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ORIGINAL PAPER

Expression of full-length human pro-urokinase in mammary glands of transgenic mice

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Abstract Human pro-urokinase expressed in the mammary glands of transgenic animals is quickly activated and converted to urokinase by proteases that are present in the milk. Thus, it is nearly impossible to isolate full-sized pro-urokinase from the milk of transgenic animals. To solve this problem, we constructed transgenic mice that express human pro-urokinase and modified ecotin, which is a potent serine protease inhibitor from *E. coli*, in their mammary glands. The gene encoding ecotin was modified so as to enhance its specificity for the human urokinase-type plasminogen activator. Co-expression of modified ecotin and human pro-urokinase in the mammary glands allows for purification of full-length human pro-urokinase from these transgenic mice. The results described here suggest a general way of preventing the activation of zymogens that are expressed in the mammary glands of transgenic animals by co-expression of a zymogen along with a protease inhibitor.

Keywords Pro-urokinase · Ecotin · Transgenic animals · Milk · Inhibitor · Protease · Thrombolytic agents

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Introduction

Urokinase [SWISS-PROT: P00749] is a potent thrombolytic agent that is currently used for the treatment of acute ischemic stroke and acute peripheral arterial occlusion (Ouriel and Kandarpa 2004). As a therapeutic agent, pro-urokinase, which is a natural precursor of urokinase, has some advantages over urokinase because of a lower probability of systemic bleeding and improved fibrin specificity, which is due to the formation of a plasminogen-pro-urokinase zymogen complex (Lijnen et al. 1986; Petersen 1997; Ouriel et al. 1999; Tanahashi and Fukuuchi 2002; Ouriel and Kandarpa 2004).

Recombinant pro-urokinase may be produced in *E. coli* cells; however, it accumulates in insoluble inclusion bodies that must be denatured and renatured in vitro to acquire activity (Ratzkin et al. 1981; Jacobs et al. 1985; Hua et al. 1996; Zhu et al. 2001). This puts serious limitations on the commercial utilization of *E. coli* cells for producing recombinant pro-urokinase. Alternatively, recombinant pro-urokinase may be produced in mammalian cells. Although

expression in cultured human cells is simple in principle, the use of cultured cells as a bioreactor can be very expensive. Thus, the method of choice seems to be expression of pro-urokinase in the mammary glands of transgenic animals. Targeted expression of recombinant proteins in the mammary glands of transgenic animals is currently used for the commercial production of several pharmaceutically important human proteins (for a review, see Goldman et al. 2004). As biological reactors, transgenic animals are an order of magnitude more economical than microbial or expensive cell reactors. No extensive capital investment in the construction of transgenic animals is necessary, and transgenic animal farming is not energetically expensive (Velander et al. 1997; Lubon 1998). In model experiments, active human urokinase was successfully expressed in the mammary glands of transgenic mice (Meade et al. 1990; Brandazza et al. 1991); however, the production of pro-urokinase in the milk of transgenic animals presents a more difficult task. The main problem is that pro-urokinase is readily converted to urokinase following cleavage of its polypeptide chain between Lys158 and Ile159. Plasmin mediates this cleavage in the plasma, but other proteolytic enzymes can also mediate activation of pro-urokinase (Ichinose et al. 1986; Marcotte and Henkin 1993). We previously found that full-size human pro-urokinase expressed in the mammary glands of transgenic mice is quickly converted to urokinase.

Here we are presenting a new strategy to suppress the premature activation of pro-urokinase and its conversion to urokinase in the milk of transgenic animals. The problem of premature cleavage of recombinant pro-urokinase by proteolytic enzymes present in milk was solved by the co-expression of a human gene encoding pro-urokinase and an *E. coli* gene encoding ecotin in the mammary glands of transgenic mice. Ecotin is a wide-range inhibitor of serine proteases (Chung et al. 1983). It has been genetically engineered to redirect its specificity (Yang et al. 1998). We have demonstrated that mutated ecotin protects pro-urokinase in vivo and allows for the purification of intact human pro-urokinase from the milk of transgenic mice. The proposed approach can be used to suppress premature cleavage of other zymogens that are expressed in the mammary glands of transgenic animals.

Materials and methods

Gene constructs

DNA constructs for microinjection were made using the commercial vectors pCAT3 (Promega) and pBC1 (Invitrogen). The construction of a partially spliced gene encoding human pro-urokinase was described previously (Shevelev et al. 1992). The *E. coli ecotin* [SWISS-PROT: P23827] gene was amplified from *E. coli* K-12 JM109 DNA using the primers 5′-ATgTTAAgC TTCAgCgACATCATCggT-3′ and 5′-TgCggTACCTCACggCAgTTTAgCgAACTACC-3′. The mutations M84R and D70R (Yang and Craik 1998) were introduced by PCR. The T63A mutation, which removes the putative N-glycosylation site, was introduced by replacing the *Bsm*AI–*Bst*EII fragment of the *M84RD70R* gene with a synthetic one that contains the substituted codon. All genetic engineering manipulations were carried out according to previously described methods (Maniatis et al. 1982). All enzymes were purchased from New England Biolabs.

