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Purification and Characterisation of 6 and 58 kDa Forms of the Endogenous Serine Proteinase Inhibitory Proteins of Ovine Articular Cartilage

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The major ovine articular cartilage (AC) serine proteinase inhibitory protein (SPI), a 58 kDa glycoprotein (SPI-58), was purified to homogeneity by sequential Sephacryl S-300 gel permeation, concanavalin A affinity, Mono Q anion exchange and Superose 12 FPLC. If precautions to prevent degradation of the native 58 kDa SPI were not undertaken during the early stages of its purification a SPI of ~6 kDa (SPI-6) was generated. SPI-6 could also be generated from SPI-58 by chymotrypsin affinity chromatography, suggesting that SPI-6 could be produced from SPI-58 *in vivo* by proteolytic processing within the tissue. SPI-6 was indistinguishable from the Kunitz inhibitor, bovine pancreatic trypsin inhibitor (BPTI) by SDS-PAGE under both reducing and non reducing conditions and showed a strong homology to BPTI in N-terminal sequence. These data suggest that the BPTI-like 6 kDa SPI constituted the inhibitory domain of the native 58 kDa SPI of ovine AC. Detection of [^{14}C]-lysine-SPI-6 and SPI-58 in the serum free culture medium from ovine chondrocytes cultured in alginate beads in the presence of [^{14}C]-lysine indicated that these SPIs were chondrocyte biosynthetic products. The inhibitory profiles of SPI-58 and SPI-6 differed somewhat suggesting that each may have an independent role *in vivo*.

Key words: Bovine pancreatic trypsin inhibitor / Ovine articular cartilage / Serine proteinase inhibitor.

Introduction

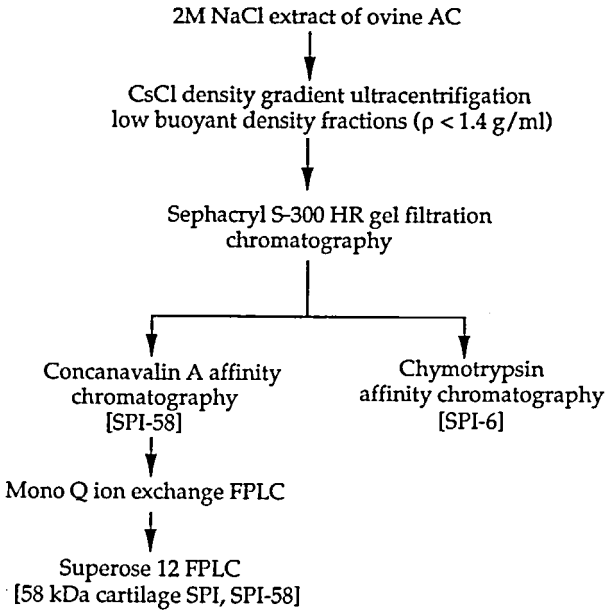
Serine proteinase inhibitors (SPIs) have been extracted from a number of mammalian cartilages (Roughley *et al.*, 1978; Knight *et al.*, 1979). The most extensively studied are the SPIs of human articular cartilage (AC) which contains both high (M_r > 50000) and low relative molecular mass (M_r < 20000) species. The low M_r cationic inhibitors of human AC have been purified to homogeneity (Andrews and Ghosh, 1990) and identified as members of the mucus proteinase inhibitor (MPI) family of serine proteinase inhibitors (Böhm *et al.*, 1991). While Böhm *et al.* (1991), reported that the low M_r cationic SPI of human AC may have been derived from leukocytes by sequestration [corresponding to secretory leukocyte proteinase inhibitor (SLPI)], Jacoby *et al.* (1993) demonstrated that chondrocytes also express the SLPI gene. This finding was consistent with the earlier work of Andrews and Ghosh (1990) who isolated a radiolabelled low M_r SPI from media of human AC chondrocytes cultured in the presence of ^{3}H-leucine. The low M_r cationic SPI of human AC would therefore appear to be potentially derived from both chondrocytes and exogenous sources. The principal high M_r SPI of human AC has been identified as α_1-proteinase inhibitor (α_1PI) (M_r ~55000) (Ghosh *et al.*, 1986).

In contrast to human AC, the major SPI of bovine cartilage is the low M_r Kunitz inhibitor bovine pancreatic trypsin inhibitor (BPTI) (Rifkin and Crowe, 1977; Roughley *et al.*, 1978). This was based on the similarities in the spectrum of inhibition, amino acid composition, molecular weight and the immunoreactivity of the two proteins. A second low M_r SPI inhibitor of ~10000 – 25000 has also been isolated from bovine AC (Sorgente *et al.*, 1976; Arsenis *et al.*, 1986; DiMuzio *et al.*, 1987). This partially characterised inhibitor was a potent inhibitor of human leukocyte elastase (Arsenis *et al.*, 1986; DiMuzio *et al.*, 1987) and chymotrypsin (Sorgente *et al.*, 1976) but did not share immunoreactivity with BPTI (DiMuzio *et al.*, 1987). No high M_r bovine AC SPIs have so far been described. In the present study we describe the isolation and characterisation of the endogenous SPIs of ovine AC.

