Sheep mast cell proteinase-1: characterization as a member of a new class of dual-specific ruminant chymases

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Sheep mast cell proteinase 1 (SMCP-1), which is abundantly expressed in gastrointestinal but not skin mast cells, was isolated and its substrate specificity was investigated. Peptide substrates, including angiotensin I, substance P, bradykinin and oxidized insulin B chain were hydrolysed at P1 Phe, Leu or Tyr residues, conforming to the known chymotrypsin-like properties of the enzyme. However, SMCP-1 was found to hydrolyse some chromogenic substrates with P1 Lys and Arg residues. The enzyme also demonstrated trypsin-like activity against protein substrates, cleaving BSA at Lys^{114}-Leu^{115}, Lys^{238}-Val^{239}, Lys^{260}-Tyr^{261} and Lys^{376}-His^{377}. Bovine fibrinogen β-chain was cleaved at Lys^{28}-Lys^{29}. To ensure homogeneity of the enzyme, the ratio of chymotrypsin-like to trypsin-like activity was observed; it was found to be constant during purification and between different preparations of SMCP-1. Treatment of SMCP-1 with a range of inhibitors decreased chymotrypsin-like and trypsin-like activities by similar extents, supporting the assertion that both activities are the property of a single enzyme. In terms of activity, and by N-terminal amino acid sequencing, SMCP-1 strongly resembles the similarly dual-specific bovine duodenal proteinase, duodenase. It is proposed that SMCP-1 and duodenase represent a new class of ruminant chymases with unusual dual specificities.

INTRODUCTION

Mast cells store large amounts of serine proteinases in secretory granules [1], which are released extracellularly and systemically during inflammatory responses. These serine proteinases fall into two main classes: the so-called tryptases and chymases. Tryptase is a molecule composed, in man, of four subunit serine proteinases of 32–34 kDa each, giving a total size of 134 kDa [2]. The tetramer has trypsin-like substrate specificity, but dissociated subunits are inactive. Tryptase has been described in man, dog, mouse, rat and cow [3–7]. It is present in most human mast cells but is absent from rodent intestinal mucosal mast cells [1].

Mast cell granule chymases are, in contrast, active in monomeric form, exhibiting chymotrypsin-like specificity [8]. Chymases have been described in human, rat, mouse and dog mast cells [9–12], and sheep mast cell proteinase (SMCP) isolated from sheep gastrointestinal mucosal mast cells was also characterized as a chymase [13–15]. To distinguish SMCP from another sheep mast cell chymase (SMCP-2), 80% similar to human chymase, which we have partly cloned and sequenced (S. McAleese, unpublished work), the term SMCP-1 will be used. The chymases share a high degree of sequence similarity with cathepsin G [16] and the granzymes, the granule proteinases found in peripheral blood leucocytes [17].

The functions of mast cell chymases *in vivo* are poorly understood. However, several functions of these enzymes have been demonstrated, such as angiotensin conversion [18] and the induction of mucosal permeability in the gastrointestinal tract during parasite expulsion [19]. The object of this study was to investigate the substrate specificity of SMCP-1, to improve our understanding of how it might function when released systemically and into the gut lumen after nematode infection [20]. Our results show that SMCP-1 has both trypsin- and chymotrypsin-like activity and many similarities to the recently described bovine duodenase [21], a dual-specific chymase isolated from bovine intestine.

MATERIALS AND METHODS

Materials

Equine α_1-proteinase inhibitor (L Spi1 variant) and equine neutrophil elastase 2B were purified as described previously [22,23]. Bovine pancreatic trypsin and bovine lung aprotinin were supplied by Boehringer Mannheim UK. HPLC-grade solvents were obtained from Rathburn Chemicals (Walkerburn, Scotland, U.K.). All other solvents and Coomassie Brilliant Blue R250 were obtained from BDH Laboratory Supplies (Lutterworth, Leics., U.K.). Unless otherwise stated, all chromatography columns and packing materials were supplied by Pharmacia Biotech (St. Albans, Herts., U.K.). Human serum albumin fraction V was obtained from Advanced Protein Products (Brierley Hill, W. Midlands, U.K.). All other reagents were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.).

