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Glycosylation of Prourokinase Produced by *Pichia pastoris* Impairs Enzymatic Activity but Not Secretion

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Both glycosylated and nonglycosylated forms of recombinant human prourokinase were produced to the level of 20 mg/L by yeast *Pichia pastoris* in BMMY medium after 2 days of culture. The expressed pro-UK was 98% secreted into the culture medium and easily purified by carboxymethyl cellulose chromatography. More than 99% of pro-UK in the culture medium was found in single-chain form. This was contradictory to a previous finding which found that glycosylation of pro-UK by yeast inhibited its secretion. The absence of glycosylation at Asn302 of pro-UK has no measurable effect on its secretion from the yeast cells. However, the nonglycosylated pro-UK was much less stable in the culture medium, probably due to proteolysis. Nonglycosylated pro-UK from yeast had a clot lysing activity comparable to that of *Escherichia coli*-derived or mammalian cell-derived recombinant pro-UK. By contrast, the glycosylated yeast pro-UK was less activatable by plasmin and had a lower enzymatic activity against plasminogen and a lower clot lysing activity than nonglycosylated pro-UK from yeast, while their amidolytic activity against S2444 was equivalent. It was concluded that glycosylation of pro-UK by yeast *P. pastoris* interferes with the catalytic site but not secretion of this protein.

Human urokinase-type plasminogen activator (uPA)[2] is a serine protease that is a physiological plasminogen activator. It is involved in a number of biological functions, including fibrinolysis, embryogenesis, cell migration, tissue remodeling, ovulation, and wound healing (1–5). The two-chain form of uPA (tc-uPA) prepared from human urine or kidney cell culture has been used as a thrombolytic agent for more than two decades. More recently, the single-chain form of uPA (sc-uPA), also called prourokinase (pro-UK), has been developed for thrombolysis because of its fibrin specificity.

Recombinant pro-UK has been produced by a number of different host cells, including *Escherichia coli* (6), mammalian cells (7), and yeast (8). Production in *E. coli* is problematic due to its accumulation in an unfolded state as inclusion bodies, from which recovery of biologically active pro-UK by refolding is low in efficiency. The mammalian cell expression system is high in cost and low in yield and requires stringent control procedures to detect infectious agents. Since yeast can be grown rapidly on simple media to high cell density, secreting expression of foreign proteins by yeast has advantages over other expression systems.

Pro-UK was produced in *Saccharomyces cerevisin* using the GAL7 promoter and the prepeptide sequence of a fungal aspartic proteinase, *Mucor pusillus rennin* (8). However, it was reported that most of pro-UK accumulated inside the cells and only trace amounts were secreted (8). *Pichia pastoris* is an industrial methylotrophic yeast which is readily cultured to a very high density and has been developed as a host for expression of foreign proteins as an alternative to *S. cerevisin* (9). Therefore, *P. pastoris* was chosen to express pro-UK in this study.

Human pro-UK normally has an N-glycosylation site at Asn302. The N-linked oligosaccharides attached to the recombinant glycoprotein in yeast can consist of mannan structures containing more than 50 mannose monomers (10), which was reported to interfere with secretion of pro-UK by yeast cells (8). In this study, glycosylated pro-UK produced by yeast *P. pastoris* was studied for secretion, enzymatic properties, and stabil-

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[2] Abbreviations used: uPA, human urokinase-type plasminogen activator; tc-uPA, two-chain form of uPA; sc-uPA, single-chain form of uPA; pro-UK, prourokinase; OD, optical density.

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ity in culture medium. The findings were compared with nonglycosylated pro-UK from the same yeast strain using a variant of human pro-UK lacking the N-glycosylation site at Asn302.

MATERIALS AND METHODS

Materials

Restriction enzymes and DNA-modifying enzymes were purchased from New England Biolabs (Beverly, MA). The DNA sequencing kit was obtained from U.S. Biochemicals (Cleveland, OH). The recombinant nonglycosylated human pro-UK expressed by *E. coli* was obtained from Farmitalia CarloErba (Milan, Italy). The recombinant glycosylated human pro-UK expressed by mammalian cells was obtained from Abbott Laboratories (Chicago, IL). ^{35}S-Labeled dATP was obtained from DuPont (Boston, MA). Oligonucleotides were custom synthesized by Amitof Biotech (Boston, MA). *P. pastoris* KM 71, expression vector pPICZ-α, yeast culture medium BMGY (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, 1% glycerol, 100 mM potassium phosphate, pH 6.0), and yeast culture medium BMMY (1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 4×10^{-5}% biotin, 0.5% methanol, 100 mM potassium phosphate, pH 6.0) were purchased from Invitrogen (Carlsbad, CA). S2444 (L-pyroglutamylglycyl-L-arginine-*p*-nitroaniline hydrochloride), a chromogenic substrate for urokinase, and S2251 (H-D-valyl-L-leucyllysine-*p*-nitroaniline dihydrochloride), a chromogenic substrate for plasmin, were obtained from Kabi (Franklin, OH). Human plasmin was purchased from American Diagnostica Inc. (Greenwich, CT). Carboxymethyl cellulose, sorbitol, and all other reagents were obtained from Sigma (St. Louis, MO).