Construction of transgenic mice

The DNA constructs, which were diluted in 0.1 mM EDTA and 10 mM Tris (pH 7.4), were microinjected into the male pronuclei of one-cell embryos using a Narishige microinjector (Japan) using a needle with a 1.5 μm outer diameter. Microinjection was monitored using the Axiovert 35 (Germany) inverted microscope. The DNA solution (~4 pl), which contained 400–800 copies of a linear construct, was injected using a standard experimental protocol. Transgenic mice were identified by PCR analysis with pro-urokinase-specific primers (5′-AgAgCAggCAggAgTTAggA-3′ and 5′-AAggggACAggAggATgAgA-3′, PCR fragment = 534 bp) and ecotin (5′-CgACTCCAAAggCAgCAATg-3′ and 5′-AATCACCTgACgCTTCAT CCC-3′, PCR fragment = 124 bp).

Purification of human pro-urokinase from the milk of transgenic mice

To isolate pro-urokinase, the milk from transgenic mice was thawed on ice and immediately diluted with 20 volumes of ice-cold loading buffer [0.2 M NaCl, 10 mM Na-phosphate, 1 mM benzamidine-HCl (pH

7.0)]. The mixture was centrifuged twice at 10,000*g* at 4°C, and the supernatant was loaded onto a 0.4 ml affinity column (carbohydate matrix conjugated with monoclonal antibody to human urokinase, ImTek, Russia). The column was washed with 20 volumes of loading buffer (1 mM EDTA + 0.01% Triton X-100) and then with five volumes of washing buffer [1 M NaCl and 10 mM Na-phosphate (pH 7.0)]. The column was saturated with low-ionic strength buffer [1 mM Na-phosphate (pH 7.0)], and the pro-urokinase was eluted with 3% acetic acid. The peak fractions were neutralized with Tris solution, combined and dialyzed against a 10 mM Na-phosphate (pH 6.0)–50 mM NaCl buffer. The protein solution was loaded onto a 0.2 ml CM-cellulose column that had been equilibrated with a 10 mM Na-phosphate (pH 6.0)–50 mM NaCl solution. The column was washed with five volumes of the same buffer, and the bound proteins were eluted with a 10 mM Na-phosphate (pH 6.0)–500 mM NaCl solution. The peak fractions were pooled, and the protein concentration was determined. After the addition of mannitol (Aldrich) to a final concentration of 100 mM, we found that the protein could be freeze-dried with only a 10–20% loss in activity.

Analytical methods

The fibrin plate assay for the detection of urokinase activity was carried out as previously described (Astrup and Mullertz 1952). Bovine fibrinogen, human plasminogen and human plasmin were purchased from Sigma. Before testing of the fibrinolytic activity, lyophilized urine urokinase (Calbiochem, cat. No. 672112, certified to be 10,000 IU/vial with a specific activity of 100 IU/μg protein) was dissolved in 100 μl of a 10 mM Na-phosphate (pH 7)–100 mM NaCl solution supplemented with BSA (100 μg/ml), with subsequent dilutions in the same buffer. Samples of recombinant pro-urokinases that were produced in *E. coli* and in mammary glands were prepared in the same buffer.

The chromogenic substrates S2224 (pyro-Glu-Gly-Arg-para-nitroaniline) for urokinase and S2251 (H-D-Val-Leu-Lys-para-nitroaniline) for plasmin were purchased from Roche and Chromogenix. The reaction mixtures for the control amidolytic assays contained 10 mM Na-phosphate buffer (pH 7.2), serial dilutions of the standard preparation of pro-urokinase (activated) or plasmin (final concentrations 0, 10, 20, 50 μM) and 250 μM of the chromogenic substrate. Aliquots were withdrawn before the addition of the enzyme and after different durations of incubation (usually 60, 120, 240, 360, 480 and 600 s). The reactions were stopped by the addition of acetic acid to a final concentration of 10%, and the enzymatic release of colored para-nitroaniline was monitored by the measurement of absorption at 405 nm. Hydrolysis of the chromogenic substrates by milk fractions and standard purified enzymes was assayed in parallel. Human plasmin (Sigma) was considered to have standard plasmin activity. Human pro-urokinase that was produced in *E. coli*, purified to homogeneity and activated by human plasmin (1:200 molar ratio, 30 min at 37°C) was considered to have standard urokinase activity. The specific activity of this enzyme was found to be similar to that of commercially available human urine urokinase (Calbiochem, cat. No. 672112).