Electrophoresis and N-terminal sequencing of proteins

Analytical SDS/PAGE was performed with the Mini-Protean II format (Bio-Rad), in accordance with the manufacturer's protocols. In addition, non-reducing sample loading buffer was prepared by omitting 2-mercaptoethanol from the recommended constituents of SDS/PAGE sample loading buffer. Substrate gels, using co-polymerized azocasein, were run as described by

Abbreviations used: Cbz, benzyloxycarbonyl; C:T ratio, ratio of chymotrypsin-like to trypsin-like activity; NA, 4-nitroanilide; PBS/Brij, PBS containing 0.1% Brij-35; SBzl, thiobenzyl ester; SMCP, sheep mast cell proteinase; Suc, succinyl; Tos-Lys-CH_2Cl, tosyl-lysylchloromethane.

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Todorova et al. [24]. Native PAGE to separate basic proteins was performed with the method of Thomas and Hodes [25]. Purified or semi-purified proteins to be sequenced were first separated by SDS/PAGE, typically with 10 % (w/v) polyacrylamide Tris/Tricine gels, as described by Schagger and von Jagow [26]. The resolved proteins were transferred to Immobilon-P membrane by semi-dry blotting with 10 mM 3-cyclohexylamino-1-propranesulphonic acid in 10 % (v/v) methanol buffer at 75 mA per minigel for 1 h. Protein bands were revealed by staining the blot briefly with Coomassie Brilliant Blue R250 and destaining with methanol/water/acetic acid (5:5:1, by vol.). Blotted proteins of interest were sequenced by B. Dunbar (University of Aberdeen Protein Sequencing Facility, Aberdeen, Scotland, U.K.) and P. Barker (Microchemical Facility, Babraham Institute, Cambridge, U.K.).

SMCP-1 purification

SMCP-1 was purified from the gastric mucosa of sheep, a rich source of mucosal mast cells when immunized by infection with the nematode parasite *Telodorsagia circumcincta* [13]. Samples were collected at post-mortem and stored at −70 °C until required. The procedure followed was similar to that described by Huntley et al. [13], with the addition of a final chromatographic step on a Mono S cation-exchange column in 20 mM phosphate buffer (pH 7.0)/0.1 % Brij 35. SMCP-1 was eluted at approx. 150 mM NaCl during a 50–400 mM NaCl gradient. During purification, fractions were assayed for chymotrypsin-like and trypsin-like activity, for example with Suc-Phe-Leu-Phe-SBzl and Cbz-Lys-SBzl (where Suc represents succinyl, SBzl thiobenzyl ester and Cbz benzyloxycarbonyl) respectively (see below).

Degradation of peptides by SMCP-1

The peptides to be analysed (angiotensin I, angiotensin II, substance P, bradykinin, Met-Lys-bradykinin and oxidized insulin B chain) were dissolved to a concentration of 2.5 mg/ml in distilled water (except insulin B chain, which was dissolved in 0.1 M NH_4OH, pH then adjusted to 7.5 by the addition of an appropriate volume of 0.1 M acetic acid, and buffered by the addition of Tris/HCl, pH 7.5, to a final concentration of 25 mM Tris and 2.5 mg/ml peptide). Digestions were performed by adding 10–30 μl of SMCP-1 [0.3 mg/ml in PBS (pH 7.5)/0.1 % Brij-35 (PBS/Brij)] to 100 μl of peptide at 2.5 mg/ml, and incubating at room temperature for 140–210 min. Peptide fragments were separated by reverse-phase HPLC on a Spherisorb ODS-2 5 μm column (125 mm × 4.6 mm; Sigma) in a linear gradient from 0.1 % trifluoroacetic acid in water to 0.1 % trifluoroacetic acid in acetonitrile/water (3:1, v/v). Fractions were concentrated by centrifugal evaporation under reduced pressure. The identity of peptide fractions was determined by a combination of amino acid analysis and mass spectrometry (I. Davidson, University of Aberdeen Protein Sequencing Facility, Aberdeen, Scotland, U.K.).