Methods

Recombinant plasmid constructs. The plasmid pSAT-UK containing the coding region of pro-UK (11) was used as a template for subcloning into the yeast expression vector pPICZα by PCR. Vector pPICZα has an AOX1 promoter that allows methanol-inducible, high-level expression in *Pichia*, an α-factor signal sequence for secretion, and a Zeocin resistance gene (12) for positive clone selection. The following primers were used in the PCR amplification. As shown in Fig. 1, primer 1 (5′-CTCTCGAGAAAAGAAGCAATGAACTTCATCAAGTTC CATCG-3′) was incorporated to introduce the *Xho*I restriction site, α-factor signal peptide sequence, and a Kex2 cleavage site before the first codon of pro-UK. Primer 2 (5′-AATCTAGATCAGAGGGCCAGGCCATTCTC-3′) was incorporated to include an *Xba*I restriction site after the stop codon.

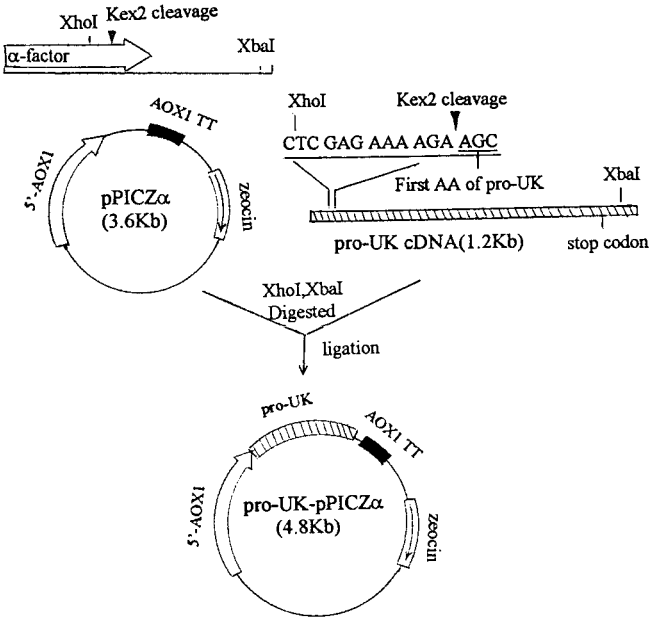

FIG. 1. Construction of pro-UK-pPICZα expression plasmid.

The amplified pro-UK DNA was digested with *Xho*I and *Xba*I, and then purified and ligated with vector pPICZα that was pretreated with the above enzymes. The initial transformation was performed with the host strain TOP 10F. Positive clones were selected and sequenced to confirm the above gene modifications.

Site-directed mutagenesis for nonglycosylated pro-UK gene. The Stratagene Quickchange site-directed mutagenesis kit was used to create the Asn302 to Ala302 mutation. Briefly, the oligonucleotide primers containing the desired mutation (Asn302 to Ala302), each complementary to opposite strands of the vector, were extended during temperature cycling by using *Pfu*Turbo DNA polymerase. This polymerase has been shown to significantly increase PCR product yields and replication fidelity compared with *Taq* DNA polymerase. Incorporation of oligonucleotide primers generated a mutated plasmid containing staggered nicks. Following temperature cycling, the PCR product and the parental DNA template were treated with *Dpn*I (target sequence: 5′-Gm6ATC-3′), which is specific for methylated and hemimethylated DNA, in order to select for mutation containing synthesized DNA. The nicked vector DNA incorporating the desired mutations was then transformed into *E. coli* XL-Blue supercompetent cells. Positive clones were selected and their DNA sequences were determined to confirm the expected mutation.

