Characterization of two serpins from bovine plasma and milk

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An inhibitor of pancreatic elastase (EI), which can also inhibit chymotrypsin, and an inhibitor of trypsin (TI), which can also inhibit plasmin, have been isolated from bovine plasma. EI and TI belong to the serpin family of inhibitors. The size of both inhibitors is approx. 60 kDa and they are able to form SDS-stable complexes with proteinases. Curiously, TI dimerizes in the presence of SDS, a feature which has been observed previously only in non-denaturing gels of human α_1 -antitrypsin (α_1 PI). EI and TI are glycosylated [16% and 19% (w/w) respectively] and their amino acid compositions are similar to those of other plasma serpins. Neither EI nor TI is the equivalent of bovine α_1 PI, as revealed by partial sequence analysis of their N-termini

and reactive sites. Rather, both inhibitors appear to be related to human α_1 -antichymotrypsin. Inhibition of pancreatic elastase and chymotrypsin by EI occurs with a $k_{\text{ass.}} \sim 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$. TI inhibits trypsin with a $k_{\text{ass.}} \sim 10^5 \text{ M}^{-1} \cdot \text{s}^{-1}$. Plasmin is inhibited by TI with a $k_{\text{ass.}} \sim 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$. The values of the kinetic constants are similar to those determined for the well-studied human serpins. Antibodies to EI and TI reveal a set of four antigenically related proteins of similar size in plasma. In addition, they detect the same set of proteins in milk. The inhibitors isolated from milk are identical to EI and TI from plasma. EI could control the activity of chymotrypsin-like proteinases in milk. In contrast, no target proteinases of TI in milk can be suggested.

INTRODUCTION

The serpin family is a large group of proteins, most of which are plasma proteinase inhibitors. Inhibitory serpins are involved in regulation of blood coagulation, fibrinolysis, complement activation and inflammatory reactions.

The mechanism of proteinase inhibition is not fully understood. However, an exposed reactive-site loop (RSL) in the C-terminal part of the serpin is known to interact with the active site of the proteinase. In general, the RSL sequence contains a residue (P_1) which meets the primary substrate specificity of the serpin's target protease(s). Thus, site-directed mutagenesis of human α_1 -antitrypsin (α_1 PI), changing P_1 from Met to Arg, results in an effective thrombin inhibitor (Carrell et al., 1982). Further, the conformation of the RSL is believed to determine whether a serpin functions as an inhibitor or is merely cleaved (Mast et al., 1992; Schulze et al., 1992).

The well-characterized α_1 PI has been described in 11 mammalian species, but α_1 -antichymotrypsin (α_1 ACT) in only two, namely humans and pigs. Orthologous serpins, as exemplified by α_1 PIs, have highly conserved reactive-site sequences; in contrast, paralogous serpins have few if any identically placed residues at their reactive sites (Patterson, 1991).

Bovine milk contains plasmin and plasminogen activators (Grufferty and Fox, 1988), but, generally, only minor proteolysis of the milk proteins is seen, probably reflecting the presence of regulatory inhibitors. However, milk proteases are reported to be responsible for the partial cleavage of foreign proteins expressed in the mammary gland of transgenic animals (Bishoff et al., 1992). A Kunitz-type trypsin inhibitor has previously been isolated from bovine milk (Laskowski and Laskowski, 1951). However, the inhibitors present during the main lactation period have never been characterized.

As part of a study of protease inhibitors in bovine milk, we report here the isolation, amino acid composition, N-terminal-

and reactive-site sequences of an elastase inhibitor (EI) and a trypsin inhibitor (TI) from bovine plasma and milk. EI can also inhibit chymotrypsin and TI inhibits plasmin as well. Both inhibitors show substantial structural differences from the putative bovine α_1 PI (Sinha et al., 1992), and they are probably related to human α_1 ACT and a previously described trypsin inhibitor from sheep (Sinha et al., 1988).

MATERIALS AND METHODS

Materials

Porcine pancreatic elastase type IV (PPE), thermolysin and human α_1 PI were obtained from Sigma. Subtilisin was obtained from Novo and *Staphylococcus aureus* protease from Miles. Bovine blood was obtained from a local slaughterhouse and anticoagulated with trisodium citrate. After centrifugation the plasma was stored at -20°C . Bovine plasminogen was purified by the method of Deutsch and Mertz (1970) and midiplasmin (kringles 4–5 disulphide bound to the light chain) was purified as detailed by Christensen et al. (1994). Succinyl-Ala-Ala-Ala-*p*-nitroanilide (AAApNA), succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (AAPFPNA) and D-Val-Leu-Lys-*p*-nitroanilide (VLKpNA) were from Chromogenix, and *N*-benzoyl-L-arginine-*p*-nitroanilide (BAPNA), *p*-nitrophenyl-*p*'-guanidinobenzoate and trypsin were from Boehringer. Diaminobenzidine and peroxidase-conjugated swine anti-(rabbit immunoglobulin) antibodies were from Dako A/S. Column materials and cyanogen bromide-activated Sepharose were from Pharmacia.

