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Isolation of Trypsin from Bovine Pancreas Using Immobilized Benzamidine and Peptide CTPR Ligands in Expanded Beds

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Abstract: Peptide CTPR and p-amino benzamidine (PAB) immobilized on Streamline™ were utilized as the chromatographic matrices for trypsin purification from bovine pancreas. By using a clarified pancreas extract, maximum capacity for CTPR-Streamline was 47.4 mg/mL and for PAB-Streamline 78.9 mg/mL while Kd values were 0.39 and 0.38 respectively. Dynamic capacity was 23.0 and 46.0 mg/mL for CTPR- and PAB-Streamline respectively. When the purification process was applied to unclarified pancreas extract in the expanded-bed adsorption mode, 80% trypsin recovery with a purification factor of 18.7 was achieved. Cationic and anionic trypsin obtained from the affinity column were separated by ion-exchange chromatography.

Keywords: Affinity, expanded bed, p-aminobenzamidine, peptide, trypsin

INTRODUCTION

Trypsin is a pancreatic serine protease with substrate specificity based on its positively charged lysine and arginine side chains. Its recovery and

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purification from pancreas is of industrial importance. Partially purified preparations of trypsin are used as a meat tenderizer and in the leather industry. With stringent purity criteria, it is used as a digestive aid. A high-purity product is utilized in the cell culture industry, in proteomics and in the preparation of immunoglobulin fragments. Owing to the proneness of trypsin to undergo autodigestion during purification (1), the development of rapid and easy-to-scale-up purification methods is of special interest.

Conventional isolation and purification of proteins from mammalian tissue homogenates involves a complex sequence of clarification, recovery, and purification steps, thus resulting in a significant loss of the desired product. Expanded bed adsorption (EBA) with affinity matrices combines clarification, coarse, and fine purification in only one step. The drastic reduction in the overall number of steps leads to a higher product yield, shorter process time and more favourable process economy (2, 3).

Various inhibitors and substrate analogues have been used as ligands for trypsin purification by affinity chromatography. Short competitive inhibitors are more advantageous than larger protein inhibitors since they are less prone to biodegradation and can be manufactured aseptically in large quantities under good manufacture practices at a low cost. Synthetic benzamidine derivates are attractive as they are chemically stable and behave as specific inhibitors for several serine proteases (4, 5). Hixson and Nishikawa (4) employed *m*-aminobenzamidine-agarose for bovine trypsin purification. Makriyannis and Clonis (6) utilized a tripeptide ligand (Thr-Pro-Arg-OH) immobilized on Ultrogel A6R.

In this paper we report a method for trypsin affinity purification from a bovine pancreas homogenate using the peptide amide Cys-Thr-Pro-Arg-NH_2 (CTPR) or p-aminobenzamidine (PAB) immobilized on a particulate matrix suited for expanded bed adsorption techniques (Streamline^{TM}).

MATERIALS AND METHODS

Materials

N-benzoyl-L-arginine-ethyl ester hydrochloride (BAEE), N-benzoyl-L-tyrosine-ethyl ester (BTEE), N,N′disuccinimidyl carbonate (DSC), 6-aminocaproic acid (6-AC), bovine trypsin (SA 10,980 BAEE U/mg), bovine chymotrypsin (SA 50 BTEE U/mg), p-aminobenzamidine (PAB), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) were from Sigma-Aldrich (St. Louis, MO, USA). Fluorenylmethyloxy amino acids (Fmoc-amino acids) and Rink-Amide-MBHA resin were from Peptides International (Louisville, KY, USA). S-Sepharose, Sepharose 4B, and Streamline AC were from Amersham Biosciences (Uppsala, Sweden). All other reagents were AR grade.

Peptide Synthesis

The CTPR peptide was prepared by Fmoc solid-phase chemistry as described by Chan and White (7).

Peptide analysis was carried out by HPLC and MALDI mass spectrometry.