Results

Purification of the Native 58 kDa SPI from Ovine Articular Cartilage

A summary of the purification procedure for the native high M_r SPI from ovine articular cartilage is shown in Scheme 1 and Table 1. Sephacryl S-300 chromatography resolved the cartilage extract into several protein peaks and one peak of inhibitory activity against chymotrypsin (Kav 0.48) (Figure 1). The Sephacryl S-300 SPI pool was then applied to a column of concanavalin A Sepharose 4B, elution from this column was by a linear gradient of methyl α-D-glucopyranoside and the SPI eluted at a methyl α-D-glucopyranoside range of 0.04 to 0.08 M (result not shown). The concanavalin A SPI pool was further purified by Mono Q anion exchange FPLC. The NaCl gradient employed for elution



Scheme 1 Preparation of the 6 and 58 kDa Forms of the Ovine Cartilage SPIs.

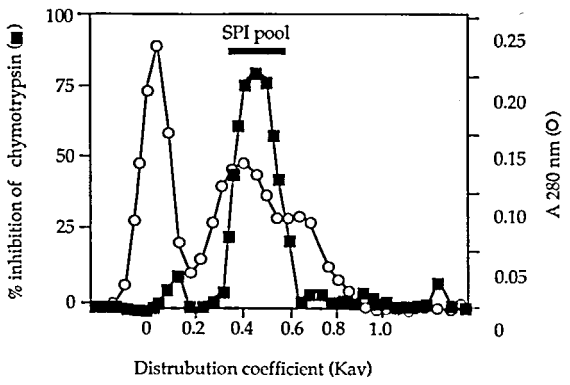


Fig. 1 Sephacryl S-300 HR Gel Permeation Chromatography of the Low Buoyant Density SPI Fractions (p < 1.4 g/ml) from Ultracentrifugation of the 2 M NaCl Extract of Ovine AC.
Fractions were monitored for protein (○, A 280) and for inhibition of chymotrypsin (■, 50 μl versus 0.1 μg of enzyme) and fractions containing serine proteinase inhibitory activity were pooled (—).

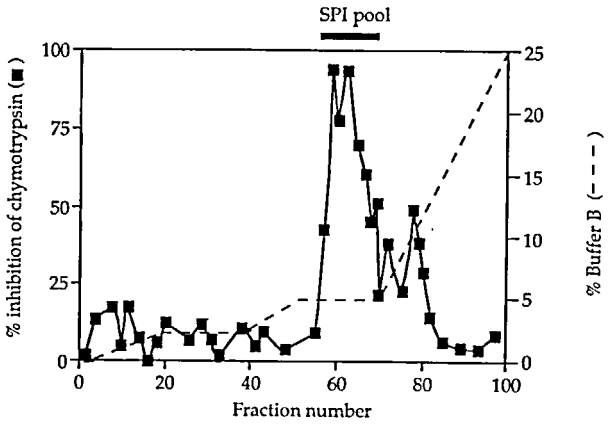


Fig. 2 Ion Exchange Chromatography of the Pooled Inhibitory Fractions from Concanavalin A Chromatography.
The concentrated inhibitory fractions from the concanavalin A affinity column were equilibrated in 20 mM Tris, pH 7.2 starting buffer and applied to a Mono Q HR 5/5 column (~ 2.5 ml bed volume) equilibrated in the same buffer. Non-bound material was eluted with starting buffer till a steady base-line was achieved. Bound material was then eluted using a linear gradient of NaCl (100% buffer B = 1 M NaCl in starting buffer). Eluant fractions (0.5 ml) were monitored for inhibition of chymotrypsin (■, 20 μl versus 0.1 μg of enzyme).

was chosen to give optimal separation of the SPI from other non-inhibitory proteins. The SPI eluted as a major peak at a NaCl concentration of ~0.05 M (Figure 2). When subjected to Superose 12 FPLC the Mono Q SPI pool was resolved into two protein peaks one of which exhibited inhibitory activity against chymotrypsin and trypsin (results not shown). When the inhibitor containing fractions were re-applied to the column a single SPI peak with a Kav of 0.45 was evident (Figure 3). A constant ratio of protein to SPI activity was evident in the fractions eluting across this peak demonstrating the purity of the SPI.

The increasing purity of the SPI through successive steps of the purification scheme was confirmed by SDS-PAGE (Figure 4A) and by Western blotting using biotinylated trypsin (bT) for the detection of SPIs (Figure 4B), and was also reflected by the specific activity of respective SPI pools (Table 1). On SDS-PAGE the SPI migrated as a protein of ~58 kDa on comparison with protein standards

Table 1 Relative Purity of the Native 58 kDa Serine Proteinase Inhibitor from Ovine Articular Cartilage (SPI-58) through Successive Steps in the Purification Scheme (Scheme 1).

Purification step	Protein (mg)	Activity (Units)*	Specific activity (Units/mg of protein)	Recovery (%)	Relative purification
AC Extract	61	1342	22	100	1
Ultracentrifugation D3	33	1188	36	89	1.6
Sephacryl S300	13	855	66	64	3
Concanavalin A	1.12	364	325	27	14.7
Mono Q	1.157	85	541	6	24.6
Superose 12	0.055	50	910	4	41.4

* One unit of inhibitory activity was defined as the amount of SPI required to produce 50% inhibition of chymotrypsin (0.2 μg) using conditions described in the text.

