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Causes of Proteolytic Degradation of Secreted Recombinant Proteins Produced in Methylotrophic Yeast *Pichia pastoris*: Case Study With Recombinant Ovine Interferon-τ

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Abstract: It was observed that during fermentative production of recombinant ovine interferon-τ (r-oIFN-τ) in *Pichia pastoris,* a secreted recombinant protein, the protein was degraded increasingly after 48 h of induction and the rate of degradation increased towards the end of fermentation at 72 h, when the fermentation was stopped. Proteases, whose primary source was the vacuoles, was found in increasing levels in the cytoplasm and in the fermentation broth after 48 h of induction and reached maximal values when the batch was completed at 72 h. Protease levels at various cell fractions as well as in the culture supernatant were lower when glycerol was used as the carbon source instead of methanol. It can be concluded that methanol metabolism along with cell lysis towards the end of fermentation contributes to increased proteolytic activity and eventual degradation of recombinant protein. © 2004 Wiley Periodicals, Inc.

Keywords: proteolytic degradation; secreted recombinant interferon-τ; methanol; *Pichia pastoris*

INTRODUCTION

Proteolytic degradation has been a perpetual problem when yeasts are employed to express recombinant proteins (Van Den Hazel et al., 1996; Kobayashi et al., 2000; Gimenez et al., 2000). Yeast vacuoles contain various proteases whose levels vary according to the nutritional conditions (Hansen et al., 1977). *Saccharomyces cerevisiae* is known to express several proteases, some of which have been well identified (Van Den Hazel et al., 1996). In comparison, the proteases in *Pichia pastoris* are not well characterized, although there have been several reports of proteolytic degradation of recombinant proteins produced in *P. pastoris* (Sinha et al., 2003; Kobayashi et al., 2000; Zhou and Zhang, 2002; Werten et al., 1999). A few remedial measures like addition of casamino acids, addition of protease inhibitors, or use of protease-deficient strains have been suggested as means to circumvent proteolytic degradation of the recombinant protein (Goodrick et al., 2001; Kurokawa et al., 2002; Brankamp et al., 1995). However, no in-depth analysis on the conditions that promote proteolysis or the nature of the proteases acting on the desired protein is exactly known. There have been no reports of extracellular proteases in yeasts, the main source of proteases in *Saccharomyces* being vacuolar proteases, followed by proteasome and the proteases of the secretory pathway (Yasuhara et al., 1994). In *S. cervisiae* the major vacuolar proteases are proteinase A (PrA), proteinase B (PrB), carboxypeptidases, and aminopeptidases (Van Den Hazel et al., 1996). It has been observed that when PrA is overexpressed in yeast, a part of the enzyme can be secreted (Rothman et al., 1986). In general, these soluble proteases are contained within the vacuoles, although both vacuolar and secretory proteins move through endoplasmic reticulum and Golgi complex during their process of synthesis (Yoshihisa and Anraku, 1990).

Proteolytic degradation of secreted recombinant proteins can be caused by degradation of the cell membrane due to cell lysis in high cell density fermentation. Yeast cells are known to be stressed by starvation, change of carbon sources, heat and pH changes, or toxic chemicals. Proteins damaged by oxidative stress and heat-shock response also elicit a proteolytic response (Hilt and Wolf, 1992). In this regard, proteins damaged by oxidative stress are a probability, since methanol metabolism demands high oxygen and a by-product is hydrogen peroxide. Excess production of the vacuolar proteases resulted in a secretion to cytosol and eventually to the culture medium (Stevens et al., 1986). In the ongoing research, proteases were found to act on several recombinant proteins during fermentation, most prominently on recombinant ovine interferon-τ (r-oIFN-τ). The proteases were not detected during the glycerol growth phase and their activity increased after the first 48 h of the methanol induction phase and reached a maximum between 60–72 h of fermentation (Sinha et al., 2003). Since, the recombinant proteins are also produced during the methanol induction period, the presence of proteases was deleterious to the success of the process. The purpose of this research is to understand the nature of the

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proteases and the circumstances which promote proteolysis of recombinant protein with a view to improvement of the recombinant protein production.

MATERIALS AND METHODS

Strain and Inoculum

A Mut^+ recombinant strain of *Pichia pastoris* X-33 transformed with the oIFN-τ gene integrated to the *P. pastoris* chromosome having expression under the control of the AOX1 promoter was obtained from Pepgen (Alameda, CA). *Pichia pastoris* X-33 is an His^+ mutant obtained from restoring the His gene in *P. pastoris* GS115 which is HIS4. The frozen culture was thawed and added to previously sterilized BMGY medium (1% yeast extract, 2% peptone, 0.1 M potassium phosphate buffer, pH 6.0, 1.3% yeast nitrogen base, and 1.2% glycerol) in shake flasks. The culture was grown for 48 h approximately to an optical density of 4–5; 150 ml of the seed culture was transferred aseptically to 2 L of the fermentation medium.

Fermentation Conditions

Fermentations were performed in 5-L Bioflo III/3000 stirred-tank fermentors (New Brunswick Scientific, Edison, GA) having a 2 L starting volume interfaced with computer using Biocommand 32 software (New Brunswick Scientific) for data acquisition and control. Methanol was fed to the fermentor using a closed loop control system with methanol feed pump (Model 101 U/R, Watson-Marlow, UK), balance (Model PG 12001-S, Mettler Toledo, Switzerland) for weighing the methanol bottle interfaced with the computer using Biocommand 32 software. The cells were grown exponentially at a desired specific growth rate by controlling the methanol feed using an exponential feed model (Sinha et al., 2003). The dissolved oxygen was controlled at 40% saturation using a D.O.-agitation cascade system between 200 and 800 rpm at the beginning of fermentation. When a maximum rpm of 800 was reached, pure oxygen was supplied through a gas blender to control dissolved oxygen at 40% saturation. Methanol concentration in the off gas was analyzed with a methanol concentration monitor and controller (Model MC-168, PTI Instruments, Kathleen, GA).

The fermentation can be divided into various phases, namely, the glycerol batch phase, glycerol fed-batch phase, and a brief transition phase followed by the methanol fed-batch induction phase (Zhang et al., 2000). Fermentations were performed at 30°C. The pH during growth (glycerol batch and fed-batch phase) was maintained at 5.0, and then ramped up to 6.0 over a 2-h period starting from induction on methanol (beginning of the transition phase). Saturated aqueous ammonium hydroxide was used to control the pH at 6.0 until the end of fermentation. FM22 medium (Stratton et al., 1998) with citrate (10 mM) was employed for growth and recombinant protein production. Glycerol (40 g/l) was also added during the batch phase. Glycerol feed was started coinciding with a dissolved oxygen spike and fed at 20 g/l/h for a specified time before being ramped down to 0 g/l/h at a uniform rate over a 3-h period. The ramping down of glycerol marked the beginning of the transition phase and 1.5 g/l of methanol was added to the bioreactor to allow the cells to adjust to methanol. A continuous exponential feed of methanol with 2 ml/L of PTM4 salts (methanol fed-batch phase) was started as soon as the cells adapted to the new carbon source, which was typically 2 h from the point of induction. The fermentation was continued for 72 h using an exponential methanol feed profile. However, for establishing the effect of glycerol on protease activity, in certain experiments 50% v/v glycerol was used as carbon source instead of methanol, while the exponential feeding profile and specific growth rates remained the same as carried out with methanol as the sole carbon source. Samples were taken at regular intervals and analyzed for various proteases, r-oIFN-τ and total protein.

