Rapid Purification of DesPro(2)-Val15-Leu17-Aprotinin from the Culture Broth of a Recombinant *Saccharomyces cerevisiae*

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Received February 25, 1993/Accepted July 27, 1993

A rapid two-step procedure has been developed for the purification of Despro(2)-Val15-Leu17-aprotinin from the culture supernatant of a recombinant yeast by affinity and ion-exchange chromatography. DesPro(2)-Val15-Leu17-aprotinin was purified to homogeneity, as demonstrated by dodecylsulfate gel electrophoresis and analysis of the N-terminal amino acid sequence. © 1993 John Wiley & Sons, Inc.
Key words: affinity chromatography • chymotrypsin Sepharose • DesPro(2)-Val15-Leu17-aprotinin • elastase inhibitor • protease inhibitor

INTRODUCTION

Aprotinin, also known as bovine pancreatic trypsin inhibitor, is a small protein with a molecular weight of 6.5 kDa. It consists of 58 amino acid residues in a single polypeptide chain, crosslinked by three disulphide bridges.[1]

Aprotinin inhibits, besides trypsin, a broad range of proteases.[6,9,11,16,17,21,23] Elastase is only weakly inhibited, however.[18]

The inhibition constant, K_i, of the aprotinin : elastase complex (K_i: $3.5 * 10^{-6}$ M) is considered too high for medical applications. The inhibition of human leukocyte elastase (HLE) is of considerable medical interest. The enzyme is believed to be normally involved in the intracellular degradation of proteins. Upon stimulation of the leukocyte, it is released into the plasma, where it is blocked normally by the α_1-proteinase inhibitor (α_1-PI). In case of septic shock or an inherited α_1-PI deficiency, the released HLE is no longer properly balanced, so that it can degrade connective tissue proteins such as elastin or proteoglycans, leading to many organic dysfunctions (edema, shock lung.)[14,15] One possible approach to limit such damages would be to decrease the level of active circulating HLE by administration of appropriate inhibitors, e.g., DesPro(2)-Val15-Leu17-aprotinin (rec. aprotinin), which is a modified recombinant aprotinin, a strong elastase inhibitor with a $K_i < 10^{-10}$ M and negligible inhibition of trypsin.[2–4,19,20,22] Because of the low molecular weight of the rec. aprotinin, clearance through the kidney is much better as for the α_1-PI. Rec. aprotinin can be produced by a recombinant strain of *Saccharomyces cerevisiae* and secreted into the culture medium. Here we describe a fast process designed for the purification of rec. aprotinin, taking advantage of the interaction with chymotrypsin to isolate the recombinant inhibitor by a simple two-step procedure from yeast culture supernatants.

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MATERIALS AND METHODS

Strains and Cultivation

The recombinant strain of *Saccharomyces cerevisiae* WHL 292 PS 7041 was obtained from Bayer AG, Wuppertal. The strain was maintained on agar (0.67% Bacto Yeast Nitrogen Base (Difco, Detroit, MI), 2% glucose at 4°C, and transferred monthly. The growth medium contained (per liter): 20 g Difco yeast extract, 1.4 g KH_2PO_4, 2 g $(NH_4)_2SO_4$, 0.25 g $MgSO_4 * 7\ H_2O$, 0.25 g $CaCl_2 * 2\ H_2O$, 0.1 mL Antifoam SAG 471 (Union Carbide, Düsseldorf, Germany). The initial fermentation medium was sterilized by heating at 121°C for at least 30 min. The final pH of the medium was adjusted to 5.5. The stirrer speed was set to 600 rpm, temperature 28°C, aeration rate 0.5 vvm, and pH was controlled at 5.5 by automatic addition of 2.5N NaOH and 2.5N H_2SO_4. Yeast extract (5 g/L) was fed in daily increments. In order to prevent growth inhibiting ethanol formation, the cultivation was performed under RQ control, keeping the respiratory quotient at a value of 1.0. This way the anaerobic fermentation of glucose (Crabtree effect[8]) could be prevented in favor of the respiration during an extended fed batch. The critical variable for RQ control was the flow rate for glucose addition. In order to achieve optimal control, the fermentor was equipped with an IBM-AT computer, PI control, a computer-controlled pump for glucose addition, measuring devices for O_2 (Magnos 4 G, Hartmann und Braun AG, Frankfurt, Germany), CO_2 concentrations (mass spectrometer Quadrex 100 Fc, Leybold AG, Köln, Germany), and a gas flow meter. MEAS software for on-line monitoring and evaluation was a gift from Ch. Wandrey (IBT 2 Forschungszentrum Jülich, Germany). At the end of cultivation, cells were separated by a steam sterilizable CSA 1 Westfalia separator (Westfalia, Oelde, Germany). The supernatant obtained was filtered in a Minitanfilter, equipped with 0.1-μm (VVLP) membranes (Millipore, Eschborn, Germany), and served as starting material.