Transformation of yeast cells. The plasmid pro-UK-pPICZα was linearized with *Pme*I enzyme and used for homologous recombination into the yeast host strain

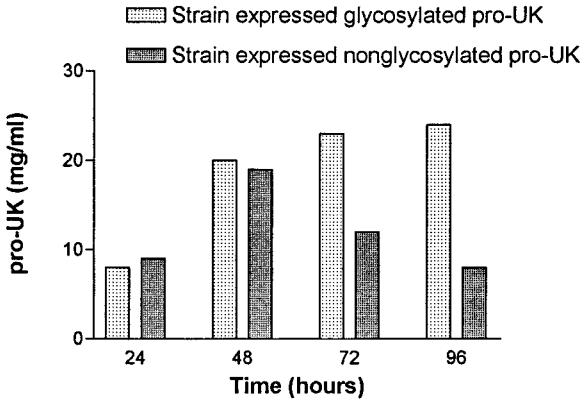

FIG. 2. Secretion of pro-UK in shake-flask cultures of BMMY medium.

KM 71 by the EasyComp transformation kit (Invitrogen). Positive clones were selected in Zeocin resistance YPDS plates (1% yeast extract, 2% peptone, 2% glucose, 1 M sorbitol, 2% agar, 100 μg/ml Zeocin). Transformants were monitored for secretion of fibrinolytic activity. The clones with high production of glycosylated pro-UK and nonglycosylated pro-UK were selected, respectively.

Expression and purification of pro-UK. The time courses of secretion of glycosylated pro-UK and nonglycosylated pro-UK were examined for 96 h in BMMY medium at 30°C. First, the yeast strains for expressing glycosylated pro-UK and nonglycosylated pro-UK were inoculated respectively in BMGY medium until the cultures reached the logarithmic phase of growth. The cultures were then concentrated in BMMY to one-fifth their original sizes and allowed to continue to grow at 30°C with shaking. Pure methanol was added to the medium to a concentration of 0.5% every 24 h. Culture supernatants from the BMMY medium were harvested and applied to carboxymethyl cellulose columns equilibrated with 50 mM acetic acid, 0.2 M NaCl, 0.01% Tween 80. After the column was washed with the equilibrium buffer, pro-UK was eluted with 50 mM HAc, 0.5 M NaCl, 0.01% Tween 80. Purified pro-UK samples were analyzed by 12% reducing and nonreducing SDS–PAGE.

Urokinase amidolytic activity assay. The tc-uPA was obtained by pretreating sc-uPA with plasmin as previously described (13). The amidolytic activity of tc-uPA was assayed against the synthetic substrate S2444 (0.6 mM) in the assay buffer (0.05 M Tris, 0.1 M NaCl, 0.2% BSA, and 0.01% Tween 80, pH 7.4) and standardized against the International Reference Preparation of UK (National Institute for Biological Standards and Controls, London, UK).

The kinetic parameters of amidolysis were determined by incubating tc-uPAs (2 nM) at room temperature with S2444 (30–300 μM) in the assay buffer. The reaction rate was calculated using the increase in optical density (OD) over time at 410 nm against a reference wavelength of 490 nm on a microplate reader (MR 5000; Dynatech Laboratories, Alexandria, VA) as previously described (14). The Michaelis constant (K_M) and catalytic rate constant (k_{cat}) were determined from plots with a computerized program (Enzfitter, Elsevier–Biosoft, Cambridge, UK).

Glu-plasminogen activation assay. Time–absorbance curves of Glu-plasminogen activation were obtained by measuring the OD increase of the reaction mixture over time squared at the wavelength 410 nm and a reference wavelength 490 nm on a microplate reader. The reaction mixture contained S2251 (1.2 mM), Glu-plasminogen (0.2–4.0 μM), and tc-uPAs (0.1 nM) and was incubated at room temperature. The reaction rates were determined as previously described (14).

Plasmin activation of pro-UK. Pro-UK (0.0–3.0 μM) was incubated with plasmin (0.1 nM) in the presence of 1.2 mM S2444 in assay buffer at room temperature. The amount of tc-uPA generated was measured OD increase over time at 410 nm against a reference wavelength of 490 nm on a microplate reader. The reaction rate was determined as previously described (14).