Localization of Proteases

Preparation of Intracellular Extract

The fermentation broth was centrifuged at 4,000*g* for 10 min at 4°C and the pellets containing the yeast cells were washed once with 20 mM Pipes-KOH and recentrifuged. A wet cell pellet of 0.4 g was taken in each 2 ml tube along with 1 ml of 20 mM Pipes-KOH and 0.8 g silica zirconia beads and homogenized in a bead beater (Biospec Products, Bartlesville, OK) with 8 cycles of 50 sec each. The homogenate was centrifuged at 50000*g* for 20 min at 4°C and the supernatant was taken as the cell free extract.

Preparation of Spheroplasts and Isolation of Periplasmic Fraction

Cells were harvested at 4,500*g* for 5 min, the resulting pellet was washed once with 10 mM DTT, 20 mM Tris-SO_4, pH 9.4, centrifuged at 4,500*g* for 5 min at 4°C, and the pellet was resuspended in 10 mM DTT, 20 mM Tris-SO_4, pH 9.4, and incubated on a mild rocking shaker at 30°C for 15 min. The treated cells were centrifuged at 4,500*g* for 5 min at 4°C and the resulting pellet was resuspended in 1 M sorbitol, 20 mM PIPES-KOH, pH 6.8, with 1 U/OD_{600} lyticase and incubated at 30°C for 30 min with mild shaking. Generation of spheroplasts was ensured by monitoring the decrease in absorbance at OD_{600}. As a confirmatory test, a small sample of the generated spheroplasts was suspended in 20 mM PIPES-KOH, pH 6.8, when the spheroplasts were found to lyse upon microscopic observation. The pellet containing the spheroplasts was harvested by centrifugation at 1,000*g* for 5 min. The supernatant from centrifugation was taken as the crude

periplasmic extract. All centrifugations were carried out in refrigerated centrifuges at 4°C.

Isolation of Vacuolar Enzymes and Cytoplasmic Extract

The spheroplasts were resuspended in prechilled 200 mM sorbitol in an ice-bath, 20 mM Pipes-KOH, pH 6.8, along with 50 μg/OD_{600} DEAE and then quickly transferred to an ice-bath and kept for 5 min. The spheroplast suspension was then transferred to 30°C and incubated for 15 min at that temperature. The bursting of the spheroplasts was ensured by microscopic observation. The resulting suspension was centrifuged at 5,000*g* for 5 min. The supernatant was taken as the cytoplasmic extract. The pellet was taken as the vacuolar fraction and was suspended in 15% w/v ficoll in 200 mM sorbitol, 20 mM Pipes-KOH at OD_{600} of 40–50. Ultracentrifuge tubes of 13 mL each was layered successively from bottom upwards with 4 ml of vacuolar suspension in 15%, 8%, 4%, and 2% ficoll in 200 mM sorbitol, 20 mM Pipes-KOH, and centrifuged at 100,000*g* for 90 min at 4°C. Vacuoles were collected from the 4% to 2% ficoll interface. The collected vacuoles were washed with 200 mM sorbitol, 20 mM Pipes-KOH, and recentrifuged at 50,000*g* for 20 min. The resulting pellet was suspended in 20 mM Pipes-KOH buffer and sonicated for 3 cycles of 30 sec each using a pen sonicator using an ice-bath for rapid cooling of the samples.

Protease Assays

Total protease in fermentation samples was assayed using Bodipy-casein (Molecular Probes, Eugene, OR) as the fluorescent substrate (Jones et al., 1997) at a concentration of 1 μg/l. The protease sample was added at a dilution of 1:40 to 1:10 depending on activity. The enzyme-substrate mixture was incubated at 30°C for 4 h in the dark and then stopped by addition of 1 M perchloric acid. The samples were neutralized by 1 M NaOH and 1 ml of 20 mM MES was added to make up the volume and adjust pH. The fluorescent intensity from the protease samples was read at 480 nm (excitation) and 530 nm (emission). One unit of activity was defined as the amount of enzyme required to obtain 10 units of fluorescence activity.

Enzyme assays specific to yeast proteases, proteinase A, proteinase B, carboxypeptidase Y, and aminopeptidase activities were carried out on various fermentation samples as described below and the results were determined spectrophotometrically. Proteinase A activity was assayed by measuring the release of tyrosine-containing peptides from acid denatured hemoglobin at a pH of 3.2 (Lenney et al., 1974). The reaction was carried out by incubating a suitably diluted sample of 0.1 mL with 0.4 mL of hemoglobin working solution prepared from mixing equal volumes of acid denatured hemoglobin stock solution with 0.2 M glycine-HCl, pH 3.2, for 1 h 30 min at 37°C. The mixture was centrifuged at 2000*g* for 5 min. The released peptides in the supernatant were measured as tyrosine equivalent using tyrosine as standard. Acid denatured hemoglobin was previously prepared by incubating 25 g/L hemoglobin in 1 N HCl, pH 1.8, for 1 h at 35°C, after which the pH was increased to 3.2 with NaOH and the concentration was adjusted to 20 g/L. One unit of proteinase A activity corresponded to the amount of enzyme that produced 1 μg of tyrosine equivalent per minute at 750 nm wavelength.

Protease B activity was assayed by measuring the blue dye released from Hide powder azure as substrate (Rinderknecht et al., 1968). The reaction was carried out by incubating 0.2 ml of diluted sample of the enzyme with 12 mg of Hide powder azure along with 0.375 ml of 0.1 M Tris-HCl, pH 7.2, 0.125 mL of 1% w/v Triton X-100 and 40 μL of 20% w/v SDS at 37°C for 1 h under constant shaking. The reaction was stopped by placing the reaction mixture in an ice-bath followed by centrifugation at 2,000*g* for 5 min. The supernatant was diluted with 2 ml of distilled water and the absorbance was read at 595 nm wavelength against a suitable reaction blank. One unit of proteinase B activity was defined as the amount of enzyme that produced an increase in absorbance of 1 OD per minute at 595 nm under the assay conditions.

Carboxypeptidase Y activity was assayed by measuring the amount of L-leucine released upon enzymatic digestion of N-CBZ-Phe-Leu (*N*-benzyloxycarbonyl-L-phenylalanyl-L-leucine) as substrate (Kase et al., 1990). The reaction was carried out by incubating 0.1 ml of a diluted sample with 1 ml of 5 mM N-CBZ-Phe-Leu in 0.1 M MOPS buffer, pH 7.0, for 1 h, 30 min at 37°C with intermittent shaking. The reaction was stopped by removing 0.2 mL aliquots to 1 mL ninhydrin reagent in a boiling water bath and heated for 5 min at 80–85°C. The reaction mixture was cooled to room temperature and the absorbance was read at 570 nm wavelength against a reagent blank. The amount of L-leucine released was estimated from a standard curve of L-leucine. One unit of CpY activity was defined as the amount of enzyme that released 1 mmol of L-leucine per minute under assay conditions.

