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Downstream Processing of Recombinant Hirudin Produced in *Saccharomyces cerevisiae*

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Abstract A recombinant form of hirudin, a potent thrombin-specific inhibitor derived from the bloodsucking leech, was expressed as a secretory product in *Saccharomyces cerevisiae* under the control of *GAL10* promoter and the mating factor α pre-pro leader sequence. In an attempt to produce recombinant hirudin (r-Hir) of therapeutic purity in large quantities, the fed-batch fermentation was carried out by using this recombinant yeast, and subsequently downstream processing was developed with the preparative-scale column chromatography systems. About 234 mg/l of biologically active r-Hir was produced as a secretory product by the fed-batch fermentation strategy developed for an efficient downstream processing. Using a two-step chromatography process (an anion exchange chromatography followed by the reverse phase HPLC), the r-Hir was purified to >98% with an overall recovery yield of 84%. According to the N-terminal amino acid sequencing, the purified r-Hir was found to have the predicted N-terminal amino acid sequence. The biological activity of the purified r-Hir to inhibit thrombin was also identical to that of the commercial hirudin.

Key words: Recombinant hirudin, downstream processing, preparative-scale, *Saccharomyces cerevisiae*

Hirudin is a specific and potent inhibitor of thrombin which is isolated from the bloodsucking leech *Hirudo medicinalis* [1, 6, 16]. It blocks thrombin-mediated conversion of fibrinogen to fibrin in the clot formation. According to animal studies, hirudin exhibits efficacy in preventing various thrombosis. Due to these biological activities, hirudin attracts considerable interest in the pharmaceutical industry and is currently being developed as a therapeutic agent for the treatment or prophylaxis of various thrombotic diseases.

To economically produce hirudin on a large scale, the gene coding for hirudin has been synthesized and cloned into a yeast expression vector, YEGα-1, which contains the mating factor α pre-pro leader sequence and galactose inducible promoter, *GAL10* [15]. A significant amount of biologically active hirudin was found to be secreted into the extracellular medium by recombinant *S. cerevisiae*. Using this recombinant yeast, we have optimized the fermentation processes for the overproduction of r-Hir [9, 10, 14]. In addition, the purification schemes have also been developed on a laboratory scale [14].

In the present work we have developed the downstream processing for r-Hir with preparative scale column chromatographies in an attempt to obtain r-Hir of therapeutic grade in large quantities. The biologically active r-Hir was purified to >98% with a high recovery yield using a simple two-step chromatography procedure. It is expected that the downstream processing developed in this study can be efficiently used for the commercial production of r-Hir.

Materials and Methods

Strains and Plasmid

Haploid *S. cerevisiae* 2805 (*MATα pep4::HIS3 prb1-δ can1 GAL2 his3 δ ura3-52*) was used as the host for gene expression and secretion of hirudin. *E. coli* HB101 [*F⁻ hsdS20(r⁻ m⁻) recA13 proA2 galK2*] or DH5α [*F⁻ lacZ ΔM15 hsdR17(r⁻ m⁻) gyrA36*] was used for the propagation of the plasmid. YEGα-HIR5 is a plasmid in which the synthetic hirudin gene is transcribed under the control of *GAL10* promoter and secreted by the pre-pro leader sequence of the mating factor α1 (*MFα1*) [15]. The plasmid YEGα-HIR5 was transformed into the *S. cerevisiae* strain by the method of Ito *et al.* [8].

Media and Fed-Batch Fermentation

Minimal YNBCAD medium containing 6.7 g/l yeast nitrogen base without amino acids, 5 g/l casamino acid and

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20 g/l dextrose was used for the selection of transformed cells and for the seed culture. A single yeast transformant on the YNBCAD agar plate was inoculated into 10 mL YNBCAD medium and was incubated overnight at 30°C. For the inoculum development, the seed culture at 10% (v/v) level was transferred into an Erlenmeyer flask containing the same medium and was incubated for 24 h at 30°C. This culture was used as an inoculum for the fed-batch fermentation. In the fed-batch cultures, 10% (v/v) inoculum level was used.

The production medium used for the fed-batch fermentation consists of 40 g/l yeast extract, 5 g/l casamino acid, 20 g/l glucose, and 10 g/l KH_2PO_4. Galactose concentration was maintained at a maximum level of 30 g/l using the feed solution containing galactose. All substrates used in this study were of an industrial grade. Fed-batch fermentation was carried out using a step-wise feeding of galactose into the growing culture in a 5 liter jar fermentor (Bioflo III, New Brunswick Scientific Inc., U.S.A.) with a working volume of 3.5 l as described by Rao *et al.* [9].

