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High-Level Production of Prourokinase-Annexin V Chimeras in the Methylotrophic Yeast *Pichia pastoris*

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Prourokinase (proUK)-annexin V chimeras expressed by the methylotrophic yeast *Pichia pastoris* in a synthetic medium as part of a system designed to yield a novel thrombolytic agent are degraded, as it is thought, by various yeast proteases present in the culture supernatant. Minimization of proteolysis was therefore investigated to increase the yield of intact proUK-annexin V. Protease inhibitor screening study indicated several proteases including at least serine protease like chymotrypsin were involved in the proteolysis. Addition of more than 10% of peptone or more than 0.2 mol l^{-1} of arginine to the medium was effective in minimizing proteolysis in shake-flask culture. Culture condition of higher pH was also effective, however, induced a cell death. Cell improvement by increasing the methanol utilization ability yielded greater tolerance to high pH. As a result, the culture condition with highly concentrated peptone solution fed under controlled conditions of pH 8.0 was established, which greatly reduced proteolytic degradation in fed-batch fermentation. These optimal conditions, which enabled fibrinolytic activity to reach 7800 IU ml^{-1}, could easily be applied in industrial scale production.

[**Key words:** proteolysis, protease, *Pichia pastoris*, urokinase, annexin V]

Urokinase (UK), a trypsin-type serine protease, activates plasminogen to plasmin. As plasmin hydrolyzes the fibrin which constitutes thrombi, UK is used for thrombolytic therapy. However, the insufficient binding affinity of UK for fibrin clots can cause serious side effects including resistance to reperfusion, coronary reocclusion and intracerebral bleeding (1). It is therefore important in the development of novel thrombolytic agents to improve their binding affinity for fibrin clots.

Annexin V, a member of the annexin family of phospholipid-binding proteins (2), consists of a single-chain polypeptide with a phospholipid binding site on the C-terminal side (3). It binds strongly to phosphatidylserine (PS) in the presence of Ca^{2+} and inhibits coagulation by competing with factor Xa and prothrombin (4). PS is located mainly on the inner membrane of both erythrocytes and blood platelets (5). In platelet activation, the normal asymmetric distribution of PS is lost, resulting in its relocation to the activated platelet surface, exposed to the blood stream (6). Intravenously administered annexin V also accumulates selectively in arterial and intracardiac thrombi *in vivo* (7, 8).

Given these characteristics, certain studies have attempted to use annexin V as a thrombus-targeting component in thrombolytic agents such as UK and proUK, a precursor of active UK: proUK-annexin V chimeras expressed in *Escherichia coli* show similar membrane-binding activity and thrombolytic activity to the parent molecules (9), while disulfide-linked conjugates of annnexin V with the B-chain of UK show three- to four-fold stronger thrombolytic activity than UK in a rat pulmonary embolism model (10). ProUK-annexin V chimeras secreted by the methylotrophic yeast *Pichia pastoris* show similar phospholipid-binding affinity to annexin V (11).

For purposes of clinical study and commercial production, hitherto reported expression levels of the novel thrombolytic agent represented by proUK-annexin V chimera are insufficient and need to be improved. In general, the *P. pastoris* expression system has the advantage of high-level production but the serious disadvantage that the expressed protein appears to be degraded by yeast proteases during cultivation (12). The present paper reports on the optimization of medium composition and culture conditions to minimize the proteolysis of proUK-annexin V chimeras secreted by *P. pastoris* and thus increase production volume.

MATERIALS AND METHODS

Strains The fused cDNA of proUK-annexin V chimera consisted of four elements, (i) a modified pre-sequence of *Mucor*

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Abbreviations: UK, urokinase; proUK, prourokinase; PS, phosphatidylserine; *AOX1*, gene encoding alcohol oxidase 1; *AOX2*, gene encoding alcohol oxidase 2; OD_{540}, optical density at 540 nm wavelength; IU, international units; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DFP, diisopropyl fluorophosphate; PMSF, phenylmethylsulfonyl fluoride; z-Glu, benzyloxycarbonyl-glutamic acid; TPCK, *N*-tosyl-L-phenylalanyl chloromethyl ketone; TLCK, *N*-tosyl-L-lysyl chloromethyl ketone; *p*-CMBA, *p*-chloromercuriobenzoic acid; TIU, trypsin inhibitor units.

