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AFFINITY CHROMATOGRAPHY OF RECOMBINANT *RHIZOMUCOR MIEHEI* ASPARTIC PROTEINASE ON Si-300 BACITRACIN

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SUMMARY

An high-performance affinity chromatography column was made by activation of coated silica Si-300 polyol with tresyl chloride and coupling it with the antibiotic bacitracin. The column was used to purify recombinant *Rhizomucor miehei* aspartic proteinase.

INTRODUCTION

Isolation of proteinases by affinity chromatography is a rapid and selective chromatographic procedure. The peptide antibiotics bacitracin, bacilliquin and gramicidin S have shown to be suitable for such methods both on soft gels (Sepharose)[1] and on silica-based materials such as "Silochrom"[2]. Especially when working with endoproteinases, which are autoinactivated[3,4], affinity chromatography is of great value. The matrix used most often is agarose[5] in the form of Sepharose or its cross-linked forms, but other forms of entirely synthetic gel materials containing hydroxyl groups are available. We used a coated silica material Si-300 polyol, which is excellent for rapid purification processes. The matrix can be activated with cyanogen bromide or its less harzardous variants[6], the epoxy, aldehyde, vinyl sulphone, carbonylimidazole activation[5], or the recently introduced activation reagents tresyl chloride[7] and fluoro-1-methylpyridinium toluene-4-sulphonate (FMP)[8],

We describe the construction of an high-performance affinity chromatography column coupled with bacitracin by the tresyl chloride method. By use of this column, recombinant *Rhizomucor miehei* aspartic proteinase was purified.

MATERIALS AND METHODS

Reagents

Si-300 (30 μm) polyol was from Serva (Heidelberg, F.R.G.), tresyl chloride (2,2,2-trifluoroethanesulphonyl chloride) from Fluka (Buchs, Switzerland) and bacitracin (zinc free) and Endo H from Sigma (St. Louis, MO, U.S.A.).

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Activation of resin

The Si-300 material was activated by the tresyl chloride method[1]. A 10-g amount of Si-300 polyol was washed three times with 100 ml acetone (dried with molecular sieve 4A, overnight). The moist gel was added to a dry beaker, containing 10 ml of dry acetone and 500 μl dry pyridine. With magnetic stirring, 400 μl tresyl chloride were added to the suspension. After 15 min at 0°C, the gel was washed with 100 ml of each of the following: acetone, 30, 50 and 70% of 5 m*M* HCl in acetone (v/v) and 1 m*M* HCl. The product was stored at +4°C until used.

Coupling with bacitracin

A 2-g amount of bacitracin was dissolved in 100 ml coupling buffer (0.2 *M* sodium phosphate +0.5 *M* NaCl, pH 8.2) and added to the activated Si-300 polyol, which had been briefly washed with cold coupling buffer. Coupling proceeded with gentle rotation on a Celloshaker (Chemetron, Italy) for 20 h at +4°C. The gel was treated with 0.2 *M* Tris (pH 8.5) for 5 h at room temperature and washed with 20 m*M* Tris–1 *M* NaCl–25% *n*-propanol and 20 m*M* Tris (pH 7.0). The slurry was poured into a steel column 12 cm × 1.6 cm (Knauer, Berlin, F.R.G.) and allowed to stand for 30 min. The column was then packed under pressure at a flow-rate of 8 ml/min in 20 m*M* Tris (pH 7.0).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE on gradient gels (7.5–20%) was performed essentially as described by Anderson *et al.*[9].

Sequence analysis

N-terminal sequence analysis of purified recombinant *Rhizomucor miehei* aspartic proteinase was carried out by automated Edman degradation, using an Applied Biosystems (Foster City, CA, U.S.A.) Model 470A gas-phase sequencer, and the phenylthiohydantoin (PTH)-amino acids were analysed by high-performance liquid chromatography (HPLC)[10].

Analysis of recombinant Rhizomucor miehei aspartic proteinase enzymatic activity

Clotting activity, determined as kilorennet units (KRU), was measured according to British Standard 3624:1963. One unit of milk-clotting activity is the amount of proteinase that clots 10 ml of reconstituted skim milk in 100 s at 30°C[11].

