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Suppression of proteolytic degradation of recombinant hirudin from *Saccharomyces cerevisiae* using the O_2-enriched air

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Abstract

A novel process using O_2-enriched air supply was used to suppress the C-terminal proteolytic degradation of recombinant hirudin (r-hirudin) from *Saccharomyces cerevisiae.* When dissolved O_2 was controlled above 20% saturation level using normal air, inactive forms of C-terminally truncated hirudin were observed in culture broth from 48 h of fermentation. The use of O_2-enriched air giving above 40% saturation of dissolved O_2 suppressed the proteolytic degradation and hence the formation of truncated forms of inactive r-hirudin until 60 h of fermentation.

Introduction

Hirudin variant 2 (HV2) from the salivary gland of the blood-sucking leech, *Hirudo medicinalis*, is a polypeptide which composed of 65 amino acids (Markwardt 1970). The limited availability of natural hirudin has encouraged the development of various fermentation processes using recombinant microbial sources for its large-scale production. In a previous study, we reported the production of HV2 using *Saccharomyces cerevisiae* harboring *GAL 10* promoter (Sohn *et al.* 1991, Jagannadha Rao *et al.* 1998). In this case, the antithrombin activity of r-hirudin in the culture broth was sharply decreased at the late phase of cultivation due to the proteolytic degradation of C-terminal by extracellular/or cell-surface bound proteases. The C-terminal degradation of intact r-hirudin (hir65) leads to the formation of inactive truncated forms of r-hirudin lacking the last amino acid glutamine (hir64) and/or glutamine-leucine (hir63) (Heim *et al.* 1994). The genotype studies are being carried out to produce mutants capable of resisting the endoprotease activity (Loison *et al.* 1988, Riehl-Bellon *et al.* 1989). However, there is no report on the suppression of this proteolytic activity and on the formation of truncated forms of r-hirudin by means of physical process developments. Although, use of an expression cassette is an important parameter in the expression of a foreign gene, the importance of nutritional and metabolic parameters cannot be undermined (Bhattacharya & Dubey 1995). In this connection, O_2 plays a crucial role and thus on the formation of foreign gene product. In this communication, we report for the first time a physical process development by supplying O_2-enriched air for the suppression of proteolytic degradation of r-hirudin from *Saccharomyces cerevisiae*.

Materials and methods

Recombinant strain and growth conditions

The recombinant strain used for this study is haploid *Saccharomyces cerevisiae* KCTC 2805 (*Matα pep4:: HIS3 prb 1 can 1 his3 ura3-52*) with plasmid YEGα-HIR525. The plasmid contains the *GAL10* promoter, *MFα1* signal sequence, hirudin structural gene and GAL7 terminator (Sohn *et al.* 1991).

The growth medium employed consists of 40 g yeast extract/l, 5 g casamino acids/l, 20 g glucose/l, 10 g KH_2PO_4/l. Galactose was maintained at a maximum of 30 g/l using the feed solution containing 500 g galactose/l. Fermentation was carried out in a 5 l jar fermenter (Bioflo III, New Brunswick Scientific Inc., USA) with a working volume of 3.5 l.

Fermentation was conducted at an agitator speed of 600 rpm and under the controlled conditions of pH and temperature at 5.4 and 30 °C, respectively. Fed-batch fermentations were carried out using stepwise feeding of galactose into the growing culture in a 5 l fermenter as previously reported by us (Jagannadha Rao *et al.* 1998). Aeration rate was maintained at 1.0 vvm. Enrichment of inlet air with pure O_2 was done intermittently in order to maintain the dissolved O_2 (DO) level above 40% saturation.

Analytical methods

The growth of yeast cells was monitored turbidometrically at 600 nm. The dry cell mass was estimated by a predetermined conversion factor 0.208 g dry cell (DCW)/l × O.D. determined from the standard plot of dry cell mass of yeast versus O.D. at 600 nm. Glucose concentrations were determined by using a glucose kit (Sigma kit). Galactose concentrations were measured by 3,5-dinitrosalicylic acid method (Miller 1959). The ethanol concentration was measured using a gas chromatograph equipped with a flame-ionization detector. The hirudin activity in culture supernatant was determined using thrombin and a chromogenic substrate, chromozyme TH, as described by Sohn *et al.* (1991).

HPLC analysis of r-hirudin

Quantitative analysis of r-hirudin in fermentation broth was done by Reverse Phase-HPLC on a C8 column (YMC A-203, 5 μm particle size, 250 × 4.6 mm i.d., 12 nm). The column was initially equilibrated with 0.1% trifluoroacetic acid (TFA). After sample injection, it was operated isocratically with the same solvent for 5 min. The r-hirudin was eluted with a linear gradient of acetonitrile (15–30%)/0.1% TFA, at 1 ml/min for 30 min. The absorbance was monitored at 220 nm. The concentration of r-hirudin in sample fluids was determined by comparing the sample peak areas to a purified r-hirudin reference standard.

