluble carbodiimide according to Hixson and Nishikawa[5]. Ovomucoid and antilysine were coupled to cyanogen bromide-activated Spheron[2]. Ovomucoid was purchased from Koch-Light (Colnbrook, Great Britain) and antilysine (polyvalent lung trypsin inhibitor) was purchased from Léčiva (Prague, Czechoslovakia).

Chromatography of crude pancreatic extract on ovomucoid-Spheron

A sample of active pancreatic extract (100 ml) was placed on a column (10 × 2 cm) which was subsequently eluted with an aqueous solution of ammonium formate (0.05 *M* formic acid adjusted to pH 8.0 with 5% aqueous ammonia). The course of the chromatography is shown in Fig. 2(I).

Chromatography of fraction (a), eluted from an ovomucoid-Spheron column [cf., Fig. 2(I)], on a column of antilysine-Spheron

The fraction of material not adsorbed (180 ml) was placed directly on the antilysine-Spheron column (10 × 2 cm). The course of the chromatography is shown in Fig. 2(II).

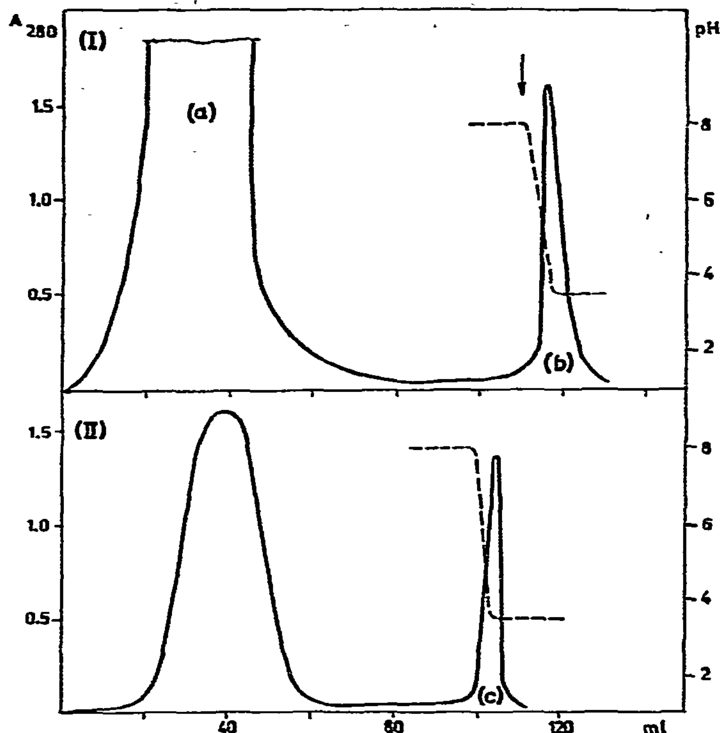

Fig. 2. Chromatography of crude pancreatic extract on ovomucoid-Spheron (I) and antilysine-Spheron (II). (I) A sample of active pancreatic extract (100 ml) was placed on a column of ovomucoid-Spheron (10 × 2 cm), which was subsequently eluted with an aqueous solution of ammonium formate (0.05 *M* formic acid adjusted to pH 8.0 with 5% aqueous ammonia). Fractions (6 ml) were collected at 20-min intervals. The arrow designates the change in pH from 8.0 to 3.5 (0.1 *M* formic acid adjusted to pH 3.5 with ammonia). (II) The fraction (a) of material not adsorbed (180 ml) was placed directly on the antilysine-Spheron column (10 × 2 cm). The course of the chromatography was analogous to that described for (I). (a) Contaminants and chymotrypsin; (b) trypsin; (c) chymotrypsin; ———, absorbance at 280 nm; - - -, pH.

Chromatography of crude pancreatic extract on N-benzyloxycarbonylglycyl-D-phenylalanine-NH_2-Spheron

A sample of active pancreatic extract (100 ml) was applied to the column (6.0 × 1.5 cm). The course of the chromatography is shown in Fig. 3(I).

Chromatography of fraction A, filtered through a column of N-benzyloxycarbonylglycyl-D-phenylalanine [cf., Fig. 3(I)], on a column of NH_2-benzamidine-NH_2-Spheron (25 × 1 cm)

The course of chromatography is shown in Fig. 3(II).

RESULTS AND DISCUSSION

To isolate chymotrypsin and trypsin from a crude pancreatic extract we used first specific adsorbents prepared by coupling naturally occurring, high-molecular-weight protease inhibitors to Spheron 300 activated with cyanogen bromide. Fig. 2(I) shows the isolation of trypsin on Spheron P 300 with attached ovomucoid. The capacity of the column was 1.5 mg of trypsin per millilitre of gel. Because volatile buffers were used for desorption, the enzyme fraction after lyophilization yielded a trypsin preparation containing less than 5% of salts, thus conforming to standards of commercial preparations. The activity of the trypsin preparation obtained was 31.3 units/g, assayed with lysine ethyl ester as substrate. This activity is considerably higher than the activity prescribed by the standard for commercial preparations.