Aminopeptidase activity was assayed by measuring the amount of p-nitroaniline liberated upon enzymatic digestion of leucine-p-nitroanilide (Trumbly and Bradley, 1983). The reaction was carried out by incubating 50 μL of a suitably diluted sample with 100 μL of leucine-p-nitroanilide stock solution (10 mM in 5 mM H_2SO_4), 50 μM $ZnCl_2$, and 950 μL of 50 mM Tris-HCl, pH 7.2, for 15 min at 37°C in a constant temperature water bath. The liberated yellow p-nitro aniline was measured at 405 nm wavelength against a suitable blank and estimated from a p-nitro aniline standard curve. One unit of aminopeptidase activity was defined as the amount of enzyme that liberated 1 μM of p-nitro aniline per minute under the assay conditions.

SDS-PAGE and Zymogram Analysis

The protein profile in various samples were determined by running 12% Tris-glycine precast gels (Novex, Invitrogen)

according to the method of Laemmli (1970) under reducing conditions using SeeBlue molecular weight marker standards (Invitrogen, Carlsbad, CA). For zymogram gels, protein samples were incubated with an equal volume of 2× nonreducing SDS loading buffer for 10 min and then applied to either precast (Novex, Invitrogen) 12% Tris-glycine gels containing β-casein as substrate or 10% Tris-glycine prepared gels containing r-oIFN-τ as substrate. Gel electrophoresis was carried out and then the proteins in the gel were renatured with Triton-X for 30 min. The resulting gel was washed with double-deionized water (using Branstead's nano-pure diamond UV system that provides water with 105 parts per billion (ppb) TOC (total organic carbon) and up to 18.2 megohm-cm resistivity water) and incubated for 1 h in a Tris-HCl developing buffer. The developed gel was stained with Coomassie brilliant blue for 2 h and then destained until transparent zones caused by proteolytic digestion of the protein substrate in the gel are visible against a blue background.

Western Blotting of Interferon-τ

Recombinant-oIFN-τ was produced from *P. pastoris* X-33 recombinant cell line and purified by DEAE (diethylaminoethyl) ion-exchange chromatography and size exclusion chromatography to a final purity of >98% as determined by HPLC, Coomassie brilliant blue stained SDS-PAGE, and silver-stained 2D gel electrophoresis. The pure product was stored frozen in Tris-HCl buffer, then thawed just prior to use. Protein bands were separated on a 12% Tris-glycine gel as described above and then transferred to a PVDF membrane using a semidry transfer apparatus (Bio-Rad, Hercules, CA). The PVDF membrane was soaked with blotto (5% w/v skimmed milk powder in Tris buffer saline) and treated sequentially with anti r-oIFN-τ antibodies derived from rabbit for 2 h at a dilution of 1:60,000, washed three times with Tris-buffered saline (TBS) followed by treatment with goat-antirabbit secondary antibodies (Kirkland and Perry, Gaithersburg, MA) at a dilution of 1:40,000 for 30 min. The membrane was again washed three times with TBS and the image was developed using the ECL+plus Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ). Previously, antibodies to r-oIFN-τ from three production and one sanguination bleed were retained, aliquoted, and stored frozen at −20°C in serum by Covance (Virginville, PA). Prior to storage the antibodies were semi-purified by using sequential protein A and protein G chromatography. To minimize cross-reactivity with *Pichia* proteins, the rabbits were fed a yeast-free diet.

Assay of Interferon-τ

The yield of r-oIFN-τ was determined by high-performance liquid chromatography (Waters 600E, Milford, MA), using a 259 VHP polymer (C-4) reversed phase (5 μm, 4.6 × 150 mm; Vydac, Hesperia, CA) equipped with four-eluent autoblending pump (Waters 600), UV-visible detector (Waters 486), and an autosampler (Waters 717) with a combination of acetonitrile, iso-propanol, and trifluoroacetic acid as the mobile phase. Chrom Perfect software (v. 3.54, Mountain View, CA) was used for instrument control, data collection, and processing.

Cell Viability

Cell viability was studied using methylene blue dye exclusion technique (Martinez de Maranon, 1999). Fermentation samples were taken at regular intervals and a suitably diluted cell suspension (approximate OD_{600} = 20−30) was mixed with an equal volume of methylene blue dye solution for 1 min and then mounted on a hemocytometer to count the percentage of live cells in the total population. Cells which take up methylene blue and appeared deep blue were considered dead compared to live cells, which appear translucent. In a similar manner, neutral red was used to stain the vacuoles of the yeast cells and the vacuoles were stained red against a transparent cytoplasm.

RESULTS AND DISCUSSION

Action of Proteases on IFN-τ

The level of r-oIFN-τ produced during methanol induction of the Mut^+ strain of *P. pastoris* X-33 drops typically after 50−55 h (Sinha et al., 2003). Protease activity of the culture supernatant was found to increase substantially after 48 h into methanol induction and reached a maximum value between 60 h and 72 h of fermentation. Protease activity was below detectable limits during the glycerol batch and glycerol fed-batch phase. Hence, there is a probable relationship between the increase in proteases and the disappearance of r-oIFN-τ. To look more closely into this aspect, purified r-oIFN-τ was incubated in vitro with fermentation culture supernatant at 30°C and it was observed that the r-oIFN-τ concentration decreased with time compared to a control having only r-oIFN-τ and no protease (Table I). This suggests that r-oIFN-τ is degraded by the proteases in the fermentation culture supernatant. To further confirm this observation, a zymogram gel was prepared which contained r-oIFN-τ as substrate. While the zone clearing in lane 1 was from the proteases present in fermentation supernatant, the zone clearing in lane 2 resulted from a partially enriched protease from the same source. Cleared zones against the blue background confirmed the proteolytic degradation of r-oIFN-τ (Fig. 1).

Fermentation Conditions and Proteolytic Activity

Proteolytic activity in the fermentation culture supernatant varied with changes in the in the culture conditions and

medium composition. It was observed that pH 5.0 elicits increased proteolytic response (an average of 2,836 U/mL protease or 1,243.9 U of protease/mg protein in fermentation supernatant) and results in lower r-oIFN-τ production. However, at pH 6.0 the proteolytic activity and IFN-τ degradation was substantially reduced (Table II). Use of 5 g/L casamino acids reduced proteolysis of IFN-τ over the original FM22 medium, perhaps by acting as a preferential substrate (Table II). When the level of total phosphate present in original FM22 medium was reduced to 0.6 times the level of phosphate in original FM22 medium, protease activity increased more than twice than that obtained using original FM22 medium; while protease activity was reduced slightly when 1.4 times the level of total phosphates present in original FM22 medium was used (Table II). With variations in levels (0.4 times higher or lower of original FM22) of either the entire FM22 medium (Table II) or other individual medium components; however, no clear relationship emerged between protease levels in the fermentation supernatant and the media composition (data not shown). An intermittent feed of nitrogen (designated "High nitrogen feed" in Table II) in the form of ammonium sulfate (200 g/L) was fed at the rate of 2 mL every 3 h beginning at induction until the end of the batch. This reduced proteolytic activity, but not with any remarkable differences with the originally described process using FM22 medium (Table II). Higher concentrations of nitrogen feed did not show any further reduction in protease activity (data not shown). It was concluded that nitrogen limitation is not the prominent cause of proteolytic activity and the medium composition has adequate nutrients in the form of nitrogen, phosphorus, or minerals and has little to do with increased proteases. Since, the proteolytic activity was detected after the carbon source transition from glycerol to methanol and increased both with time of induction as well as with increasing growth rates on methanol, it was suspected that either the process of transition or methanol itself as carbon source or presence of recombinant protein gene elicits proteolytic action.