Degradation of BSA by SMCP-1

SMCP-1 (4 μl of 0.5 mg/ml in PBS/Brij) was added to 100 μl of BSA (1.0 mg/ml in 10 mM Tris/HCl, pH 8.0) and incubated at 37 °C for 4 h, then heated at 95 °C for 1 min to denature the enzyme. SDS/PAGE loading buffer (1 vol.) lacking 2-mercaptoethanol was added and heated at 95 °C for 3 min to ensure complete denaturation of the enzyme. Reducing SDS/PAGE loading buffer (3 vol.; containing 2-mercaptoethanol) was then added and the solution was heated at 95 °C for a further 3 min. The digested BSA fragments were separated by SDS/PAGE and sequenced N-terminally as described above.

Cleavage of bovine fibrinogen by SMCP-1

Bovine fibrinogen (1 ml of 5 mg/ml in PBS) was incubated with 50 μl of SMCP-1 (330 μg/ml) for 15 min at 15 °C. Trifluoroacetic acid (10 %; 10 μl) in water was added, causing precipitation. After centrifugation, the supernatant was injected on a reverse-phase HPLC column (Spherisorb ODS-2 5 μm; 125 mm × 4.6 mm; Sigma) and eluted in a gradient from 0.1 % trifluoroacetic acid in water to 0.1 % trifluoroacetic acid in acetonitrile/water (3:1, v/v). Fractions were collected and freeze-dried, then analysed by mass spectrometry and amino acid analysis.

Addition of SMCP-1 to serum albumins: analysis by SDS/PAGE

To 10 μl of SMCP-1 (0.27 mg/ml in PBS/Brij) or buffer (50 mM Tris/HCl, pH 8.0) were added solutions (100 μl) containing bovine, equine, human or sheep serum albumin at 1.0 mg/ml in 50 mM Tris/HCl, pH 8.0. The mixtures were incubated at 37 °C for 4 h, then prepared for SDS/PAGE as described above.

Kinetic studies

SMCP-1 concentration was determined by active-site titration by the method of Jameson et al. [27] for chymotrypsin-like enzymes. Equine α_1-proteinase inhibitor and human α_1-antichymotrypsin were titrated against known concentrations of equine neutrophil elastase 2B and chymotrypsin respectively. Aprotinin and soybean trypsin inhibitor were titrated against trypsin of known concentration. Michaelis–Menten kinetic parameters with the substrates Suc-Ala-Ala-Pro-Phe-NA (where NA represents 4-nitroanilide), Suc-Phe-Leu-Phe-SBzl, Tos-Gly-Pro-Lys-NA, Tos-Gly-Pro-Arg-NA, Cbz-Arg-NA, Cbz-Gly-Gly-Leu-NA and Cbz-Lys-NA were determined by nonlinear regression analysis on a Beckman DU-600 spectrophotometer, using the onboard enzyme mechanism analysis software, which generally gave similar results to classical Lineweaver–Burk treatment of the data. Incubations were performed in 95 % PBS (containing 0.1 % Brij-35)/5 % DMSO at 20 °C and, for thiobenzyl esters, 5,5′-dithiobis-(2-nitrobenzoic acid) was present at a concentration of 0.5 mM. Molar extinction coefficients at 405 nm were determined under the experimental conditions. The values used in calculations of k_{cat} were 9900 $M^{-1} \cdot cm^{-1}$ with nitroanilide substrates, and 15000 $M^{-1} \cdot cm^{-1}$ with thiobenzyl ester substrates. The inhibitory effects of tosyl-lysylchloromethane (Tos-Lys-CH_2Cl), soybean trypsin inhibitor, aprotinin, equine α_1-proteinase inhibitor and human α_1-antichymotrypsin on SMCP-1 were studied by incubating with an excess of each inhibitor, and the residual trypsin-like and chymotrypsin-like activity was measured after addition of the substrates Tos-Gly-Pro-Lys-NA and Suc-Ala-Ala-Pro-Phe-NA respectively.

RESULTS

Purification of SMCP-1

The purified enzyme was judged to be a single band by native PAGE and by SDS/PAGE with both Coomassie Brilliant Blue R250 (see Figure 1) and silver staining (approximate molecular mass 28 kDa). A single band of proteolytic activity was visible on azocasein substrate gels (results not shown). The yield of SMCP-1 from parasite-infected abomasal tissue was typically in the range 100–400 μg of SMCP-1 per g of wet tissue. During

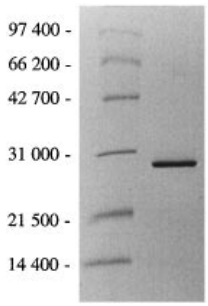

Figure 1 SDS/PAGE gel (12% T) showing purified SMCP-1

Positions of molecular mass markers are indicated at the left, in Da. The gel was stained with Coomassie Brilliant Blue R-250.