Ligand Immobilization

Streamline AC was activated with DSC as per Wilchek and Miron (8). 6-AC was used as the spacer, and peptide CTPR and PAB were immobilized using the EDAC-mediated bond formation as described by Hermanson et al. (9).

Enzyme and Protein Assays

Trypsin Assay

A 0.2 ml enzyme solution was added to a 3.0 mL 0.5 mM BAEE in 50 mM Tris-HCl buffer, pH 7.6, 10 mM $CaCl_2$. One BAEE unit of trypsin is defined as the amount producing a 0.001/min absorbance increase at 253 nm, at pH 7.6 and 25°C.

Chymotrypsin Assay

A 0.2 mL enzyme solution was added to a 3.0 mL 0.5 mM BTEE in 50 mM Tris-HCl buffer, pH 7.8, 10 mM $CaCl_2$. One BTEE unit of chymotrypsin is defined as the amount of enzyme required to hydrolyze 1 μmol of BTEE/min. The molar absorption coefficient of BTEE at 256 nm was taken as $964\,M^{-1}cm^{-1}$.

Pure trypsin concentration was determined by its absorbance at 280 nm using pure trypsin as the standard ($E1\%$ 14.3 at 280 nm).

Protein concentration was measured by the Bradford's method (10).

SDS-PAGE and Isoelectric Focusing

SDS-PAGE was performed as per Laemmli (11) and stained with Coomassie Blue or silver nitrate following the standard procedure.

3–9 isoelectric focusing was performed with PhastSystem™ as indicated by the manufacturer (Amersham Biosciences), and stained with silver nitrate according to the standard procedure.

Preparation of Bovine Pancreas Crude Extract

It was prepared from fresh tissue as described by Clonis et al. (12). The tissue (350 g) was cut into small pieces, homogenized with 700 mL 100 mM sodium acetate buffer, pH 4.0, and left overnight under stirring at 4°C. Proteolytic activation of the extract was effected by adding $CaCl_2$ up to 30 mM and 14 mg pure trypsin. pH was adjusted to 8.0 with Tris and NaOH to obtain a 500 mM Tris-acetate buffer, the extract was then incubated at 25°C under shaking for 3 h and filtered through cheese-cloth. The final volume of the extract was 1300 mL and its trypsin activity was 9600 BAEE units/mL.

Adsorption Isotherm Measurement

Adsorption isotherms for trypsin binding to the affinity adsorbents were measured as described by Chase (13). Values for the dissociation constant (K_d) and the maximum adsorption capacity (q_m) were calculated as per Chase (13) and are given as the mean $\pm$ SE.

Breakthrough Curves in Packed Bed

They were measured in experiments where pancreas extract clarified by centrifugation (10000 $\times$ g) and filtration (0.22 μm membrane) was pumped through packed columns of CTPR-Streamline or PAB-Streamline. The eluate was monitored for trypsin activity in all the 1-mL fractions collected. The adsorbent dynamic capacity was measured at 10% breakthrough.

Chromatography in Expanded-bed PAB-Streamline

Expanded bed adsorption was performed at 190 cm/h fluid velocity. 1092 mL unclarified bovine pancreas crude extract (9600 BAEE trypsin units/mL and 20% biomass content, wet weight) was the sample. After adsorption, the bed was allowed to settle and elution was performed with 10 mM $CaCl_2$ in 100 mM acetic acid in the fixed-bed mode at 110 cm/h fluid velocity.

Cationic and Anionic Trypsin Fractionation by Ion-exchange Chromatography

Cationic and anionic bovine trypsin were separated in a 3-mL packed-bed S-Sepharose column equilibrated with 20 mM Tris-HCl buffer, pH 5.0, 10 mM $CaCl_2$ (adsorption buffer). The elution was accomplished by

addition of 1 M NaCl to the adsorption buffer. Fractions of 2 mL were collected and their absorbance at 280 nm and enzyme activity was measured. Pass-through and eluted fractions were analyzed by isoelectric focusing.