CCC 0006-3592/93/01101331-06

Determination of Rec. Aprotinin

HLE was purchased from Elastin Products Co. (Pacific, MO). The concentration of rec. aprotinin was determined by inhibition studies. A fixed quantity of HLE (100 μL of a solution with a concentration of 1 μg/mL) was mixed with varying amounts of rec. aprotinin (100 μL) in 0.2 *M* Tris-HCl (pH 9) + 0.5 *M* NaCl + 0.05% Tween-80. After incubation for 30 min at 37°C, the enzyme reaction was started by addition of 100 μL 2.5 m*M* Methoxy-Succ-Ala-Ala-Pro-Val-pNA (Bachem, Heidelberg). The residual enzyme activity was determined following the release of *p*-nitroanilide (pNA) from the substrate at 405 nm in a spectrophotometer (Thermo Max microplate reader, Molecular Devices Corp., Menlo Park, CA). The assay was calibrated using known amounts of pure rec. aprotinin. Straight inhibition curves were obtained from which unknown inhibitor concentrations were read. Pure rec. aprotinin was a gift from Bayer AG (Wuppertal).

Purification of DesPro(2)-Val15-Leu17-Aprotinin

The pH of the filtered supernatant was adjusted to 8 with Tris base (at 4°C) and applied to a chymotrypsin Sepharose column equilibrated with a 20 m*M* Tris-HCl buffer pH 8, at a flow rate of 77 cm/h (referring to the geometric cross section). The column (5 cm * 6.1 cm) was washed with starting buffer to remove unbound protein, and the absorption of the column effluent was monitored at 280 nm. The rec. aprotinin was firmly bound to the affinity matrix. Elution was carried out with 30 m*M* citric acid buffer, pH 3, at a flow rate of 19 cm/h. The product-containing pool was further purified by cation-exchange chromatography (S-Sepharose, column 2.6 cm * 34 cm), equilibrated with citric acid buffer, pH 3.5. After adsorption of the sample, the column was rinsed with 12 column volumes of 20 m*M* Bicine-NaOH, pH 9, and five column volumes 30 m*M* citric acid, pH 3.5. Then elution was performed with six column volumes of a linear gradient of 0 to 1 *M* NaCl in the equilibration buffer. The flow rate during chromatography was 68 cm/h. The effluent was followed at 280 nm and collected in 2-mL fractions. Separated proteins were checked for N-terminal amino acid sequence (Protein Sequenator, Model 477A and HPLC 120 A, Applied Biosystems, Weiterstadt, Germany). Only fractions containing correctly processed DesPro(2)-Val15-Leu17-aprotinin were combined and submitted to further controls by SDS–polyacrylamide gel electrophoresis.

Electrophoretic Methods

SDS–polyacrylamide gel electrophoresis and silver staining was performed using the Phast System (Pharmacia-LKB, Freiburg, Germany) according to the instruction of the manufacturer for HD-Phastgels (Development Technique File No. 211).

Immobilization of Chymotrypsin on Sepharose

The activated matrices, EAH-Sepharose 4B, CNBr-Sepharose 4B, and Tresyl-Sepharose 4B, were purchased from Pharmacia LKB.

Chymotrypsin was obtained from Boehringer Mannheim, Germany. Coupling of chymotrypsin to the preactivated Sepharose 4B was carried out according to instructions (Pharmacia-LKB).