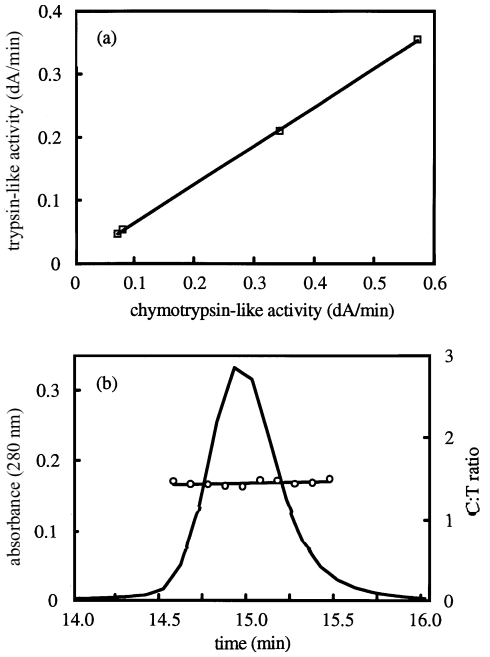

Figure 2 Analysis of trypsin-like and chymotrypsin-like activities of SMCP

(**a**) Relation of chymotrypsin-like to trypsin-like activity (dA, ΔA) in four separate preparations of SMCP-1 from parasite-infected abomasal tissue (two each from two different sheep). Linear regression analysis gave $r^2 = 0.99$. (**b**) Mono-S cation-exchange chromatography of SMCP-1 (45 μg) run in 20 mM phosphate (pH 7.0)/0.1% Brij-35 at 1 ml/min with a 100–200 mM NaCl gradient, increasing at 5 mM/min. Sequential 0.1 ml fractions were collected, and C:T ratios are shown. The chymotrypsin and trypsin-like activities were measured at 405 nm, with Suc-Phe-Leu-Phe-SBzl (0.5 mM) and CBS-Lys-SBzl (0.4 mM) respectively, in the presence of 0.5 mM 5,5′-dithiobis-(2-nitrobenzoic acid).

purification, the ratio of chymotrypsin-like to trypsin-like activity (C:T ratio) was constant. Moreover,the same ratio was observed in four separate SMCP-1 preparations from two different sheep (Figure 2a).

When purified SMCP-1 (45 μg) was rechromatographed on a Mono-S cation-exchange column (20 mM phosphate/0.1% Brij-35/pH 7.0) with a 5 mM/ml NaCl gradient, the C:T ratio remained the same in all fractions collected from the peak (Figure 2b).

N-terminal sequencing of SMCP-1 (Table 1) showed a single sequence and demonstrated almost complete identity (92%) with a recently described bovine duodenal proteinase, duodenase [21,28] over the first 25 residues, and close similarity with other mast cell chymases and granzymes (Table 1). Sequence identity was lower with the tryptase, mouse mast cell proteinase 6 [5]. The N-terminal sequence for SMCP-1 reported here has a glutamine residue at position 19, rather than a glutamic residue as reported by Miller et al. [20]. As the samples of enzyme were derived from different sheep, this might reflect a polymorphism.