RESULTS AND DISCUSSION

Peptide Synthesis

Makriyannis and Clonis (6) reported that the peptide TPR-OH bound to agarose was an effective affinity matrix for trypsin purification. In this work, the peptide amide was synthesized to prevent peptide polymerization during EDC-mediated coupling and a cysteine residue was added to the N-terminus to allow the peptide to be coupled only through the side-chain sulphydryl group, this enhancing its orientation on the support.

Adsorption Isotherms

Figure 1 shows the adsorption isotherms for trypsin binding to PAB-Streamline and CTPR-Streamline, at 4°C. A Langmuir-type isotherm was obtained in both cases. Maximum capacity for trypsin was 47.4 ± 1.0 mg/mL for CTPR-Streamline and 78.9 ± 4.9 mg/mL for PAB-Streamline while K_d values were 0.39 ± 0.04 mg/mL and 0.38 ± 0.09 mg/mL respectively.

Breakthrough Curves

In order to estimate the dynamic capacity of the CTPR-Streamline and PAB-Streamline for trypsin, breakthrough curves were first measured with clarified pancreas extract in packed-column systems. Loading clarified fluid on a packed bed accurately predicts the dynamic capacity of the expanded bed loaded with an unclarified sample. This significantly decreases the experimental complexity associated with running an expanded bed (14), (15). Figure 2 shows breakthrough curves obtained at two different fluid velocities. A fluid velocity decrease from 190 to 100 cm/h brought about an increase in curve sharpness and column dynamic capacity due to its impact on mass transfer. Fluid velocities of 50 and 75 cm/h did not result in higher dynamic capacities. Moreover, increasing residence time by decreasing fluid velocity had a negative effect on bed stability and lesser expansion (Table 1). As trypsin concentration was determined through its enzymatic activity, only the active enzyme was measured. SDS-PAGE of the product obtained at low fluid velocities did not show bands of low molecular weight, even after the sample treatment with dithiotreitol.

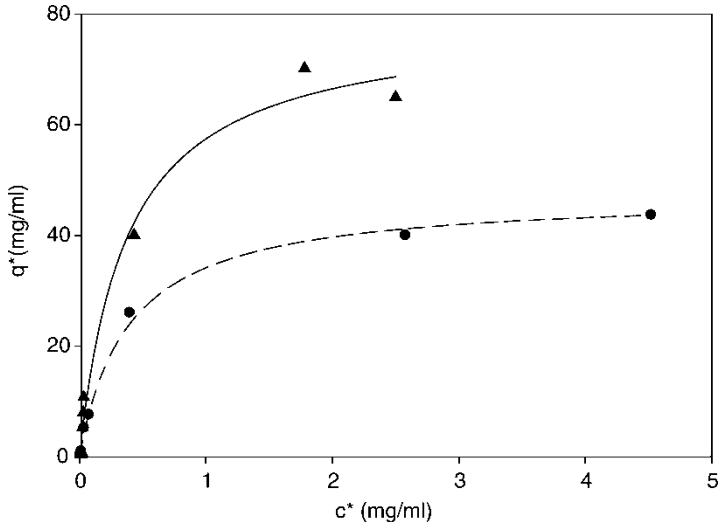

Figure 1. Equilibrium adsorption isotherms for the binding of trypsin to CTPR- and PAB-Streamline. Adsorption buffers were 100 mM sodium acetate, pH 5.5, 10 mM $CaCl_2$, and 50 mM Tris-acetate, pH 8.0, 100 mM NaCl, 10 mM $CaCl_2$, for CTPR- and PAB-Streamline respectively. 0.2 mL of a 50% suspension of the adsorbent in adsorption buffer was added to tubes containing increasing amounts of bovine trypsin in a final volume of 1 mL. The suspension was gently stirred and trypsin activity at equilibrium (c^*) was assayed in the supernatant after a 24-h incubation. The equilibrium concentration of trypsin bound to the matrix per unit of total adsorbent volume (q^*) was calculated as the total amount of trypsin present at the beginning of the experiment less the amount still in the soluble phase at equilibrium. (▲) PAB-Streamline, (●) CTPR-Streamline.

