Journal of Chromatography, 261 (1983) 138–141
Elsevier Science Publishers B.V., Amsterdam — Printed in the Netherlands

CHROM. 15,719

Note

Affinity chromatography of trypsin on thermally modified casein

IVO ŠAFAŘÍK
Department of Biochemistry and Microbiology, Institute of Chemical Technology, Suchbátarova 5, 166 28 Prague (Czechoslovakia)
(Received January 19th, 1983)

Animal, plant and microbial proteases can be isolated and purified using a variety of methods. Affinity chromatography is of special interest because it allows the desired protease(s) to be isolated in a single step. In this method various substrates, substrate analogues and inhibitors (both natural and synthetic) or antibodies are used as ligands for immobilization on various types of insoluble supports[1–3]. In contrast, an insoluble protein substrate such as collagen can be used for the affinity chromatography of collagenase itself without immobilization on to an insoluble matrix[4]. This is a simplification of the chromatographic method because the coupling procedure may be a critical step.

To simplify the affinity chromatography of trypsin, a method was developed using thermally modified casein as a water-insoluble protease substrate. The method is inexpensive because only granulated casein is used to prepare an affinity chromatographic sorbent.

EXPERIMENTAL

Materials

Casein after Hammarsten, which is supplied in a granulated form (the diameter of most particles is less than 1 mm) was obtained from Reanal (Hungary) and was used without any pre-treatment. Azocasein was prepared in the laboratory[5]. Trypsin was purchased from Léčiva (Czechoslovakia), enzyme casein hydrolysate from Imuna (Czechoslovakia) and ammonium sulphate and other common chemicals from Lachema (Czechoslovakia).

Preparation of affinity chromatographic sorbent

Casein was heated in a thin layer at 190°C for 30, 60, 90 and 180 min and at 230°C for 120 min in a hot-air thermostat. The resultant material had a yellow to dark brown colour, depending on the temperature and time used. For washing the thermally modified casein 0.5–1% sodium hydroxide solution, water, 1 *M* ammonium sulphate solution and water again were used. This procedure was repeated until the absorbances of the water and ammonium sulphate washings were lower than 0.01 at 280 nm in a 1-cm cuvette.

0021-9673/83/$03.00

Affinity chromatography of trypsin

Glass columns (250 × 10 mm I.D.) with a sintered-glass disc at the bottom were filled with the prepared affinity sorbent in the usual way. Bed dimensions of 200 × 10 mm were used throughout. After sample application, the ballast proteins were eluted with water. The adsorbed trypsin was eluted from the column with 1 *M* ammonium sulphate or 1 *M* sodium chloride solution until no enzyme activity could be detected in the effluent. The flow-rates ranged from 0.5 to 4 ml/min. The separations were carried out at laboratory temperture.

Other procedures

The protein content in the eluted fractions was monitored spectrophotometrically at 280 nm, and the Warburg and Christian method[6] was used for quantitative determinations.

The proteolytic activity in the eluted fractions was determined with azocasein as substrate. The reaction mixture contained 1 ml of 1% azocasein solution in 0.2 *M* phosphate buffer (pH 7.2) and 0.5 ml of the tested solution. After 30 min at 37°C the reaction was stopped with 1.5 ml of 5% trichloroacetic acid. After centrifugation the absorbance at 366 nm (against a blank) was read, which is proportional to the protease activity.

RESULTS

In the first experiment, 10 ml of a model mixture containing 100 mg of enzyme casein hydrolysate and 5 mg of trypsin were used for chromatography on casein heated at 230°C for 120 min. Fig. 1 shows the distribution of total proteins and the