Purification of EI and TI from bovine plasma

Bovine plasma (100 ml) was fractionated with $(\text{NH}_4)_2\text{SO}_4$. Proteins precipitating at between 50% and 80% saturation were dissolved in approx. 40 ml of 30 mM sodium phosphate, pH 6.7 (buffer A). After dialysis (4°C) against buffer A, the sample

Abbreviations used: PPE, porcine pancreatic elastase; EI, plasma elastase inhibitor; TI, plasma trypsin inhibitor; RSL, reactive-site loop; AAPNA, succinyl-Ala-Ala-Ala-*p*-nitroanilide; AAPFPNA, succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide; VLKpNA, D-Val-Leu-Lys-*p*-nitroanilide; BAPNA, benzoyl-L-arginine-*p*-nitroanilide; α_1 PI, α_1 -antitrypsin; α_1 ACT, α_1 -antichymotrypsin; TFA, trifluoroacetic acid.

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was passed through a 5 cm × 38 cm Blue-Sepharose column equilibrated with buffer A. Fractions with inhibitory activity against PPE and trypsin were pooled and applied to a column (5 cm × 20 cm) of DEAE-Sephacel equilibrated with 30 mM sodium phosphate, pH 6.5. The column was washed with equilibration buffer until non-adsorbed proteins were eluted. Bound material was eluted with a 4 l gradient from 0 to 0.2 M NaCl in 30 mM sodium phosphate, pH 6.5. Inhibitory fractions (identified as described below) were pooled, dialysed against buffer A, concentrated and applied to a Blue-Sepharose column (2.5 cm × 90 cm) equilibrated and eluted with buffer A. The eluate was analysed by SDS/PAGE (Laemmli, 1970) and for inhibitory activity. Fractions inhibiting PPE and containing an approx. 60 kDa component were finally purified on an HR 5/5 Mono Q column in 20 mM piperazine/HCl, pH 9.8, using a 20 ml 0–0.3 M NaCl gradient (flow rate 0.5 ml/min). Fractions inhibiting trypsin, holding three protein components of approx. 40 kDa, 60 kDa and 120 kDa, were pooled, concentrated and applied to a column of Sephacryl S-200 (2.5 cm × 91 cm) equilibrated and eluted with 30 mM piperazine/HCl, 0.3 M NaCl, pH 6.0.

Purification of EI and TI from milk

Fresh bovine pooled milk was skimmed by centrifugation and the caseins were precipitated by adjusting the pH to 4.6 with 1 M HCl. After centrifugation, the supernatant (acid whey, 350 ml) was dialysed against 20 mM piperazine/HCl, pH 6.0, and applied to a column of DEAE-Sephacel (2.5 cm × 37 cm) equilibrated with the same buffer. Bound proteins were eluted with a 2 l gradient of NaCl (0–0.5 M). Fractions with inhibitory activity against PPE and trypsin or plasmin were pooled and dialysed against 30 mM sodium phosphate, pH 6.7. After passage through a Sephadex G-100 column (2.5 cm × 100 cm) in the same buffer, also containing 0.5 M NaCl, the final purification was performed on Blue-Sepharose and Mono Q as described above for the plasma inhibitors.

For immunosorbent purification of EI and TI, acid whey (500 ml) was dialysed against 30 mM sodium phosphate, pH 7.5, and centrifuged. The supernatant was then applied to a 1.5 cm × 12 cm column containing immobilized antibodies against EI at a flow rate of 30 ml/h. The column was washed with 30 mM sodium phosphate, 0.5 M NaCl, pH 7.5. Bound proteins were eluted with 0.1 M glycine, pH 2.5, and the pH of the fractions was immediately brought back to 7.5 by addition of 1 M Tris/HCl, pH 9.0.

Monitoring of inhibitory activity in column fractions

Samples (50 µl) from the column fractions were mixed with 50 µl of PPE (40 µg/ml), trypsin (20 µg/ml) or plasmin (0.1 mg/ml) and incubated for 30 min at 25 °C in a microtitre plate, before addition of 150 µl of substrate (0.1 mM AAPNA for PPE, 1 mM BAPNA for trypsin and 0.2 mM VLKpNA for plasmin, final concentrations). The change in absorbance at 405 nm was followed for 15 min using an EL-340 Bio-tel Instruments plate reader.