Table I. Action of fermentation broth proteases on purified IFN-τ at 30°C.

Time of incubation h	Pure r-oIFN-τ (control) μg/ml	Pure r-oIFN-τ + r-oIFN-τ in FS μg/ml
0	360 (360)	516 (514.5)
6	367 (360)	449 (514.5)
12	374 (360)	402 (514.5)
24	360 (360)	364 (514.5)
48	358 (360)	238 (514.5)
72	354 (360)	185 (514.5)

Pure IFN-τ (control) ≡ Pure IFN-τ stock solution: nanopure water = 1:1.

Pure IFN-τ + FS ≡ Pure IFN-τ stock solution: fermentation supernatant (FS) (72-h sample) = 1:1.

Values in parentheses indicate theoretical values assuming no degradation.

Results presented are average of data points from two independent experiments, $n = 2$.

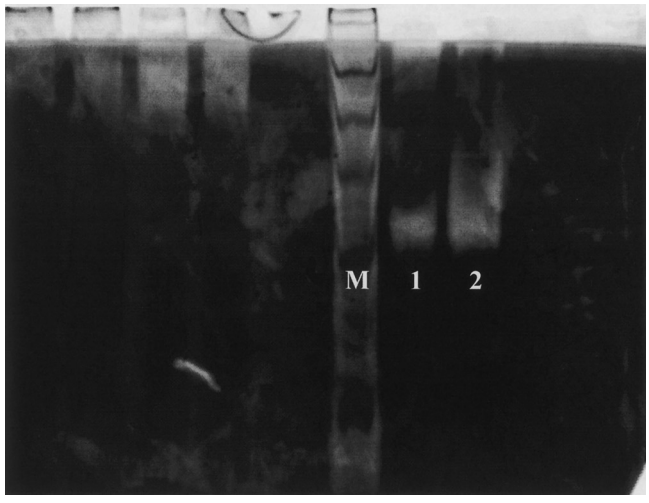

Figure 1. Zymogram showing digestion of IFN-τ by proteases from *Pichia pastoris*. Lane markings: M = Marker, 1 = culture supernatant, 2 = affinity purified sample.

Intracellular Proteases in *P. pastoris*

To further understand what causes proteolysis, the fermentations were carried out, as described above, using methanol as substrate. For comparison, *P. pastoris* X-33 recombinant cell line was cultivated on glycerol as the sole carbon source following the same exponential feed profile ($\mu = 0.025$) as described using methanol as the carbon source. This helped to understand the effect of methanol on protease production during fermentation. The time course of protease activity in *P. pastoris* culture supernatant was studied. The proteases were assayed for total protease activity as well as four prominent vacuolar proteases, PrA, PrB, CpY, and aminopeptidase, in order to understand the mechanisms related to proteolytic degradation of the recombinant protein. It was observed that when cells were either induced on methanol or grown on glycerol to the same cell density, both intracellular levels and activity in the fermentation broth of proteinase A, proteinase B, carboxypeptidase Y, and aminopeptidases were higher using methanol as the substrate compared to using glycerol as the substrate (Figs. 2–5). A maximum aminopeptidase activity of 1,556.9 U/ml cell was measured in the intracellular extract on methanol grown cells compared to 1,021.2 U/ml cells in the intracellular extract from cells cultivated on glycerol as the sole carbon source. In general, the levels of the four proteases obtained from the intracellular extract increased rapidly from the beginning of induction and reached a steady level from 24 h, often decreasing towards the end of fermentation at 72 h (Figs. 2–5). Similarly, the intracellular levels of the total proteases, as measured by fluorescent casein assay, increased rapidly after transition from glycerol

Table II. Effect of the level of medium components on proteases estimated from r-oIFN-τ fermentation culture filtrate.

Medium	IFN-τ yield mg/l	Protease U/ml	Total protein mg/ml	Specific activity U/mg protein
High FM22[a]	139.1 (54 h)	1,368 (71 h)	4.88 (71 h)	280.3
Low FM22[b]	607.8 (43 h)	2,720 (72 h)	1.88 (72 h)	1,446.8
High phosphate[c]	356.4 (60 h)	828 (70 h)	5.86 (70 h)	141.3
Low phosphate[d]	174.2 (55 h)	2,484 (67 h)	0.86 (67 h)	2,888.4
Casamino acids[e]	166.8 (60 h)	696 (48 h)	5.13 (48 h)	135.7
High nitrogen feed[f]	320.2 (54 h)	1,528 (69 h)	7.30 (69 h)	209.3
Original FM22*	330.9 (54 h)	1,052 (71 h)	6.36 (71 h)	165.4
Original FM22 (glycerol)[g]	BDL	390 (72 h)	3.91 (72 h)	99.8

Specific growth rate, $\mu_{set} = 0.025\ h^{-1}$ for all fermentation experiments.

Results presented are average of data points from at least two independent experiments, $n = 2$, $*n = 5$, % coefficient of variation ((std. dev./average) × 100) < 6.

Protease activity was determined by fluorescent assay using Bodipy casein as substrate and was performed as described in Materials and Methods.

[a]1.4 × (all components in original FM22 medium).

[b]0.6 × (all components in original FM22 medium).

[c]1.4 × (total phosphates in original FM22 medium).

[d]0.6 × (total phosphates in original FM22 medium).

[e]Casamino acids (5 g/L) in original FM22 medium.

[f]Ammonium sulfate (200 g/L) fed 2 mL every 3 h from induction.

[g]Glycerol (50% v/v) is fed in place of methanol.

to methanol, then remained constant and decreased towards the later stages of fermentation.

Protease Activities Found in *P. pastoris* Culture Supernatant

The time course of proteases in *P. pastoris* culture supernatant was studied. The same enzyme activities which were detected in the intracellular extract were present in the fermentation culture supernatant. The protease levels were near detectable limits at the beginning of induction and gradually increased towards the end of fermentation at 72 h (Figs. 2–5). The levels of proteinase A were high in the culture supernatant, while the level of aminopeptidase was low. However, the levels of these proteases in the culture supernatant were on average 10–40 times lower than their corresponding levels inside the cell. It is most likely that these proteases are primarily intracellular but move out of

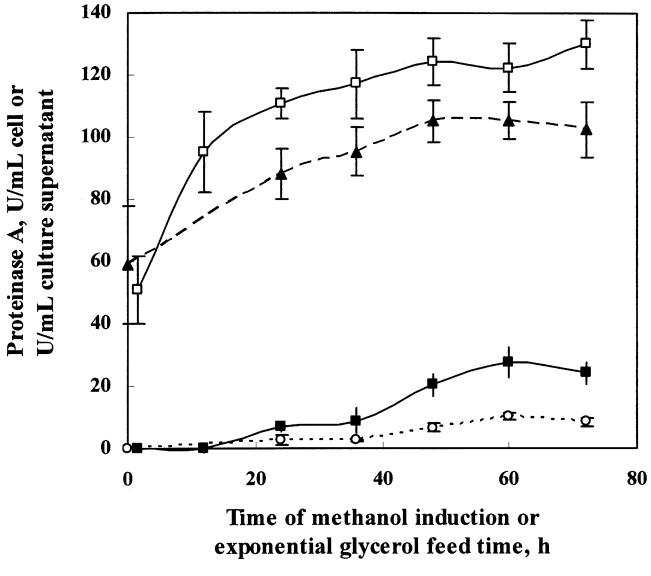

Figure 2. Time course of proteinase A activity during r-oIFN-τ production by *Pichia pastoris*: □ cell free extract-methanol; ■ culture supernatant-methanol; ▲ cell free extract-glycerol; ○ culture supernatant-glycerol.