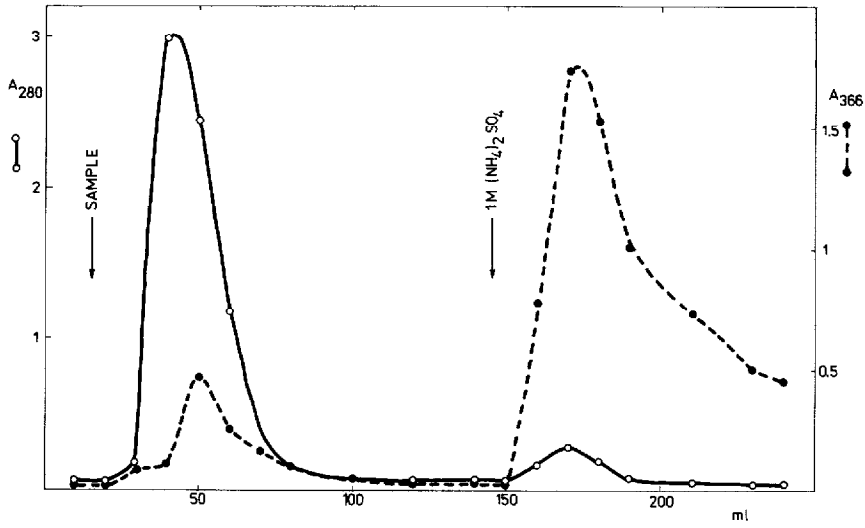

Fig. 1. Chromatography of 10 ml of a model mixture containing 100 mg of enzyme casein hydrolysate and 5 mg of trypsin on a 200 × 10 mm I.D. column of casein heated at 230°C for 120 min. The column was washed with water and 1 *M* ammonium sulphate solution. The flow-rate was 1–2 ml/min. Solid line, absorbance at 280 nm; broken line, absorbance at 366 nm.

protease activity in the effluent. Of the applied protease activity, 54% was found in the first 50 ml of effluent after changing the elution conditions (in the fractions between 150 and 200 ml) and the recovery was 75% from the fractions between 150 and 240 ml; 13.4% of the total protease activity was eluted with water together with ballast proteins and the remaining 11.6% was slowly eluted from the column into fractions not shown in Fig. 1 (over 240 ml). The degree of purification based on specific activity was 10.6-fold in a 75% yield.

Similar results were obtained when 15 mg of trypsin (approximately 5-year-old preparation) in 10 ml of water were applied to the same column. Of the applied protease activity, 81.2% was found in the first 140 ml of effluent after changing the elution conditions, 10.6% was eluted with ballast proteins and 8.2% was slowly eluted from the column after elution of the majority of the protease activity. The specific activity increased 2.2-fold after chromatography.

To determine the capacity of the sorbent, 700 mg of trypsin in 50 ml of water were applied to the same column. After elution of non-bound trypsin and ballast proteins with water the adsorbed trypsin was eluted with 1 *M* ammonium sulphate solution. The capacity is approximately 0.5 mg of pure trypsin per millilitre of the sorbent.

To compare the effects of various modification conditions on the chromatographic separation, beds of dimensions 200 × 10 mm were prepared from the thermally modified casein samples heated at 190°C for 30, 60, 90 and 180 min, and at 230°C for 120 min. A 10-ml volume of the model mixture containing 100 mg of enzyme casein hydrolysate and 10 mg of trypsin was applied to each column. Elution was carried out under conditions similar to those described above. The elution profiles were similar, indicating that there is no simple dependence between the degree of modification of casein and the chromatographic behaviour. The recoveries were higher than 75% in all instances. The specific activities increased 10- to 15-fold after chromatography.

An approximately 20–30% reduction in bed volume was observed when water was replaced with 1 *M* ammonium sulphate or sodium chloride solution for elution.

DISCUSSION

Many affinity chromatographic materials have been developed for the isolation and purification of proteases. Nevertheless, no cheap and simply made material for preliminary experiments or for use by non-specialists has been available. The method presented here employs one of the commonest and cheapest biochemical materials, casein. The modification of casein into a water-insoluble affinity chromatographic sorbent can be easily effected by heating.

The resultant material is far from ideal. As can be seen from Fig. 1, tailing occurred in all instances when elution was carried out with ammonium sulphate solution. The recovery of the protease activity was approximately 75%, which is comparable to that with other affinity chromatographic sorbents. Some properties of this material (*e.g.*, capacity) could probably be improved by pre-treatment of casein (selection of particles with the most suitable diameter).

Probably other proteins could be used for the preparation of sorbents in a

similar way, if they were supplied (or prepared in the laboratory) in a granulated form.

REFERENCES

1 C. R. Lowe and P. D. G. Dean, *Affinity Chromatography*, Wiley, Chichester, 1974.
2 V. S. Vesa, *Prikl. Biokhim. Mikrobiol.*, 16 (1980) 629.
3 J. Turková, *J. Chromatogr.*, 91 (1974) 267.
4 P. M. Gallop, S. Seifter and E. Meilman, *J. Biol. Chem.*, 227 (1957) 891.
5 J. Charney and R. M. Tomarelli, *J. Biol. Chem.*, 171 (1947) 501.
6 O. Warburg and W. Christian, *Biochem. Z.*, 310 (1942) 384.