Purification of r-Hir

After the cells were separated by centrifugation at 5,000×g, the supernatant was filtered again using a 2 µm-filter. A 545 ml culture supernatant was desalted by the treatment of 10% (wet wt./v) IonClear BigBead (Sterogene Bioseparations Inc., U.S.A.) for 10 min, centrifuged at 5,000×g to remove it and then diluted with distilled water to 2,000 ml to further reduce the conductivity to 4.3 mS/min. The desalted solution was loaded on a glass column (3.2×15 cm, Amicon Co., U.S.A.) containing Q-Sepharose XL anion exchange resin (Pharmacia, Sweden). The resin was eqilibrated with 50 mM sodium phosphate buffer (pH 5.5). The r-Hir was eluted with a linear gradient of 0-0.5 M NaCl in the equilibration buffer at 10 ml/min. Fractions were collected and assayed by rp-HPLC. In addition, fractions containing r-Hir were collected and concentrated by an ultrafiltration membrane (M.W. cut-off=3,000, Amicon Co., U.S.A.). The concentrated sample was loaded onto a preparative C8 rp-HPLC column (2.2× 25 cm, 10 µm, Vydac, U.S.A.). An elution was performed by using a linear gradient of 15~30% acetonitrile at a flow rate of 15 ml/min in the presence of 0.1% trifluoroacetic acid (TFA). Fractions containing r-Hir were pooled and lyophilized to dry.

Analytical Methods

Galactose concentration was measured by the DNS method [12]. The antithrombin activity of hirudin was measured by a colorimetric assay using Chromozyme TH as a thrombin chromogenic substrate as described by Sohn *et al.* [15]. Quantitation of r-Hir was also completed by rp-HPLC on a YMC-Pack C8 column (250×4.6 mm, 5 µm, YMC Co. Ltd., Japan). The column was initially equilibriated with 15% acetonitrile containing 0.1% (TFA). After sample injection, it was operated isocratically with the same solvent for 5 min. The r-Hir was eluted with a linear gradient of 15-30% acetonitrile containing 0.1% TFA at a flow rate of 1 ml/min for 30 min. The absorbance was monitored at 220 nm. The commercial hirudin (Accurate Co., U.S.A.) was used as a standard.

RESULTS AND DISCUSSION

Fed-Batch Fermentation Strategy for Efficient Downstream Processing

Fed-batch fermentation was carried out for the overproduction of r-Hir using a step-wise feeding of galactose as previously reported by us [9]. The step-wise feeding of galactose was found to be a suitable feeding strategy for the production of r-Hir by *S. cerevisiae*. The reason is due to its advantages over other feeding strategies such as the easy control of fermentation parameters, reproducibility and high yields. We have previously developed various feeding strategies for fed-batch cultures of *S. cerevisiae* carrying a galactose-inducible expression system [3, 4]. Although a high productivity can be achieved by fed-batch cultures employing well-controlled and sophisticated feeding strategies, these culture methods are often rather complicated in practice, and they do not always result in reproducible productivity.

HPLC analysis showed that the truncated forms of r-hirudin, which were presumed to be formed due to C-terminal proteolytic degradation, started forming after 48 h of fermentation (Fig. 1). The C-terminal proteolytic degradation of r-Hir in *S. cerevisiae* was already known to be mediated by carboxypeptidases such as YscY and Yscα [7]. Not only the hirudin activity increased after 48 h to 60 h of fermentation, but the concentration of truncated forms in the culture broth also increased. Hinnen *et al.* [7] isolated the degraded products (d1 and d2 in Fig. 1) of r-Hir from the recombinant yeast culture broth, and d1 and d2 were identified as C-terminally truncated forms lacking the C-terminal glutamine (r-Hir(1-64)) and lacking in addition the penultimate leucine (r-Hir(1-63)). As shown in Fig. 1, the r-Hir(1-63) became the major degraded product as the culture proceeded. For an efficient recovery of a single active form of the product, it is recommended to have fewer degradation products and contaminants in the culture broth. This will enable the adoption of a less expensive downstream processing to recover a highly purified active form of the product. Even though the final concentration of r-Hir at 48 h of fermentation was lower than that obtained at later stages of fermentation [9], it was highly preferable to harvest the culture broth at 48 h of fermentation. The advantages are: a) negligible formation of truncated forms of r-Hir, b) low conductivity and less

