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Note

Immobilized Bowman–Birk inhibitor for selective isolation of chymotrypsin B from bovine pancreas

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Currently, affinity chromatography is both the most simple and effective technique of enzyme purification. This is also true for serine proteolytic enzymes, which most often are purified with immobilized inhibitors. Most available inhibitors of these enzymes however are able to interact with two or more proteinases of different specificities, which makes difficult their selective separation from the bulk of serine proteinases. In spite of this, in such cases there is a possibility of taking advantage either of chemical modification which changes the specificity of an inhibitor or the use of selective elution to separate a desired enzyme from a mixture of proteinases bound to the immobilized inhibitor. The use of the first method for purification of chymotrypsin with immobilized pancreatic trypsin inhibitor (BPTI) was suggested by Fritz *et al.*[1]. The modified inhibitor lost its ability to react with trypsin, plasmin and kallikrein but preserved its reactivity with chymotrypsin A. Lin *et al.*[2] separated trypsin from chymotrypsin activity by use of immobilized Bowman–Birk inhibitor followed by selective elution with different buffers and salt concentrations. Bovine pancreas contains both chymotrypsinogen A and chymotrypsinogen B. The latter is usually separated by the method of Guy *et al.*[3] in which the sulphuric acid-soluble proteins are fractionated by means of ammonium sulphate followed by ion-exchange chromatography and zymogen activation with trypsin.

Here we present the use of immobilized Bowman–Birk inhibitor and selective elution to separate chymotrypsin B from a mixture of other pancreatic proteinases.

MATERIALS AND METHODS

N-Acetyl-L-tyrosine-*p*-nitroanilide (ATPNA) and Amido Black 10B were from Merck (Darmstadt, F.R.G.). N-Benzoyl-L-tyrosine ethyl ester (BTEE), N-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), trypsin inhibitor from soy bean (STI), trypsin inhibitor from lima bean (LBI) and chymotrypsin A_α, crystallized three times, were from Sigma. White soluble casein was obtained from BDH, acrylamide, N,N,N′,N′-tetramethylethylenediamine (TEMED) and N,N′-methylenebisacrylamide from Fluka (Buchs, Switzerland). Chymotrypsin B (standard preparation) was isolated according to Guy *et al.*[3]; basic pancreatic trypsin inhibitor (BPTI) was prepared according to Wilusz *et al.*[4]; Bowman–Birk trypsin inhibitor (BBI) was isolated

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according to the method of Kassell[5]. All other reagents were analytical grade products of PPH POCH-Gliwice (Poland). Fresh bovine pancreases were obtained from a slaughterhouse not later than 2 h after the animals were killed.

Protein was determined at 280 nm using the following values of $E^{1\%}_{280}$:15.0 for trypsin[6]; 18.4 for chymotrypsin B[7]; 20.2 for chymotrypsin A_α[8]; 8.3 for BPTI[9]; 4.8 for BBI[10] and 9.9 for STI[10].

The esterase activity of chymotrypsin was determined according to Hummel[11], using BTEE as a substrate. One unit of esterase activity, is defined as that amount of enzyme which hydrolyses 1 μmol of BTEE per min at pH 7.8 and 25°C.

Trypsin and chymotrypsin amidase activities were determined according to Erlanger *et al.*[12], using BAPNA as a substrate for trypsin (at 25°C) and ATPNA for chymotrypsin (at 35°C). One unit of amidase activity is defined as the amount of enzyme yielding an increase in absorbance at 410 nm of 0.1 in 10 min.

Proteolytic activity was determined according to Kunitz[13] with casein as a substrate. One unit of proteolytic activity is defined as that amount of enzyme which under the given conditions gives an increase of E_{280} of 1.0.

Deoxyribonuclease activity was determined according to Anfinsen *et al.*[14].

Amino acids were determined with an automatic amino acid analyzer (Microtechna CSSR, Type AAA 881) by the mathod of Spackman *et al.*[15]. Protein samples of 1 mg were hydrolyzed at 110°C for 24, 48 and 72 h. Half-cystine was estimated after oxidation to cysteic acid by use of performic acid[16]. The concentrations of serine, threonine and tyrosine were calculated by extrapolation to zero time hydrolysis. Tryptophan was estimated according to Goodwin and Morton[17].

STI, BBI, LBI and BPTI were immobilized on cyanogen bromide-activated Sepharose 4B according to March *et al.*[18]. Two hundred mg of each inhibitor were bound to 7.5 ml activated Sepharose 4B gel, with a yield of about 70%.

Affinity chromatography of the preparations of trypsin, chymotrypsin A_α and chymotrypsin B (standard, separated according to Guy *et al.*[3]) was performed on immobilized BBI, LBI, BPTI and STI. Just prior to application to the column, the enzymes were dissolved in 0.05 *M* Tris–HCl buffer, pH 7.5 containing 0.05 *M* calcium chloride and 0.5 *M* sodium chloride (starting buffer). The same buffer was used to equilibrate the column and to wash out the non-adsorbed proteins. The adsorbed enzymes were eluted with 10^{-2} *M* hydrochloric acid containing 0.5 *M* sodium chloride followed by 10^{-2} *M* hydrochloric acid without NaCl.