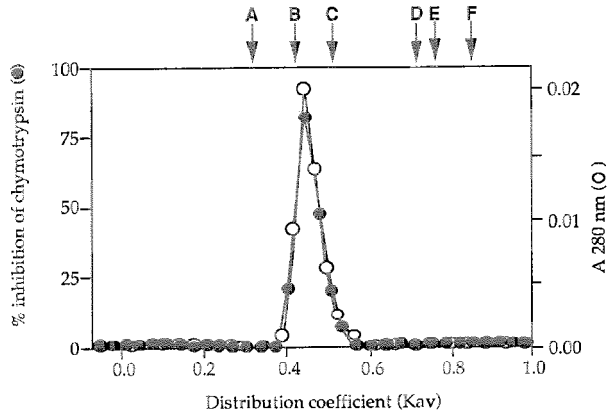


Fig. 3 Superose 12 HR 10/30 FPLC of the Inhibitory Pool Obtained from the Initial Fractionation of the Mono Q HR 5/5 Inhibitory Pool by Superose 12 HR 10/30 FPLC.
The SPI pool was subjected to Superose 12 FPLC in 50 mM Tris, pH 7.2 containing 0.15 M NaCl and 0.05% Tween 20. Eluant fractions (0.25 ml) were collected at a flow rate of 24 ml per hour and monitored for protein (○, A 280 nm) and for chymotrypsin inhibitory activity (■, 20 µl versus 0.1 µg of enzyme). The vertical arrows indicate the elution positions of the globular protein standards (A) catalase (232 kDa), (B) bovine serum albumin (67 kDa), (C) ovalbumin (43 kDa), (D) chymotrypsinogen A (25 kDa), (E) ribonuclease A (13.7 kDa), (F) bovine pancreatic trypsin inhibitor (6.5 kDa).

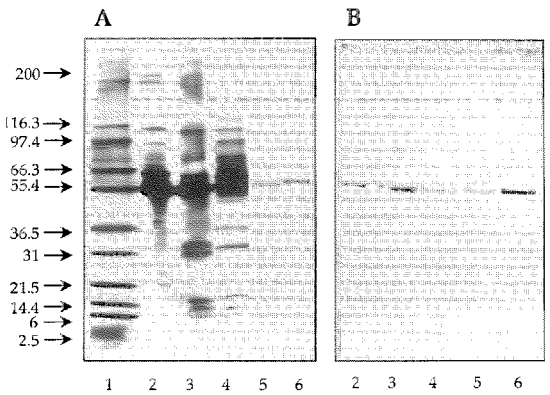


Fig. 4 Demonstration of the Increasing Purity of the Ovine Cartilage SPI after Successive Steps in the Purification Scheme by (A) 10 – 20% Polyacrylamide Gradient SDS-PAGE Using the Tris-Tricine Buffer System and (B) Western Blotting Using Biotinylated Trypsin to Detect Active SPIs.
The samples electrophoresed were: lane 1, Novex broad range protein standards (myosin, rabbit muscle 200 kDa; β-galactosidase, *E. coli*, 116.3 kDa; phosphorylase b, rabbit muscle, 97.4 kDa; bovine serum albumin, 66.3 kDa; glutamic dehydrogenase, bovine liver, 55.4 kDa; lactate dehydrogenase, porcine muscle, 36.5 kDa; carbonic anhydrase, bovine erythrocytes, 31.0 kDa; trypsin inhibitor, soybean, 21.5 kDa; lysozyme, chicken egg white, 14.4 kDa; aprotinin, bovine lung, 6 kDa; insulin B chain, bovine pancreas, 3.5 kDa; insulin A chain, bovine pancreas, 2.5 kDa), these are indicated at the left-hand side of the figure. Lane 2, an aliquot of a 2 M NaCl extract of ovine articular cartilage. Lane 3, SPI pool from Sephacryl S 300 chromatography. Lane 4, SPI pool from concanavalin A affinity. Lane 5, SPI pool from Mono Q ion exchange. Lane 6, SPI pool from Superose 12 FPLC. Proteins were stained using Coomassie R 250.

(SPI-58). A single inhibitory species of apparent M_r 58 000 was present in the neutral salt extract, and was also evident through all subsequent purification steps, demonstrating that no change in molecular size of SPI-58 had occurred during the isolation procedure. Using the protocol outlined, 55 µg of SPI-58 was purified from 20 g (wet weight) of ovine AC, this represents a 4% recovery on a protein basis of SPI from the cartilage extract. Adsorption of the SPI to plastic appeared to contribute significantly to losses in the latter stages of purification, this was reduced by the inclusion of Tween 20 (0.05%) in the buffers and collection and storage of samples in polypropylene minisorb tubes. Significant losses were also incurred (< 20%) when samples were concentrated by diafiltration but alternatives such as lyophilisation and precipitation using ethanol or acetone resulted in > 30% loss in inhibitory activity, presumably due to denaturation.