Amino acid and carbohydrate analysis

Hydrolysis was performed in 6 M HCl/0.1 % (w/v) phenol/1 % (v/v) thioglycolic acid for 16 h, 24 h and 72 h at 110 °C. To determine half-cystine the samples were oxidized with 10 µl of

1 M performic acid for 30 min before hydrolysis. Amino acids were determined by h.p.l.c. cation-exchange chromatography as described by Barkholt and Jensen (1989) and modified by Sottrup-Jensen (1993). Sialic acid (Liu, 1972) and glucosamine (hydrolysis with 6 M HCl/0.1 % (w/v) phenol/1 % (v/v) thioglycolic acid for 3 h at 110 °C) were determined on the amino acid analyser.

For analysis of neutral sugars, proteins were desalted by passage through a 1 cm × 30 cm TSK G2000 SW column equilibrated and eluted with 0.1 % (v/v) aqueous trifluoroacetic acid (TFA) and hydrolysed (50–100 pmol protein) with 2 M TFA for 2 h at 100 °C. Neutral sugars were separated on MCI CK10U resin (3 mm × 100 mm) using isocratic elution with 88 % (v/v) acetonitrile as described by Honda and Suzuki (1984). Rhamnose was used as internal standard.

Sequence analysis

Edman degradations were performed in an AB 477A sequencer, and phenylthiohydantoin-derivatives were analysed on-line with a 120A h.p.l.c. system. The Normal-1 reaction and conversion cycles were used.

Measurement of association rates

The active-site titre of trypsin was determined with *p*-nitrophenyl-*p*'-guanidinobenzoate (Chase and Shaw, 1970). Trypsin (10 nM) was incubated for 15 min at 25 °C with human α_1 PI (1–20 nM) and residual tryptic activity determined with BAPNA (1 mM). Human α_1 PI thus titrated was subsequently used as a secondary standard to determine the concentrations of active PPE, chymotrypsin and midiplasmin (Beatty et al., 1980).

All kinetic experiments were done in 50 mM Tris/HCl, 0.1 M NaCl, pH 8.0. Second-order rate constants for the association of EI with PPE and TI with trypsin were determined (Beatty et al., 1980) by incubating equimolar amounts (100 nM) of enzyme and inhibitor for given periods of time, before adding 20 µl of substrate to the mixture (1020 µl final volume). The substrates for trypsin were either 225 mM BAPNA or 10 mM VLKpNA, and for PPE 30 mM AAPNA was used.

The pseudo-first-order rate constants were determined in the same way, except that the concentrations of inhibitors were 10-fold higher than the enzyme concentrations (midiplasmin 120 nM, chymotrypsin 25 nM). The substrates were 10 mM VLKpNA for midiplasmin and 20 mM AAPFPNA for chymotrypsin.

In all kinetic experiments the steady-state rate of substrate hydrolysis was determined by recording the change in absorbance at 405 nm for 2 min.

Proteolytic inactivation of inhibitors

Inhibitors (400 µg) were incubated with 1/100 (w/w) of various proteases at 37 °C in a total volume of 175 µl. Reactions with trypsin, PPE, thermolysin, subtilisin and *S. aureus* protease were done in 50 mM Tris/HCl, 0.1 M NaCl, pH 8.0. Reactions with papain were done in 0.1 M sodium phosphate, 2 mM EDTA, 0.04 mM dithioerythritol, pH 6.8. Aliquots of 30 µl were withdrawn at different times and analysed for inhibitory activity. Proteases able to inactivate EI or TI were used to produce cleaved inhibitors by incubation of 400 µg of inhibitor until approx. 90 % of the inhibitory activity was lost. The cleaved inhibitor was desalted by passage through a 24 ml Superdex 75 column equilibrated and eluted with 0.1 M NH_4HCO_3 , freeze-dried and subjected to sequence analysis. In the same way

complexes of EI and PPE, and of TI and trypsin were prepared (molar ratio of inhibitor to enzyme = 3:2). In an attempt to prepare the 1:1 complex between EI and chymotrypsin, EI was found to be largely inactivated. The mixture was processed as described above.

Isolation of tryptic peptides from EI and TI

Approx. 400 μ g of EI and TI were separately reduced and carboxamidomethylated. After treatment with 8 μ g of trypsin in 0.1 M NH_4HCO_3 for 7 h at 37 °C the digests were separated on a 4 mm $\times$ 250 mm Nucleosil 100-5 C18 column. The peptides were eluted at 50 °C with a 4–76% (v/v) gradient of acetonitrile in 0.1% (v/v) TFA over 60 min. The flow rate was 1 ml/min and the separation was monitored by recording the absorbance at 229 nm.