Dynamic capacity for trypsin adsorption to CTPR-Streamline from clarified pancreas extract was 27 mg/mL and 23 mg/mL at 100 and 190 cm/h respectively, and that for adsorption to PAB-Streamline was 69 mg/mL and 46 mg/mL at 100 and 190 cm/h respectively. In both cases, dynamic capacity at a 190 cm/h fluid velocity is approximately 50% of the maximum capacity for trypsin obtained from the isotherm.

As dynamic capacity of PAB-Streamline at 190 cm/h fluid velocity is 100% higher than that of CTPR-Streamline, PAB-Streamline was utilized for further experiments.

Bovine Trypsin Purification from an Unclarified Pancreas Homogenate by Expanded-bed Chromatography with PAB-Streamline

The process was carried out at room temperature and fractions collected were kept in ice. Fluid velocity during equilibration, sample load and wash was

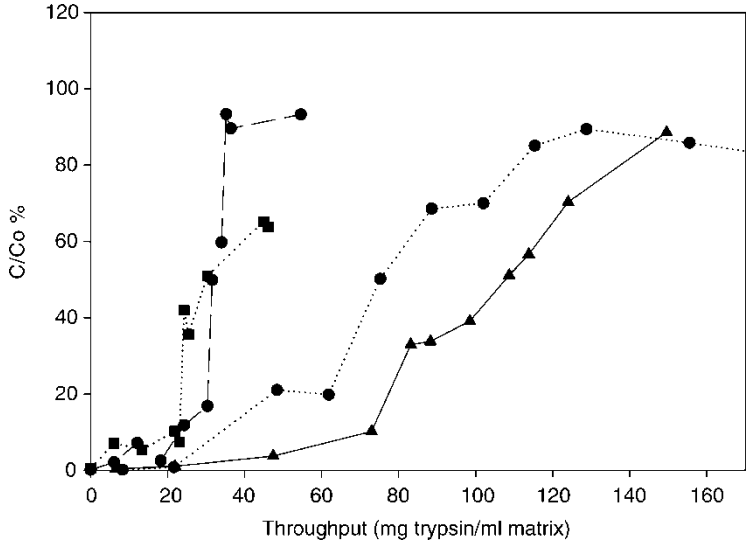

Figure 2. Breakthrough curves for PAB- and CTPR-Streamline in packed beds. Clarified bovine pancreas extract was pumped into packed columns (1.0 × 2.6 cm) of PAB- or CTPR-Streamline. Adsorption buffers were 100 mM sodium acetate, pH 5.5, 10 mM $CaCl_2$ and 50 mM Tris-acetate, pH 8.0, 10 mM $CaCl_2$, for CTPR- and PAB-Streamline respectively. The outlet of the column was monitored for trypsin activity in all the 1-mL fractions collected at fluid velocities of 100 and 190 cm/h. (···●···) CTPR-Streamline, 100 cm/h, (■) CTPR-Streamline, 190 cm/h, (▲) PAB-Streamline, 100 cm/h, (– – –●– – –) PAB-Streamline, 190 cm/h.

190 cm/h. Despite the fact that fluid velocity increase brought about a decrease in column dynamic capacity, this high speed was utilized to reduce sample application time. The expansion degree (ratio of expanded bed height to settle-bed height) was 2.0 during equilibration and increased

Table 1. Bed expansion and bed stability of PAB-Streamline and CTPR-Streamline at different pancreas crude extract velocities