In an attempt to improve the recovery of the purified SPI-58 from ovine AC, affinity chromatography on immobilised chymotrypsin was investigated following Sephacryl S-300 chromatography (Scheme 1). Examination of SPI fractions by SDS-PAGE and Western blotting using the bT detection system, revealed that fragmentation of SPI-58 had occurred with concomitant generation of two low M_r active inhibitory species (Figure 5, lane 4). Thus chymotrypsin affinity chromatography was not a viable means of purifying the native cartilage 58 kDa SPI.

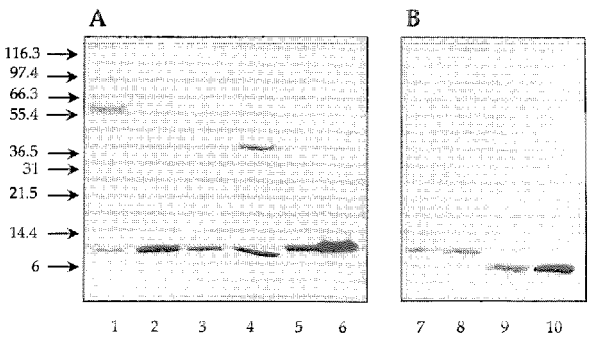


Fig. 5 Comparison of BPTI (Trasylol®) and the Ovine Articular Cartilage SPIs by 10 – 20% Gradient (Tris-tricine) SDS-PAGE Followed by (A) Biotinylated Trypsin Detection Methodology and (B) Staining with Coomassie R 250.
Electroblotting to nitrocellulose and detection of the SPIs using: The migration positions of the Novex broad range standards (see Figure 5) are shown on the left of the figure. The samples electrophoresed were: lane 1 and 2, Kav 0.45 (lane 1) and Kav 0.85 (lane 2) SPI pools from Superose 12 FPLC of stored ovine cartilage SPI pool. Lane 3, Kav 0.45 pool as described for lane 2 following further storage. Lane 4, Sephacryl S-300 SPI pool following chymotrypsin affinity chromatography. Lane 5, SPI from chondrocyte conditioned medium. Lane 6, BPTI (0.1 µg). Lane 7, the 6 kDa cartilage SPI (SPI-6) (1 µg). Lane 8, BPTI (1 µg). Lane 9, 6 kDa cartilage SPI (1 µg). + 0.1% DTT. Lane 10, BPTI (1 µg) + 0.1% DTT.

Generation of a Low M_r SPI upon Storage of Partially Purified SPI-58 and the Relationship of the Low M_r SPI to SPI-58

Examination by Superose 12 FPLC of SPI samples which had been purified up to the concanavalin A affinity step (Scheme 1), and stored for one month at 4°C revealed that a small M_r SPI species (Kav 0.85) was now present in addition to the 58 kDa SPI (Kav 0.45) (data not shown). SDS-PAGE and Western blot analysis revealed that the low M_r SPI pool (Kav 0.85) contained a SPI of ~12 kDa while the high M_r pool (Kav 0.45) contained both 58 kDa and 12 kDa species (Figure 5A, lanes 1 and 2) with prolonged storage only the 12 kDa species was detectable in the Kav 0.45 pool (Figure 5A, lane 3). The SPI samples prepared for Western blotting and detection with bT were not reduced in order to preserve the tertiary structure and biological activity of the inhibitors. In these unreduced samples both BPTI and the ovine low M_r SPI migrated with an apparent M_r of 12000 (Figure 5B). When the samples were reduced however, both BPTI (M_r 6512) and the ovine low M_r SPI migrated with an apparent M_r of ~6000 thus we consider the low M_r ovine cartilage SPI to be a protein of ~6 kDa (SPI-6) (Fig. 5B). The SPI-6 focussed at the limit of the pH 3–10 gradient used in IEF (result not shown) and thus the exact pI of this protein was not discernible by this technique, however it is clearly a highly basic protein of pI ≥10. Furthermore, SPI-6 displayed a strong homology to BPTI upon amino terminal sequencing (Table 2). SPI-6 however was not detectable in a normal cartilage extract when precautions were taken to minimise degradation of SPI-58 by including synthetic proteinase inhibitors. SPI-6 was not evident in purified SPI-58 samples even after prolonged storage, suggesting that the proteinases responsible for conversion of SPI-58 to SPI-6 were now absent from these samples. Collectively these results suggest that SPI-6 was generated from SPI-58 by proteolytic processing by proteases which co-extracted from the cartilage. Furthermore, chymotrypsin affinity chromatography could be used to generate the 6 kDa SPI from the purified native 58 kDa SPI (Scheme 1).

Table 2 Comparison of the N-terminal Sequence of the 6 kDa Ovine Cartilage Serine Proteinase Inhibitor (SPI) with that of Basic Pancreatic Trypsin Inhibitor (BPTI) and Spleen Inhibitor II (SII).

Residue number	1	2	3	4	5	6	7	8	9	10	11	12
BPTI	R	P	D	F	C	L	E	P	P	Y	T	G
SII	R	P	D	F	C	L	E	P	P	Y	T	G
Ovine AC 6 kDA SPI	R	P	D	F	X	L	E	P	P	Y	X	Y

X – signifies a blank cycle
The enumeration sequence indicated is that of BPTI with the other SPIs aligned appropriately (residue 1 = amino terminal residue).