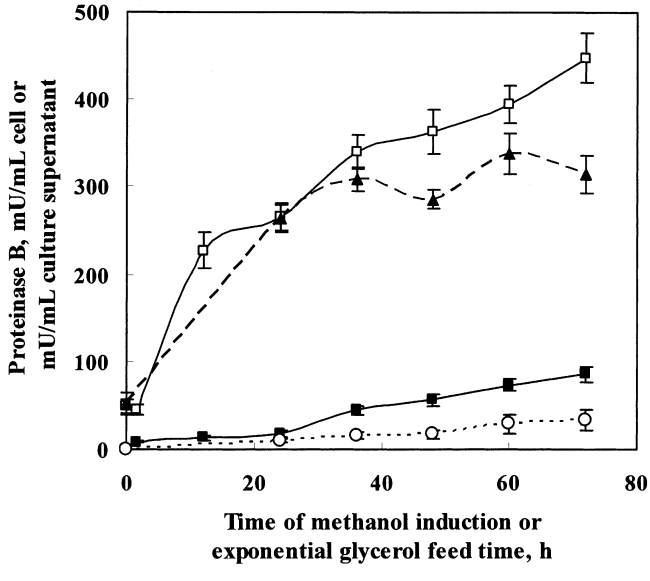

Figure 3. Time course of proteinase B activity during r-oIFN-τ production by *Pichia pastoris*: □ cell free extract-methanol; ■ culture supernatant-methanol; ▲ cell free extract-glycerol; ○ culture supernatant-glycerol.

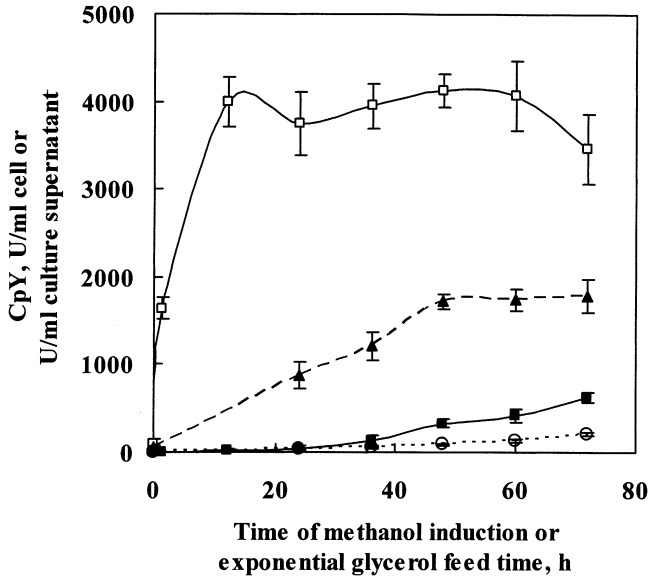

Figure 4. Time course of carboxypeptidase Y activity during r-oIFN-τ production by *Pichia pastoris*: □ cell free extract-methanol; ■ culture supernatant-methanol; ▲ cell free extract-glycerol; ○ culture supernatant-glycerol.

the cell between 60 h and 72 h or onset of stationary phase. Thus, the proteases, which were found on methanol as substrate were also found on glycerol as substrate, its levels were much reduced when glycerol was used (Figs. 2–5). Using glycerol as the carbon source, the total proteases were also lower than that obtained from growth on methanol as the sole carbon source (Table II). For comparison, fermentations were carried out with *P. pastoris* (X-33 WT) and induced in a similar fashion on methanol. Analysis of protease activity revealed types of proteases similar to

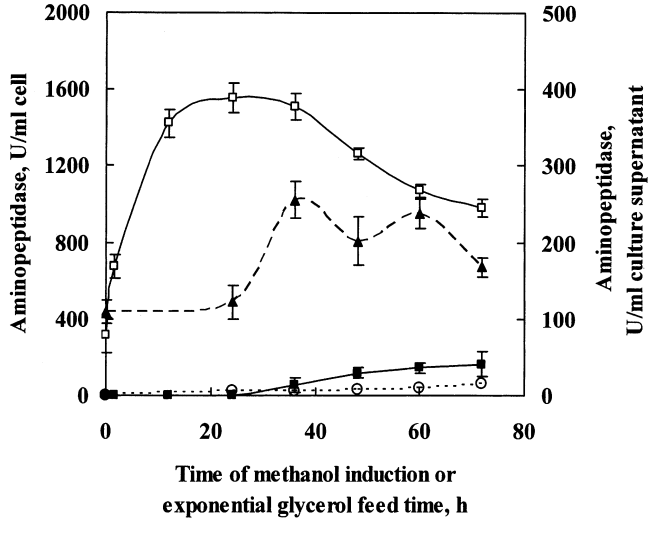

Figure 5. Time course of aminopeptidase activity during r-oIFN-τ production by *Pichia pastoris*: □ cell free extract-methanol; ■ culture supernatant-methanol; ▲ cell free extract-glycerol; ○ culture supernatant-glycerol.

P. pastoris recombinant clone (Fig. 7). Also, no significant difference in the levels of proteases were obtained between the wildtype and the recombinant clone (data not shown). Further, zymogram analysis of the periplasmic, cytoplasmic, and vacuolar extracts of both *P. pastoris* wildtype and the recombinant clone show comparable protease activity of the corresponding fractions from two clones (Fig. 7). Hence, it can be concluded that the presence of the IFN-τ gene did not create any metabolic burden on the cell triggering excess proteolytic activity.

These observations suggest that most of these proteases active on r-oIFN-τ in *P. pastoris* are intracellular and not secreted (Figs. 2–7). The literature reports suggest similar proteases are present in other industrially important yeasts like *Saccharomyces* and *Kluyveromyces* species, their primary source being vacuoles (Yasuhara et al., 1994; Flores et al., 1999). Western blots of the proteolytic degradation pattern of the recombinant protein, r-oIFN-τ,