Results and discussion

The culture broth of *Saccharomyces cerevisiae* secreting the r-hirudin was analyzed using HPLC. One major peak and two minor peaks could be distinguished, which are absent in the untransformed control yeast strains. These minor peaks are presumed to be truncated forms of matured r-hirudin and detected from 48 h of fermentation onwards. The formation of truncated forms appears to be due to the proteolytic degradation by extracellular or cell-surface bound proteases. However, the genotype of host strain (*MAT α pep4::HIS3 prb 1 can 1 his3 ura3-52*) used to develop a recombinant strain has the ability to repress the secretion of extracellular proteases. Consequently, it suggests that the observed proteolytic degradation might be due to the cell-surface bound proteases. The C-terminal degradation of r-hirudin in *Saccharomyses cerevisiae*, mediated through carboxypeptidases yscY and yscα was reported by Heim *et al.* (1994). Also according to them, minimal medium supplemented with Casamino acids strongly promoted the formation of C-terminally truncated by-products, with almost exclusive occurrence at later stages of fermentation. This finding pointed to an involvement of proteases creating C-terminal degradation products. From our preliminary studies, Casamino acids proved to enhance the productivity of r-hirudin and hence, included as one of the component of production medium. It further suggests that the formation of truncated forms of r-hirudin also might be due to the carboxypeptidases yscY and yscα mediated proteolytic degradation.

The amount of truncated forms of r-hirudin in the culture broth increased after 48 h of fermentation (Figures 1a–c). It is presumed that the proteolytic activity of endoproteases could be influenced by change in physical environment of the system. In order to minimize the proteolytic degradation, different process changes were attempted, like variation in culture pH and dissolved O_2 (DO) level. Initial fermentation studies were made at different culture pH ranging from 4.5–7.0. Shifting the culture pH to a higher or lower level did not influence the formation of C-terminally degraded products, indicating that the proteolytic activity of endopeptidases had not been affected (data not shown). On the other hand an important parameter change observed was the rapid consumption of DO by the yeast cells after post-logarithmic growth phase. The DO in the culture broth was measured using O_2 sensor (Metler-Toledo). The DO rapidly decreased below 20% saturation level even up on continuous supply