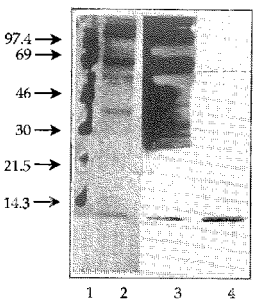


Fig. 6 Demonstration of the Synthesis of a 6 kDa SPI by Ovine Chondrocytes in Culture.
10 – 20% gradient (Tris-tricine) SDS-PAGE with evaluation by: Lane 1 and 2. Phosphor Screen Autoradiography to detect [^{14}C] labelled proteins, Lane 3 Silver Staining, and Lane 4, Western blotting using biotinylated trypsin to detect active SPIs.
The samples electrophoresed were: lane 1, Amersham [^{14}C] protein molecular weight markers myosin (200 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa) carbonic anhydrase (30 kDa), soya bean trypsin inhibitor (21.5 kDa) and lysozyme (14.3 kDa). Lanes 2 – 4, concentrated ovine chondrocyte culture medium.

Demonstration of Purity of SPI-6 and SPI-58

Three criteria were used to demonstrate that the purification of the ovine cartilage SPIs to homogeneity had been achieved using the present procedure.

(i) A single band was evident by SDS-PAGE of SP-6 and SPI-58 (Figure 4A, 5B);
(ii) a single unimodal monodisperse protein peak was evident upon rechromatography of the purified SPIs by Superose 12 FPLC (SPI-58, Figure 3, SPI-6 result not shown);
(iii) only one amino acid was identified in each cycle step during amino terminal sequencing of SPI-6 and SPI-58.

While only a limited number of residues could be identified for SPI-58 (NH_2-L-Q-P-G-R-K-R), twelve amino terminal residues were successfully identified in SPI-6 and these showed > 90% sequence homology with BPTI (Table 2).

SPIs Isolated from Ovine Chondrocyte Conditioned Medium

Western blot analysis employing bT for detection revealed the presence of a low M_r SPI in the chondrocyte conditioned medium from alginate bead culture (Figure 6, lane 4). Silver staining demonstrated a single 12 kDa protein band (without reduction), incorporation of [^{14}C]-lysine into this protein was also evident by phosphorImage analysis (Figure 6, lane 2). Analysis of more concentrated medium samples showed that a second SPI was also present which eluted with a Kav of ~0.45 on Superose 12 FPLC, corresponding to the 58 kDa SPI from the ovine AC extract (result not shown). The 58 kDa SPI was present at very low levels however in comparison to the 12 kDa SPI. Analysis by SDS-PAGE and Western blotting using bT for detection revealed that the low M_r SPI in the chondrocyte culture medium migrated to the same extent as SPI-6 and BPTI (Figure 5, lanes 5 and 6).

The SPI-58 and SPI-6 from chondrocyte culture media or isolated from ovine articular cartilage showed no cross-reactivity with anti-sera to human ITI or α_1PI on Western blots (result not shown).

Table 3 Comparison of the Relative Inhibition of Serine Proteinases by the 58 kDa and 6 kDa Serine Proteinase Inhibitors (SPIs) Purified from Ovine Cartilage Extracts and of the 6 kDa SPI Purified from Ovine Chondrocyte Conditioned Medium with the Inhibitory Activity of Bovine Pancreatic Trypsin Inhibitor (BPTI).

Proteinase	Substrate	% Inhibition by 1 unit of SPI*			
		58 kDa SPI from AC extracts	6 kDa SPI from AC extracts	6 kDa SPI from culture medium	BPTI
Porcine pancreatic chymotrypsin	suAAPFna	50	50	50	50
Porcine pancreatic trypsin	bFVAna	96	97	100	100
Human leukocyte cathepsin G	suAAPFna	100	11	10	0
Human leukocyte elastase	suAAVna	93	40	38	38
Porcine pancreatic elastase	suAAAna	52	0	0	0
Plasmin (porcine blood)	VLKna	35	98	98	88
Kallikrein (porcine glandular)	VLRna	0	100	100	100

* One unit of inhibitory activity was defined as the amount of SPI required to give 50% inhibition of active site titrated chymotrypsin (0.2 μg). Each proteinase (0.2 μg) was incubated with one unit of inhibitor and the residual enzyme activity assessed using the substrates indicated.

Relative Inhibitory Activity of SPI-6 and SPI-58

Insufficient amounts of purified SPI-58 and SPI-6 were available to determine dissociation constants (K_i) for specific proteinases, however, sufficient was available to determine the relative inhibitory activities against 7 active site titrated serine proteinases. These data were compared to the inhibition of these proteinases by purified BPTI. The inhibitory activity of the purified ovine AC SPI-6 or the 6 kDa SPI isolated from ovine culture medium were indistinguishable (Table 3). Chymotrypsin and trypsin were inhibited to a similar extent by both SPI-58 and SPI-6 however, of these enzymes trypsin was the most strongly inhibited. SPI-58 was a strong inhibitor of the human leukocyte proteinases, elastase and cathepsin G and showed significant inhibitory activity pancreatic elastase (PPE). In contrast, SPI-6 was a weaker inhibitor of the leukocyte proteinases and showed no activity against PPE, but unlike SPI-58 it showed potent plasmin and glandular kallikrein inhibitory activity. The inhibitory spectrum of SPI-6 and BPTI were very similar against all the proteinases examined except leukocyte Cathepsin G, however in this case the inhibition of this proteinase was weak (Table 3).