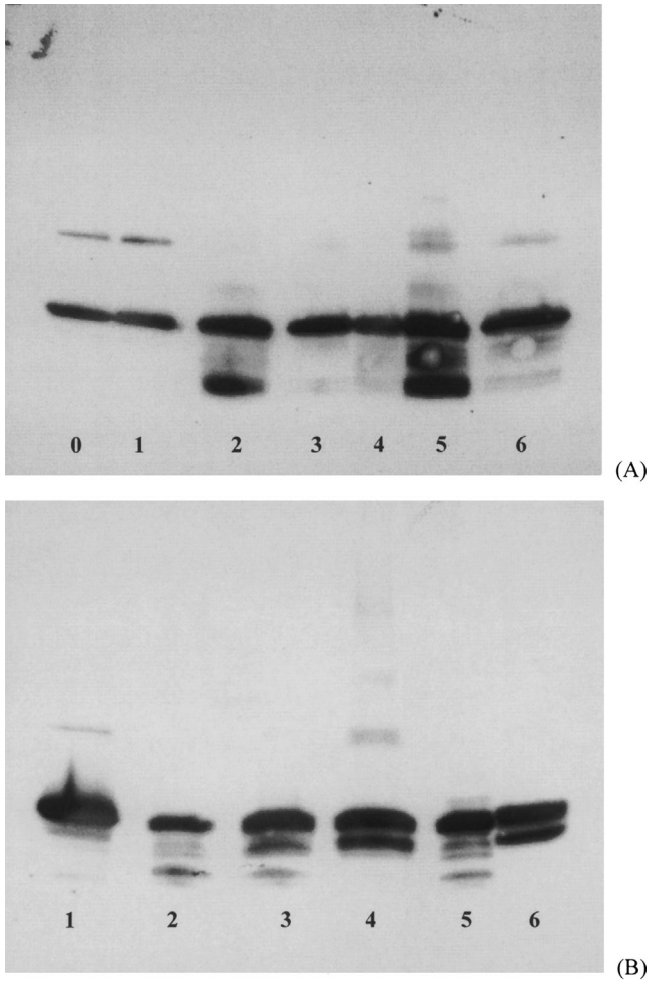

Figure 6. Western blots of r-oIFN-τ digested with (**A**) fermentation culture supernatant and (**B**) cell-free extract in the presence of inhibitors with no inhibitor as the control. From left: r-oIFN-τ in buffer (Lane 0 and 1 in **A**; Lane 1 in **B**); Lane 2: r-oIFN-τ (τ) with protease source (P); Lane 3: τ + P + PMSF; Lane 4: τ + P + pepstatin; Lane 5: τ + P + EDTA; Lane 6: τ + P + cocktail of PMSF, pepstatin, and EDTA.

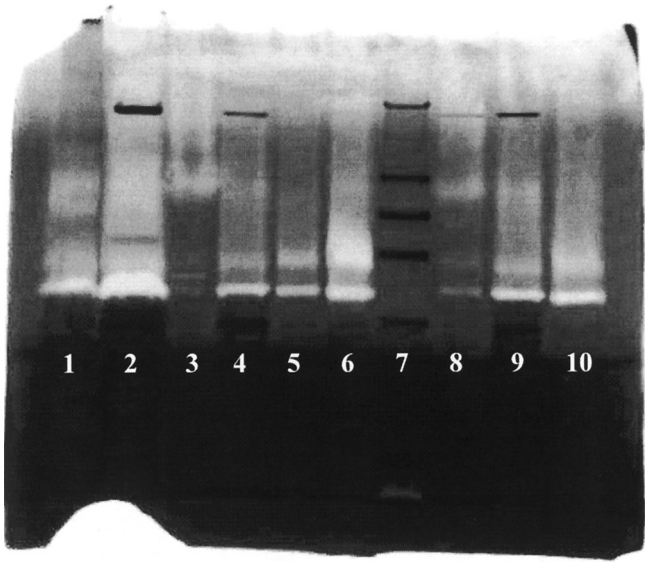

Figure 7. Casein zymogram of distribution of protease activity in culture supernatant, cell-free extract, and vacuolar fractions during fermentation. On methanol (recombinant oIFN-τ cell line) 1: culture supernatant; 2: cell-free extract; 3: periplasmic extract; 4: cytoplasmic extract; 5, 6: vacuolar extracts from two consecutive ficoll layers; 7: Marker; on methanol (WT) 8: periplasmic extract; 9: cytoplasmic extract; 10: vacuolar extract.

with in vitro studies using both cell-free extracts and culture supernatant revealed similar degradation patterns (Fig. 6). Thus, it may be concluded that proteases, which are generally intracellular, are found in the fermentation broth during high-density cell growth, especially on methanol as substrate. Since the protease levels increased slowly during induction, reaching maximal values towards the end of fermentation at 72 h, which also corresponds to the sustainable limit of fermentor capacity, growth on methanol itself as also cell lysis may be one of the possible mechanisms behind release of protease activity in the culture supernatant.

Cell Viability Studies

The methylene blue dye exclusion technique was used to differentiate between live and dead cells during fermentation and, in turn, to determine if cell lysis is contributing to increased proteolytic activity in the fermentation broth. Neutral red is actively taken up and internalized by live vacuoles, which appear as purple spherical bodies against a clear cytoplasm, whereas dead cells appear as a red smear. Cell count measurements indicated that the percentage of dead cells is minimal on both glycerol and methanol as the carbon source until the first 48 h of induction. However, the morphology of the cells was different. The cells grown on methanol were generally smaller and denser compared to cells grown on glycerol as the sole carbon source. The cells on either substrate tend to grow larger as fermentation progressed, with the vacuoles also increasing in size and numbers. The amount of live cells on methanol was on average 95% and 91%, respectively, at 60 h and 72 h of induction. However, on staining with neutral red more cells appeared as a red smear compared to red-stained vacuoles present at 48 h. No appreciable amount of dead cells was observed with cells grown on glycerol until 72 h of fermentation. These observations suggest that cells remain more viable on glycerol compared to methanol. It has been reported that only a small fraction of *Pichia* cells actually lyse even if they become nonviable at any earlier stage; however, this still accounts for significant release of host cell protein to the fermentation medium (Hohenblum et al., 2003). However, leaky cell membranes due to the action of hydrogen peroxide and formaldehyde formed as a by-product of methanol metabolism is a possibility. Assessment of cell viability during *P. pastoris* fermentation using flow cytometry has shown that almost 35% of the cells become nonviable (Hohenblum et al., 2003). They concluded that dead cells retain the cell proteins as long as they are not lysed. Further research is necessary to understand the exact mechanism of release of intracellular proteases to the fermentation medium in the presence of methanol.

Studies on Inhibition of Proteases

Inhibitors belonging to four different classes of enzymes, namely, serine, cysteine, aspartic, and metallo, were added separately to the enzyme source (fermentation culture supernatant) and incubated for 30 min before starting the fluorescent enzyme assay using Bodipy casein as substrate. The enzyme source, which was the 72 h fermentation culture supernatant from an IFN-τ fermentation, without inhibitors was used as control. It was found that, of the various inhibitors employed, phenyl methyl sulfonyl fluoride (PMSF) (1 mM) reduced the total protease activity by 78%, while 1 mM EDTA reduced the activity by 45% (Table III). A combination of 1 mM EDTA and 1 mM PMSF reduced protease activity by 94.2% (Table III). When the PMSF concentration was increased from 1 mM to 10 mM, precipitate was seen; however, no further increase in inhibition was observed, which proves that 1 mM is an effective concentration to inhibit all serine proteases in the culture supernatant. It was further observed that a 1 mM PMSF-treated fermentation culture supernatant did not degrade IFN-τ in an in vitro experiment, as described above (Fig. 6). However, the inhibition profile differed between fluorescent casein and IFN-τ as substrate (as with Bodipy casein), its hydrolysis was reduced when EDTA, a metalloprotease inhibitor, was added to the culture supernatant while r-oIFN-τ degradation was not prevented by EDTA. This showed that while Bodipy casein was susceptible to metalloproteases, IFN-τ was not. The extent of inhibition with a combination of PMSF, pepstatin, EDTA, and E64 was similar to the amount of inhibition with PMSF and EDTA, but no significant inhibition in protease activity was noted with pepstatin or E64 or their combination (Table III). The lack of inhibition with pepstatin was not unexpected, as aspartic proteases are generally active at pH 3.0 and the assay was done at pH 6.