Clotting activity in column eluates was determined by a microtitre plate assay. An 100-μl volume of a diluted sample from each fraction was pipetted into a microtitre plate well. The diluting buffer (buffer D) was 50 m*M* 2-morpholinoethanesulphonic acid–15 m*M* $CaCl_2$–10 m*M* NaCl (pH 6.1). An 100-μl volume (0.5%) dried skim milk in buffer D was added, and the milk-clotting activity was followed by measuring the change in absorbance at 540 nm in a Perkin-Elmer (Norwalk, CT, U.S.A.) Lambda reader at 1-min intervals.

HPLC

HPLC was performed with a Series 4 Perkin-Elmer HPLC pump, an LC95 detector and a PE7700 data controller. The columns were an Altex Spherogel TSK G3000SWG, 600 mm × 21.5 mm (Beckman Instruments, Berkeley, CA, U.S.A.) and

the bacitracin affinity column described in Materials and Methods. Proteins were detected at 280 nm. Buffers: A, 0.1 *M* sodium acetate (pH 4.5); B, 0.1 *M* sodium acetate–1 *M* NaCl–25% *n*-propanol (pH 4.5); C, 50 m*M* 2-morpholinoethanesulphonic acid (pH 6.1).

RESULTS AND DISCUSSION

Purification and characterization of rRMP

Aspargilus oryzae was transformed with *Rhizomucor miehei* aspartic proteinase and grown as described by Christensen *et al.*[12]. Recombinant *Rhizomucor miehei* aspartic proteinase was recovered from the spent culture medium. The amount of extracellular recombinant *Rhizomucor miehei* aspartic proteinase was measured by clot analysis to be 3.3 g/l. *A. oryzae* culture supernatant was dialysed against distilled water and lyophilized. The recombinant *Rhizomucor miehei* aspartic proteinase enzyme was purified by affinity chromatography on a Si-300 HPLC bacitracin column (Fig. 1). The sample of 317 mg lyophilized culture supernant, dissolved in 30 ml buffer A, was applied to the affinity column (12 cm × 1.6 cm), equilibrated in buffer A at a flow-rate of 5 ml/min. Non-adsorbed material passed through the column with buffer A. Then proteinase was eluted with buffer B. The fractions containing the active enzyme were desalted on a G-25 column (Pharmacia, Uppsala, Sweden) equilibrated in 10 m*M* ammonium bicarbonate and lyophilized. The final purification procedure consisted of size-exclusion chromatography on a TSK G3000SWG column (60 cm × 2.15 cm), equipped with a precolumn TSK-GSWGP (7.5 cm × 2.15 cm) (Fig. 2). Aliqouts of 1.5 ml were loaded on the column equilibrated with buffer C. A flow-rate of 5 ml/min and fraction size of 5 ml were used.

TABLE I

PURIFICATION OF RECOMBINANT *RHIZOMUCOR MIEHEI* ASPARTIC PROTEINASE

Step	*Amount (ml)*	*Specific acitivity (KRU/g)*	*Total activity (KRU)*	*Units/Abs. 280 nm*	*Purification factor*
Supernatant	30	16.5	496	0.54	1.0
Bacitracin affinity chromatography	15	31.0	465	4.35	8.1
Size-exclusion chromatography	50	9.1	452	5.72	10.6

Dialysis and lyophilization of the active fractions from size-exclusion chromatography resulted in a product with a specific activity of 4950 KRU/g. The apparent molecular weight of recombinant *Rhizomucor miehei* aspartic proteinase determined by SDS-PAGE was slightly larger than that obtained for *Rhizomucor miehei* aspartic proteinase[13] (Fig. 3). After treatment of both recombinant *Rhizomucor miehei* aspartic proteinase and *Rhizomucor miehei* aspartic proteinase with Endo H, the apparent molecular weights, determined by SDS-PAGE, were identical (data not shown). *Rhizomucor miehei* aspartic proteinase is reported to be either homogeneous with one N-terminal Ala-Ala-Ala-Asp-Gly-Ser-[14] or heterogeneous, with 3 N-terminals,