The fraction of the material not adsorbed to ovomucoid-Spheron was directly applied to Spheron with attached antilysine [Fig. 2(II)]. Chymotrypsin was then specifically adsorbed to this polyvalent protease inhibitor at an alkaline pH. The capacity of the column was 1.4 mg of chymotrypsin per millilitre of gel. The fraction of desorbed chymotrypsin afforded, after lyophilization, a preparation with less than 5% of salts. Its activity, 46.5 units/g, determined with tyrosine ethyl ester as substrate, was again higher than that required by standards.

The activity of ovomucoid-Spheron dropped to 20% after ten runs. Regeneration by washing with 4 *M* urea restored the original capacity of the specific adsorbent to 90%, yet after four runs a considerable decrease in the capacity was again observed. The capacity of antilysine-Spheron decreased to 23% after ten runs. Washing of the adsorbent with 4 *M* urea regenerated 94% of the original capacity, yet a rapid decrease was observed after repeated use of the regenerated adsorbent. Because of the low stability and high cost of naturally occurring protein inhibitors, we focused our attention on the use of specific adsorbents prepared from low-molecular-weight synthetic inhibitors.

NH_2-Spheron was prepared by coupling of hexamethylenediamine to cyanogen bromide-activated Spheron P 300. Subsequently, N-benzyloxycarbonylglycyl-D-phenylalanine or aminobenzamidine were attached to NH_2-Spheron. Fig. 3(I) shows the affinity chromatography of a crude pancreatic extract on Z-Gly-D-Phe-NH_2-Spheron. The capacity of the column was 1.35 mg of enzyme per millilitre of gel. The activity of the lyophilized preparation obtained was 49.5 units/g, determined with tyrosine ethyl ester as substrate.

The material emerging in the first peak was applied to a column of NH_2-benzamidine-NH_2-Spheron [Fig. 3(II)]. The capacity of the column was 1.3 mg per millilitre of gel. The activity of the lyophilized preparation was 33 units/g, determined with lysine ethyl ester.

The data presented here show that identical results were obtained with specific adsorbents prepared both with naturally occurring protease inhibitors and with low-molecular-weight synthetic inhibitors. Unlike the naturally occurring inhibitors, which undergo denaturation because of their protein character and thus irreversibly lose their activity, the synthetic low-molecular-weight inhibitors are completely stable. The capacity of specific adsorbents prepared with these inhibitors can be regenerated almost infinitely, *e.g.*, by washing with 6 *M* guanidine hydrochloride. Synthetic low-molecular-weight inhibitors coupled to both chemically and mechanically stable

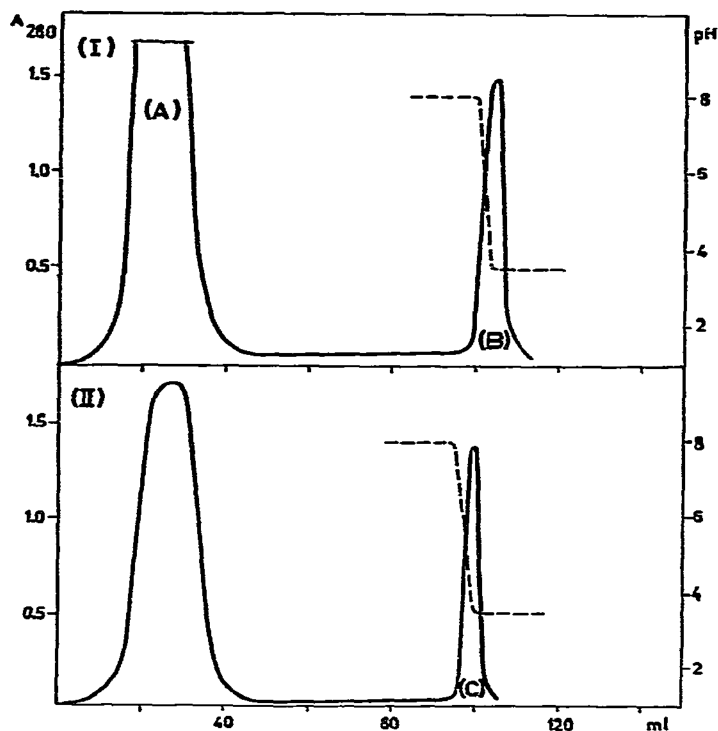

Fig. 3. Chromatography of crude pancreatic extract on N-benzyloxycarbonylglycyl-D-phenylalanine-NH_2-Spheron (I) and NH_2-benzamidine-NH_2-Spheron (II). (I) A sample of active pancreatic extract (100 ml) was applied to the column of N-benzyloxycarbonylglycyl-D-phenylalanine-NH_2-Spheron (6.0 × 1.5 cm). The course of the chromatography was identical with that shown in Fig. 2. (II) Fraction (A), filtered through a column of N-benzyloxycarbonylglycyl-D-phenylalanine, was placed directly on a column of NH_2-benzamidine-NH_2-Spheron (25 × 1 cm). The course of chromatography is identical with that shown in Fig. 2. (A) Contaminants and trypsin; (B) chymotrypsin; (C) trypsin; ———, absorbance at 280 nm; – – –, pH.

hydroxyalkyl methacrylate gels therefore represent specific sorbents suitable for the isolation of enzymes even on a large scale.

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