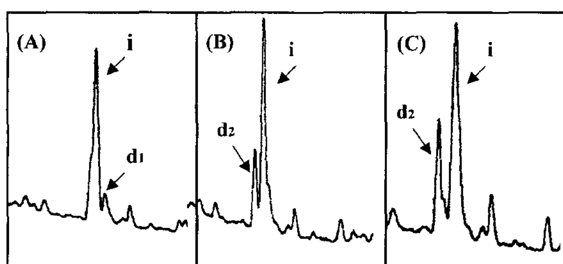

Fig. 1. HPLC chromatograms of secreted hirudin species after 48 h (A), 56 h (B), and 60 h (C) of fed-batch culture.
The peaks i, d_1, and d_2 represent the r-Hir(1-65), r-Hir(1-64), and r-Hir (1-63), respectively.

color intensity of the culture broth, which are highly essential at purification stages in order to minimize product loss.

Development of Downstream Processing

Schemes for purification of r-Hir were first developed on a laboratory scale, and then scaled-up to obtain large amounts of r-Hir from the culture broth harvested after 48 h of culture. After the cells were separated by centrifugation, 545 ml of the culture supernatant was used as a starting feed solution to purify r-Hir. About 234 mg/l of r-Hir was contained in this solution. We have previously separated various types of r-Hir using immobilized metal affinity chromatography (IMAC) [2] and metal-affinity aqueous two-phase partitioning [5]. Among different types of column chromatographies tested, IMAC showed the most efficient performance with respect to the purification fold and yield [14]. Furthermore, we continued to carry out the optimization studies to further improve the chromatography efficiency and consequently found that Q-Sepharose XL resin is the preferred resin, on the basis of binding capacity and cost.

In general, direct capture of proteins onto ion exchangers from the fermentation broth is hampered by the high ionic strength of the initial broth. Therefore, desalting or reducing ionic strength is often required prior to the ion exchange chromatography. For this purpose, desalting was carried out using Ion Clear BigBead (Sterogene Bioseparations Inc., U.S.A.) in the batch mode, with the ionic strength being reduced from 19.9 mS/min to 12.4 mS/min. However, since the ionic strength was still too high to directly apply this sample solution to the ion exchange chromatography, it was thus further reduced to 4.3 mS/min by diluting it to 3.6-fold with distilled water. The process time was greatly reduced by this desalting method compared to the diafiltration that had previously employed for r-Hir purification [14]. Reducing the downstream processing time was crucial for r-Hir produced by *S. cerevisiae* because r-Hir was very labile to proteolysis.

Figure 2 shows the elution profiles of the feed solution applied to a preparative scale Q-Sepharose XL column.

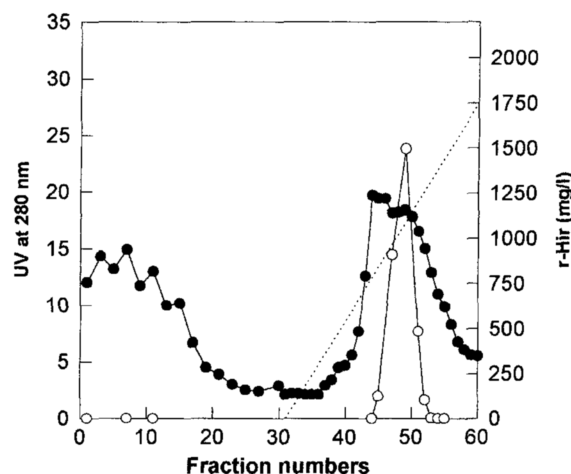

Fig. 2. Anion exchange chromatogram: (●) UV at 280 nm; (○) r-Hir concentration.
The elution conditions are described in Materials and Methods. The r-Hir concentration was analyzed by an analytical rp-HPLC. The dotted line represents a linear gradient profile of NaCl (0- 0.5 M).

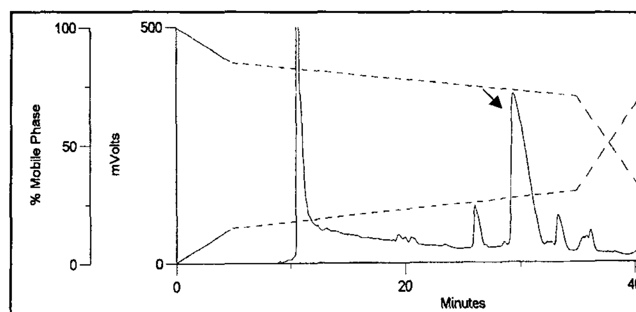

Fig. 3. Preparative scale rp-HPLC chromatogram for r-Hir purification.
The elution conditions are described in Materials and Methods. The arrow indicates the peak of r-Hir.

