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RESOLUTION IN AFFINITY CHROMATOGRAPHY

THE EFFECT OF THE HETEROGENEITY OF IMMOBILIZED SOYBEAN TRYPSIN INHIBITOR ON THE SEPARATION OF PANCREATIC PROTEASES

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SUMMARY

By affinity chromatography, trypsins and chymotrypsins from mouse pancreas homogenates have been separated using soybean trypsin inhibitor immobilized on Sepharose. The effects of the functional heterogeneity of the adsorbent have been investigated in terms of the resolution obtained. Heterogeneity has been found to originate from the following sources: heterogeneity of the ligand before immobilization; alteration of the ligand by immobilization; and modification of the ligand after immobilization by molecules to be fractionated. Only when the heterogeneity of the adsorbent was minimized could the resolution of closely related enzyme species be achieved. The elution conditions for different enzymes depended on the amount of enzyme applied, as no complete homogeneity could be obtained. In addition, it was found that the adsorbent was partly degraded by the pancreas extract, reducing its fractionating capacity.

INTRODUCTION

In affinity chromatography, biomolecules are separated according to their varying ability to interact with active ligands immobilized on insoluble matrices[1]. Desorption can be carried out in two ways: all adsorbed molecules with related (but not necessarily identical) activity can be eluted stepwise regardless of the strength of their interaction with the ligand, or alternatively adsorbed counter ligands can be desorbed by a suitable gradient (pH, ionic strength, biochemically active solutes, etc.). In the latter case, differences in association constants of the ligand–counter ligand

complexes under the elution conditions employed can be used to separate molecules that have quantitative differences in activity. Differences in elution conditions are then assumed to reflect differences in biological activities, and can be used to characterize the molecule. Under such premisses, the applicability of the adsorbent depends on the functional homogeneity of the immobilized ligand.

Heterogeneity of the adsorbent in terms of association constants can be caused by (i) heterogeneity of the biospecific ligand used for the preparation of the adsorbent, (ii) varying alteration of the ligand due to the immobilization process and (iii) varying modification of the ligand by molecules to be fractionated.

Heterogeneity of the ligand before immobilization

Functionally different mutants of proteins used as ligands have been isolated and characterized[2,3]. In fact, high-molecular-weight ligands of biological origin, such as proteins, nucleic acids and carbohydrates used for the preparation of the adsorbent, may be genetically heterogeneous. Such heterogeneity may be found in both commercial and non-commercial ligand preparations.

It is obvious that the presence of impurities in the ligand preparation with a similar or stronger affinity for the molecules to be separated will endanger the use of the adsorbent in gradient separations.

Alteration of the ligand by immobilization

The effective activity of the ligand can be changed due to the immobilization in various ways, because the micro-environment provided by the matrix (charge density, steric hindrance, etc.) may affect the interaction of the immobilized ligand with the molecules to be fractionated in various ways and also affect the structure of the ligand[4]. By immobilization, the molecular properties of the ligand may be changed *e.g.* by modification of the chemical structure of the ligand or cross-linking between the ligand and the matrix[5].

Modification of the ligand after immobilization

The material to be fractionated may contain components that modify the activity of the bound ligand. These components may or may not be similar to the components to be separated. Thus, enzymes (proteases, nucleases) present in crude extracts may digest the bound ligand (proteins, nucleic acids), thus reducing the capacity of the adsorbent to bind its specific counter ligand. In addition to non-specific degradation of the ligand, enzymes and other chemicals present in the mixture to be fractionated may modify specifically the properties of the bound ligand, giving rise to forms with retained, but altered, activity[6].

In this study, the above three effects on the capacity of immobilized ligands used as adsorbents in affinity chromatography were investigated. Soybean trypsin inhibitor (Kunitz) immobilized on Sepharose was used to fractionate chymotrypsins and trypsins present in homogenates of mouse pancreas after activation with immobilized trypsin.

EXPERIMENTAL

*Materials**

Soybean trypsin inhibitor (Worthington, SI; and Sigma, Type I-S), bovine α-chymotrypsin (Worthington, CDI), Sepharose® 4B (Pharmacia, Uppsala, Sweden), N-benzoyl-L-tyrosine ethyl ester and N-tosyl-L-arginine methyl ester (BDH, Poole, Great Britain) were used as purchased. All other chemicals were of reagent grade.

Preparation of STI–Sepharose

STI was coupled to Sepharose 4B at pH 8.5 or 7.2 using the cyanogen bromide-method described earlier[4,7]. The amount of coupled protein was determined either by amino acid analysis or from the difference between the amount of STI added and the amount of protein eluted from the gel after the coupling procedure.

Modification of coupled STI with CT

The coupled STI was incubated at pH 8.0 (0.2 *M* sodium chloride, 0.05 *M* Tris–hydrochloric acid) with CT (molar ratio of STI to CT = 1:50) for 24 h using a peristaltic pump (flow-rate 10 ml/h) and a column packed with the adsorbent[8]. After the incubation, the CT was eluted with buffer of pH 2.5 (0.2 *M* sodium chloride, 1.0 *M* acetic acid).