pussilus rennin, (ii) a human proUK gene in which the Asn302 codon had been changed to a Gln codon to avoid glycosylation-based interference with secretion (13), (iii) a hirudin-derived spacer sequence and (iv) a human annexin V. *P. pastoris* GS115 was transformed with an expression vector by placing the fused proUK-annexin V gene under the control of a methanol-inducible *AOX1* promoter. The most productive clone, which displayed a Mut^s (methanol utilization slow) phenotype and possessed three copies of the expression unit of proUK-annexin V chimera, was chosen and designated as *P. pastoris* HB225 (11). Subsequently, another expression vector was designed to place the fused proUK-annexin V gene under the control of a mutated *AOX2* promoter (14). *P. pastoris* GS115 was transformed with the expression vector using the lithium acetate method (15). These transformants displayed a Mut$^+$ (methanol utilization high) phenotype, in which the expression vector was integrated into the *his4* locus of the chromosome. The transformant with the highest expression level in the shake-flask culture described below, which possessed two copies of the expression unit of proUK-annexin V chimera, was designated as *P. pastoris* UMP2-33.

Media and culture conditions For the preparation of a seed culture, a single colony of each clone, HB225 and UMP2-33, was inoculated from the stock plate into an Erlenmeyer flask containing 50 ml of YPD medium (1% yeast extract, 2% peptone, 2% dextrose) and the culture incubated at 30°C for 24 h with shaking at 120 rpm.

In the conventional shake-flask culture experiment, 1 ml of the seed culture was inoculated into Erlenmeyer flasks each containing 50 ml of one of the media and the culture incubated at 30°C for 72 h with shaking at 120 rpm. In order to reflect high-cell-density cultures in shake-flask cultures, cells proliferated from seed culture in Y5P10G5 medium (5% yeast extract, 10% peptone, 5% glycerol) were collected by centrifugation, washed and suspended in sterile saline. The concentrated culture was inoculated into an Erlenmeyer flask containing 30 ml of Y5P10M3 medium (5% yeast extract, 10% peptone, 3% methanol) to adjust initial OD_{540} to approximately 200 and incubated at 30°C for 72 h with vigorous shaking at 160 rpm.

In the fed-batch fermentation experiment, the seed culture was inoculated into a 3-*l* fermentor (Bio Master D type; Able, Tokyo) containing 1000 ml of PBM medium (16) or Y5P10G2 medium (5% yeast extract, 10% peptone, 2% glycerol). Air-flow rate and agitation rate were set at 1 vvm and 1000 rpm, respectively, and culture temperature maintained at 30°C. In cultures with PBM medium, pH was maintained at 5.85 by addition of 28% aqueous ammonia. In other cases, 2 N NaOH and/or 2 N HCl were added to control pH. After the complete consumption of the glycerol in the batch medium, continuous feeding with PFM medium (16) or methanol with or without 32% peptone solution was started. The feeding rate was controlled to maintain the methanol concentration in the culture broth at less than 2% (v/v) (16).

Cell concentration and viability Cell concentration was monitored by measurement of optical density at 540 nm wavelength using a Model UV-1200 spectrophotometer (Shimadzu, Kyoto).

The culture broth was diluted with phosphate buffer, stained with 0.02% methylene blue solution and cells counted with a microscope. Viability was determined as the ratio of living cells to total cells.

Protease activity assay Culture samples were centrifuged at 8000×*g* for 5 min and protease activity in the supernatants measured using the casein plate method (17) without Amidoblack staining. Human plasmin (Sigma-Aldrich, Tokyo) was used as the standard and protease activity expressed in plasmin units according to the specifications for plasmin.

Fibrinolytic activity assay Culture samples were centrifuged at 8000×*g* for 5 min and fibrinolytic activity in the supernatants measured using the bovine fibrin plate method (18). Urokinase Reference Standards (Mitsubishi Parma Corp., Osaka) were used and fibrinolytic activity expressed in IU.

Western blot analysis SDS–PAGE was performed using a 10–20% acrylamide gradient slab gel (Multigel 10/20; Daiichi Pure Chemicals, Tokyo) according to the method of Laemmli (19). The proteins were then electrophoretically transferred to a PVDF membrane (Immobilon P; Millipore, Tokyo) as described by Towbin (20). The membrane was incubated in a casein solution (Vector Laboratories, Burlingame, CA, USA) for 2 h at room temperature, then in a casein solution containing horse anti-proUK antibody (Mitsubishi Pharma Corp.) overnight at 4°C. The membrane was washed three times with TTBS (100 mM Tris–HCl [pH 7.5], 0.9% NaCl, 0.01% [v/v] Tween 20) and then incubated in TTBS containing biotinylated goat anti-horse antibody (Vector Laboratories) for 30 min at room temperature. The membrane was washed three times with TTBS, and then incubated in TTBS containing avidin-biotin-horseradish peroxidase complex (Vectastain ABC kit; Vector Laboratories) for 30 min at room temperature. After washing with TTBS, the proteins were visualized using the ECL detection system (Amersham Biosciences, Tokyo). Biotinylated SDS–PAGE Standards (Low Range; Bio-Rad, Hercules, CA, USA) were used as the molecular weight marker.