Table 4 Reactivity of the Ovine Articular Cartilage Serine Proteinase Inhibitors SPI-58 and SPI-6 with Biotinylated Lectins on Western Blots.

	Inhibitor	
	SPI-58	SPI-6
Wheat Germ Agglutinin	+	–
Pisum Sativum agglutinin	–	–
Concanavalin A	+	–
Ricinus communis agglutinin 1	–	–
Peanut agglutinin	–	–
Dolichos Biflorus	–	–

\+ strongly reactive
– no detectable reactivity

Carbohydrate Content of SPI-58 and SPI-6

Biotinylated lectins were used to provide information on the carbohydrate content of the ovine cartilage inhibitors. Of the 6 lectins tested only Con A and Wheat Germ Agglutinin bound strongly to SPI-58 on Western blots (Table 4). Demonstrating that SPI-58 contains α-linked mannose or α-linked glucose residues and N-acetyl glucosamine. None of the lectins bound to SPI-6 suggesting that it may not be glycosylated.

Discussion

This study has shown that the major SPI present in ovine AC is a glycoprotein with an apparent M_r of 58 000 on SDS-PAGE (SPI-58). The sensitivity of the biotinylated trypsin detection system facilitated its identification in crude cartilage extracts in which it was unlikely that any artifactual modifications of the inhibitor had occurred. The 58 kDa SPI species therefore apparently represented the native tissue form of the cartilage SPI. We have already demonstrated using SDS-PAGE, native PAGE and isoelectric focussing that SPI-58 is distinct from α_1PI which is present in ovine serum (Rodgers *et al.*, 1995). In addition, SPI-58 may be a more potent inhibitor of cathepsin G and a weaker inhibitor of pancreatic elastase than ovine α_1PI (Mistry *et al.*, 1991). The native cartilage SPI also differs from the ~62 kDa SPI isolated from ovine serum by Sinha *et al.* (1988) which unlike the cartilage SPI was a poor inhibitor of leukocyte elastase. No significant homology in the N-terminal sequence to either of these ovine serum SPIs and the ovine cartilage 58 kDa SPI could be found.

Preparations of the SPI-58 rapidly processed through the stages i to iv of the purification scheme (Scheme 1) were stable on prolonged storage at < 4 °C. However if precautions to prevent degradation of the native 58 kDa SPI were not undertaken early in the purification scheme a second SPI with an apparent M_r of 6000 (when reduced)

was generated. Significantly, chymotrypsin affinity chromatography converted the SPI-58 into two active forms with molecular weights corresponding to 6000 and 40000. This finding suggested that the 6 kDa cartilage SPI (SPI-6) could have arisen by proteolytic modification of the native SPI by co-extracted endogenous proteinases.

SPI-6 was also found to be present in culture medium from ovine chondrocytes cultured in alginate beads. We employed alginate bead culture techniques at it has been reported that chondrocyte phenotypic stability is preserved when they are encapsulated in this matrix (Guo *et al.*, 1989; Häuselmann *et al.*, 1994). When [^{14}C]-lysine was included in the alginate bead culture media [^{14}C]-SPI-6 was synthesised by chondrocytes confirming its cellular origins [^{14}C]-SPI-58 was also detected in the medium but this was present at very low levels in comparison to [^{14}C]-SPI-6. Since we cultured the chondrocytes under serum-free conditions, in order to simplify the subsequent purification of ovine SPIs, it is likely that most of the 58 kDa SPI present may have been degraded by proteinases which are also synthesised by the ovine chondrocytes and released into the media under these culture conditions (Rodgers *et al.*, 1995). The SPI-6 from ovine AC was indistinguishable from the Kunitz inhibitor BPTI by SDS-PAGE under both reducing and non reducing conditions and showed a strong homology to BPTI in the N-terminal sequence.

In addition to BPTI, a family of Kunitz inhibitors closely related to BPTI (Spleen inhibitors I, II and III) have been identified in a number of bovine (Fioretti *et al.*, 1983) and ovine (Fioretti *et al.*, 1990) organs and tissues. BPTI and the bovine spleen inhibitors (Si) originate from two closely related genes (Kingston and Anderson, 1986). The most variable regions are those coding for the mature protein which has 7 substitutions (Creighton and Darby, 1989). Three of these substitutions occur in the active site of the inhibitor (Chen and Bode, 1983) but involve chemically similar amino acid side-chains thus only subtle differences in the protease specificity of these inhibitors has been reported (Creighton and Charles, 1987). In particular, the affinity of SiII for porcine pancreatic kallikrein has been shown to be decreased in comparison to BPTI at pH levels higher than pH 6.5 (Fioretti *et al.*, 1985). In contrast to BPTI, the spleen inhibitors I, II and III contain carbohydrate (Fioretti *et al.*, 1983). In the studies of Fioretti *et al.* (1983, 1990) the spleen inhibitors were isolated using trypsin affinity chromatography. On the basis of the present experiments we would suggest that proteolytic modification of a higher molecular weight precursor form of the SPI may have occurred using this experimental approach. The genes for BPTI and bovine SiII encode polypeptides of either 98 or 100 residues. The mature 58 amino acid protein has a 33 or 35 residue amino extension and a 7 residue carboxyl extension (Creighton and Charles, 1987). These N- and C-terminal extensions of BPTI do not substantially affect the protein structure, refolding or inhibitory activity thus their functional roles have yet to be determined (Creighton *et al.*, 1993).