A crude preparation of chymotrypsinogen B [a fraction dialyzed against 10^{-3} *M* hydrochloric acid and salted out between 20 and 50% saturation of ammonium sulphate] was obtained according to Guy *et al.*[3]. It was diluted in an equal volume of 0.2 *M* Tris–HCl buffer, pH 8, containing 0.1 *M* calcium chloride. Then trypsin was added (6 mg per 100 g of tissue) to activate the zymogen. The course of chymotrypsinogen B activation at 4°C was monitored by determination of the activity against ATPNA. The maximum activity was attained after 30–90 min. To the resulting enzyme solution, solid sodium chloride was added to a concentration of 0.5 *M* and a precipitate formed was discarded after centrifugation. The clear supernatant was applied to an immobilized BBI column equilibrated with the starting buffer. The procedure was than identical to that described in preceding paragraph.

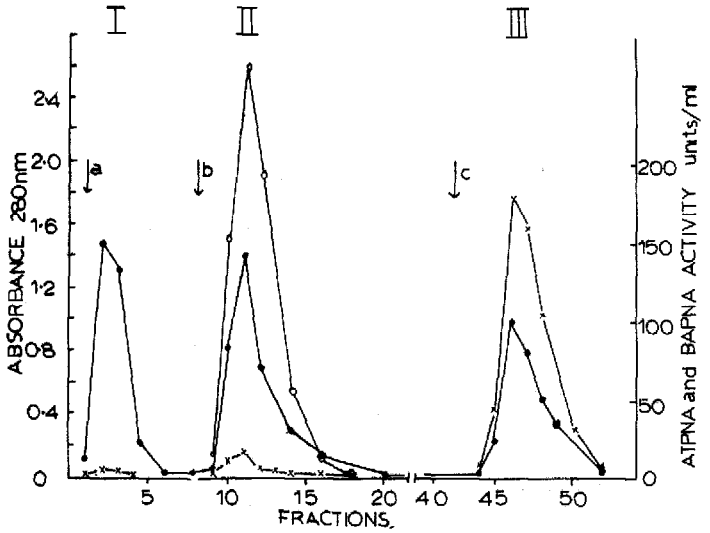

Fig. 1. Chromatography of a mixture of trypsin, chymotrypsin A_α and chymotrypsin B (standard) on BBI-Sepharose 4B column. About 14 mg of each enzyme were dissolved in 8 ml of starting buffer and applied to the column (8 × 1.5 cm). Inactive protein was eluted from the column with the starting buffer (a), the adsorbed enzymes were eluted first with 10^{-2} *M* HCl containing 0.5 *M* NaCl (b) followed by 10^{-2} *M* HCl containing no NaCl (c). Fractions of 5 ml were collected. The flow-rate was about 60 ml/h. ●—●, A_{280}; ×—×, chymotrypsin activity; ○—○, trypsin activity.

RESULTS AND DISCUSSION

The finding that the complex of chymotrypsin B with immobilized Bowman–Birk inhibitor could be dissociated only under acidic conditions at low ionic strength has been used for the separation of this enzyme from a mixture of other pancreatic proteinases. The elution profile obtained after chromatography of a mixture of trypsin, chymotrypsin A_α and chymotrypsin B on immobilized BBI is shown in Fig. 1. Non-proteinase material was washed out with starting buffer (peak I). Bound trypsin and chymotrypsin A_α emerged together as a single peak (II) when eluted with 10^{-2} *M* hydrochloric acid containing 0.5 *M* sodium chloride. In contrast, chymotrypsin B appeared to be retarded and could be eluted by means of 10^{-2} *M* hydrochloric acid containing no sodium chloride (peak III).

Similar results were obtained using immobilized LBI which belongs to the same family as the BBI inhibitor. An attempt to apply immobilized BPTI or STI (inhibitors belonging to two other families) was without success, since all three enzymes were eluted with 10^{-2} *M* hydrochloric acid containing 0.5 *M* sodium chloride as a single peak.

The results presented indicated the possibility of a significant improvement in the chymotrypsin B purification procedure. An example of the use of immobilized Bowman–Birk inhibitor for chymotrypsin B purification is presented in Fig. 2. The crude preparation of chymotrypsinogen B after activation with trypsin was applied to the Bowman–Birk inhibitor–Sepharose 4B column. One millilitre of Sepharose 4B gel containing 17 mg bound inhibitor was capable of coupling as much as 8.3 mg of