Protease inhibitor screening *P. pastoris* GS115 was inoculated into a YPM medium (1% yeast extract, 2% peptone, 3% methanol) and incubated at 30°C for 72 h. The supernatant was collected by centrifugation, filtered using a 0.22-μm pore-sized filter membrane and used as the enzyme solution. ProUK, which was isolated from culture fluid of human kidney cells (21), was added to the culture supernatant as the proteolysis substrate at a concentration of 2 μg ml^{-1} (300 IU ml^{-1}) and the product used as the reac-

TABLE 1. Screening study of protease inhibitors against proUK degradation

Group	Inhibitor	Concentration	Effect of inhibition
Serine protease inhibitor	TPCK	1 mM	+[b]
	DFP	1 mM	++[a]
	Aprotinin	0.27 TIU ml^{-1}	++
	Elastatinal	1 mM	–[c]
	Chymostatin	1 mM	++
	TLCK	1 mM	–
	Leupeptin	1 mM	+
	Benzamidine	10 mM	+
	Trypsin inhibitor	100 μg ml^{-1}	–
	PMSF	1 mM	++
Aminopeptidase inhibitor	Actinonin	1 mM	–
	Ebelactone B	1 mM	++
	1,10-Phenanthroline	10 mM	–
	Arphamenine A	1 mM	–
	Bestatin	1 mM	–
	Diprotin A	1 mM	–
Carboxyl protease inhibitor	Pepstatin A	1 mM	+
	z-Glu	10 mM	+
Thiol protease inhibitor	*N*-Ethylmaleimide	10 mM	–
	p-CMBA	1 mM	++
	Antipain	0.1 mM	++
	Iodoacetamide	1 mM	–
	Iodoacetic acid	1 mM	–
Metalloprotease inhibitor	Phosphoramidon	1 mM	–
	EDTA	50 mM	++

[a] ++, Proteolysis greately reduced.
[b] +, Proteolysis slightly reduced.
[c] –, Proteolysis not reduced.

tion mixture. Each protease inhibitor was added to the reaction mixture to adjust to the concentration described in Table 1 and incubated at 30°C for 16 h. The effect in terms of proteolysis inhibition was evaluated from the amount of degraded fragments in the western blot analysis described above.

RESULTS AND DISCUSSION

Secretion of proUK-annexin V chimeras in synthetic medium The synthetic media used in the fed-batch fermentation experiments with *P. pastoris* HB225, PBM medium as the batch medium and PFM medium as the feeding medium, both reduced protease activity in the culture broth to below the detection limit of the casein plate method (16).

A typical profile of fed-batch fermentation in synthetic medium is shown in Fig. 1. Fibrinolytic activity reached 1530 IU ml^{-1} at 98 h of cultivation, after which it disappeared rapidly. Western blot analysis showed that proUK-annexin V chimeras secreted in the medium were degraded during cultivation, even though the casein plate method did not detect protease activity in the culture broth (Fig. 1A, B). The rapid disappearance of fibrinolytic activity was thought to be caused by the degradation of proUK-annexin V chimeras. Additionally, the intact proUK-annexin V present in the culture broth at 98 h disappeared completely by the end of the cultivation period. This indicates that the proteolysis of intact proUK-annexin V in the culture medium occurred, at least partly, after secretion, although the possibility of intracellular degradation could not be excluded. The non-glycosylated form of proUK may have enhanced susceptibility to proteolysis, as it is reported to be much less stable in culture medium than the glycosylated form (22).