Substrate and inhibitor kinetics

Substrate kinetic data are shown in Table 2. SMCP-1 hydrolysed the chymotrypsin substrate Suc-Ala-Ala-Pro-Phe-NA relatively slowly, with a k_{cat}/K_m of 1.1×10^3 $M^{-1} \cdot s^{-1}$, which is similar to published values obtained with the rodent mucosal mast cell proteinases RMCP-II (from rat) and MMCP-1A (from mouse) (4.2×10^2 and 2.5×10^3 $M^{-1} \cdot s^{-1}$ respectively) [29,30]. However, Suc-Phe-Leu-Phe-SBzl was cleaved much more rapidly ($k_{cat}/K_m = 2.3 \times 10^6$ $M^{-1} \cdot s^{-1}$). Hydrolysis of Cbz-Lys-SBzl was also rapid, and the k_{cat}/K_m of 1.2×10^6 $M^{-1} \cdot s^{-1}$ was similar to that described for trypsin with this substrate ($k_{cat}/K_m = 1.5 \times 10^6$ $M^{-1} \cdot s^{-1}$) [31]. However, the k_{cat}/K_m values found for the Tos-Gly-Pro-Lys- and Tos-Gly-Pro-Arg-nitroanilides were 1/76–1/3700 of those published for trypsin ($k_{cat}/K_m = 1.3 \times 10^6$ and 4.1×10^6 $M^{-1} \cdot s^{-1}$ respectively) [31]. The catalytic rate for SMCP-1 with Tos-Gly-Pro-Lys-NA was 36 times greater than with the Arg- analogue. Incubation of SMCP-1 with the trypsin inhibitor Tos-Lys-CH_2Cl caused decreases in the rate of hydrolysis of both trypsin and chymotrypsin substrates to less than 50% (Table 3). More than 90% inhibition of trypsin-like and chymotrypsin-like activity was achieved with soybean trypsin inhibitor and equine α_1-proteinase inhibitor. Bovine lung aprotinin and human α_1-antichymotrypsin had no effect under the conditions used.

Substrate specificity of SMCP-1

SMCP-1 hydrolysed angiotensin I and II, bradykinin, Met-Lys-bradykinin, oxidized insulin B chain and substance P after Phe, Leu or Tyr residues (Table 4). With BSA as the substrate, the cleavages observed were C-terminal to Lys residues. Alignment of the SMCP-1 cleavage sites of BSA (Table 5) revealed similarities in the extended substrate sequence, i.e. acidic residues (Asp or Glu) at the P4 and P4′ positions. Time-course incubation of BSA with SMCP-1 (results not shown) indicated that cleavage of BSA was a function of time, and not sample preparation, and also that the rate of cleavage after Lys^{114} was greatest, followed by cleavage after Lys^{238}. Pretreatment of SMCP-1 with soybean trypsin inhibitor prevented the cleavage of serum albumins (results not shown).

The most prominent peptide fragment (by HPLC) from cleavage of bovine fibrinogen by SMCP-1 was analysed by mass spectrometry, giving m/z 3281.3 (100%; M^+), 3202.4 (81%). The molecular ion is consistent with a fragment (1–28) of bovine fibrinogen β-chain (QFPTDYDEGQDDRPKVGLGARGHR-PYDK), taking into account the reported pyrrolidone carboxylic acid formation at Glu^1 and sulphation at Tyr^6 [32] (calculated for $C_{139}H_{206}N_{42}O_{49}S = 3281.5$). Amino acid analysis confirmed this identification, implying cleavage at Lys^{28}-Lys^{29} (Table 4).

Cleavage of serum albumins by SMCP-1

Bovine, equine and sheep serum albumins were cleaved to give distinct peptide fragments (Figure 3). Human serum albumin was apparently cleaved to a much smaller extent. The major cleavage product with equine and sheep serum albumins had

Table 1 N-terminal amino acid sequence of SMCP-1 compared with related proteinases

Sequences compared are SMCP-1 {(a), [20]; (b), this study}, bovine duodenase [21], human skin mast cell chymase (h-MC chymase; [41]), rat mast cell proteinases I and II (RMCP-I [35] and RMCP-II [36]), mouse mast cell proteinase 1 (MMCP-1 [11]), human cathepsin G [16], human granzyme A [34], human lung chymotrypsin-like serine proteinase (h-LCSP [42]) and the mouse tryptase, mouse mast cell proteinase 6 (MMCP-6 [5]).