Clot lysis and fibrinogenolysis. Clots in 5 ml of pooled bank plasma were incubated 37°C with different concentrations of pro-UK (up to 10 μg/ml). Clot lysis was monitored every 0.5 h for 6 h. At the end of the experiment, 1.0 ml of plasma was transferred to a tube containing 0.04 ml of aprotinin (10,000 KIU/ml) and plasma fibrinogen was measured by a modification

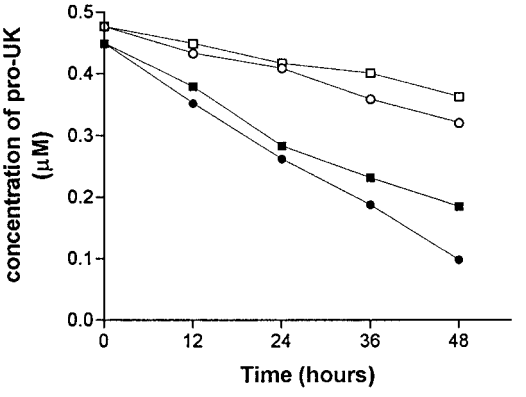

FIG. 3. Stability of glycosylated pro-UK and nonglycosylated pro-UK in culture supernatant after expression. Pro-UK levels in culture supernatant were measured every 12 h. The supernatant was stored at 4°C (glycosylated pro-UK, □; nonglycosylated pro-UK, ■) and room temperature (glycosylated pro-UK, ○; nonglycosylated pro-UK, ●) separately.

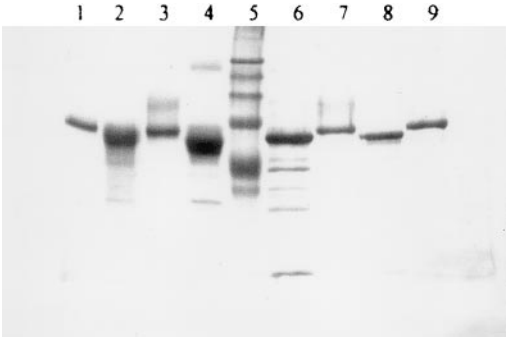

FIG. 4. SDS–PAGE analysis of purified pro-UK. Lanes 1–4, samples were under the nonreduced condition. Lanes 6–9, samples were under the reduced condition. Lane 5, protein molecular mass standards (112, 86, 70, 57, 39.5, and 36 kDa). Lanes 1 and 9, mammalian cell-derived glycosylated pro-UK. Lanes 2 and 8, *E. coli*-derived nonglycosylated pro-UK. Lanes 3 and 7, yeast glycosylated pro-UK. Lanes 4 and 6, yeast nonglycosylated pro-UK.

of the method of Rampling and Gaffney (15). Briefly, 0.1 ml of the aprotinin–plasma solution was transferred to 0.9 ml of 10.5% (w/v) Na_2SO_3 solution, incubated at 37°C for 15 min, and then centrifuged (11,000*g*) for 12 min. The supernatant was aspirated and the precipitate mixed with 0.4 ml of Na_2SO_3 solution followed by centrifugation again for 12 min. After removal of the supernatant, the precipitate was dissolved by adding 1.0 ml of 4 M urea in 0.1 M NaOH and incubating at 100°C for 20 min. The absorbance at 280 nm was measured and expressed as the percentage of fibrinogen remaining in control incubations (without the addition of pro-UK).

Statistical analysis. Each experiment was done in triplicate and the values recorded represent means from at least three independent experiments.

RESULTS AND DISCUSSION

Plasmid Construction and Gene Expression in a Yeast System

A recombinant vector able to synthesize α-factor signal peptide and pro-UK was constructed (Fig. 1). The initiation codon ATG in the α-factor signal sequence in pPICZα corresponds to the native initiation codon of the AOX1 gene. The open reading frame of the mature gene of pro-UK was cloned in frame and downstream of the α-factor signal sequence. The protease cleavage site Kex2 for the α-factor signal sequence was put immediately before the first codon of pro-UK. The recombinant plasmid pro-UK-pPICZα was transformed *to P. pastoris* KM 71 and highly expressed clones were selected by assaying urokinase amidolytic activities of the expressed products.

The secretion of glycosylated pro-UK and nonglycosylated pro-UK by *P. pastoris* was studied. The strains were grown in BMGY medium for 24 h. When the cell density reached 4.0 OD_{600}, the culture medium was changed to BMMY for expression. Every 24 h, 1 ml of expression culture medium was taken to assay pro-UK expression. As shown in Fig. 2, the amount of pro-UK in the culture medium gradually increased and reached a maximum of 20 mg/L at 48 h of cultivation for both strains expressing glycosylated pro-UK and nonglycosylated pro-UK. After 48 h, the pro-UK level in the culture medium continually increased to 23 mg/L for the glycosylated pro-UK strain. In contrast, it rapidly decreased to 12 mg/L for the nonglycosylated pro-UK strain, which was consistent with previous findings by others (16). This was probably due to nonspecific proteolysis in the yeast culture.