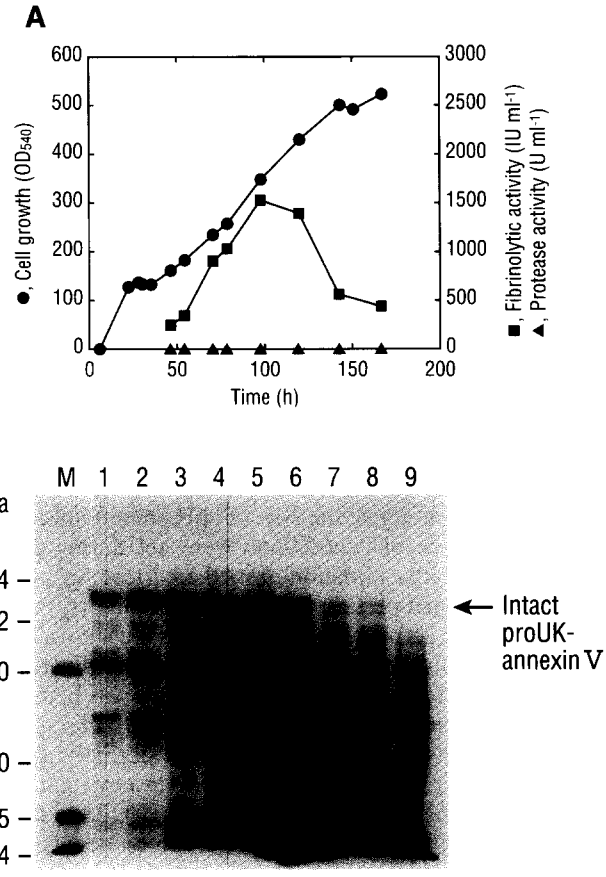

FIG. 1. (A) Fed-batch fermentation of *P. pastoris* HB225 in synthetic media. PBM medium for the batch medium and PFM medium for the feeding medium were used. Symbols: circles, cell concentration (OD_{540}); squares, fibrinolytic activity; triangles, protease activity. (B) Western blot analysis of proUK-annexin V chimeras secreted in synthetic medium. Culture supernatants collected by centrifugation at different timepoints during cultivation were mixed with equal volume of 2 × SDS sample buffer and heated at 95°C for 5 min. 10 μl of sample was dispensed into each well. Lane M, Molecular weight marker; lane 1, 47 h of cultivation; lane 2, 55 h; lane 3, 71 h; lane 4, 79 h; lane 5, 98 h; lane 6, 120 h; lane 7, 143 h; lane 8, 151 h; lane 9, 167 h.

Characterization of yeast proteases responsible for proUK degradation To confirm the presence of proteases in the culture supernatant, either proUK or annexin V, which was purified from human placenta (23), was incubated in the culture supernatant of the host strain, *P. pastoris* GS115. ProUK was completely degraded after 16 h at 30°C, while annexin V was stable in the culture supernatant for 20 h at 30°C (data not shown). These results indicate that the proUK domain of proUK-annexin V chimeras is highly susceptible to proteolysis compared to the annexin V domain.

In order to characterize the proteases responsible for proUK-annexin V degradation, a series of protease inhibitors was added to the culture supernatant containing proUK. Eight of the 25 protease inhibitors showed strong inhibition of proUK degradation (Table 1). The target protease of each inhibitor is different. Some have wide spectrum, and others narrow. As for serine protease inhibitors, wide-spectrum inhibitors, such as DFP, chymostatin and PMSF, effectively protected proUK-annexin V from proteolysis. On the other hand, relatively narrow-spectrum ones, such as elastatinal, TLCK and trypsin inhibitor, showed no effect on protection, with exceptions such as aprotinin and TPCK. These results clearly indicate that some of serine proteases, but not all of them, are responsible for the degradation. The species of problem proteases can be elucidated, at least partially, by scrutinizing the spectrum of each inhibitor. For example, TPCK, DFP and chymostatin reduced proteolysis of products. Common target proteases of these inhibitors include chymotrypsin. It is thus likely that chymotrypsin-like protease may be one of the problem proteases. Wide-spectrum inhibitor, leupeptin, which does not inhibit chymotrypsin activity, reduced the degradation slightly. This indicates that serine proteases other than chymotrypsin-like ones are also responsible for proteolysis of the products.

No single inhibitor completely protected products from proteolysis. Each five group included at least one effective inhibitor. Common target proteases of all groups are very few. These phenomena suggest that several proteases are involved in proUK-annxin V degradation.

It was hypothesized that proUK-annexin V degradation might be reduced by addition of a combination of different protease inhibitors to the culture medium. This method however turned out to be impractical because of the great cost and the possible inhibition of cell growth.

Effect of medium composition on minimization of proteolysis The effect of medium composition on minimization of proteolytic degradation was investigated using a shake-flask culture. First, both yeast extract and peptone concentrations were varied between 1–7% and 2–14%, respectively. It was found that yeast extract and/or peptone concentrations did affect the degree of minimization of proteolysis (Fig. 2A). Specifically, medium containing more than 5% yeast extract and more than 10% peptone (Y5P10 medium) produced satisfactory reduction of proteolysis.