One gram of dry CNBr-Sepharose or Tresyl-Sepharose was swollen for 15 min in 1 m*M* HCl and washed with 200 mL 1 m*M* HCl on a glassintered funnel. Excess liquid was drained and the wet preactivated resin mixed with twice the volume of coupling buffer (0.1 *M* $NaHCO_3$, 0.5 *M* NaCl, pH 8.3) containing various amounts of chymotrypsin. The resulting suspension had a pH of 8.0 and was agitated on a rotary shaker at room temperature. After 2 h, glycine was added to a final concentration of 0.2 *M* and incubation continued for 2 h in order to quench reactive groups on the resin. Then the resin was washed sequentially with 200 mL coupling buffer; 200 mL 0.1 *M* sodium acetate; 200 mL 30 m*M* citric acid/NaOH, pH 3.0; 100 mL sodium acetate; 100 mL coupling buffer; and 50 mL starting buffer. EAH-Sepharose was suspended in 0.5 *M* NaCl. The swollen resin was washed with 200 mL 0.5 *M* NaCl on a glassintered funnel followed by 100 mL H_2O. Chymotrypsin was dissolved in water and titrated to a pH between 4.5 and 6.0. *N*-ethyl-*N*-(3-dimethylaminopropyl)-carbodiimide (EDC) was also dissolved in water and the pH adjusted to about 6. The drained gel was suspended in the chymotrypsin solution and mixed with the EDC solution (final concentration of EDC 0.1 *M*). The ratio of the fluid and gel was about 2 : 1. The suspension was agitated on a rotary shaker for 12 h at room temperature. Then the gel was separated and washed with 200 mL of 0.1 *M* sodium acetate. The resin was incubated for 5 h in the sodium acetate solution to quench reactive groups. Finally, the affinity matrix was washed as described above.

Breakthrough Curves

One hundred fifty milliliters of 70 m*M* Tris-HCl buffer, pH 8, containing 14.6 mg/L of pure rec. aprotinin was applied to a small affinity column (1 cm * 1.6 cm) at a linear flow rate of 0.13 cm/min. The effluent from the column was collected in 1-mL fractions and the content of rec. aprotinin determined as described above. An adsorption isotherm was obtained, plotting the adsorbed amount versus free solute concentration in the effluent.

RESULTS AND DISCUSSION

Selection of the First Processing Step

Rec. aprotinin was obtained in the culture supernatant at rather low concentrations, typically in the range of 12 to 15 μg/mL. The first aim, therefore, must be to concentrate the desired product. Because of the low mo-

lecular mass of rec. aprotinin (6.5 kDa), ultrafiltration was not the first choice; instead, adsorption processes were investigated. In order to combine the concentration with a purification step we looked for a suitable ligand for an affinity-based adsorption. Chymotrypsin was selected because it is readily available at reasonable prices and does not degrade the protease inhibitor. The dissociation constant of the rec. aprotinin: chymotrypsin complex was estimated from inhibition studies, titrating a known amount of chymotrypsin with increasing concentrations of pure rec. aprotinin. Figure 1 shows a plot for the determination of the apparent K_i value.

According to Bieth,[5] the following relations exist:

$$[I_0]/(1 - a) = K_i * 1/a + [E_0]$$

$[I_0]$ = inhibitor concentration used

$[E_0]$ = enzyme concentration used, here chymotrypsin concentration

a = relative remaining enzyme activity

$$K_i = [E] * [I]/[E]$$

Plotting $[I_0]/(1 - a)$ versus $1/a$, a straight line results with the slope $K_i = 2 * 10^{-7}$ (see Fig. 1). The y-intercept corresponds to the constant chymotrypsin concentration in the tests. Here the y-axis is crossed at 0.205 μM, and the value is nearly identical to the constant amount of chymotrypsin used in the experiments (0.2 μM), indicating that the evaluation is consistent.

The dissociation constant observed is low enough to expect that the rec. aprotinin will be captured in good yield from the culture supernatant, even considering the fact that immobilization of chymotrypsin may result in a lower binding efficiency.

Preparation and Evaluation of the Immobilized Chymotrypsin

Chymotrypsin was immobilized to agarose using three different chemistries: cyanogen bromide coupling (A) and tresyl coupling (B) were carried out at pH 8, and carbodiimide coupling (C) at around pH 6.