Although we originally considered that the ovine cartilage SPIs may have arisen from fragmentation of serum derived ovine inter-α-trypsin inhibitor (ITI) which is well known to breakdown into a range of biologically active SPI species, this possibility was found not to be the case for several reasons. The inhibitory domain of ovine ITI has been isolated by limited proteolysis and the amino acid sequence fully determined (Rasp *et al.*, 1987). The 6 kDa SPI we isolated from ovine AC showed less than 10% sequence homology to the inhibitory domain of ovine ITI and neither the SPI-58 or SPI-6 cross-reacted with antibodies raised against ITI.

The principle SPI inhibitor in human AC is a low M_r cationic inhibitor (SLPI) (Andrews and Ghosh, 1990; Böhm *et al.*, 1991) whereas its counterpart in ovine AC (SPI-58) is a high M_r inhibitor with an acidic pI. However, from the present studies it was evident that proteolytic cleavage of the native ovine AC SPI could generate a low M_r cationic SPI form which exhibited many physico-chemical similarities to its human counterpart. This difference in molecular size and charge of the SPI-6 and SPI-58 of ovine AC may permit them to each access different regions of the extracellular matrix and thus provide a more effective protection of resident proteins. For example, it is likely that the 58 kDa anionic SPI species (pI ~4.8 – 5.2) would readily diffuse throughout the highly anionic (proteoglycan rich) pericellular cartilage matrix. However, conversion of the 58 kDa SPI to the 6 kDa form by limited proteolysis *in situ* would generate a cationic form which now would be expected to bind strongly to the highly negatively charged proteoglycans of the cartilage extracellular matrix. An argument could therefore be made that the synthesis of the anionic native SPI molecule (SPI-58) by the chondrocyte, provides it with a means of dispersing this protein to regions remote from its pericellular matrix. Another significant and noteworthy feature of SPI-6 was its capacity to inhibit plasmin while the higher M_r species, SPI-58, was a relatively poor plasmin inhibitor (Table 3). Plasmin has been implicated as a physiological activator of matrix metalloproteinases (MMPs) which are capable of degrading matrix components including proteoglycans and collagens (Murphy *et al.*, 1992; Nagase *et al.*, 1992). Plasmin is thus an important enzyme to regulate for effective control of tissue homeostasis. It is intriguing to speculate on the physiological and regulatory significance of this process whereby SPI-58, a relatively poor plasmin inhibitor is converted to SPI-6, a relatively potent plasmin inhibitor. This may represent an alternative means of control of extracellular matrix (ECM) proteolysis rather than the *de novo* synthesis of SPIs. This proposal is consistent with the studies of Niemann *et al.* (1992) who reported that an active fragment of α_1PI in human placenta (SPAAT) could be generated *in vitro* by proteolytic cleavage of α_1PI. Furthermore, SPAAT was found to display a different inhibitory spectrum to the parent inhibitor. These authors proposed that α_1PI was deposited in the ECM, and when cleaved by a tissue proteinase may then release the N-terminal peptide while SPAAT remained bound to the ECM molecules.

Materials and Methods

Materials

Bovine pancreatic trypsin (EC 3.4.21.4, type XIII, TPCK treated), bovine pancreatic chymotrypsin (EC 3.4.21.1, Type II), porcine pancreatic elastase (EC 3.4.21.36), porcine pancreatic kallikrein (EC 3.4.21.35) succinyl-ala-ala-pro-phe-4-nitroanilide (suAAPFna), N-benzoyl-phe-val-arg-4-nitroanilide (bFVAna), succinyl-ala-ala-ala-4-nitroanilide (suAAAna), D-val-leu-arg-4-nitroanilide (VLRna), D-val-leu-lys-4-nitroanilide (VLKna), CBZ-arg-4-nitroanilide (cbzRna), Tris (hydroxymethyl) aminomethane (free base), Tricine [N-tris (hydroxymethyl) methylglycine], pepstatin A, phenylmethylsulfonylfluoride (PMSF), iodoacetamide, α_1-proteinase inhibitor (α_1-PI), Tween®-20, avidin labelled alkaline phosphatase conjugate and DME/F12 culture media were purchased from the Sigma Chemical Co. Ltd., (MO, USA). Succinyl-ala-ala-val-4-nitroanilide (suAAVna) was purchased from Bachem, (Bubendorf, Switzerland). Plasmin (EC 3.4.21.7, human plasma) was purchased from Boehringer-Mannheim (Castle Hill, NSW, Australia). Human neutrophil elastase (EC 3.4.21.37) and cathepsin G (EC 3.4.21.20) were obtained from Novo-Calbiochem (Alexandria, Sydney, NSW, Australia). A biotinylated lectin sampler kit was purchased from Pierce (Rockford, IL, USA). Precast 10 x 10 x 1mm, 10 – 20% polyacrylamide (Tris-tricine) gradient gels, nitrocellulose membranes, and broadrange electrophoresis standard proteins were purchased from Novex, (French's Forest, NSW, Australia). Immobilon-P polyvinylidine difluoride membranes were purchased from Millipore, (Lane Cove, NSW, Australia).