Western blot analysis was carried out as described previously (Crisp and Dunn 1994). The controls for loading and transfer were performed by staining membranes with Ponso. Polyclonal antibodies against ecotin and human pro-urokinase were raised in rabbits. For immunization, we used M84RD70R T63A-N-terminal-6His ecotin that had been purified from *E. coli* using conventional methods and human pro-urokinase that had been produced in *E. coli* for pharmacological purposes (Purolase, Technogene Ltd, Russia). The goat-anti-rabbit POX conjugate was purchased from ImTek, Russia.

Results

Construction of transgenic mice carrying a hybrid ovine *β*-lactoglobulin/human pro-urokinase gene

To produce transgenic mice that expressed human pro-urokinase in their milk, we used an ovine *β-lactoglobulin* promoter and a partially spliced human *pro-urokinase* gene with the three initial introns excised (Shevelev et al. 1992). A 777 bp fragment of the upstream region of the ovine *β-lactoglobulin* gene, beginning 772 bp upstream of the transcription start site and including the TATA box (34–30 bp upstream of the transcription start) and the first five (non-translated) nucleotides of the

β-lactoglobulin gene, was PCR-amplified using genomic ovine DNA as a template. The primers were designed based on the sequence of the ovine *β-lactoglobulin* gene (GenBank Accession No. X12817). The PCR-amplified DNA fragment was cloned into Nhe I–Bgl II sites of the pCAT3 basic vector (Promega). The fragment containing the *CAT* gene was then excised from the construct by digestion with *Hind* III and *Xba* I restriction enzymes and replaced by a 4.75 kb DNA fragment containing the human *pro-urokinase* gene, from which the first three introns and the end of non-coding exon 11, which contains the polyadenylation site, were excised. Polyadenylation of the mRNA that was encoded by the artificial *pro-urokinase* gene was driven by an SV40 polyadenylation signal in the construct. The scheme of the final construct is shown in Fig. 1. The DNA fragment used to construct transgenic mice was excised by digestion with MluI and *Sal*I restriction endonucleases. Transgenic mice were produced by microinjection of this DNA fragment into the male pronucleus (see "Materials and methods"). On average, about 15% of new-born mice that were obtained after implantation of the injected ovicells were found to carry the transgene(s), as confirmed by PCR analysis (data not shown). The expression level of pro-urokinase/urokinase in the milk of transgenic female mice was determined in the fibrin plate and by amidolytic assays and then confirmed by western blotting. The expression level varied from 20 to 1,000 μg/ml. The expression level of ecotin was determined by the same assays, but based on inhibitory effect on urokinase.

Human pro-urokinase is rapidly converted to urokinase in the milk of transgenic mice

The high level of observed amidolytic activity and the western blotting results (Fig. 2, lanes 1, 2) indicated the presence of a completely activated form of pro-urokinase (i.e., urokinase) in the milk of the transgenic mice. Attempts to reduce the decay of activity during storage by analyzing milk immediately after its release from the milk gland were not successful. Therefore, human pro-urokinase is most likely rapidly converted to active urokinase in the milk of transgenic mice. In order to evaluate this hypothesis, we examined the proteolytic activity in the milk of non-transgenic mice, and its potential protease-inhibiting activity was assayed in parallel experiments. Active proteases

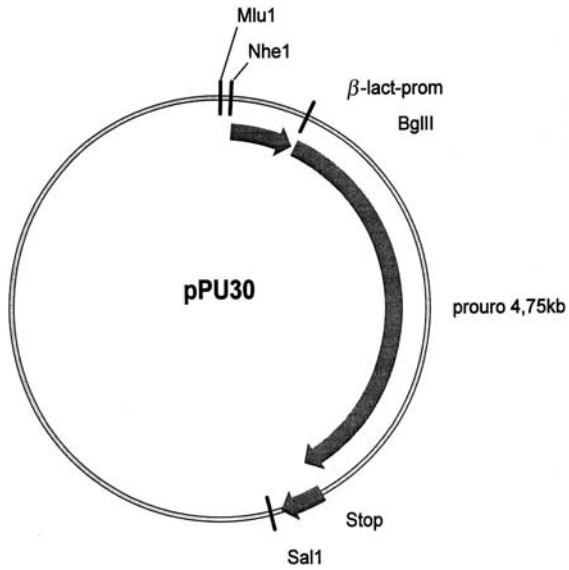

Fig. 1 A scheme of the construct that was used to obtain transgenic mice expressing human pro-urokinase

[activated human recombinant pro-urokinase (from *E. coli*) or human plasmin (Sigma)] or protease precursors [human recombinant pro-urokinase (from *E. coli*, <2% active) and human plasminogen (Sigma)] were added to the whey fraction of non-transgenic mouse milk for subsequent kinetic analysis of the activation of inactive protease precursors or the inhibition of active proteases.