Excess culture supernatant was added to the gels and the binding capacity of rec. aprotinin per milliliter of gel determined. In preliminary experiments, we observed that matrix A adsorbed about twice as much rec. aprotinin as matrix B (0.22 μg/mL vs. 0.1 μg/mL), while adsorbing very little of the colored byproducts. In contrast, carbodiimide-activated EAH-Sepharose (matrix C) bound negligible amounts of rec. aprotinin but a major portion of the colored byproducts. The binding of chymotrypsin to CNBr-activated agarose was optimized, as summarized in Table I. About 90 mg chymotrypsin can be immobilized per gram (dry) gel = 3.75 mL, wet gel within about 1 h incubation at pH 8.3, as only 6 mg chymotrypsin could be immobilized on Tresyl-activated Sepharose (data not shown). Washed affinity gel, 1.25 mL, was then incubated with 80 mL of culture supernatant at 4°C with shaking. After 3 h, the resin was filled into a small column (1.0 cm diameter) and the rec. aprotinin concentrations determined in the flow through, the wash fraction (20 mM tris, pH 8.0), and the eluate (30 mM citrate buffer, pH 3.0). The results are shown in Table II. The binding capacity approached an optimum at around 90 mg ($\sim 3.6 * 10^{-6}$ mol). Increasing the ligand density threefold resulted in five-times-higher binding capacities for rec. aprotinin. This may be explained by the fact that the number of covalent bonds between chymotrypsin and the preactivated matrix decreases with higher ligand density, and also, the probability of unproductive ligand immobilization.

The mass balance for rec. aprotinin was very good. In a series of experiments, employing the same affinity matrix, six times the average recovery was 96% (σ = 7%). No trend could be observed in the yield during the repetitive uses. Elution at pH 3.0 was quantitative, but was found to be incomplete at pH 4.0 (~75% yield).

Breakthrough Curve and Adsorption Isotherm

Breakthrough curves were recorded in order to determine the amount of rec. aprotinin that could be adsorbed per unit

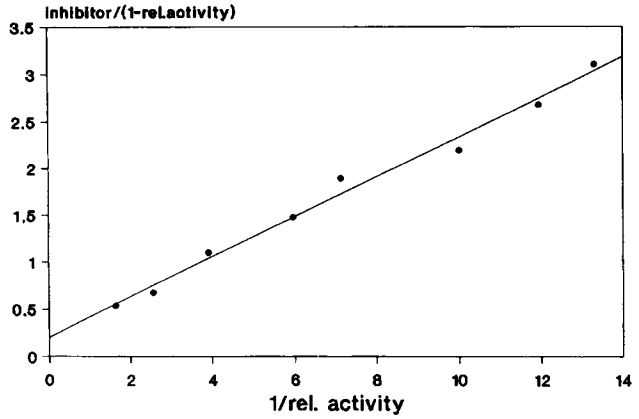

Figure 1. Determination of the apparent K_i value of the DesPro(2)-Val15-Leu17-aprotinin: chymotrypsin complex.

Table I. Optimization of chymotrypsin immobilization to CNBr-activated agarose.[a]

	Chymotrypsin in solution after:		
Chymotrypsin (mg/g dry gel)	30 min (mg/mL)	1 h (mg/mL)	2 h (mg/mL)
30	0.0	0.0	0.0
60	0.0	0.0	0.0
90	0.1	0.04	0.0
150	69.0	67.0	67.0

[a] At 30 min, 1 h, and 2 h after initiating the coupling reaction, probes were withdrawn and the remaining protein mass (mg) in the supernatant was determined according to Bradford,[7] using chymotrypsin for calibration of the assay.

Table II. Binding capacities as a function of chymotrypsin concentration in the matrix.

	Milligrams bound chymotrypsin/g dry gel		
	30	60	90
Capacity (μg rec. aprotinin eluted/mL gel)	126	472	641
Yield (eluted rec. aprotinin related to total rec. aprotinin applied	13%	50%	69%

volume of chymotrypsin-Sepharose under chromatographic conditions.