Rabbit antibodies to EI and TI

Antisera to purified EI and TI were raised by standard protocols in rabbits by Dako. Antibodies were purified by passage of 10 ml of antiserum through a 2 ml protein-A Sepharose column equilibrated with 0.1 M sodium phosphate, pH 7.0. After elution with 0.1 M glycine, pH 2.5, the pH of all fractions was immediately adjusted to 7.0 by addition of 1 M Tris/HCl, pH 9.0.

Western blot analysis

Samples for Western blot analysis were separated by SDS/PAGE and subsequently transferred to poly(vinylidene difluoride) membranes by electroblotting (Matsudaira, 1987). The secondary antibodies were peroxidase-conjugated swine anti-(rabbit immunoglobulin) diluted 500-fold and the immunoreactive bands were visualized by staining with diaminobenzidine according to standard procedures from Dako.

RESULTS

Purification of TI and EI from bovine plasma

Fractionation of plasma by $(\text{NH}_4)_2\text{SO}_4$ followed by Blue-Sepharose and ion-exchange chromatography (Figure 1a) resulted in a preparation containing three major proteins with sizes of approx. 40 kDa, 60 kDa and 120 kDa (Figure 2, lane 2) and able to inhibit both PPE and trypsin. Further separation could not be achieved by ion-exchange chromatography on Mono Q. However, passage through a second Blue-Sepharose column resulted in the separation of the EI and TI (Figure 1b). EI was present in the front fractions and after a final Mono Q step it was homogeneous in SDS/PAGE (Figure 2, lane 3) and was able to form stable complexes with PPE, but not with chymotrypsin (Figure 2, lanes 4 and 5).

TI was found to be retarded by the Blue-Sepharose column and eluted together with the 40 kDa component (Figure 1b). After removal of the latter on Sephacryl S-200 (Figure 1c), a final Mono Q step removed trace contaminants from TI. Since PPE and trypsin were not inhibited by the 40 kDa compound, and since no phenylthiohydantoin-amino acids were obtained when subjected to Edman degradation (not shown), this protein was not investigated further.

The TI preparation showed in addition to the 60 kDa band a 120 kDa species (Figure 2, lane 6). Enhanced formation of the

120 kDa species was observed when larger amounts were applied in SDS/PAGE. From electroblotted samples the N-terminal sequences of the 60 kDa and 120 kDa species were identical (14 residues determined). Gel chromatography on Sephacryl S-200 would be expected to resolve a 120 kDa and a 60 kDa protein. Since the sample eluted as a single symmetrical peak on this column, corresponding to a 60 kDa protein, the 120 kDa component was judged to be a dimer of the 60 kDa component formed during SDS/PAGE sample preparation. Furthermore, addition of catalytic amounts of PPE or excess of trypsin to the TI preparation before SDS/PAGE completely prevented the formation of the 120 kDa species and caused a slight reduction in the size of TI (Figure 2, lane 7), whereas neither reduction, alkylation nor addition of the nucleophilic compound methylamine had any effect on dimer formation in SDS/PAGE (not shown). Occasionally, when incubating equimolar amounts of TI

Figure 1 Purification of bovine plasma inhibitors

(a) The proteins from $(\text{NH}_4)_2\text{SO}_4$ fractionation were, after removal of albumin by Blue-Sepharose chromatography, separated by ion-exchange chromatography on DEAE-Sepharose in a sodium chloride gradient (.....), and the absorbance at 280 nm was monitored (—). (b) Separation of TI (I) and EI (II) on Blue-Sepharose. (c) Separation on Sephacryl S-200 of TI (I) from the 40 kDa contaminant. Horizontal bars indicate the pooled fractions.