Table III. Effect of inhibitors on proteolytic activity of *Pichia pastoris* culture filtrate using fluorescent Bodipy-casein as substrate.

Inhibitor	Concentration	Residual % activity
Control (no inhibitor)		100
PMSF	1 mM	22.1
EDTA	1 mM	54.9
E-64	20 μM	104
Pepstatin	10 μM	98.3
PMSF + Pepstatin	1 mM, 10 μM	18.5
PMSF + EDTA	1 mM, 1 mM	5.8
PMSF + E-64	1 mM, 20 μM	22.5
EDTA + E-64	1 mM, 20 μM	53.8
EDTA + Pepstatin	1 mM, 10 μM	56.1
E-64 + Pepstatin	20 μM, 10 μM	108
PMSF + EDTA + E-64	1 mM, 1 mM, 20 μM	6.0
PMSF + EDTA + Pepstatin	1 mM, 1 mM, 10 μM	5.5
PMSF + E-64 + Pepstatin	1 mM, 20 μM, 10 μM	19.2
PMSF + EDTA + E-64 + Pepstatin	1 mM, 1 mM, 20 μM, 10 μM	5.2

The enzyme source was treated with each inhibitor for 30 min prior to enyme assay.

Results presented are average of data points from three independent experiments.

However, when the culture supernatant was treated with pepstatin, 10% of PrA activity remained (assayed at pH 3.2) compared to control, indicating the presence of aspartic proteases in the medium.

Purified r-oIFN-τ was then incubated separately with fermentation culture supernatant and cell-free extract which was pretreated with inhibitors like PMSF, pepstatin, EDTA, E-64, and a cocktail of all of these inhibitors. It was observed both from Western blots as well as HPLC analysis that PMSF and pepstatin prevents the digestion of IFN-τ to a maximum extent, followed by EDTA. Pepstatin and PMSF prevent most degradation; however, a part of r-oIFN-τ is broken down to a lower molecular weight fragment which is possibly from inactivation of these inhibitors in an aqueous reaction environment over a 48-h period. It may be concluded that IFN-τ is acted on by various proteases, a serine and aspartic protease being maximally active on the protein (Fig. 6). Various mechanistic classes of proteases are present in the *Pichia* fermentation culture supernatants that are also present in the intracellular extract. These proteases have different specific activities against various proteins and, hence, individual proteins needs to be tested for the kind of inhibitor suitable to prevent its degradation.

Localization of Proteases

Fermentation samples were taken at regular intervals during the induction phase and various cellular fractions (periplasmic extract, the cytoplasmic extract, vacuolar sap) were isolated as described above and examined for proteolytic activity by assaying for total proteases and the major vacuolar proteases, proteinase A (PrA), proteinase B (PrB), carboxypeptidase Y (CpY), and aminopeptidases. The localization studies were based on time-course samples taken from *P. pastoris* recombinant cell line cultivated on methanol or glycerol as a comparative carbon source. This experimental plan gave us the opportunity to examine the changes in the nature and levels of proteases for the changeover of the carbon source from glycerol to methanol for r-oIFN-τ induction during the course of fermentation with respect to using glycerol as the sole carbon source throughout the fermentation. Inhibitors were employed to inhibit proteolytic activity at various stages to ensure validity of the observations. Pepstatin was found to inhibit proteinase A, PMSF almost completely inhibited proteinase B and carboxypeptidase reactions, while EDTA inhibited aminopeptidase (Ap) activity. Detailed analysis of the time course of periplasmic extracts revealed that although total proteolytic activity could be detected by fluorescence assay, PrA and CpY activity was below the detectable limit in the periplasmic extracts. Very little PrB and aminopeptidase activity was detected at random in some of the experimental repeats, which might result from lysis of a few cells during the process of spheroplast formation. Detection of proteolytic activity in fluorescence assay indicated the possibility of membrane-bound proteases being released during the process of spheroplast formation. However, they were not likely contributors to proteolysis, since in vitro incubation of the periplasmic extract with r-oIFN-τ was not found to digest the protein from Western blot analysis. Moreover, it was unlikely that membrane-bound proteases might be released during fermentation, considering the fact that no degeneration of the cell wall was noticed during fermentation and, as described above, cell death was noticed only during the later stages of fermentation, between 60–72 h.

PrA, PrB, CpY, and Ap were all detected to varying extents in the cytoplasmic extracts. As a general trend, higher levels of these enzymes were found from 48 h of induction when methanol was used as a carbon source. When the cells were not induced, but grown on an exponential growth profile on glycerol ($\mu = 0.025\ h^{-1}$), the levels of vacuolar proteases in the cytoplasmic extract were lower than the levels of these proteases obtained when methanol was used (Table IV). The levels of the vacuolar proteases assayed increased rapidly from 0–24 h of induction and then decreased slowly after 48 h until batch termination at 72 h of induction. In contrast, the levels of cytoplasmic proteases increased during 48 h and 72 h of induction, although proteases measured in cell-free extracts generally increased at 0 h of induction and stayed at relatively constant levels (Figs. 2–5).

These trends revealed that PrA, PrB, CpY, and Aps are of a vacuolar origin similar to other yeasts (Yasuhara et al., 1994; Flores et al., 1999), but can be found in cytoplasm as well in the fermentation broth, mostly during the later part of induction on methanol, and to a much lesser extent on glycerol. Analysis of the protease profile on a casein zymogram also revealed common protease bands between

Table IV. Localization of various proteases from *Pichia pastoris* recombinant cell culture.

	Periplasmic extract *U/mg		Cytoplasmic extract *U/mg		Vacuolar extract *U/mg		Culture filtrate *U/mg	
Enzyme assayed	Methanol	Glycerol	Methanol	Glycerol	Methanol	Glycerol	Methanol	Glycerol
Proteinase A	BDL	1.69	0.62	0.30	73.2	41.0	2.2	2.7
Proteinase B	4.6	3.6	21.2	9.7	303.5	73.4	13.4	5.7
CpY	19.2	9.8	592.3	380.9	3,719.4	856.4	80.5	27.2
Aminopeptidase	2.6	3.4	20.5	23.4	144.6	92.8	40.6	15.0
Total protease	68.2	99.8	325.3	210.1	6264	3954	677	224

Based on maximum proteolytic activity in each extract, which was at 72 h.
Results presented are average of data points from at least two independent experiments, % coefficient of variation ((std. dev./average) × 100) < 5.
*U/mg: U/mg protein in the extract.
BDL: below detection limit.

various cellular extracts (namely, cytoplasmic extract, vacuolar extract, and medium supernatant), showing the presence of proteases of vacuolar origin in the fermentation culture supernatant (Fig. 7).

It is probable that cell growth on methanol as carbon source for induction of recombinant protein production causes stress on the cell machinery to elicit higher protease activity. The degradation of secreted recombinant proteins might be a combined action of overproduction of proteases which results in mislocalization to the fermentation medium, with leaky membranes resulting from cultivation on methanol and cell lysis adding to the overall process of proteolysis. This suggests that the induction time of *P pastoris* growing on methanol as the sole carbon source is a critical factor which controls protease activity during *P. pastoris* fermentation. Hence, the kinetics of product accumulation versus its degradation due to proteolysis needs to be figured out for determining the optimal induction time for production of a recombinant protein from a Mut^+ strain of *P. pastoris*. In this regard, induction at high cell-density after extended glycerol fed-batch phase to enhance cell growth, followed by short induction times on methanol, should prevent proteolytic degradation of the target protein. In general, mixed feeds of methanol and glycerol using Mut^s (methanol utilization slow) strain or in some cases Mut^+ strain should be a better choice over using methanol as the sole carbon source to prevent proteolysis during fermentation (Zhang et al., 2003). However, protease deficient (SMD series) strains may not be the strain of choice, as they have typically reduced growth rates compared to their wildtype counterpart (Gleeson et al., 1998).