Secondly, the concentration of peptone, derived from animal tissues, in the medium was varied: higher concentrations increased the amount of intact proUK-annexin V and decreased the amount of smaller fragments (Fig. 2B). Peptone alone was therefore considered to be effective in minimizing proteolysis. Additionally, peptones prepared from casein and from soybean were tested, and the former was effective, but the latter was not (data not shown). Addition of casamino acids to medium is reported to be effective in minimizing the degradation of mouse epidermal growth factor secreted by *P. pastoris* (24) and of gelatins secreted by *P. pastoris* (25). In the present study, addition of casamino acids did not overcome the proteolysis of proUK-annexin V, although it did reduce the proteolysis slightly (data not shown). Yeast extract is a water-soluble portion of autolyzed yeast, and contains vitamins, nucleic acids and amino acids. Peptone is prepared from protein materials by completely or partially digested with proteases. Ones from casein or animal tissues contain large amount of polypeptides, dipeptides and amino acids, while one from soybean contains carbohydrates rather than peptides. Casamino acids are acid-hydrolysed casein and contain amino acids without peptides. Considering the difference of compounds in above supplements, large amount of peptides are thought to protect proUK-annexin V from the attack of several yeast proteases by acting as substrate.

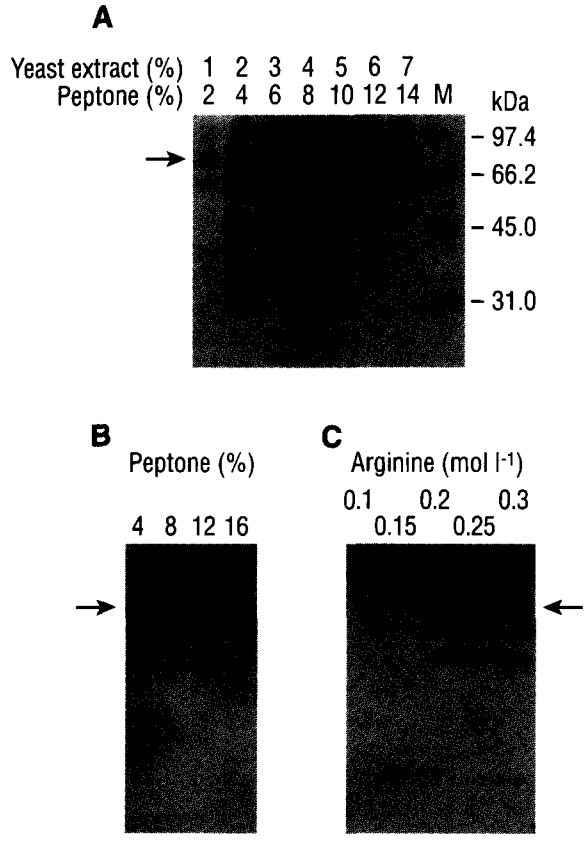

FIG. 2. Western blot analyses to investigate the effect of medium composition on minimization of the proteolytic degradation of proUK-annexin V. The position of intact proUK-annexin V is marked with an arrow. (A) Effect of yeast extract and peptone concentrations. Upper numbers above each lane show concentrations of yeast extract and lower numbers peptone concentrations. Lane M shows molecular weight marker. (B) Effect of peptone concentration. Except for peptone concentration, which was as indicated above each lane, each medium was the same, containing 2% yeast extract and 3% methanol. (C) Effect of arginine addition. Arginine concentrations are indicated above each lane.

Thirdly, each of 20 amino acids was added to YPM medium with culture pH adjusted to 6.8±0.3. YPM medium supplemented with more than 0.2 mol l^{-1} of arginine was found to eliminate proteolysis (Fig. 2C), while the addition of other amino acids did not do so completely (data not shown). This finding was consistent with the secretion of human UK by *P. pastoris* (26) and with the secretion of human serum albumin by *Saccharomyces cerevisiae* (27). Trypsin cleaves the carboxyl side of arginine or lysine. High concentration of arginine might inhibit trypsin-like protease by acting as competitor. However, the result that trypsin inhibitor and TLCK, chemical synthetic inhibitor against trypsin, did not reduce proUK degradation as shown in Table 1, indicates that trypsin-like protease is not related to proUK-annexin V degradation. Therefore, it is not likely that arginine acts as competitor for protease responsible for proUK-annexin V degradation.

Effect of pH on minimization of proteolysis Y5P10 medium failed to reduce proteolysis in fed-batch fermentation. Except for pH, the same culture conditions had been set for the shake-flask culture and the fed-batch fermentation, with pH maintained at 7.0 in the fed-batch fermentation but changed from 6.6 to 8.3 in the shake-flask culture.