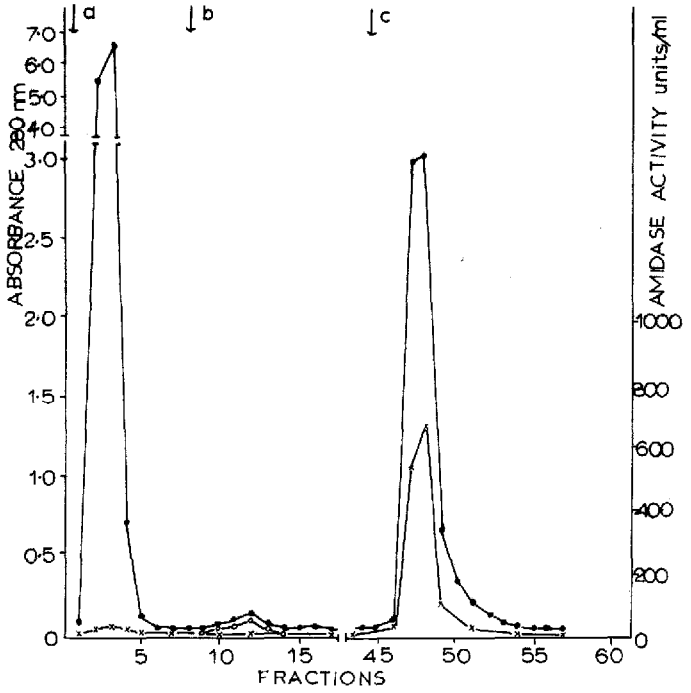

Fig. 2. Chromatography of crude chymotrypsinogen B preparation, after activation by trypsin on immobilized BBI. 20 ml of trypsin-activated mixture were applied to the column (8 × 1.5 cm). For details see Fig. 1. ●—●, A_{280}; ×—×, chymotrypsin activity; ○—○, trypsin activity.

chymotrypsin B. Non-bound material was removed with starting buffer. Both trypsin used for chymotrypsinogen B activation and chymotrypsin A_α present in the preparation were eluted with 10^{-2} *M* hydrochloric acid containing 0.5 *M* sodium chloride. To remove all traces of chymotrypsin A_α, the elution must be continued until the absorbance at 280 nm drops below 0.02. Then chymotrypsin B was eluted by means of 10^{-2} *M* hydrochloric acid containing no sodium chloride, and stored frozen or lyophilized after being dialyzed against 10^{-3} *M* hydrochloric acid. From 100 g of fresh bovine pancreas, 40–45 mg of chymotrypsin B, essentially free of trypsin, chymotrypsin A_α and deoxyribonuclease were obtained.

TABLE I

ESTERASE, AMIDASE AND PROTEOLYTIC ACTIVITIES OF CHYMOTRYPSINS

Chymotrypsin preparation	*Activity (units/mg of protein)*		
	Esterase	*Amidase*	*Caseinolytic*
B, purified according to ref. 3	201.25	321.42	6.02
B, purified on immobilized BBI	210.69	303.03	6.02
A_α*	81.76	100.00	6.09

* Additionally purified on immobilized STI.

TABLE II

AMOUNTS (μg) OF INHIBITORS REQUIRED FOR 50% INHIBITION OF AMIDASE ACTIVITY OF 1 μg DIFFERENT CHYMOTRYPSIN PREPARATIONS

Inhibitor	*Enzyme*		
	Chymotrypsin B purified according to ref. 3	*Chymotrypsin B purified on immobilized BBI*	*Chymotrypsin A_α**
BPTI	23.30	23.50	0.25
BBI	0.22	0.22	0.30
STI	0.70	0.71	1.10

* See Table I.

TABLE III

AMINO ACID COMPOSITION OF BOVINE CHYMOTRYPSIN B

Amino acid	*Residues (mol/mol)*	
	Preparation obtained in this work	*According to Parkes and Smillie*[19]
Lys	10.9	11.0
His	2.0	2.0
Arg	4.8	4.9
Asp	19.8	20.0
Thr	22.1	21.5
Ser	20.0	20.0
Glu	18.0	18.0
Pro	14.5	13.0
Gly	22.6	22.0
Ala	22.7	22.0
1/2 Cys	7.6	9.7
Val	24.1	24.1
Met	3.6	3.7
Ile	8.1	8.3
Leu	18.5	17.7
Tyr	3.4	2.9
Phe	7.0	7.0
Trp	8.5	8.0*

* Data of Smillie *et al.*[20].

Some properties of isolated chymotrypsin B were compared with those of a chymotrypsin B preparation obtained by the method of Guy *et al.*[3] and with chymotrypsin A_α (Tables I and II). Both preparations of chymotrypsin B show similar esterolytic, amidase and caseinolytic activities and inhibitors like STI, BPTI and BBI inhibit them to the same extent. In contrast, chymotrypsin A_α shows a three-fold lower esterolytic and amidase activity and a ten-fold lower amount of BPTI causes 50% inhibition. The data presented here and the amino acid composition (Table III) of chymotrypsin B isolated in the course of this work showed that this protein was identical with chymotrypsin B separated by other methods. The lower amount of half-cystine is probably due to incomplete oxidation of half-cystine to cysteic acid.

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