Chromatographic matrix	Fluid velocity (cm/h)	Bed expansion (h/h_0)	Bed stability (visual inspection)
PAB-Streamline	50	1.30	Not acceptable
PAB-Streamline	75	1.46	Acceptable
PAB-Streamline	100	1.67	Acceptable
PAB-Streamline	190	2.60	Acceptable
CTPR-Streamline	50	1.26	Not acceptable
CTPR-Streamline	75	1.47	Acceptable
CTPR-Streamline	100	1.61	Acceptable
CTPR-Streamline	190	2.48	Acceptable

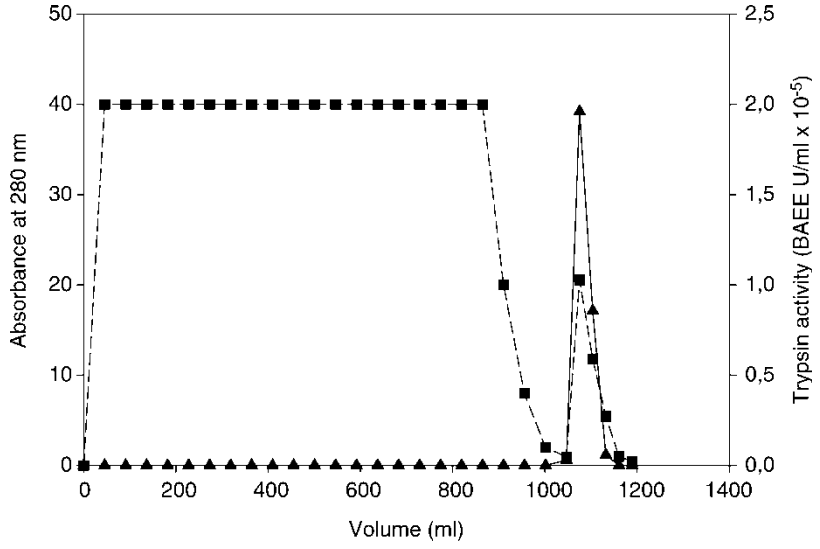

Figure 3. Trypsin purification from unclarified bovine pancreas homogenate by expanded bed adsorption. It was performed in a 2 cm inner diameter column. 40 mL PAB-Streamline was filled into the column and equilibrated in an upward flow with 50 mM Tris-acetate, pH 8.0, 10 mM $CaCl_2$, (adsorption buffer) at a 190 cm/h fluid velocity. 1092 mL unclarified bovine pancreas crude extract was loaded and the column was washed with adsorption buffer until there were no more particles and protein in the effluent. The bed was then allowed to settle and trypsin elution was performed in the fixed-bed mode with 10 mM $CaCl_2$ in 100 mM acetic acid, at a 110 cm/h fluid velocity. Trypsin activity (▲) and absorbance at 280 nm (■) were measured in all the 35-mL fractions collected.

to 2.6 when the high viscous crude feed was pumped into the column. Figure 3 shows the corresponding chromatographic pattern. The purification factor was 18.7 and the yield 80%. Fluid velocities over 190 cm/h caused overexpansion and adsorbent beads packed tightly against the adapter net.

SDS-PAGE and IEF

SDS-PAGE analysis shows the high effectiveness of the method for trypsin purification developed (Figs. 4A and 4B). IEF analysis of the fractions eluted from those columns showed two bands corresponding to the anionic (pI = 4.9) and cationic (pI = 8.5) trypsin (not shown).

Cationic and anionic bovine trypsin eluted from CTPR and PAB-Streamline columns were fractionated by ion-exchange chromatography on S-Sepharose. The pass-through and eluted fractions obtained with 1 M NaCl displayed trypsin activity. Analysis by IEF evidenced that the