In order to investigate the effect of pH on minimization of proteolysis, three pH conditions were set during the induction phase (after initiation of methanol feeding): uncontrolled, pH 7.0 and pH 7.7. Cell concentration, pH, viability and fibrinolytic activity during cultivation are shown in Fig. 3. When uncontrolled, pH was stable at approximately 6.6 while the glycerol in the Y5P10G2 medium was being consumed by *P. pastoris*, but rose to over 8.3 subsequently. High pH decreased viability and reduced fibrinolytic activity and cell growth. This suggests that an environment of more than pH 7.7 is too severe for strain HB225 to survive in and express proUK-annexin V. Western blot analyses are shown in Fig. 4. Under higher pH conditions, *i.e.*, uncontrolled (pH 8.3) and pH 7.7, fewer degraded fragments were observed than under controlled pH 7.0 conditions, indicating that high pH strongly suppresses protease activity. It is concluded that a high pH environment is not optimal for the yeast proteases responsible for proUK-annexin V degradation and conversely is conducive to maintaining the proUK-annexin V molecule intact.

Control of pH value is reportedly effective in minimizing proteolytic degradation of foreign proteins expressed by *P.*

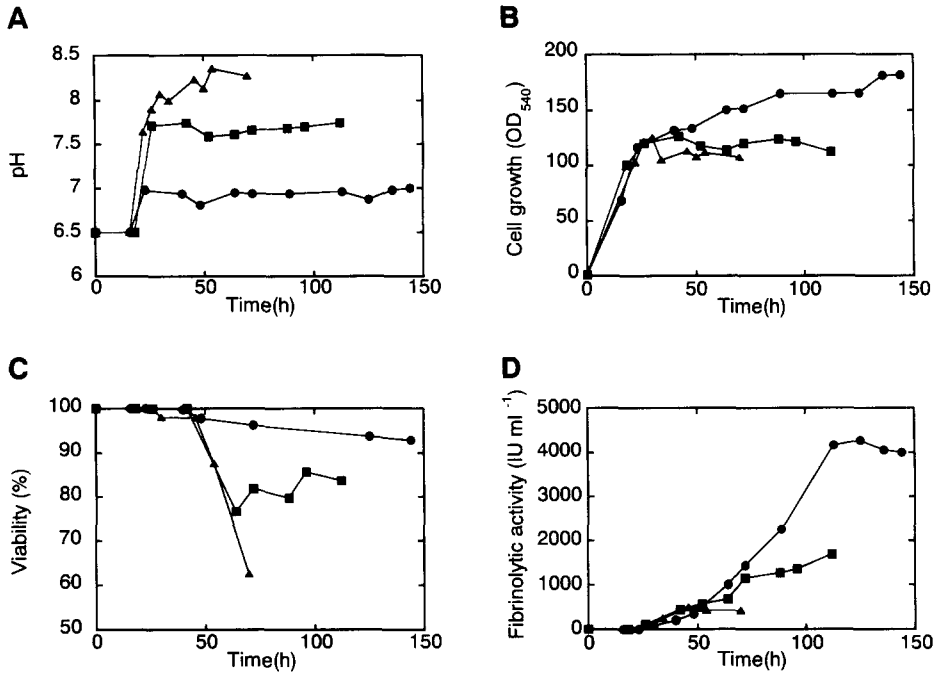

FIG. 3. Fed-batch fermentation of *P. pastoris* HB225 in Y5P10G2 medium. ProUK-annexin V expression was induced by methanol feeding under controlled conditions of pH 7.0 and pH 7.7, and without pH control. (A) Time course of culture pH. (B) Time course of cell concentration (OD_{540}). (C) Time course of viability. (D) Time course of fibrinolytic activity. Symbols: circles, pH 7.0; squares, pH 7.7; triangles, pH not controlled.

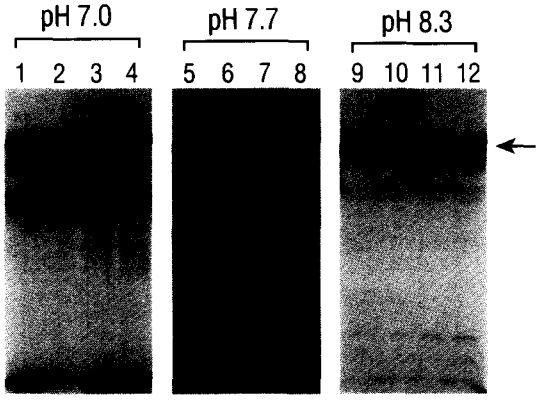

FIG. 4. Western blot analyses to investigate the effect of culture pH on minimization of the proteolytic degradation of proUK-annexin V. Culture supernatants were collected by centrifugation from each culture, as shown in Fig. 3. Lanes 1 and 5, 64 h of cultivation under controlled conditions of pH 7.0 and 7.7, respectively; lanes 2 and 6, 72 h; lanes 3 and 7, 89 h; lanes 4 and 8, 112 h; lane 9, 46 h of cultivation without pH control; lane 10, 50 h; lane 11, 54 h; lane 12, 70 h. The position of intact proUK-annexin V is marked with an arrow.

pastoris, with low range values of pH 3.0 to 5.0 effective for gelatins (25) and HIV-1 envelope proteins (28), and mid-range values of pH 5.6 to 6.0 for human serum albumin (16) and mouse epidermal growth factor (24). Cell growth is not inhibited in these ranges. In the present study, it is important to establish within the higher range a pH level which represents an appropriate compromise between cell growth and minimization of proUK-annexin V degradation.