CONCLUSION

Methanol, which induces AOX1 promoter for recombinant protein induction, also creates conditions which trigger excess protease production and cell lysis when grown exponentially on methanol resulting in proteolytic degradation of the secreted protein during high cell density fermentation. The proteolytic activity increases with the time of induction as well as the specific growth rate on methanol. Hence, it is important to determine the optimum induction time and substrate feed strategies for recombinant protein production. The exact mechanism of increase in protease activity in the fermentation broth is not known, but it is clear that use of methanol as carbon source results in release or misdirection of proteases of vacuolar origin to the fermentation medium, causing degradation of secreted proteins.

References

Brankamp RG, Sreekrishna K, Smith P, Blankenship DT, Cardin AD. 1995. Expression of a synthetic gene encoding the anticoagulant-antimetastatic protein ghilanten by the methylotropic yeast *Pichia pastoris*. Prot Exp Purif 6:813–820.

Flores MV, Cuellas A, Voget CE. 1999. The proteolytic system of the yeast *Kluyveromyces lactis*. Yeast 15:1437–1448.

Gimenez JA, Monkovic DD, Dekleva ML. 2000. Identification and monitoring of protease activity in recombinant *Saccharomyces cerevisiae*. Biotechnol Bioeng 67:245–251.

Gleeson MA, White CE, Meininger DP, Komives EA. 1998. Generation of protease-deficient strains and their use in heterologous protein expression. Methods Mol Biol 103:81–94.

Goodrick JC, Xu M, Finnegan R, Schilling BM, Schiavi S, Hoppe H, Wan NC. 2001. High-level expression and stabilization of recombinant human chitinase produced in a continuous constitutive *Pichia pastoris* expression system. Biotechnol Bioeng 74:492–497.

Hansen RJ, Switzer RL, Hinze H, Holzer H. 1977. Effects of glucose and nitrogen source on the levels of proteinases, peptidases, and proteinase inhibitors in yeast. Biochim Biophys Acta 496:103–114.

Hilt W, Wolf DH. 1992. Stress-induced proteolysis in yeast. Mol Microbiol 6:2437–2442.

Hohenblum H, Borth N, Mattanovich D. 2003. Assessing viability and cell-associated product of recombinant protein producing *Pichia pastoris* with flow cytometry. J Biotechnol 102:281–290.

Jones LJ, Upson RH, Haugland RP, Panchuk-Voloshina N, Zhou M, Haugland RP. 1997. Quenched BODIPY dye-labeled casein substrates for the assay of protease activity by direct fluorescence measurement. Anal Biochem 251:144–152.

Kase R, Itoh K, Takiyama N, Oshima A, Sakuraba H, Suzuki Y. 1990. Galactosialidosis: simultaneous deficiency of esterase, carboxy-terminal deamidase and acid carboxypeptidase activities. Biochem Biophys Res Commun 172:1175–1179.

Kobayashi K, Kuwae S, Ohya T, Ohda T, Ohyama M, Ohi H, Tomomitsu K, Ohmura T. 2000. High-level expression of recombi-

nant human serum albumin from the methylotrophic yeast *Pichia pastoris* with minimal protease production and activation. J Biosci Bioeng 89:55–61.

Kurokawa T, Lee C, Shiomi N, Nakano A, Katoh S. 2002. Secretion of α-amylase from *Saccharomyces cerevisiae* and *Pichia pastoris* and characterization of its C-terminus with an anti-peptide antibody. J Chem Eng Jpn 35:1277–1281.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. Nature 227:680–685.

Lenney JF, Matile P, Wiemken A, Schellenberg M, Meyer J. 1974. Activities and cellular localization of yeast proteases and their inhibitors. Biochem Biophys Res Commun 60:1378–1383.

Martinez de Maranon I, Chaudanson N, Joly N, Gervais P. 1999. Slow heat rate increases yeast thermotolerance by maintaining plasma membrane integrity. Biotechnol Bioeng 65:176–181.

Rinderknecht H, Geokas MC, Silverman P, Haverback BJ. 1968. A new ultrasensitive method for the determination of proteolytic activity. Clin Chim Acta 21:197–203.

Rothman JH, Hunter CP, Valls LA, Stevens TH. 1986. Overproduction-induced mislocalization of a yeast vacuolar protein allows isolation of its structural gene. Proc Natl Acad Sci USA 83:3248–3252.

Sinha J, Plantz BA, Zhang W, Gouthro M, Schlegel V, Liu C-P, Meagher M. 2003. Improved production of recombinant ovine interferon-τ by Mut^+ strain of *Pichia pastoris* using an optimized methanol feed profile. Biotechnol Prog 19:794–802.

Stevens TH, Rothman JH, Payne GS, Schekman R. 1986. Gene dosage-dependent secretion of yeast vacuolar carboxypeptidase Y. J Cell Biol 102:1551–1557.

Stratton J, Chiruvolu V, Meagher M. 1998. High cell-density fermentation. In: Higgins DR, Cregg JM, editors. Methods in Molecular Biology, vol. 103 (Pichia Protocols). Totowa, NJ: Humana Press. p 107–120.

Trumbly RJ, Bradley G. 1983. Isolation and characterization of aminopeptidase mutants of *Saccharomyces cerevisiae*. J Bacteriol 156: 36–48.

Van Den Hazel HB, Kiellanbrandt MC, Winther JR. 1996. Biosynthesis and function of yeast vacuolar proteases: review. Yeast 12:1–16.

Werten MW, van den Bosch TJ, Wind RD, Mooibroek H, de Wolf FA. 1999. High-yield secretion of recombinant gelatins by *Pichia pastoris*. Yeast 15:1087–1096.

Yasuhara T, Nakai T, Ohashi A. 1994. Aminopeptidase Y, a new aminopeptidase from *Saccharomyces cerevisiae*. J Biol Chem 269: 13644–13650.

Yoshihisa T, Anraku Y. 1990. A novel pathway of import of α-mannosidase, a marker enzyme of vacuolar membrane, in *Saccharomyces cerevisiae*. J Biol Chem 265:22418–22425.

Zhang W, Bevins MA, Plantz BA, Smith LA, Meagher MM. 2000. Modeling *Pichia pastoris* growth on methanol and optimizing the production of a recombinant protein, the heavy-chain fragment C of botulinum neurotoxin, serotype A. Biotechnol Bioeng 70:1–8.

Zhang W, Potter KJ, Plantz BA, Schlegel VL, Smith LA, Meagher MM. 2003. *Pichia pastoris* fermentation with mixed-feeds of glycerol and methanol: growth kinetics and product improvement. J Ind Microbiol Biotechnol 30:210–215.

Zhou X-S, Zhang Y-X. 2002. Decrease of proteolytic degradation of recombinant hirudin produced by *Pichia pastoris* by controlling the specific growth rate. Biotechnol Lett 24:1449–1453.