To isolate the whey fraction, fresh non-transgenic mouse milk was diluted fivefold with 10 mM Na-phosphate (pH 7.0) and 100 mM NaCl and centrifuged at 10,000*g* for 10 min. The active proteases or their precursors were added to the whey, and, after different preincubation times at 37°C, aliquots were withdrawn and stored in liquid nitrogen for subsequent measurement of the amidolytic activity using the urokinase (S2444) or plasmin (S2251) substrates. We often use both substrates for each protease for the two following reasons. First, plasmin has limited specificity and catalyzes background hydrolysis of the urokinase substrate S2444 with about 10% activity on S2251. Second, the addition of urokinase to plasminogen leads to the appearance of plasmin activity and vice versa. The results of the experiments are presented in Table 1. The whey fraction itself exhibits no detectable endogenous plasmin or urokinase activity (Table 1, rows 9, 19, 20). A low level of plasmin activity is probably masked by caseins and

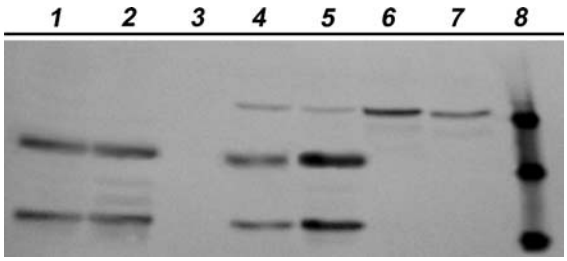

Fig. 2 Western blot analysis of pro-urokinase expressed in *E. coli* and in the mammary glands of transgenic mice. The blot was stained with a rabbit antibody against human uPA and developed with a goat anti-rabbit POX conjugated to H_2O_2 and 4-Chloro-1-Naphtol substrates (*lanes 1, 2*)—whey fractions from the milk of urokinase (+) transgenic mice; (*lane 3*)—whey fraction from the milk of a wild-type mouse; (*lanes 4, 5*)—whey fractions from the milk of class 1 pro-urokinase (+) ecotin (+) transgenes; (*lanes 6, 7*)—whey fractions from the milk of class 2 pro-urokinase (+) ecotin (+) transgenes; (*lane 8*)—a mixture of pro-urokinase from *E. coli*, 0.5 μg (*upper band*), and 1 μg of pro-urokinase from *E. coli* digested with plasmin (2 *lower bands*). In *lanes 1–7*, 0.2 μl of the whey fraction was loaded per lane

lactalbumins, which are weak inhibitors of plasmin (Politis et al. 1993). In fact, the activity of plasmin was inhibited by about twofold by the whey as compared to the buffer solution (Table 1, rows 1 and 2).

When added to the whey fraction of mouse milk, pro-urokinase is rapidly transformed to urokinase, with more than 50% conversion occurring over the course of 20 min (compare rows 16 and 8 in Table 1). Plasmin activity appears soon after the addition of pro-urokinase or urokinase to the whey (rows 14 and 6). It is necessary to note that plasminogen, one of the major components of plasma, is also present in milk whey (Politis et al. 1993). Thus, the addition of pro-urokinase to the milk resulted in the formation of a system with two reciprocally activating pro-enzymes. Minimal activation of at least one of them immediately resulted in a burst of autocatalytic activity that generated more and more active enzymes. The initial steps of these reactions may be triggered by activation of pro-urokinase by kallikreins and cathepsins (Ichinose et al. 1986; Marcotte and Henkin 1993).

Addition of ecotin bearing a triple mutation to the whey fraction prevents pro-urokinase and plasminogen activation

We reasoned that protease inhibitors may prevent the activation of zymogens in the milk of transgenic animals. A specific inhibitor of human urokinase-type plasminogen activator has been described in the literature (Yang and Craik 1998). This inhibitor was engineered by targeted modification of the active center of ecotin, which is a serine protease inhibitor from *E. coli*. We PCR-amplified the *ecotin* gene from *E. coli* DNA and introduced two mutations, M84R and D70R, into this gene, which were originally described by Yang and Craik (1998). The T63A mutation was introduced in order to eliminate a putative glycosylation site. Finally, a 6-histidine tag and a signal sequence that is necessary for secretion of the recombinant protein from *E. coli* and eukaryotic cells were inserted into the cDNA.

6-His tagged M84R, D70R, T63A ecotin and 6-His tagged wild-type ecotin were purified after expression in *E. coli*. The ability of these recombinant proteins to inhibit in vitro trypsin, activated pro-urokinase and plasmin and to form 1:1 complexes with active proteases was confirmed (data not shown). In contrast, modified ecotin as well as wild-type ecotin were not able to form complexes with pro-urokinase and plasminogen. This feature distinguishes these two zymogens from trypsinogen, which has been reported to bind ecotin (Lengyel et al. 1998).