Capacity of STI–Sepharose

STI-Sepharose, packed in a column, was saturated with CT (*ca.* 10 mg/ml) in buffer of pH 8.0 (described above) until the activity in the eluate amounted to the corresponding activity in the CT solution applied. The column was washed with the same buffer until no CT activity could be detected in the eluate. Adsorbed CT was released by buffer of pH 2.5 (described above). The amount eluted was determined from UV absorbance using a molar absorption coefficient of $\varepsilon_{280\ nm} = 4.9 \cdot 10^4$ $mole^{-1}cm^{-1}$.

Isolation and fractionation of the chymotrypsins from mouse pancreas

The pancreases from several animals (strain A/Sn, obtained from the Institute of Genetics and Plant Breeding, University of Uppsala, Sweden) were pooled and homogenized[9]. The proteases were prepared by treatment of the homogenate with immobilized trypsin[9] and the activated homogenate was stored at −20 °C.

Six millilitres of STI–Sepharose were packed into a column (I.D. = 9 mm), washed with buffer of pH 2.5 and equilibrated with buffer of pH 5.8 (0.2 *M* sodium chloride, 0.05 *M* acetic acid–sodium acetate). A flow-rate of 0.9 ml/min was maintained by a peristaltic pump and the absorbance at 280 nm was monitored. After equilibration of the STI–Sepharose, the sample was thawed and introduced into the column. When the first peak, containing inactive proteins, had been eluted, the chymotrypsins were eluted with a pH and ionic strength gradient of 5.2–4.0 (linear

* The following abbreviations are used: STI = soybean trypsin inhibitor; STI(W) = soybean trypsin inhibitor obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A.; STI(S) = soybean trypsin inhibitor obtained from Sigma, St. Louis, Mo., U.S.A.; CT = α-chymotrypsin; BTEE = N-benzoyl-L-tyrosine ethyl ester; TAME = N-tosyl-L-arginine methyl ester.

in pH) formed by a gradient mixer (Gilson MixoGrad) from the buffers of pH 5.8 and 2.5 described above. When the CTs had been eluted, the trypsins were eluted with buffer of pH 2.5.

Determination of enzyme activity

The enzyme activities were determined with BTEE and TAME at 25.0 °C using a pH-stat[9].

RESULTS

Aliquots, each corresponding to one mouse pancreas, of a homogenate of several mouse pancreases, were separated on different STI–Sepharose preparations under otherwise identical conditions. Different resolutions were obtained.

Using unmodified STI(W)–Sepharose, coupled at pH 7.2, for the separation

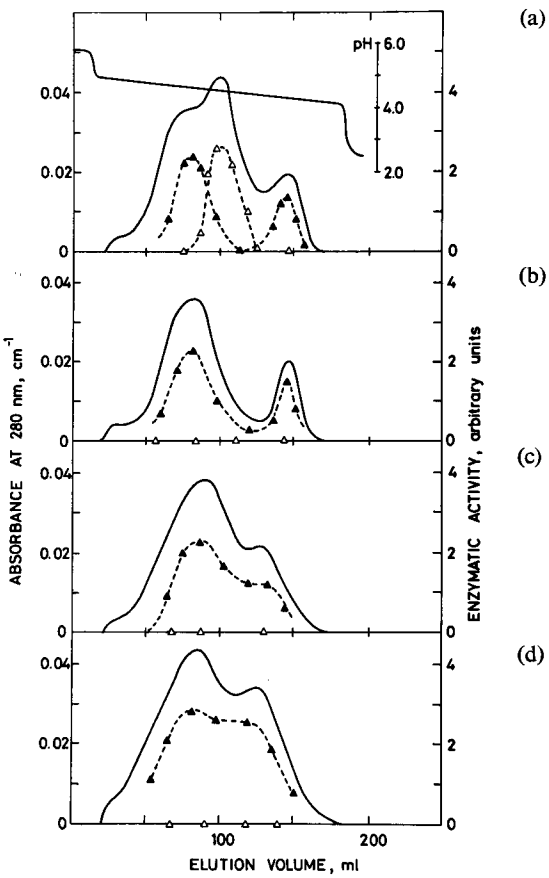

Fig. 1. Separation patterns of mouse chymotrypsins on different preparations of STI–Sepharose. ———, Absorbance or pH; ▲---▲, chymotryptic activity; △---△, tryptic activity. (a) Unmodified STI(W)–Sepharose, coupled at pH 7.2; (b) chymotrypsin-modified STI(W)–Sepharose, coupled at pH 7.2; (c) chymotrypsin-modified STI(W)–Sepharose, coupled at pH 8.5; (d) chymotrypsin-modified STI(S)–Sepharose, coupled at pH 7.2.