As shown in Figure 2, the breakthrough curve is quite steep. About 80 mL of the product solution could be applied to the small affinity column before product appeared in the effluent. The amount of inhibitor completely adsorbed ranged between 0.8 and 1 mg aprotinin per milliliter affinity gel for the different chymotrypsin agarose batches prepared. From the data of Figure 2, an adsorption isotherm may be constructed (Fig. 3). Even at a relatively high loading of the matrix (1000 μg/mL) there were only low aprotinin concentrations in the solution. From the molar ratio of chymotrypsin and rec. aprotinin it can be estimated that 20% of the immobilized chymotrypsin is capable of binding rec. aprotinin. The dynamic capacity of the chymotrypsin agarose was not altered when the flow rate was increased tenfold (77 cm/h) (data not shown). Affinity chromatography on chymotrypsin-Sepharose led to a considerable concentration and purification of the rec. aprotinin, as seen in Figure 4. The affinity technique does not discriminate, however, between molecules incompletely processed at the N-terminus, a problem that was known to occur with

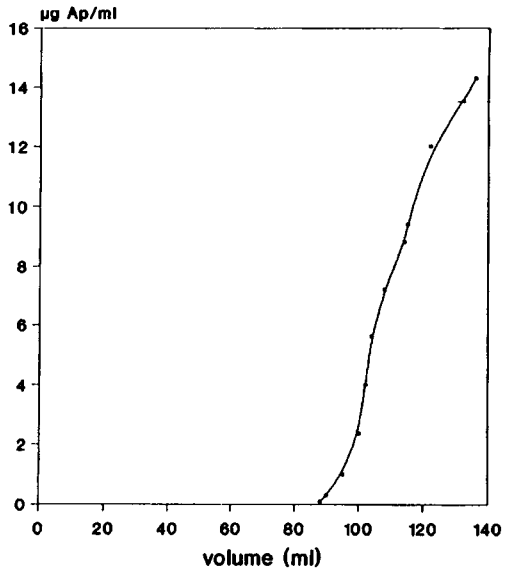

Figure 2. Breakthrough curve of chymotrypsin-Sepharose using purified DesPro(2)-VAl15-Leu17-aprotinin as a sample.

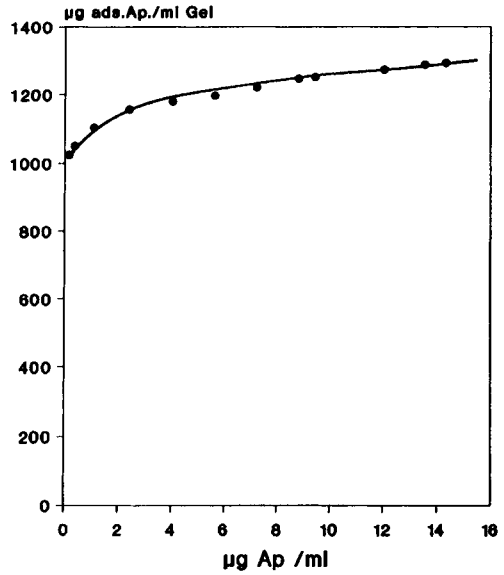

Figure 3. Adsorption isotherm for chymotrypsin-Sepharose binding to purified DesPro(2)-VAl15-Leu17-aprotinin at pH 8, 4°C.

the recombinant yeast strain employed for production.[10] The separation of the different forms of rec. aprotinin by cation-exchange chromatography has been described by Ebbers et al.,[10] and was verified in a small-scale experiment using S-Sepharose and the effluent of the affinity column as probe (data not shown). From these data, and results discussed above, an approximately 100-fold scale-up of the purification process was attempted.

Large-Scale Purification of Rec. Aprotinin

The ion-exchange column was scaled-up increasing the cross section and the volume of the gel by a factor of 6.7, holding the other parameters such as height of the gel bed and linear flow rate nearly constant. For the affinity adsorption step, the diameter of the column was increased from 1 to 5 cm, and the bed height was increased 3.8-fold resulting in a 96-fold larger gel bed. The linear

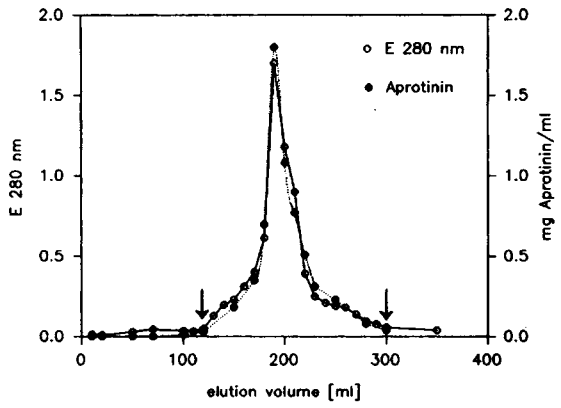

Figure 4. Elution diagram during the purification of rec. aprotinin on chymotrypsin-Sepharose. Fractions pooled for further processing are indicated by the arrows.