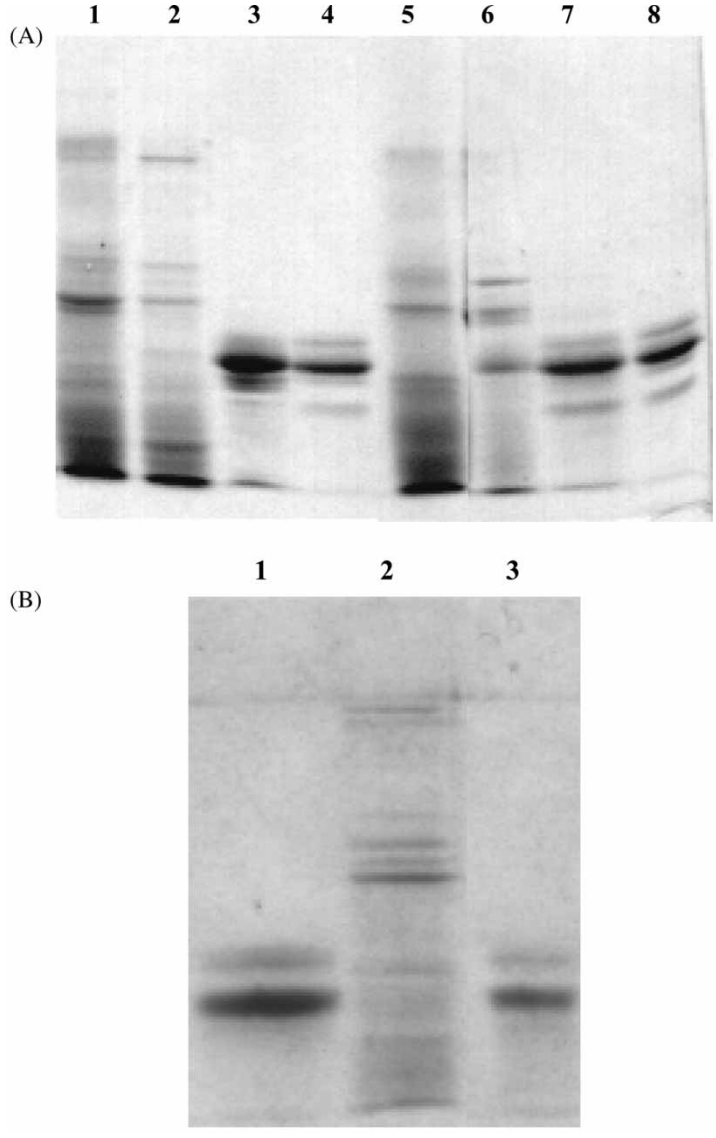

Figure 4. PAGE-SDS of trypsin fractionation. A) PAGE-SDS of the clarified crude extract fractionation with PAB-Streamline and CTPR-Streamline in the packed-bed adsorption mode: PAB-Streamline: lane 1, clarified bovine pancreas extract; lane 2, pass-through fraction; lane 3, eluted fraction; lane 4, bovine trypsin standard. CTPR-Streamline: lane 5, clarified bovine pancreas extract; lane 6, pass-through fraction; lane 7, eluted fraction; lane 8, bovine trypsin standard. B) PAGE-SDS of the unclarified crude extract fractionation with PAB-Streamline in the expanded-bed adsorption mode: lane 1, bovine trypsin standard; lane 2, unclarified bovine pancreas extract; lane 3, eluted fraction.

pass-through and eluted fractions corresponded to anionic and cationic trypsin respectively.

CONCLUSIONS

Streamline with PAB or peptide CTPR as trypsin ligands can be used for affinity purification of bovine trypsin from pancreas extracts in the expanded-bed adsorption mode. PAB-Streamline in the expanded bed adsorption mode allows a trypsin recovery of 80% with a purification factor of 18.7. The method combines clarification, capture, and purification in a single step thus reducing protein purification costs.

ABBREVIATIONS

PAB	p-aminobenzamidine
CTPR	peptide Cys-Thr-Pro-Arg
EBA	expanded bed adsorption
BAEE	N-benzoyl-L-arginine-ethyl ester hydrochloride
BTEE	N-benzoyl-L-tyrosine-ethyl ester
6-AC	6-aminocaproic acid
EDAC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide

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