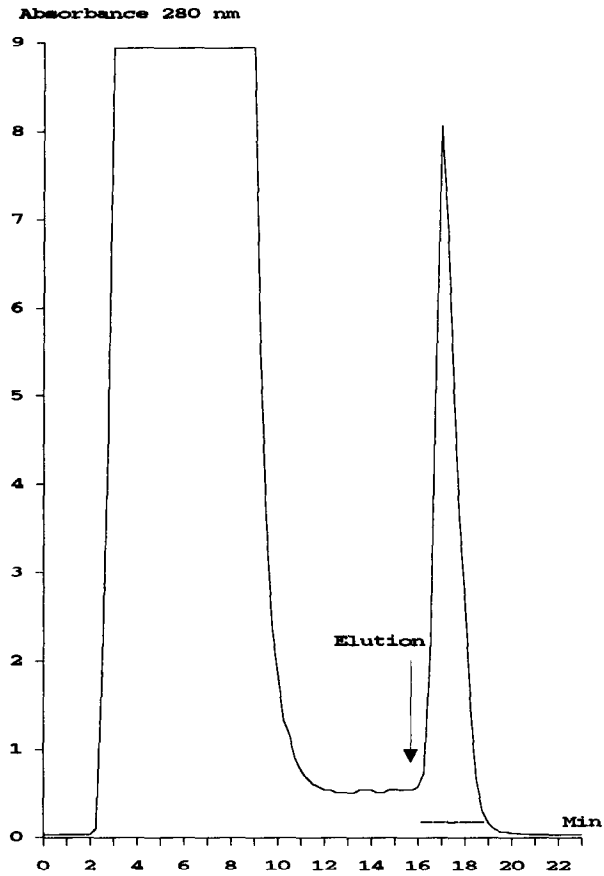

Fig. 1. Bacitracin affinity chromatography of the culture supernatant of *A. oryzae*. The sample (30 ml) was applied to the affinity column (12 cm × 1.6 cm), equilibrated in 0.1 *M* sodium acetate (pH 4.5) at a flow-rate of 5 ml/min. Non-adsorbed material passed through the column with 0.1 *M* sodium acetate (pH 4.5). Then proteinase was eluted with 0.1 *M* sodium acetate–1 *M* NaCl–25% *n*-propanol (pH 4.5). Proteinase-active fractions were packed as indicated by the horizontal bar. Proteins were detected at 280 nm.

Ala-Ala-Ala-Asp-Gly-Ser-, Gly-Ser- or Asp-Gly-Ser-[15]. We found the N-terminal sequence for recombinant *Rhizomucor miehei* aspartic proteinase determined by gas-phase sequencing on an Applied Biosystems gas-phase sequenator to be heterogeneous and consisting of two N-terminals in equal amounts: Ala-Ala-Asp-Gly-Ser- and Gly-Ser-. These differences may represent differently processed forms either during maturation or during purification.

The combination of a rigid column material and an efficient activation method with the proper ligand gives an affinity column that can be used for the rapid isolation of heterologously expressed enzymes, which otherwise would be difficult to separate from host proteins, *e.g.*, similar isoelectric point and molecular weight of a host protein.