Development of fed-batch fermentation for proUK-annexin V production During the investigation of culture conditions described above using HB225 strain, further efforts were performed to obtain strains, which express higher level of products, by noting following points. Firstly, the methanol utilization ability was changed from Mut^s to Mut^+ in order to increase the cell density, and secondly, promoter was changed from *AOX1* to mutated *AOX2*, which was successfully used for high-level expression of human serum albumin from *P. pastoris* (16). As a consequence, UMP2-33 strain was selected. In order to compare the production levels of the HB225 and UMP2-33 strains, shake-flask cultures reflecting high-cell-density cultures were performed. The fibrinolytic activity of UMP2-33, at 980 IU ml^{-1}, was found to be 1.5-fold higher than that of HB225, and it was therefore chosen for the further study of fed-batch fermentation.

As pH had been demonstrated to be an important factor affecting the proteolysis of proUK-annexin V, optimization of pH during the induction phase (after initiation of methanol feeding) was carried out. As shown in Fig. 5, viability was maintained at over 90% under controlled conditions of both pH 7.5 and pH 8.0, indicating that UMP2-33 has greater tolerance to high pH than HB225. Because, when viability decreased, intracellular proteases were released from dead cells to culture broth, maintaining viability at high level is expected to reduce proteolysis. UMP2-33 was therefore concluded to be more suitable for proUK-annexin V production. Under controlled conditions of pH 8.5, fibrinolytic activity gradually decreased after 119 h of cultivation. This was thought to be due to the accelerated release of proteases caused by inhibition of cell growth. Western blot analysis of proUK-annexin V secreted in fed-batch fermentation under controlled conditions of pH 8.0 is shown in Fig. 6: although

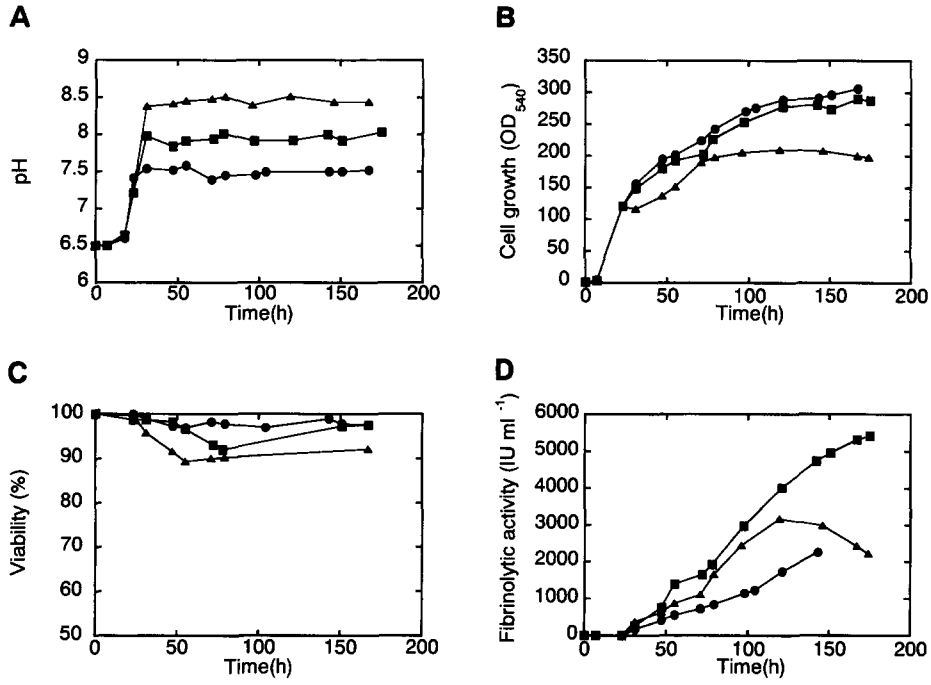

FIG. 5. Fed-batch fermentation of *P. pastoris* UMP2-33 in Y5P10G2 medium. Culture pH was maintained at pH 7.5, pH 8.0 and pH 8.5, respectively, during the induction phase of proUK-annexin V expression. (A) Time course of culture pH. (B) Time course of cell concentration (OD_{540}). (C) Time course of viability. (D) Time course of fibrinolytic activity. Symbols: circles, pH 7.5; squares, pH 8.0; triangles, pH 8.5.