Plasmin, activated pro-urokinase (urokinase), plasminogen or pro-urokinase was added to the premixed whey fraction with or without ecotin and preincubated for 20 or 40 min at 37°C. Aliquots were withdrawn and assayed for amidolytic activity using the urokinase (S2444) or/and plasmin (S2251) substrates, as described above.

Wild-type ecotin had little or no effect on urokinase and plasmin activity (data not shown). At the same time, the M84R, D70R, T63A triple mutant ecotin was an efficient inhibitor of plasmin and the activated pro-urokinase. It also prevented the activation of pro-urokinase and plasminogen in the whey (Table 2, rows 9–22). A fourfold excess of the inhibitor over the proteases (Table 2, rows 9–16) was nearly sufficient to inhibit plasmin and urokinase (rows 9–12) and to prevent the activation of those zymogens (rows 13–16). When the mixture of plasminogen and pro-urokinase was incubated in the presence of a twofold excess of modified ecotin, a leakage proteolytic activity resulted in slight activation of the zymogens (Table 2, rows 21, 22). This activity was fully suppressed in the presence of a 20-fold excess of modified ecotin (rows 23, 24).

Table 1 The dependence of the rate of the amidolytic reaction (in uM substrate hydrolyzed per second) on the reaction conditions and preincubation time

	Time of preincubation (min)	1	10	20
1	100 nM Plasmin in buffer on 1 mM S2251	0.48	0.47	0.50
2	100 nM Plasmin in whey on 1 mM S2251	0.27	0.25	0.29
3	100 nM Plasmin in buffer on 1 mM S2444	0.03	0.04	0.04
4	100 nM Plasmin in whey on 1 mM S2444	0.02	0.03	0.03
5	50 nM Activated pro-urokinase in buffer on 1 mM S2251	<0.02	<0.02	<0.02
6	50 nM Activated pro-urokinase in whey on 1 mM S2251	<0.02	0.18	0.39
7	50 nM Activated pro-urokinase in buffer on 1 mM S2444	0.87	0.90	0.86
8	50 nM Activated pro-urokinase in whey on 1 mM S2444	0.89	0.93	0.76
9	100 nM Plasminogen in buffer on 1 mM S2251	<0.02	<0.02	<0.02
10	100 nM Plasminogen in whey on 1 mM S2251	<0.02	<0.02	0.41
11	100 nM Plasminogen in buffer on 1 mM S2444	<0.02	<0.02	<0.02
12	100 nM Plasminogen in whey on 1 mM S2444	<0.02	<0.02	<0.02
13	50 nM Pro-urokinase in buffer on 1 mM S2251	<0.02	<0.02	<0.02
14	50 nM Pro-urokinase in whey on 1 mM S2251	<0.02	0.15	0.31
15	50 nM Pro-urokinase in buffer on 1 mM S2444	<0.02	<0.02	<0.02
16	50 nM Pro-urokinase in whey on 1 mM S2444	<0.02	0.23	0.62
17	Buffer on 1 mM S2251	<0.02	<0.02	<0.02
18	Buffer on 1 mM S2444	<0.02	<0.02	<0.02
19	Whey on 1 mM S2251	<0.02	<0.02	<0.02
20	Whey on 1 mM S2444	<0.02	<0.02	<0.02

"Buffer" in the table is 10 mM Na-phosphate (pH 7)—100 mM NaCl and "whey" is a mixture of the whey fractions from the milk of three non-transgenic mice isolated and diluted fivefold as described above. Error is within 15%

Co-expression of human pro-urokinase and modified ecotin of *E. coli* in the mammary glands of transgenic mice ensures production of non-activated pro-urokinase

The results described in the previous section encouraged us to use modified ecotin for the suppression of premature activation of human pro-urokinase expressed in the mammary glands of transgenic mice. In order to obtain transgenic mice that simultaneously express modified *E. coli* ecotin and human pro-urokinase in their mammary glands, transgenic mice that expressed modified ecotin in the mammary glands were obtained and crossed with mice that produced human pro-urokinase.

To express the modified ecotin in transgenic mice, a pBC1 Milk Expression Vector Kit (Invitrogen) was used. The gene encoding modified (M84R, D70R, T63A) ecotin was ligated to the eukaryotic secretion signal sequence of pre-pro-urokinase (MRALLA RLLLCVLVVSDSKg). A DNA fragment encoding this amino acid sequence in addition to the first 12 amino acids of pro-urokinase (SNELHLDASAWA) was inserted into the *BglI* site of the modified *ecotin* gene. Transgenic mice were produced using the microinjection protocol. The level of ecotin production in the milk of transgenic mice varied from 20 μg/ml to 1 mg/ml. Transgenic mice expressing ecotin were crossed with mice expressing pro-urokinase. Pro-urokinase (+) ecotin (+) transgenics were identified by PCR analysis using pro-urokinase-specific and ecotin-specific primers. Based upon the biochemical analysis of the milk of pro-urokinase (+) ecotin (+) transgenics, the animals were divided into two classes. Class 1 animals produced milk with urokinase activity, and class 2 animals produced milk with an excess of ecotin activity, which was able to inhibit the activity of exogenous urokinase. Hybrid mice of class 1 with urokinase activity in the milk produced a moderate or low amount of ecotin (1–5 μM) and partially processed pro-urokinase in excess of ecotin (4–10 μM). Both proteins were identified by western blotting (Fig. 2, lanes 4, 5 and Fig. 3, lanes 5, 6). Urokinase activity was also detected on fibrin plates. Furthermore, plasmin (200–500 nM) was detected in the milk of this group of mice.