The rigid column material is an advantage when working with organism such as

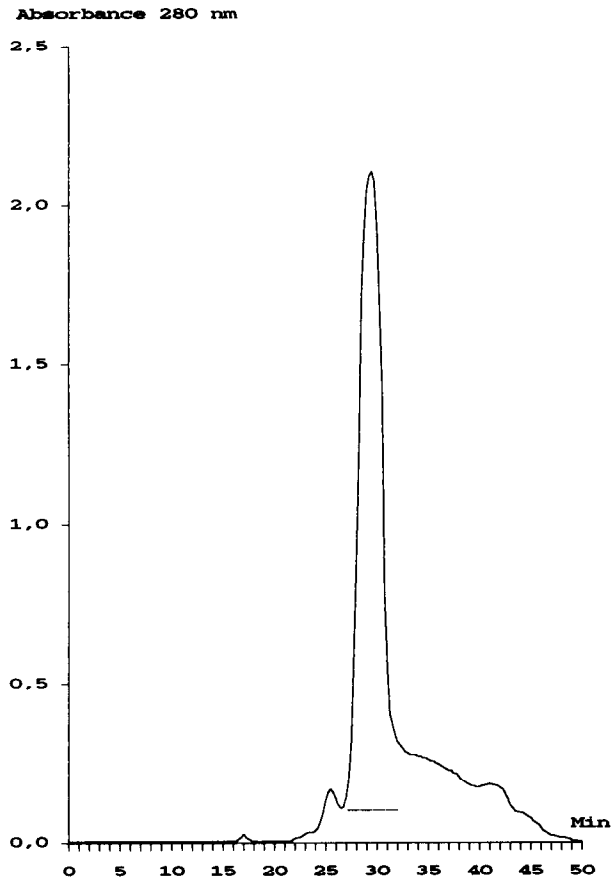

Fig. 2. Separation of recombinant *Rhizomucor miehei* aspartic proteinase from bacitracin affinity chromatography on a TSK G3000SWG size-exclusion column. The lyophilized material from bacitracin affinity chromatography was dissolved in 3 ml 50 m*M* 2-morpholinoethanesulphonic acid (pH 6.1) and the recombinant *Rhizomucor miehei* aspartic proteinase was purified by repeated chromatography on the size-exclusion column (TSK G3000SWG, 600 mm × 21.5 mm). Sample size: 1.5 ml. Proteinase-active fractions were pooled as indicated by the horizontal bar. Proteins were detected at 280 nm.

Aspergillus capable of producing Sepharose-degrading enzymes[1]. The efficiency of the affinity chromatography step is comparable to Sepharose-bacitracin[1] and Silochrom-bacitracin[1]. We did get a purification factor of 8 relative to the starting material (Table I). The binding capacity for the recombinant *Rhizomucor miehei* aspartic proteinase is *ca.* 20 mg/ml gel material.

Of special importance is the very rapid separation obtained. The enzymes are eluted rapidly and with no tailing, which implies that there is no non-specific binding to the column. Elution of enzyme can be performed using 1 *M* NaCl and *n*-propanol (20–30%). The higher the *n*-propanol content, the better is the elution.

Another important ligand for affinity chromatography of aspartic proteinases is pepstatin. This has been used for isolation of both microbial[16] and mammalian[17]

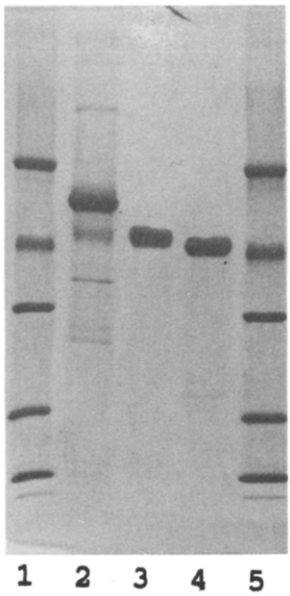

Fig. 3. SDS-PAGE of recombinant *Rhizomucor miehei* aspartic proteinase. Lanes: 1 and 5, standard proteins; MW 67000, 43000, 30000, 20000 and 14400; 2, *A. oryzae* culture supernatant; 3, purified recombinant *Rhizomucor miehei* aspartic proteinase; 4, *Rhizomucor miehei* aspartic proteinase.

aspartic proteinases. Pepstatin binds tightly to proteinases and it may be necessary to use up to 6 *M* urea to elute the enzyme[17]. The binding, however, is dependent on the type of pepstatin used, and it seems that N-acetylpepstatin may be used for isolation of microbial milk-clotting enzymes[16], although the elution volume is much larger than when using bacitracin as a ligand.

We find the tresyl chloride method to be easy and safe for activation of silica-based column material. Coupling with bacitracin gives an HPLC affinity column which is superior to conventional soft gel affinity chromatography. The use of bacitracin as a ligand, relative to pepstatin, also makes it possible to use the column to purify proteolytic enzymes other than aspartic proteinases[1].

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