The stability of glycosylated pro-UK and nonglycosylated pro-UK secreted by *P. pastoris was* analyzed. After expression, the cultured supernatants were stored at room temperature and 4°C separately. The pro-UK level was measured over time. The result showed that nonglycosylated pro-UK in culture supernatant decreased more quickly than glycosylated pro-UK (Fig. 3). The half-life of nonglycosylated pro-UK at room temperature was 30 h. In contrast, glycosylated pro-UK still had more than 80% remaining. It seems probable that the absence of glycosylation at Asn302 of pro-UK would influence the stability of pro-UK in culture supernatant. The two temperatures were not found to significantly affect the stability of pro-UK.

Secretion efficiencies of glycosylated pro-UK and nonglycosylated pro-UK were also studied. The amount of pro-UK secreted into the culture medium or

TABLE 1

Kinetic Analysis of Plasmin-Catalyzed Activation of pro-UKs

	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1} μM^{-1})
Nonglycosylated pro-UK (yeast)	7.3 ± 0.8	155 ± 17	21.3
Glycosylated pro-UK (yeast)	20.1 ± 2.1	250 ± 12	12.4
Nonglycosylated pro-UK (*E. coli*)	5.2 ± 0.9	192 ± 20	36.9
Glycosylated pro-UK (mammalian cells)	11.7 ± 1.3	211 ± 16	18.0

TABLE 2

Kinetic Analysis of Glu-Plasminogen Activation by tc-uPAs

	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1} μM^{-1})
Nonglycosylated UK (yeast)	3.97 ± 0.42	46.6 ± 2.5	11.74
Glycosylated UK (yeast)	8.54 ± 0.78	41.8 ± 3.3	4.89
Nonglycosylated UK (*E. coli*)	2.67 ± 0.16	38.9 ± 2.8	14.57
Glycosylated UK (mammalian cell)	5.17 ± 1.27	48.5 ± 4.2	9.38

accumulated in yeast cells was measured. Pro-UK from the yeast cell extract was only 1.5% of pro-UK from the culture supernatant for the stain producing glycosylated pro-UK, and it was 1.9% for the strain producing nonglycosylated pro-UK. These data indicate that glycosylation had no significant effect on the secretion of pro-UK by yeast *P. pastoris*. This was contradictory to the previous finding that glycosylation impaired secretion of pro-UK by yeast (8).

Purification and Characterization of the Secreted Product

The yeast cells were separated from the medium by centrifuging the samples at 3000 rpm for 20 min at 4°C. The supernatant was concentrated using an Amicon YM 10 membrane and the pH was adjusted to 4.5 with acetic acid. The sample was then applied to a carboxymethyl cellulose column preequilibrated with 50 mM acetic acid, 0.01% Tween 80. Pro-UK was eluted from the column by increasing the NaCl concentration to 0.5 M.

As shown in Fig. 4, purified glycosylated and nonglycosylated pro-UK appeared as single protein bands with molecular weights of 54 and 47 kDa, respectively, in SDS–PAGE under both nonreducing and reducing conditions, indicating that they were in a single-chain form.

The amidolytic activity of the culture medium prior to plasmin treatment was less than 0.7% of the activity after plasmin treatment. This also indicated that very little sc-uPA was converted to tc-uPA during the culture.

To further characterize the purified protein, the N-terminal sequence of yeast pro-UK was determined using an Applied Biosystems gas-phase sequencer for 10 cycles. The sequence analysis showed that the signal sequence originating from the vector was endogenously removed. The first 10 residues of both purified glycosylated and nonglycosylated yeast pro-UK proteins corresponded to the reported sequence of pro-UK (data not shown).

Plasmin Activation of Pro-UK

Based on the kinetic data from the activation rate study (Table 1), it was found that both yeast nonglycosylated pro-UK and *E. coli* nonglycosylated pro-UK had lower K_M and k_{cat} values than those of yeast glycosylated pro-UK and mammalian cell glycosylated pro-UK. However, their catalytic efficiencies (k_{cat}/K_M) were greater than those of glycosylated pro-UK. This confirmed our previous finding that nonglycosylated pro-UK was more sensitive to plasmin activation (17). It was suggested that polymannose attached to Asn302 of yeast pro-UK protected itself from being cleaved by plasmin at the activation site (Lys158-Ile159).