Hybrid mice of class 2 with ecotin activity in the milk were characterized by a relatively high level of ecotin production (20–60 μM) and a much lower

Table 2 Dependence of the rate of the amidolytic reaction (in μM of substrate hydrolyzed per second) on the reaction conditions, preincubation time and the presence of the inhibitor

		Time of preincubation (min) Presence of inhibitor	20	40
1	50 nM Plasmin in whey on 1 mM S2251	No ecotin	0.11	0.12
2	50 nM Plasmin in whey on 1 mM S2444	No ecotin	0.03	0.02
3	50 nM Activated pro-urokinase in whey on 1 mM S2251	No ecotin	0.21	0.46
4	50 nM Activated pro-urokinase in whey on 1 mM S2444	No ecotin	0.88	0.78
5	50 nM Plasminogen in whey on 1 mM S2251	No ecotin	0.24	0.26
6	50 nM Plasminogen in whey on 1 mM S2444	No ecotin	0.02	0.02
7	50 nM Pro-urokinase in whey on 1 mM S2251	No ecotin	0.34	0.38
8	50 nM Pro-urokinase in whey on 1 mM S2444	No ecotin	0.69	0.74
9	50 nM Plasmin in whey on 1 mM S2251	RM, DK, TA ecotin 200 nM	<0.02	<0.02
10	50 nM Plasmin in whey on 1 mM S2444	RM, DK, TA ecotin 200 nM	<0.02	<0.02
11	50 nM Activated pro-urokinase in whey on 1 mM S2251	RM, DK, TA ecotin 200 nM	<0.02	<0.02
12	50 nM Activated pro-urokinase in whey on 1 mM S2444	RM, DK, TA ecotin 200 nM	<0.02	<0.02
13	50 nM Plasminogen in whey on 1 mM S2251	RM, DK, TA ecotin 200 nM	<0.02	<0.02
14	50 nM Plasminogen in whey on 1 mM S2444	RM, DK, TA ecotin 200 nM	<0.02	<0.02
15	50 nM Pro-urokinase in whey on 1 mM S2251	RM, DK, TA ecotin 200 nM	<0.02	<0.02
16	50 nM Pro-urokinase in whey on 1 mM S2444	RM, DK, TA ecotin 200 nM	<0.02	<0.02
17	50 nM Plasminogen in whey on 1 mM S2251	RM, DK, TA ecotin 2 uM	<0.02	<0.02
18	50 nM Plasminogen in whey on 1 mM S2444	RM, DK, TA ecotin 2 uM	<0.02	<0.02
19	50 nM Pro-urokinase in whey on 1 mM S2251	RM, DK, TA ecotin 2 uM	<0.02	<0.02
20	50 nM Pro-urokinase in whey on 1 mM S2444	RM, DK, TA ecotin 2 uM	<0.02	<0.02
21	50 nM Pro-urokinase + 50 nM plasminogen in whey on 1 mM S2251	RM, DK, TA ecotin 200 nM	<0.02	0.03
22	50 nM Pro-urokinase + 50 nM plasminogen in whey on 1 mM S2444	RM, DK, TA ecotin 200 nM	0.04	0.07
23	50 nM Pro-urokinase + 50 nM plasminogen in whey on 1 mM S2251	RM, DK, TA ecotin 2 uM	<0.02	<0.02
24	50 nM Pro-urokinase + 50 nM plasminogen in whey on 1 mM S2444	RM, DK, TA ecotin 2 uM	<0.02	<0.02
25	Whey on 1 mM S2251	No ecotin	<0.02	0.03
26	Whey on 1 mM S2444	No ecotin	<0.02	<0.02

"Whey" is a mixture of the whey fractions obtained from the milk of four non-transgenic mice, isolated and diluted fivefold as described above. Error is within 15% in all cases

level of human pro-urokinase production (2.5–5 μM). The amidolytic activities of urokinase or plasmin were not detected in the milk of the class 2 mice. Milk and milk fractions also showed no detectable fibrinolytic activity on fibrin plates. We concluded that activated pro-urokinase (i.e., urokinase) was not present in the milk of the class 2 mice. At the same time, western blot analysis demonstrated that unprocessed, and thus enzymatically inactive, human pro-urokinase was present in this milk (Fig. 2, lanes 6, 7) along with ecotin (Fig. 3, lanes 7, 8). Therefore, excessive production of ecotin along with human pro-urokinase in the mammary glands of transgenic mice prevented the proteolytic activation of pro-urokinase. Correspondingly, the milk of such animals can be used as a source of human pro-urokinase.