Table 1 Amino acid and carbohydrate compositions of bovine EI and TI

Amino acid	Residues/1000 amino acid residues		Residues/molecule*	
	EI	TI	EI	TI
Asx	107.4	110.7	48	48
Thr†	74.0	70.8	33	31
Ser†	78.5	72.8	35	32
Glx	114.3	123.8	51	54
Pro	43.6	42.7	20	18
Gly	44.8	50.0	20	22
Ala	59.7	65.5	27	28
Cys‡	7.6	5.4	3	2
Val§	56.4	64.3	25	28
Met	12.8	15.4	6	7
Ile§	51.9	53.9	23	23
Leu	124.6	124.5	56	54
Tyr	24.0	22.8	11	10
Phe	64.0	52.0	29	23
His	35.2	21.2	16	9
Lys	68.0	59.3	31	26
Arg	28.3	39.9	13	17
Trp	5.0	5.1	2	2
Sum of amino acid residues			449	434

Carbohydrate	Residues/1000 amino acid residues		Residues/molecule*	
	EI	TI	EI	TI
GlcN¶	34.9	44.5	16	19
GalN	0	0	0	0
Xylose	0	0	0	0
Fucose	0.6	0.9	1	1
Mannose	17.5	21.6	8	9
Glucose	0	0	0	0
Galactose	12.6	17.2	6	7
Sialic acid	30.2	38.0	14	16

* Based on a size of approx. 60 kDa.

† Extrapolated to 0 h hydrolysis.

‡ Determined as Cys.

§ Hydrolysis for 72 h.

|| Corrected for loss, using bovine α -chymotrypsin as standard.

¶ Quantified relative to Ala and corrected for a loss of 25% during 3 h hydrolysis.

Figure 2 SDS/PAGE analysis of bovine plasma EI and TI

Protein bands were stained with Coomassie Brilliant Blue after separation in a 10–20% SDS/PAGE gel. All samples were non-reduced. Lane 1, molecular mass standards; lane 2, pool from DEAE-Sepharose containing EI and TI; lane 3, purified EI; lane 4, EI in a 1:1 complex with PPE; lane 5, EI incubated with equimolar amounts of chymotrypsin; lane 6, purified TI; lane 7, TI treated with excess of trypsin; the approx. 7–12 kDa bands are derived from trypsin.

N-terminal sequence analysis

Upon Edman degradation EI and TI yielded a single N-terminal sequence, shown in Figure 3a. The sequences of EI and TI were similar, with ten identical residues present in the first 18 cycles. The N-terminal sequences of the two inhibitors were different from that of bovine α_1 PI (Figure 3a). No other protein in the Genbank Database (release 77) showed identity with the partial EI or TI sequences. However, the N-terminal sequence of a trypsin inhibitor from sheep plasma (sheep TI), originally thought to be sheep α_1 PI (Sinha et al., 1988), is almost identical with that of TI and quite similar to that of EI. As seen from the sequences shown in Figure 3a, EI, TI and sheep TI seem to be more related to human α_1 ACT, than to human, bovine and sheep α_1 PI. This is supported by the sequences of sets of tryptic peptides of EI and TI (not shown). One peptide covering the RSL of EI is discussed below.

Kinetic analysis

The second-order rate constants for association of trypsin with TI and PPE with EI are shown in Table 2.

Incubation of EI and chymotrypsin, and TI and midiplasmin under second-order conditions resulted in a residual level of enzyme activity (approx. 70%), which did not change significantly with time. Thus, although a fraction of the enzyme was inhibited, second-order inhibition kinetics were not followed. However, pseudo-first-order conditions permitted the determination of the rate constant for the inhibition of chymotrypsin by EI and of midiplasmin by TI. The rate constants for the interaction of TI and EI with the proteases studied here are compatible with those generally seen for plasma serpins (Table 2). Compared with trypsin, the inhibition of midiplasmin by TI occurred 200-fold more slowly.

and trypsin, a faint band probably representing a stable TI–trypsin complex, was observed (not shown).

The yields of EI and TI were approx. 20 mg from 100 ml of plasma based on an $E_{1\%, 280\text{ nm}, 1\text{ cm}}$ value of 5.0 determined for the two proteins.

Compositional analysis

The amino acid and carbohydrate compositions of EI and TI were found to be similar (Table 1), with the largest differences seen for the contents of Arg, Lys, His and Phe. The composition of bovine α_1 PI deduced from its cDNA sequence (Sinha et al., 1992) was similar to EI and TI (not shown), but major differences in the content of Ala, Cys, His, Lys and Arg showed that neither EI nor TI is identical with bovine α_1 PI. The carbohydrate content of EI and TI was determined to be 16% and 19% (w/w) respectively.