	1								10								20							
SMCP-1 (a)	I	I	G	G	H	E	A	K	P	H	S	R	P	Y	M	A	F	L	E	F	K	I	S	G
SMCP-1 (b)	I	I	G	G	H	E	A	K	P	H	S	R	P	Y	M	A	x	L	Q	F				
Duodenase	I	I	G	G	H	E	A	K	P	H	S	R	P	Y	M	A	F	L	L	F	K	T	S	G
h-MC chymase	I	I	G	G	T	E	S	K	P	H	S	R	P	Y	M	A	Y	L	E	I	V	T	S	N
RMCP-I	I	I	G	G	V	E	S	R	P	H	S	R	P	Y	M	A	H	L	E	I	T	T	E	R
RMCP-II	I	I	G	G	V	E	S	I	P	H	S	R	P	Y	M	A	H	L	D	I	V	T	E	K
MMCP-1	I	I	G	G	V	E	A	R	P	H	S	R	P	Y	M	A	H	L	K	I	I	T	D	R
h-Cathepsin G	I	I	G	G	R	E	S	R	P	H	S	R	P	Y	M	A	Y	L	Q	I	Q	S	P	A
h-Granzyme A	I	I	G	G	N	E	V	T	P	H	S	R	P	Y	M	V	L	L	S	L	D	R	K	T
h-LCSP	I	I	G	G	T	E	S	K	P	D	S	R	P	Y	M	A	L	L	Q	I	V	E	P	A
MMCP-6	I	V	G	G	H	E	A	S	E	S	K	W	P	W	Q	V	S	L	R	F	K	L	N	

Table 2 Kinetic parameters for SMCP-1 acting on chromogenic substrates

Except as noted, rates were measured in 95% [10 mM phosphate (pH 7.5)/0.15 M NaCl/0.1% Brij-35]/5% DMSO at 20 °C. Abbreviation: n.h., not hydrolysed.

Substrate	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1} \cdot s^{-1}$)
Tos-Gly-Pro-Lys-NA	5.8×10^{-3}	101	1.7×10^4
Cbz-Lys-SBzl*	3.8×10^{-5}	45	1.2×10^6
Tos-Gly-Pro-Arg-NA	2.6×10^{-3}	2.8	1.1×10^3
Cbz-Arg-NA	n.h.	n.h.	n.h.
Suc-Ala-Ala-Pro-Phe-NA	1.7×10^{-3}	1.9	1.1×10^3
Suc-Phe-Leu-Phe-SBzl*	3.1×10^{-5}	70	2.3×10^6
Cbz-Gly-Gly-Leu-NA	n.h.	n.h.	n.h.

* Rates measured in 90% [10 mM phosphate (pH 7.5)/0.15 M NaCl/0.1% Brij-35]/10% DMSO, containing 0.5 mM 5,5′-dithiobis-(2-nitrobenzoic acid).

Table 3 Effect of trypsin inhibitors on SMCP-1 activity

Incubations were performed in 10 mM phosphate (pH 7.5)/0.15 M NaCl/0.1% Brij-35 at 20 °C. Trypsin-like activity was determined with the substrate Tos-Gly-Pro-Lys-NA. Chymotrypsin-like activity was determined with Suc-Ala-Ala-Pro-Phe-NA. Abbreviations: STI, soybean trypsin inhibitor; α_1PI, equine α_1-proteinase inhibitor; α_1AC, human α_1-antichymotrypsin.

Inhibitor	[Inhibitor] (μM)	[SMCP-1] (μM)	Incubation time (min)	Residual activity (%)	
				Trypsin-like	Chymotrypsin-like
Tos-Lys-CH_2Cl	500	0.05	30	37	48
STI	16	0.5	2	3	6
Aprotinin	5	0.5	10	100	100
α_1PI	4.5	0.5	30	1	0
α_1AC	1.1	0.5	30	99	101

molecular masses of approx. 53 kDa, corresponding to the BSA cleavage product (residues 115–582) of the same approximate molecular mass.

DISCUSSION

Sheep mast cell proteinase was first isolated by Huntley et al. [13] and characterized, by using simple nitrophenyl ester substrates, as a chymotrypsin-like serine endopeptidase with a pH optimum of 7.8. In this study, the same enzyme, now designated SMCP-1, was purified from sheep gastric mucosa and found to be homogeneous by all criteria of purity used, yet it hydrolysed certain substrates for trypsin as well as for chymotrypsin. The most likely explanation for this finding is that the enzyme preparation consists either of a single proteinase with both chymotrypsin and trypsin-like properties, or of a mixture of two or more proteinases with almost identical physical properties but differing specificities. If SMCP-1 is a mixture of enzymes, any variation in relative amounts of the enzymes would result in a change in the C:T ratio. The C:T ratio remained constant throughout purification and between batches of SMCP-1 (Figure 2a). Indeed, sub-fractionation of SMCP-1 during chromatography on Mono S (Figure 2b) revealed the C:T ratio to be

Table 4 Substrate specificity of SMCP-1 acting on peptide and protein substrates

Incubation conditions and analyses were performed as described in the Materials and methods section.