Glu-Plasminogen Activation by tc-uPAs

Four different forms of pro-UK (yeast glycosylated and nonglycosylated, *E. coli* nonglycosylated, and mammalian cell glycosylated) were converted to their two-chain forms by plasmin in order to measure their enzymatic activities against Glu-plasminogen. It was found that the K_M values of the two nonglycosylated tc-uPAs were lower than those of glycosylated tc-uPA. Yeast glycosylated tc-uPA had the highest K_M value

TABLE 3

Kinetic Analysis of Amidolysis of S2444 by tc-uPAs

	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M (min^{-1} μM^{-1})
Nonglycosylated UK (yeast)	64.1 ± 6.7	186 ± 21	2.9
Glycosylated UK (yeast)	79.8 ± 7.2	212 ± 18	2.7
Nonglycosylated UK (*E. coli*)	85.4 ± 8.5	242 ± 23	2.8
Glycosylated UK (mammalian cell)	78.7 ± 5.4	236 ± 19	3.0

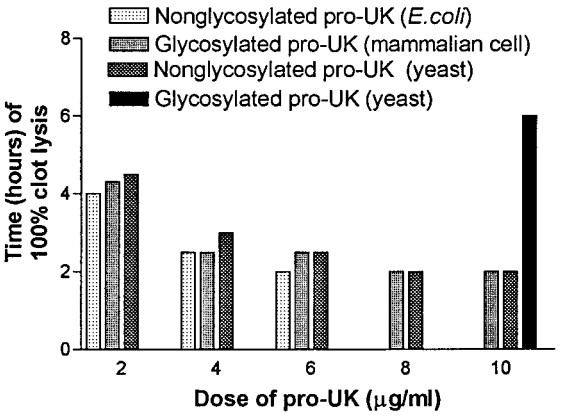

FIG. 5. Clot lysis in plasma by four different forms of pro-UK (*E. coli* nonglycosylated pro-UK; mammalian cell glycosylated pro-UK; yeast nonglycosylated pro-UK; yeast glycosylated pro-UK).

(Table 2). This suggested that polymannose at Asn302 of yeast tc-uPA also impaired its substrate binding capability for plasminogen.

Amidolysis of S2444 by tc-uPAs

Kinetic analysis of hydrolysis of S2444 showed very similar K_M and k_{cat} among the four forms of tc-uPA (Table 3), indicating that they had comparable catalytic activities against the small peptide substrate. This suggests that the loss of activity of yeast glycosylated tc-uPA against plasminogen (see above) is mainly due to spatial obstruction induced by the polymannose, which partially blocks the substrate binding pocket of yeast glycosylated tc-uPA and interferes with plasminogen binding but not that of the much smaller substrate S2444.

Clot Lysis and Fibrinogenolysis

Clot lysis assay in human plasma, which mimics *in vivo* clot lysis, was used to evaluate the fibrinolytic activities of the four forms of recombinant pro-UK. With yeast glycosylated pro-UK, 100% clot lysis in 6 h required 10 μg/ml of the protein. By contrast, with yeast nonglycosylated pro-UK, *E. coli* pro-UK, and mammalian cell pro-UK, only 2 μg/ml was required (Fig. 5). At the dose of 2 μg/ml, it was found that 80% fibrinogen remained in plasma after 6 h with all three forms of pro-UK which induced 100% clot lysis.

In summary, an efficient production system for human pro-UK (glycosylated and nonglycosylated) in yeast *P. pastoris* was established and characterized. Both the glycosylated and nonglycosylated forms of pro-UK were highly expressed and secreted into culture medium in a biologically active form. However, the yeast nonglycosylated pro-UK was unstable in culture medium due to nonspecific proteolysis. In contrast to the previous report (8), more than 98% of the expressed pro-UK by yeast, glycosylated or not, was secreted into the culture medium. Glycosylation at Asn302 had no effect on the secretion of pro-UK by yeast. More than 99% of the expressed pro-UK by yeast was in a single-chain form. Nonglycosylated yeast pro-UK had catalytic and clot lysis activity similar to that of pro-UK derived from *E. coli* or mammalian cells. Due to spatial obstruction by polymannose at Asn302, glycosylated yeast pro-UK was less activatable by plasmin and less active against its native substrate, Glu-plasminogen, but had a normal activity against the small synthetic peptide substrate S2444. Its clot lysis activity was also impaired, making it unsuitable as a thrombolytic agent.

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