Figure 3 Comparison of the N-terminal and RSL sequences of EI and TI

(a) The sequences obtained from Edman degradation of EI and TI are aligned with the sequences of a sheep TI (previously thought to be sheep α_1 PI (Sinha et al., 1988)) and human α_1 ACT (Chandra et al., 1983). The N-terminal sequences of human α_1 PI (Carrell et al., 1982) and the putative sheep (Brown et al., 1989) and bovine α_1 PI (Sinha et al., 1992) deduced from cDNA are shown for comparison. Identical residues are shown by (:), chemically similar residues by (.). (b) The partial reactive site sequences of EI and TI were determined from the cleaved and complexed inhibitors by mixture analysis and from analysis of a long tryptic EI peptide (see text). In the sequencing of the EI peptide the first residue was missed, and no sequence information was obtained beyond cycle 23. In EI the P_1 residue is tentatively identified as a Leu residue. The sequences are compared with the RSL sequences of human α_1 ACT and bovine α_1 PI. The PF sequence motif found in all inhibitory serpins is shown by (■).

Table 2 Association rate constants of EI and TI incubated with four proteases

	$k_{\text{ass}}/\text{M}^{-1} \cdot \text{s}^{-1}$	
	TI	EI
Trypsin	$3.1 \pm 0.2 \times 10^5$	No inhibition
Midipalasin	$1.4 \pm 0.1 \times 10^3$	No inhibition
Pancreatic elastase	No inhibition	$1.4 \pm 0.1 \times 10^5$
Chymotrypsin	No inhibition	$0.9 \pm 0.1 \times 10^5$

Proteolytic inactivation and partial reactive site sequences of EI and TI

The reactive site of EI was defined from sequence data of PPE in complex with EI. Three sequences were obtained resulting from EI, PPE and the new N-terminus (P_1') of the cleaved reactive site (Figure 3b). In that way, the sequence of 19 residues covering the P' sites was determined. EI was found not to be inactivated by either *S. aureus* protease, trypsin or papain. In contrast, thermolysin and subtilisin reduced the inhibitory activity by 90% in about 10 min. Sequencing of the inactivated EI yielded two sequences. Apart from the original N-terminal sequence the new sequence showed that the inactivation by both proteases was caused by a cleavage in the RSL at the $P_2'-P_3'$ bond (Figure 3b). Incubation of EI and chymotrypsin at equimolar concentrations

Figure 4 Western-blot analysis of purified EI and TI

EI (lanes 1) and TI (lanes 2) were transferred to a poly(vinylidene difluoride) membrane after electrophoresis and incubated with (a) antibodies against EI; (b) antibodies against TI.

resulted in partial inactivation of EI and cleavage at the $P_3'-P_4'$ site. No new sequence corresponding to cleavage at P_1-P_1' was observed with chymotrypsin. As shown in Figure 3b the partial sequence (22 residues determined) of a 48 residue peptide from EI, eluting at 67% acetonitrile in reverse-phase h.p.l.c. (not shown), is strongly related to the sequence of the $P_{22}-P_1$ sites of human α_1 ACT. The residue determined in cycle 23 is Leu; however, since the sequence could not be extended into the P' region, the identification of Leu as the P_1 residue of EI is tentative.

From sequence analysis of TI treated with trypsin, 16 residues covering the P' sites were determined. Cleavage resulting in inactivation of TI was possible with thermolysin, papain and *S. aureus* protease and was found to take place at the $P_1'-P_2'$, P_6-P_5 and P_2-P_1 sites respectively. Hence, the sequence of the P_5-P_1 sites of TI was obtained.

The sequence motif Pro-Phe ($P_{12}'-P_{13}'$) at the C-terminal end of the RSL is conserved among serpins and is present in both EI and TI. In the alignment shown in Figure 3b, a two-residue insertion in the RSL of TI was introduced to place both the reactive-site residues P_1-P_1' and the Pro-Phe consensus sequence in proper register. In line with the partial N-terminal sequence data of EI and TI, it is apparent from the sequences of their P' sites that EI and human α_1 ACT are more related than TI and α_1 ACT.

Immunochemical characterization

Antibodies against EI and TI were used for Western blot analysis. Both inhibitors were recognized as a single band by antibodies raised against EI (Figure 4a) and against TI (Figure 4b), demonstrating the purity of the preparations. TI is slightly larger than EI (c.f. Figure 5). The cross-reactivity reveals that the inhibitors are strongly related. When high concentrations of TI were used, the 120 kDa band was also recognized by the antibodies, compatible with the identification of this species as a TI dimer.

When plasma was analysed, four closely spaced bands were seen (Figure 5, lane 8). This is probably due to the presence of four very similar proteins as also seen in equine plasma (Potempa et al., 1991). The second band from the top was identified as EI

Figure 5 Western blot of bovine whey and plasma

Antibodies against plasma EI were used to detect cross-reactive proteins in whey after SDS/PAGE and electroblotting. Lane 1, undiluted whey; lanes 2–6, 5-, 10-, 20-, 40- and 80-fold dilutions of whey; lane 7, purified EI 2.5 $\mu\text{g/ml}$; lane 8, 500-fold diluted bovine plasma.

by comparison with the purified protein (Figure 5, lane 7) and the top band as TI based on the size difference seen in Figure 4.