Substrate	P2	P1	P1′	Bond cleaved
Angiotensin I	Pro	Phe	His	(8-9)
	Val	Tyr	Ile	(4-5)
Substance P	Gln	Phe	Phe	(7-8)
	Gly	Leu	Met	(10-11)
Bradykinin	Pro	Phe	Arg	(8-9)
Met-Lys-bradykinin	Pro	Phe	Arg	(10-11)
Oxidized insulin B chain	Ala	Leu	Tyr	(15-16)
BSA	Pro	Lys	Leu	(114-115)
	Thr	Lys	Val	(238-239)
	Ala	Lys	Tyr	(260-261)
	Leu	Lys	His	(376-377)
Bovine fibrinogen β-chain	Asp	Lys	Lys	(28-29)

Table 5 Extended sequence of BSA cleavage sites when incubated with SMCP-1

P4	P3	P2	P1	P1′	P2′	P3′	P4′
Asp^{111}	Leu^{112}	Pro^{113}	Lys^{114}	Leu^{115}	Lys^{116}	Pro^{117}	Asp^{118}
Asp^{235}	Leu^{236}	Thr^{237}	Lys^{238}	Val^{239}	His^{240}	Lys^{241}	Glu^{242}
Asp^{257}	Leu^{258}	Ala^{259}	Lys^{260}	Tyr^{261}	Ile^{262}	Cys^{263}	Asp^{264}
Asp^{373}	Lys^{374}	Leu^{375}	Lys^{376}	His^{377}	Leu^{378}	Val^{379}	Asp^{380}

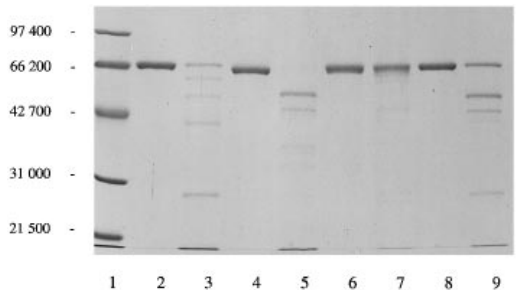

Figure 3 Degradation of serum albumins by SMCP-1: analysis by SDS/PAGE

A Coomassie Brilliant Blue R250-stained SDS/10% gel is shown. Lane 1 contained low-molecular-mass range markers (Bio-Rad) whose molecular masses are shown at the left in Da. Other lanes contained 1 μg each of serum albumins incubated at 37 °C for 4 h with buffer only or with SMCP-1 (molar ratio approx. 15:1; see the Materials and methods section for details): lane 2, BSA; lane 3, BSA + SMCP-1; lane 4, equine serum albumin (ESA); lane 5, ESA + SMCP-1; lane 6, human serum albumin (HSA); lane 7, HSA + SMCP-1; lane 8, sheep serum albumin (OSA); lane 9, OSA + SMCP-1.

constant from the leading edge to the trailing edge of the peak, indicating complete homogeneity.

It is unlikely that proteinases of differing specificity would show the same spectrum of inhibition over a range of inhibitors. Therefore the finding that both the chymotrypsin-like and trypsin-like activities of SMCP-1 were inhibited to similar extents by different inhibitors suggests that a single proteinase is responsible.

A limited range of chromogenic substrates was used to investigate the substrate specificity of SMCP-1. The results suggest that in terms of chymotrypsin substrates, SMCP-1 behaves similarly to the mucosal mast cell chymases of rat and mouse [29,30], and in terms of trypsin substrates it cleaves primarily after Lys. The trypsin-like properties of SMCP-1 were also demonstrated by inhibition by using the trypsin-specific inhibitor Tos-Lys-CH_2Cl, and the lack of inhibition by the chymotrypsin inhibitor, tosylphenylalanylchloromethane, which was described previously [14].

The hydrolysis of peptide substrates by SMCP-1 revealed a bond cleavage specificity that would be expected of a chymase, i.e. cleavage followed the aromatic residues Phe and Tyr in the P1 position. In addition, hydrolysis was also detected after Leu residues, as has been observed in the case of rat mast cell proteinase I [33]. Notably, there was no cleavage of the Lys-Arg bond in Met-Lys-bradykinin.