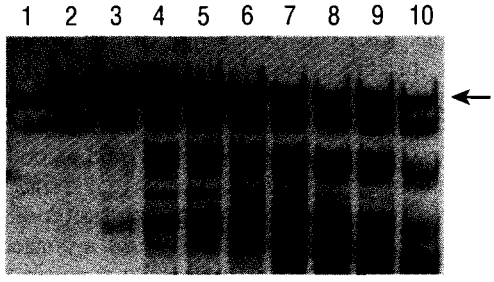

FIG. 6. Western blot analysis of proUK-annexin V secreted in fed-batch fermentation under controlled conditions of pH 8.0, as shown in Fig. 5. Lane 1, 31 h of cultivation; lane 2, 47 h; lane 3, 55 h; lane 4, 72 h; lane 5, 78 h; lane 6, 97 h; lane 7, 121 h; lane 8, 142 h; lane 9, 151 h; lane 10, 167 h. The position of intact proUK-annexin V is marked with an arrow.

fibrinolytic activity reached 5500 IU ml^{-1}, the amount of intact proUK-annexin V increased up to 97 h of cultivation, then, due to proteolysis, decreased toward the end of the cultivation period. Proteolysis was thought to occur due to a shortage of peptone, which acts as an excess substrate for the problem proteases. This shortage might in turn be due to the dilution of peptone by methanol feeding and by its consumption by yeast.

In order to prevent the concentration of peptone from decreasing, 32% peptone solution was added in parallel with methanol feeding. Based on culture volume and amount of peptone added, and ignoring consumption by yeast, the concentration of peptone was calculated at 15% at the end of the cultivation period. As expected, the degradation of intact proUK-annexin V was reduced effectively, as shown in Fig. 7. Fibrinolytic activity reached approximately 7800 IU

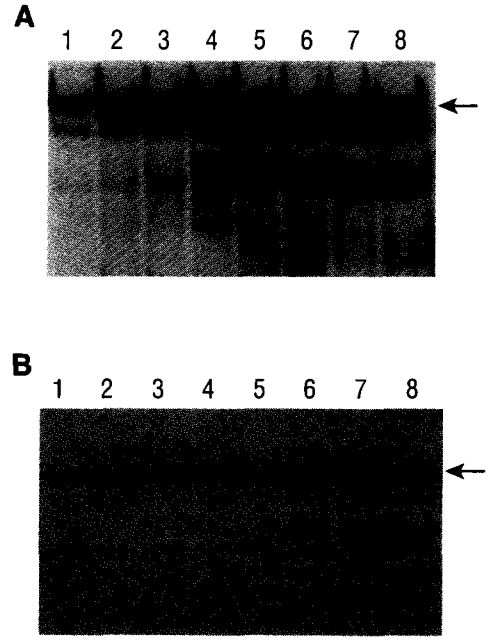

FIG. 7. Western blot analyses of proUK-annexin V secreted in fed-batch fermentation with peptone feeding. Peptone solution (32%) was added in parallel with methanol feeding. (A) Samples were not diluted so as to emphasize the presence of degraded fragments. (B) 60-fold diluted samples were dispensed into each well. Lane 1, 55 h of cultivation; lane 2, 72 h; lane 3, 78 h; lane 4, 97 h; lane 5, 121 h; lane 6, 142 h; lane 7, 151 h; lane 8, 167 h. The position of intact proUK-annexin V is marked with an arrow.

ml^{-1} at 168 h, the end of the cultivation period in fed-batch fermentation, more than ten-fold higher than in an earlier study (11).

Addition of arginine to the medium was also attempted, with concentration adjusted to 0.3 mol l^{-1}, but this led to marked cell growth inhibition under controlled conditions of pH 8.0 (data not shown). In view of the result in shake-flask culture with pH maintained at below 7.0 (Fig. 2C), the difference in cell growth between the shake-flask culture and the fed-batch fermentation appears to be due to the difference in pH. The advantage of peptone over arginine in the minimization of proteolytic degradation, even under severe conditions of high pH, was thus established.

In the present study, it was found that a combination of high pH with peptone feeding was highly effective in minimizing proteolysis. This method would not only yield enough proUK-annexin V for clinical study, but would also be easily applicable in industrial scale production. In a commercial production setting, feeding with large amounts of peptone would raise raw material costs. On the other hand, increasing production level would reduce production costs. Further improvement of culture conditions needs to be investigated under evaluation of total production cost.

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