Acid whey was also subjected to Western blot analysis (Figure 5, lanes 1–6) using antibodies against EI, and revealed a band pattern similar to plasma. The dominant band (second from top) in the plasma and whey samples corresponds to the band obtained from purified EI. The same pattern was seen when antibodies against TI were used (not shown), reflecting that EI might be the dominant species of the four.

Purification of inhibitors from bovine whey

Fractionation of acid whey on DEAE-Sephacel resulted in separation of the whey proteins in three major fractions (Figure 6). Peaks A, B and C contained α -lactalbumin, β -lactoglobulin B

and β -lactoglobulin A respectively, as identified by SDS/PAGE and amino acid analysis. Inhibitory activity against elastase, trypsin and plasmin was found mainly between peaks A and B. The combined inhibitory fractions were analysed by SDS/PAGE and gave a band pattern similar to that of the plasma inhibitors (not shown). Further processing of the inhibitory fractions proceeded as described above for the plasma inhibitors and resulted in the two inhibitors EI and TI.

By this method 2–4 mg of each inhibitor were obtained from 1000 ml of whey. By SDS/PAGE, amino-acid and N-terminal sequence analysis they were indistinguishable from EI and TI from plasma. From additional experiments it was found that immobilized antibodies against plasma EI were useful for immunosorbent isolation of active EI and TI (not shown).

DISCUSSION

We have isolated inhibitors of trypsin and PPE, TI and EI respectively, from bovine plasma. TI also inhibits plasmin and EI also inhibits chymotrypsin. The purification strategy was a modification of the method originally presented by Pannell et al. (1974) for isolation of human $\alpha_1\text{PI}$ and was intended to result in bovine $\alpha_1\text{PI}$ not previously isolated. Both inhibitors are approx. 60 kDa proteins and behave similarly during the first purification steps (Figure 1a). However, the combined effect of gel chromatography and the weak affinity of TI for Blue-Sepharose resulted in the separation of EI (front fractions) and TI (retarded fractions). TI coeluted with a 40 kDa unknown protein (Figure 1b), which was separated from TI by gel chromatography (Figure 1c).

TI and EI belong to the serpin family of inhibitors, based on the following evidence: (1) their sizes (Figure 2) and their amino acid compositions (Table 1) are similar to those of other members of the family; (2) pre-formed complexes with proteinases are stable in SDS/PAGE (regularly seen for EI–PPE, occasionally seen for TI–trypsin, Figure 2); and (3) conserved sequence motifs are present in the C-terminal part of the RSLs (Figure 3).

Figure 6 Demonstration of inhibitory activity in whey separated on DEAE-Sephacel

Whey proteins were separated on DEAE-Sephacel and analysed for inhibitory activity against PPE, trypsin and plasmin. The continuous line is the absorbance trace and the dotted lines show residual amidolytic activity of the indicated proteases: I, trypsin; II, plasmin; III, elastase. (A rate of substrate hydrolysis corresponding to a change in A_{405} of one milliabsorbance unit per minute is defined as one activity unit.)

Furthermore, the inhibition kinetics are comparable with that seen for other serpins (Table 2) (see below).

The ability of TI to form a band with the size of 120 kDa cannot be explained in terms of disulphide-bound dimers since the band was unaffected by reducing SDS/PAGE. It is known that human α_1 PI can undergo polymerization by insertion of the RSL from one molecule into the β -sheet A (Löbermann et al., 1984) of another (Mast et al., 1992). However, the polymerization of α_1 PI has previously only been observed in non-denaturing PAGE. In spite of this, we think that the 120 kDa band represents a TI dimer since its formation can be completely prevented by cleavage with small amounts of PPE or excess of trypsin, hence demonstrating the involvement of the RSL, and since its formation is concentration dependent. Occasionally multimers were observed (not shown).

The partial sequence data of EI and TI (Figure 3) show that neither protein is the equivalent of bovine α_1 PI. TI could be the bovine equivalent of sheep TI isolated by Sinha et al. (1988), since the first ten residues of each protein are identical. By comparing the sequences of the first 19 residues of EI and TI we find ten identities, and by comparing the N-termini of EI and human α_1 ACT nine residues are identical.

The extent of sequence similarity in the P' sites among EI, TI and sheep TI is less than that seen in their N-terminal regions. However, from the partial sequence of the P sites of EI we suggest that it is the bovine equivalent of human α_1 ACT. TI and the related sheep TI also appears to be structurally related to α_1 ACT.