Purification and characterization of human pro-urokinase from milk of transgenic ecotin (+) pro-urokinase (+) mice

Human pro-urokinase was purified from the milk of class 2 ecotin (+) pro-urokinase (+) transgenic mice, as described in "Materials and methods". Electrophoretic analysis of the purified protein demonstrated that unprocessed pro-urokinase had the expected mobility (Fig. 4, lanes 3, 5) and migrated slightly slower than recombinant pro-urokinase that had been

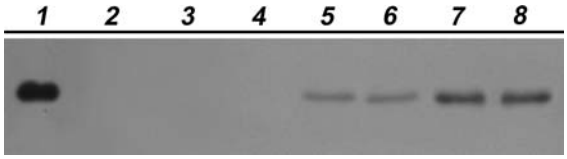

Fig. 3 Western blot analysis of pro-urokinase expressed in *E. coli* and in the mammary glands of transgenic mice. The blot was stained with a rabbit antibody against ecotin and developed with a goat anti-rabbit POX conjugated to H_2O_2 and 4-Chloro-1-Naphtol substrates (*lane 1*)—*E. coli* expressing ecotin; (*lane 2*)—whey from the milk of a wild-type mouse; (*lanes 3, 4*)—whey fractions from the milk of urokinase (+) transgenic mice; (*lanes 5, 6*)—whey fractions from the milk of class 1 pro-urokinase (+) ecotin (+) transgenes; (*lanes 7, 8*)—whey fractions from the milk of class 2 pro-urokinase (+) ecotin (+) transgenes

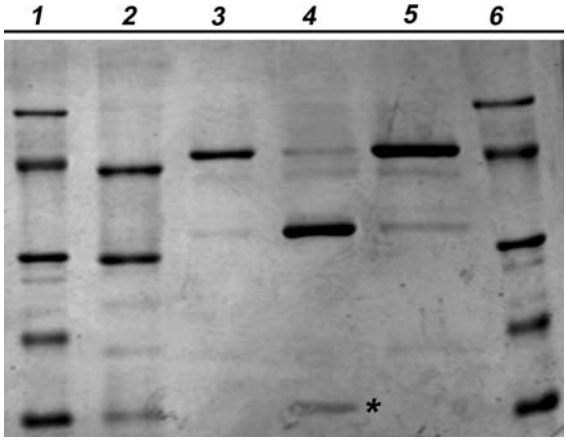

Fig. 4 Electrophoretic analysis of human pro-urokinase purified from the milk of class 2 pro-urokinase (+) ecotin (+) transgenic mice (*lanes 1, 6*)—molecular mass markers: 65, 45, 29, 20 and 14 kDa; (*lane 2*)—mixture of intact (1 μg) and plasmin-activated (1 μg) pro-urokinase expressed in *E. coli*; (*lanes 3,5*)—human pro-urokinase affinity purified from the milk of class 2 pro-urokinase (+) ecotin (+) transgenic mice; (*lane 4*)—human pro-urokinase affinity purified from the milk of class 2 pro-urokinase (+) ecotin (+) transgenic mice and digested with plasmin. After electrophoresis, the proteins were stained with Coomassie Brilliant Blue

produced in *E. coli* (Fig. 4, lane 2). This difference is likely due to glycosylation of the pro-urokinase that is produced in the mammary glands of transgenic mice, since pro-urokinase has a glycosylated glutamine at position 202 in the mature polypeptide in mammals.

We have verified that recombinant human pro-urokinase purified from the milk of transgenic mice can be activated by plasmin. In denaturing and reducing SDS PAGE gels, the activated form runs as two bands (Fig. 4, lane 4), which correspond to the heavy (~30 kDa) C-terminal fragment that contains the catalytic domain and the light (~14 kDa) N-terminal fragment (indicated by an asterisk in Fig. 4, line 4). In the purified sample, traces of activated urokinase were also detected (note the minor 30 kDa band in Fig. 4, lanes 3, 5). Since this band was not detected on the western blots of whole whey (Fig. 2, lanes 6, 7), partial processing of the zymogen likely occurred during storage or purification.