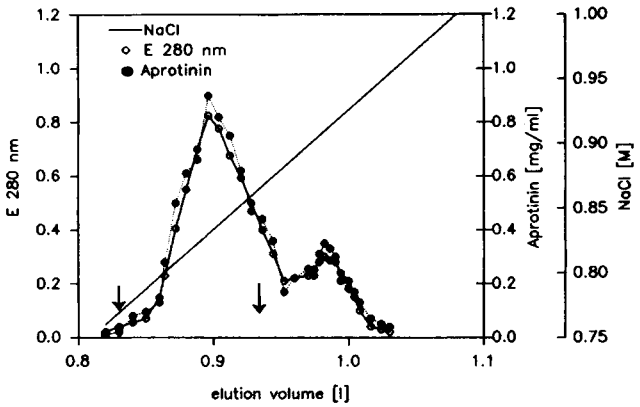

Figure 5. Final purification of rec. aprotinin on S-Sepharose elution diagram. Arrows indicate fractions containing pure rec. aprotinin.

flow rate was kept constant. The performance of the two columns is shown in Figures 4 and 5, and corresponding analytical data are summarized in Table III. Rec. aprotinin was eluted from the chymotrypsin column in 95% yield. The eluate was directly applied and further purified on the S-Sepharose cation-exchange column. Seventy percent of all active forms of elastase inhibitors were recovered, and 49% accounted for the correctly processed form of DesPro(2)-Val15-Leu17-aprotinin. The purified rec. aprotinin exhibited a single band upon SDS–polyacrylamide gel electrophoresis. In addition to the gel electrophoresis, the identity of the product was demonstrated by N-terminal amino acid sequence determination yielding arg-asp-phe-cys-leu-glu-pro-pro-tyr-thre-gly-pro-cys-val-ala, the correct sequence of the desired protein.

CONCLUSIONS

The process design presented here for the purification of rec. aprotinin has several advantages as compared with published procedures. The culture supernatant can be used directly for adsorption, because binding of the inhibitor to the affinity gel is not affected by the ionic strength. Previously, ion exchange was employed as a first step followed by gel filtration.[10] In order to bind to the cation exchanger, the culture supernatant had to be diluted with distilled water, thereby increasing the volume handled in the chromatography. The eluate from the ion-exchange column had to be concentrated to obtain good resolution and capacity during the gel-filtration step. Affinity adsorption and ion-exchange chromatography could be combined easily and without intermediate adjustments, because elution from chymotrypsin-Sepharose is carried out by a pH shift at low ionic strength, favoring binding of the product on the cation-exchange material.

The final yield of the process presented here is two- to three-fold higher than previously obtained[10] while maintaining high product quality. Improvement of strain and cultivation conditions leading to increased rec. aprotinin concentrations in the culture supernatant should present no problem for the new process design. Further scale-up appears feasible.

This work was performed within the BMFT Schwerpunktprojekt "Stoffumwandlung mit Biokatalysatoren." We thank Drs. Bennink and Goelker (Bayer AG) for advice and stimulating discussions, and Bayer AG for financial support. We gratefully acknowledge the amino acid sequence determination by S. Neumann, D. Kinzelt, and Dr. U. Jahnke.

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Table III. Purification of DesPro(2)-Val15-Leu17-aprotinin.

Step	Volume (L)	Rec. aprotinin concentration (mg/L)	Total amount rec. aprotinin (mg)	Yield
Culture supernatant	7.5	12.6	94.5	100%
Eluate affinity gel	0.19	474	90	95%
Eluate S-Sepharose				
(a) All active forms	0.19	350	66.4	70%
(b) Correctly processed rec. aprotinin	0.098	473	46.4	49%

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