In contrast with the broadly chymotrypsin-like specificity with peptide substrates, incubation of SMCP-1 with BSA revealed a remarkably specific trypsin-like activity. The bonds cleaved all possessed a Lys at the P1 position and acidic residues (Asp or Glu) at P4 and P4′. SMCP-1 was observed to cleave bovine, equine and sheep serum albumins, but human serum albumin remained largely undegraded (Figure 3). The sequence at the primary cleavage site of BSA (Lys^{114}) is similar in sheep and equine serum albumins, but Lys^{114} is replaced by Arg in the human homologue, and the human protein lacks acidic residues at P4 and P4′. Bovine fibrinogen was cleaved rapidly by SMCP-1, and analysis of HPLC separations of released peptides indicated that the β-chain was cleaved at Lys^{28}-Lys^{29}.

N-terminal amino acid sequence analysis showed that SMCP-1 belongs to the group of serine proteinases that includes cathepsin G [16], granzymes [34] and other mast cell chymases [11,35–37]. In particular, the sequence was almost identical with that for the recently discovered bovine duodenal proteinase, duodenase [21], which has dual trypsin and chymotrypsin-like properties. Duodenase hydrolysed tripeptide 4-nitroanilide substrates with Phe and Arg P1 residues and it was reported to cleave BSA primarily after Lys residues, although it also cleaved after Phe and Tyr [21]. The trypsin-like and chymotrypsin-like activities of duodenase had a similar pH optimum (7.9–8.2) to that published for SMCP (7.6–8.0) [14]. Our preliminary studies (results not shown) suggest that the C:T ratio of SMCP is sensitive to changes in pH, ionic strength and other buffer constituents; this will form the basis of a separate study.

The determinants for multiple specificity in serine proteinases have been studied in fiddler crab collagenase [38], which exhibits substrate cleavage after Arg, Leu, Phe, Lys, Gln and Ala residues. This is believed to be a consequence of the unusual charge on the residues forming the primary specificity (S1) pocket in the enzyme. Residues 189, 216 and 226 (chymotrypsin numbering) in the S1 pocket of serine proteinases are in close proximity to the side chain of the P1 amino acid. In trypsin, Asp^{189}, Gly^{216} and Gly^{226} stabilize the binding of basic amino acids, whereas in chymotrypsin, Ser^{189}, Gly^{216} and Gly^{226} can accommodate large aromatic side chains. Compared with trypsin, the juxtaposition of Asp^{189},$Gly^{226} \rightarrow Gly^{189}$,$Asp^{226}$ in crab collagenase retains trypsin-like activity, but the altered geometry also allows aromatic/hydrophobic residues to bind, rationalizing the broad specificity of the enzyme. Similarly, the dual specificity of duodenase is thought to result from the presence of the residues Asn^{189} and Asp^{226} in the substrate-binding pocket [28]. Human cathepsin G, which exhibits predominantly chymotrypsin-like properties [8], also has an acidic residue (Glu) at position 226 [16], which might explain its reported trypsin-like cleavage of the

human platelet thrombin receptor (Arg^{41}-Ser^{42}) [39] and human C3 (Arg^{748}-Ser^{749}) [40].

In summary, the substrate specificity of SMCP-1 has been defined, and shown to be both chymotrypsin-like and trypsin-like, with a preference for cleavage after Lys residues. The N-terminal sequence and properties of the enzyme are very similar to those reported for the bovine duodenal proteinase, duodenase. Therefore we suggest that duodenase is the bovine homologue of SMCP-1, i.e. a bovine mucosal mast cell proteinase. Indeed, bovine intestinal mast cells are strongly immunoreactive when detected with a polyclonal rabbit anti-SMCP-1 antibody conjugate (J. F. Huntley and H. R. P. Miller, unpublished work). Together, SMCP-1 and bovine duodenase represent a new family of ruminant mast cell proteinases that, although having close similarity to mast cell chymases, might exert some physiological effects by a trypsin-like mechanism. The physiological relevance of dual specificity is currently being investigated in this laboratory. Further studies on SMCP-1, including sequencing and site-directed mutagenesis, will be required to define more precisely the structural requirements for dual specificity.

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