The difference in specificity of EI and TI for target proteases is seen in Table 2. Strong and fast inhibition of trypsin is obtained, with TI having a P₁ Arg residue, whereas no inhibition of PPE or chymotrypsin is seen. TI also inhibits midiplasmin, although less strongly than trypsin. Efficient inhibition of midiplasmin is only achieved at a 10-fold molar excess of TI, probably due to the ability of the RSL to exist in different conformations as discussed below for the EI-chymotrypsin interaction.

In contrast to TI, EI inhibits PPE and chymotrypsin, but not trypsin or midiplasmin. Information on the sequence of the P sites of EI was obtained from the partial sequence of a large tryptic peptide extending into the P' region defined by sequence analysis of the EI-PPE complex. Although the sequence information is incomplete, we suggest that a Leu residue constitutes the P₁ residue of EI. This would be compatible with the ability of EI to inhibit chymotrypsin. However, for reasons that are unclear, strong inhibition of chymotrypsin by EI is only achieved at an approx. 10-fold molar excess of inhibitor and not at an equimolar ratio. In the latter condition (which largely leads to inactivation of EI) cleavage occurs at the P₃'-P₄' Leu-Leu bond.

Although this result could indicate that EI is a serpin with overlapping reactive sites similar to α_2 -antiplasmin (Potempa et al., 1988) and C1-inhibitor (Aulak et al., 1993), it could also reflect that EI interacts with chymotrypsin through the P₁ residue of EI and that subsequent proteolysis in the complex takes place at a secondary site. Such a reaction sequence has been observed in a recent study of the α_2 -antiplasmin-chymotrypsin interaction (Enghild et al., 1993). However, studies on human C1-inhibitor showed that a temperature-dependent fraction of the molecules in the reaction with kallikrein were cleaved rather than complexed (Patson et al., 1991). This cleavage occurred at the same site as the inhibition. Thus EI was similar to α_2 -antiplasmin and C1-inhibitor regarding the occasionally substrate-like behaviour of the RSL. The ability of the RSL to exist in distinct functional conformations not readily interchangeable is proposed to be a general feature of serpins (Hood and Gettins, 1991).

By Western blot analysis using antibodies against EI or TI we

have shown the presence of four cross-reactive species in bovine plasma (Figures 4 and 5). This probably reflects different proteins rather than serpin micro-heterogeneity, which is usually not resolved in SDS/PAGE. The pattern in bovine plasma is similar to that of equine plasma, where four structurally similar inhibitors are present (Potempa et al., 1991). EI and TI appear to be the dominant serpins in bovine plasma (levels estimated at approx. 1–2 g/l, not shown).

The trypsin-inhibiting activity of whey has previously been assigned to β -lactoglobulin (Kaminogawa et al., 1972) and to α_1 PI (Honkanen-Buzalski and Sandholm, 1981). However, the β -lactoglobulin-containing fractions have no inhibitory activity (Figure 6) and the latter assignment was tentative since bovine α_1 PI was not isolated and characterized.

The pattern of four cross-reactive serpins in bovine plasma is also seen in milk (Figure 5), and after ion-exchange chromatography (Figure 6) proteins identical with plasma EI and TI were isolated in small amounts. Using immobilized antibodies against EI, a pool containing active EI and TI could be isolated readily, and it was estimated that the level of EI and TI in milk was approx. 10 mg/l (not shown). Hence, EI and TI are likely to constitute the main protease inhibitory potential in milk.

Whether EI and TI in milk are specifically transferred from the blood or synthesized by the mammary gland is not known. In ruminants, postnatal transfer of immunity to the calf is mediated by the colostrum which has high concentrations of immunoglobulins (Brambell, 1970). Plasmin could potentially damage the immunoglobulins, and the Kunitz-type trypsin inhibitor in colostrum (Laskowski and Laskowski, 1951) is likely to provide protection against plasmin.

The presence of inhibitors in milk suggests a role in protecting not only colostrum but also milk against endogenous milk proteases throughout the lactation period. In mammary involution, increased levels of plasmin are found in milk (Politis et al., 1989). However, TI is a relatively poor inhibitor of bovine plasmin [mainly present as midiplasmin (Christensen et al., 1994)] and is probably not relevant for the control of that enzyme. Since EI is structurally similar to human α_1 ACT, which is believed to react primarily with cathepsin G and chymase, EI in milk might control the activity of proteases resulting from degranulation of leucocytes present in milk (Sordillo and Babiuk, 1991; Azzara and Dimick, 1985).

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