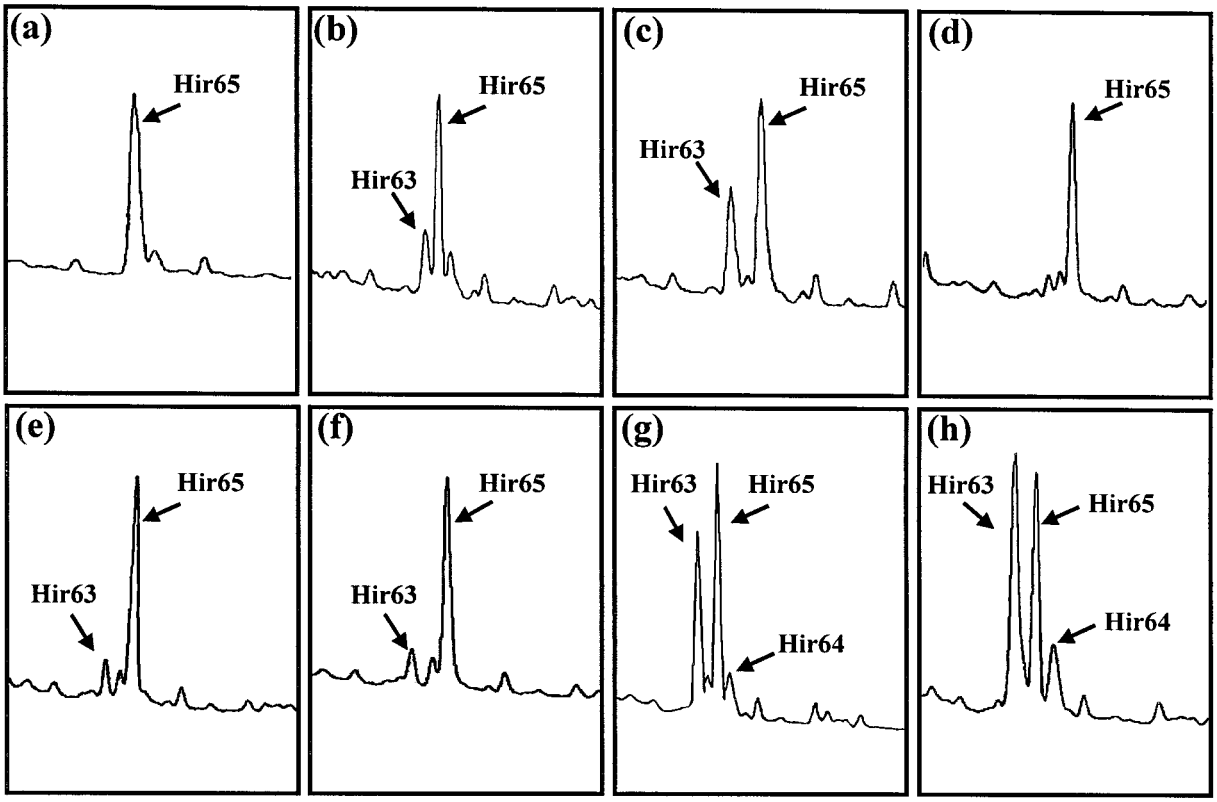

Fig. 1. HPLC profiles of secreted r-hirudin samples from (a) 48 h, (b) 56 h and (c) 60 h fermentation using normal air; and from (d) 48 h, (e) 56 h, (f) 60 h, (g) 72 h and (h) 96 h fermentation using O_2-enriched air.

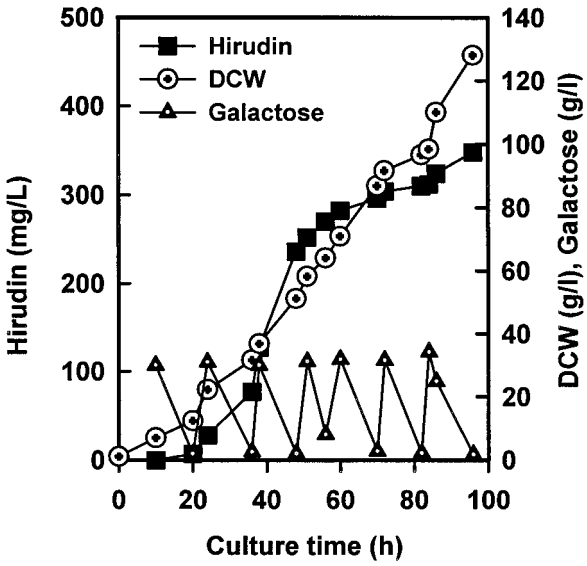

Fig. 2. Fed-batch fermentation profiles of r-hirudin production by step-wise feeding of galactose in 5-L bench scale fermenter using O_2-enriched air.

of air after 48 h of fermentation. This was due to the formation of high cell concentration and metabolic stress associated with product formation by the yeast cells after 48 h of fermentation. From then onwards more of proteolysis of r-hirudin was observed. The reason could have been due to increased redox flux due to limited availability of DO in the fermenter. This observation led us to assume that the low concentration of available DO might be promoting the proteolytic activity of the endo peptidases. To strengthen our assumption, studies were conducted by supplementing the growing cells with excess O_2 by enriching the inlet air with pure O_2. Interestingly suppression of truncated forms of r-hirudin was observed in the culture broth after 48 h of fermentation with the supply of O_2-enriched air. Pure O_2 at the flow rate of 0.3–0.5 vvm was found to be optimum range to enrich the inlet air supply from the post-logarithmic phase of cell growth. This was carried out intermittently throughout the fed-batch fermentation in order to maintain the available DO above 40% saturation level. O_2-enriched air supply suppressed the proteolytic degradation after 48 h

fermentation until 60 h of fermentation (Figures 1d–f). Only negligible amounts of truncated forms are formed during this period. But after 60 h of fermentation, the C-terminal proteolytic degradation started increasing with the simultaneous increase in total r-hirudin production until 96 h of fermentation (Figures 1g–h). The fed-batch fermentation profiles using O_2-enriched culture are given in Figure 2. The reason for the continued increase in the concentration of hirudin until 96 h of fermentation may be due to the formation of lower amounts of ethanol (below 3 g/l), indicating that excess available O_2 in the fermentation broth favoring the aerobic metabolism of the cells. Although the concentration of r-hirudin produced at 60 h of fermentation was lower than that obtained at 72 h and 96 h of fermentation, it was recommended to harvest the culture broth below 60 h of fermentation as it produced intact form of active hirudin with negligible amount of inactive truncated forms. This will also help in adopting simple and cost effective down streaming operations to purify active hirudin.

In conclusion, this is the first report on the suppression of C-terminal proteolytic degradation of recombinant hirudin by a novel process development using an O_2-enriched air supply.

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