TABLE I

ELUTION CONDITIONS FOR MOUSE CHYMOTRYPSINS (CT-I AND CT-II) OF CHYMOTRYPSIN-MODIFIED STI(W)–SEPHAROSE, COUPLED AT pH 7.2

Number of pancreases applied	*CT activity not retained (%)*	*pH of elution*	
		CT-I	*CT-II*
0.5	0	4.75	4.30
1	0	4.90	4.35
2	3	5.05	4.50
4	57	⩾5.10	4.80

of mouse CTs, only poor resolution between two peaks with CT activity (maxima at pH 4.70 and 4.30) and a peak with tryptic activity (maximum at pH 4.60) was obtained (Fig. 1a). Two additional peaks with tryptic activity could be eluted at even lower pH[9].

After modification of STI(W)–Sepharose with CT, the trypsin peak formerly eluting between the CT peaks was now eluted below pH 4.0, thus giving a good separation of the two peaks with CT activity, eluting at pH 4.70 and 4.30 (Fig. 1b).

When STI(W)–Sepharose, coupled at pH 8.5 and modified with CT, was used for separation, the two CT peaks were less well resolved, the maxima occurring at pH 4.65 and 4.40 (Fig. 1c).

Also with modified STI(S)–Sepharose (coupled at pH 7.2), only moderate resolution was obtained, the CT peaks eluting at pH 4.65 and 4.45 (Fig. 1d).

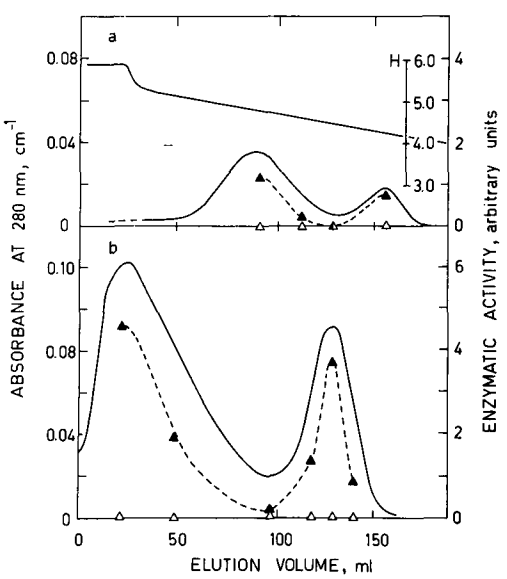

Fig. 2. Separation of different amounts of mouse chymotrypsins on STI–Sepharose. Aliquots corresponding to (a) 0.5 pancreas and (b) 2 pancreases, separated on chymotrypsin-modified STI(W)–Sepharose, coupled at pH 7.2. ———, Absorbance or pH; ▲---▲, chymotryptic activity; △---△, tryptic activity.

Different amounts of activated pancreas homogenate were fractionated on modified STI(W)–Sepharose coupled at pH 7.2. With increasing load of the adsorbent, the two peaks with CT activity eluted at higher pH values. An increasing amount of CT activity was not retained by the adsorbent at pH 8.0 and the two CT peaks were less well resolved. When the amount applied saturated the adsorbent, a trypsin peak overlapped with the last CT peak. The results are summarized in Table I and Fig. 2.

The amount of STI coupled in the preparation of STI(W)–Sepharose used in this work was 10 mg/ml, as determined both by the difference between the added and the eluted STI and by amino acid analysis. The gel had a capacity of 9 mg/ml of CT. Thus, about 75% of the bound STI molecules could interact with CT.

After the separation of a total of nine aliquots from pancreas homogenates on 4.5 ml of the adsorbent as above, the capacity for CT had decreased from 9 to 0.2 mg/ml.

DISCUSSION

In this study, the following factors were found to influence the applicability of STI–Sepharose for separating chymotrypsins and trypsins present in activated mouse pancreas homogenates:

(1) The adsorbent could be modified specifically with one of the components (CT) to be separated. The modified adsorbent showed different affinity patterns (Fig. 1a and 1c). Such modification, likewise based on limited proteolysis, has also been observed in another soybean trypsin inhibitor (Bowman–Birk)[10].

(2) Coupling of the ligand to the matrix under conditions where multi-point attachment is favoured[5,7] leads to an adsorbent with an inferior power of resolution (Fig. 1a and 1b).

(3) Preparations with ligands from different commercial sources resulted in adsorbents with different applicabilities. (Fig. 1a and 1d). This may be due to, amongst other factors, genetically different sources of raw material[2], different purification methods and varying purity of the final product[11].

(4) The elution conditions for the different components to be separated were influenced by the amount applied to the adsorbent. Therefore, when such adsorbents are used to characterize molecules by their elution conditions, care must be taken to apply comparable amounts of material (Fig. 2). The degradation of the adsorbent must also be taken into account.

The applicability of affinity chromatographic adsorbents is thus strongly influenced by the functional heterogeneity of the immobilized ligand. When the aim is not only to separate, but also to identify, closely related molecular species by their elution behaviour[9], only a small fraction of the adsorptive capacity of the adsorbent can be used.

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