The fibrin plate test was used to compare the activity of pro-urokinase that was produced in transgenic mice with that of recombinant urokinase that was produced in *E. coli* and to urokinase that was purified from human urine. Due to the presence of trace plasminogen in fibrinogen preparations and to the long incubation times, this assay is not sensitive to the activation status of the applied proteins. It should also be noted that, during the purification of pro-urokinase it was separated from ecotin prior to the fibrin plate test. The results of the fibrin plate test (data not shown) demonstrated that both recombinant pro-urokinases (produced in *E. coli* and in the mammary glands of transgenic mice) had specific activities of ~100 IU/μg of protein, as was expected for a normally folded protein.

The activities of the two recombinant pro-urokinases and the commercially available human urokinase were also compared in the amidolytic reaction test. With this test, it was possible to determine the degree of activation of the recombinant pro-urokinases. The amidobiolytic activities of the recombinant pro-urokinases were assayed both before and after activation with plasmin. The degree of activation of pro-urokinase in both samples did not exceed 2% (compare rows 1–4 with rows 7–10 in Table 3). On the other hand, the commercial preparation of urokinase from human urine mostly contained the activated enzyme.

Discussion

The production of different pharmaceutically important human proteins in the mammary gland of transgenic animals is an important segment of modern biotechnology. Many human proteins that possess

Table 3 The rates of reaction amidolytic reactions (in μM of S2444 substrate hydrolyzed per second) of pro-urokinase preparations with and without plasmin activation

	Sample	Concentration	V (μM/sec)
1	Activated pro-urokinase from *E. coli*	50 nM (2.5 μg/ml)	0.82
2	Activated pro-urokinase from *E. coli*	25 nM	0.38
3	Activated pro-urokinase from milk	50 nM	0.77
4	Activated pro-urokinase from milk	25 nM	0.41
5	Activated urokinase from urine	250 IU/ml	0.92
6	Activated urokinase from urine	125 IU/ml	0.41
7	Pro-urokinase from *E. coli*	500 nM (25 μg/ml)	0.14
8	Pro-urokinase from *E. coli*	250 nM	0.06
9	Pro-urokinase from milk	500 nM	0.16
10	Pro-urokinase from milk	250 nM	0.07
11	Urokinase from urine	2,500 IU/ml	>2
12	Urokinase from urine	1,250 IU/ml	>2

enzymatic activity are synthesized as inactive zymogens, and, in some cases, these zymogens have advantages as clinical agents over their activated forms. Human pro-urokinase has fewer side-effects than processed urokinase in the thrombolytic therapy of acute ischemic stroke and peripheral vascular occlusion. Pro-urokinase also has improved fibrin specificity (Ouriel et al. 1999). Unfortunately, different zymogens, including pro-urokinase, are quickly processed to activated enzymes in the milk of transgenic animals (Meade et al. 1990; Brandazza et al. 1991). The results described here suggest a general solution to this problem: expression of a zymogen in the mammary glands of transgenic animals along with a protease inhibitor. The strategy of co-expressing a "helper" protein that is necessary for the maturation of the product of interest in mammary glands of transgenic mice was developed more than 10 years ago (Drews et al. 1995; Paleyanda et al. 1997). These authors achieved appropriate maturation of human protein C in the mammary gland of transgenic animals by co-expression of a proteolytic enzyme (furin) that is necessary for maturation of the human protein C precursor. Minimal furin activity was sufficient for processing of the protein C precursor (Drews et al. 1995; Paleyanda et al. 1997). In our case, the task was directly the opposite, since the inhibitor must be expressed in excess of the zymogen in order to prevent premature proteolytic activation of the zymogen, pro-urokinase. Fortunately, production of excess inhibitor did not limit subsequent use of the purified zymogen. We managed to purify intact pro-urokinase that was not contaminated with the ecotin that had been used to prevent conversion of pro-urokinase to urokinase in the mammary glands of transgenic mice. We used a modified form of ecotin that had increased specificity towards the urokinase-type plasminogen activator and plasmin. The plasmin present in normal milk is likely the main source of the proteolytic activation of pro-urokinase. Since plasmin might activate other zymogens as well, this modified ecotin could be successfully used to prevent the activation of different zymogens that are expressed in the mammary glands of transgenic animals. In addition, other inhibitors of proteases could possibly be used for this purpose.

In our studies, mice that simultaneously produced human pro-urokinase and a modified *E. coli* ecotin were obtained by crossing pro-urokinase (+) and ecotin (+) mice. Unfortunately, this quick and efficient experimental approach is not optimal for the large-scale production of commercial livestock. One of the obstacles is unpredictable heritability of the transgene expression level. The transgene expression level must be determined before obtaining cross-hybrids, since the expression of ecotin in an excess over pro-urokinase is crucial for the prevention of pro-urokinase activation. Double expression cassettes in the gene construct are a potential solution to this problem. Many different promoters that provide targeted expression of transgenes in the mammary glands are currently known (for a review, see Goldman et al. 2004). Different combinations of these promoters could enable the production of transgenic constructs that would ensure the necessary relative expression levels of the genes encoding the zymogen of interest and the protease inhibitor.

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