The fractions were subjected to an analytical rp-HPLC, and the r-Hir containing fractions were collected, concentrated by ultrafiltration, and then subjected to a preparative scale rp-HPLC for final purification. Figure 3 shows the preparative scale rp-HPLC chromatogram. The major peak having the same retention time as that of the authentic hirudin was collected and lyophilized. Finally, 105 mg of the purified r-Hir was obtained from the yeast culture broth containing ca. 125 mg r-Hir with an overall recovery yield of 84%. This is the highest recovery yield ever reported for the purification of r-Hir produced by yeast [11, 13, 14]. The flow chart of the process is shown in Fig. 4.

Purity and Biological Activities of the Purified r-Hir.

Figure 5 shows the HPLC chromatograms of the starting feed solution and the purified r-Hir. The purity of the purified r-Hir was >98% as judged by an analytical rp-HPLC column (Fig. 4B). The purified protein was

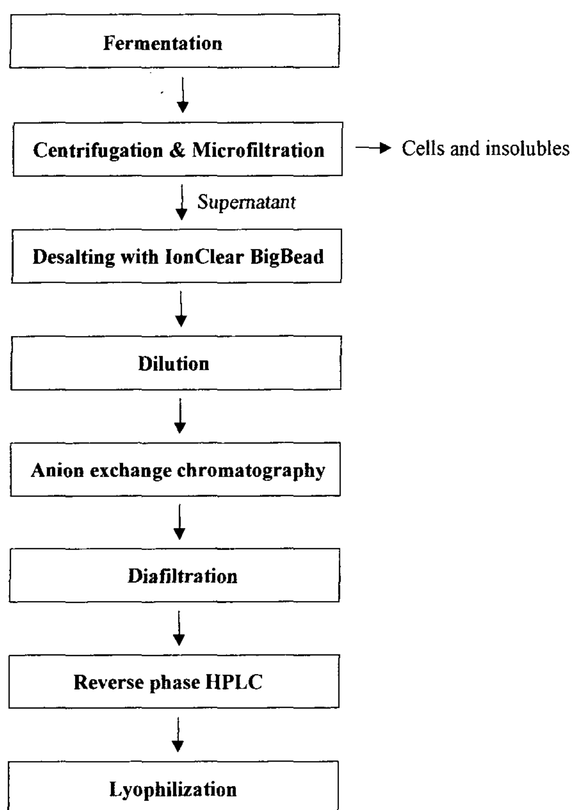

Fig. 4. Process flow diagram for purification of r-Hir.

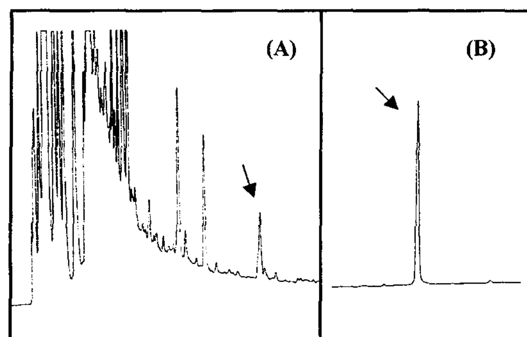

Fig. 5. HPLC analysis of the starting feed solution (A) and the purified r-Hir (B).
Arrows indicate the peaks of r-Hir(1-65).

subjected to N-terminal amino acid sequencing. The sequence of the first five amino acids was revealed to be Ile-Thr-Tyr-Thr-Asp, which is identical to the predicted N-terminus. The authenticity was confirmed by tryptic digestion and an amino acid analysis. Not only the tryptic digestion of the peptide sample and the standard hirudin yielded an identical HPLC elution profile, an amino acid composition also agreed well with the theoretical values. We have also investigated the biological activity of the purified r-Hir as previously described [15]. The biological activity of the purified r-Hir to inhibit thrombin was measured and compared to that of the commercial hirudin (Accurate Co., U.S.A.). As a result, the antithrombin activity of the purified r-Hir was identical to that of the commercial hirudin (data not shown), offering the potential for therapeutic use of r-Hir produced by the downstream processing developed in this study.

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