Methods

Tissue Extraction Articular cartilage (15 g wet weight) from the stifle joints of 2 – 3 year old pedigree merino wethers was collected, rapidly frozen under liquid nitrogen and powdered by mechanical pulverisation in a stainless steel mortar and pestle. The thawed cartilage powder was extracted with 10 volumes of 2 M NaCl, in 50 mM Tris-HCl, pH 7.2 containing PMSF (2 mM), Iodoacetamide (100 μM), Pepstatin A (10 μg/ml), EDTA (5 mM), and gentamicin (40 μg/ml) with end-over-end stirring for 48 h at 4 °C. The extract was recovered by filtration, the residual tissue washed twice with 1 volume of extraction buffer and the washings similarly collected and combined with the extract.

Purification of the Ovine Cartilage Serine Proteinase Inhibitor (Scheme 1) All the following chromatographic steps were conducted at 4 °C.

(i) Isopycnic density gradient ultracentrifugation

Briefly, sodium chloride AC extracts were brought to a starting density of 1.5 g/ml with solid caesium chloride and subjected to density gradient ultracentrifugation as indicated earlier (Rodgers *et al.*, 1995), low buoyant density fractions ($\rho < 1.4$ g/ml) were pooled and concentrated by diafiltration using a YM3 membrane (3 kDa exclusion).

(ii) Sephacryl S-300 HR gel permeation chromatography

Pooled low buoyant density fractions equilibrated in 50 mM Tris-HCl, pH 7.2 containing 1 M NaCl were chromatographed on a calibrated column of Sephacryl S-300 HR gel (100 cm x 1.6 cm, ~2% bed volume applied) eluted with the aforementioned buffer at a flow rate of 40 ml/h. Fractions (4 ml) were monitored for protein (A 280 nm) and inhibitory activity against trypsin and chymotrypsin as described below. Fractions containing SPI activity were pooled and examined further by concanavalin A affinity chromatography or chymotrypsin affinity chromatography.

(iii) Concanavalin A-Sepharose 4B or chymotrypsin affinity chromatography

Pooled SPI fractions from Sephacryl S-300 HR chromatography equilibrated in 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1mM $CaCl_2$, 1mM $MgCl_2$ and 1mM $MnCl_2$, (Con A starting buffer) were applied to a column of immobilised concanavalin A, non bound material was eluted with aforementioned buffer (10 bed vols) and the bound SPIs were eluted with a linear gradient of 0 – 0.2 M methyl α-D-glucopyranoside in Con A starting buffer and used for subsequent experiments.

A second SPI pool from Sephacryl S-300 HR chromatography was applied to a column of immobilised chymotrypsin (1 cm x 4 cm). Non-bound material was eluted step-wise with 1 M NaCl in 0.5 M sodium acetate pH 4.0, 1 M NaCl in 1 mM HCl pH 3 and the bound SPIs eluted with 3 mM HCl pH 2.0 containing 1 M NaCl (5 bed vols/step). The pH of eluant fractions were re-adjusted to 7.4 by collection into an aliquot of 1 M Tris-HCl, pH 7.5.

(iv) Mono Q HR 5/5 anion-exchange FPLC

Pooled inhibitory fractions from concanavalin A affinity chromatography were equilibrated in 50 mM Tris-HCl, pH 7.2 (buffer A) and then subjected to anion exchange FPLC on a Mono Q 5/5 column. A flow rate of 0.5 ml per min was used and 0.5 ml fractions were collected. Non-bound material was eluted with 10 bed volumes of buffer A, bound proteins were eluted with a linear gradient of 0 to 1 M NaCl in buffer A (buffer B) composed as follows, (a) 0 to 2.5% buffer B over 10 ml, (b) isocratic at 2.5% buffer B for 10 ml, (c) 2.5 to 5% buffer B over 5 ml (d) isocratic at 5% buffer B for 10 ml then, (e) a linear gradient to 100% buffer B over 15 ml (Figure 3).

(v) Superose 12 HR 10/30 FPLC

Pooled inhibitory fractions from Mono Q FPLC were further purified by Superose 12 FPLC. A flow rate of 24 ml/h was employed and 50 mM Tris-HCl, pH 7.2 containing 0.15 M NaCl and 0.05% Tween 20 was used as eluant, 0.25 ml fractions were collected into 96 well flat bottomed microtitre plates. Inhibitory activity and protein content (see below) was determined for each fraction. Inhibitory fractions were pooled, re-applied to the column and the protein content and inhibitory activity in the fractions assayed as described previously.

Protein Determination The protein content of eluant fractions was monitored by UV light absorbance (280 nm) using a Gilson Model 111 LC flow through detector. The protein content of SPI pools was determined using the bicinchoninic acid (BCA) procedure (Smith *et al.*, 1985).

Determination of Proteinase-Inhibitory Activity Chromatographic fractions (20 – 100 μl aliquots) from each successive step (i – v) of the purification scheme were assayed for inhibitory activity against bovine pancreatic chymotrypsin (0.1 μg) using the substrate Succ-Ala-Ala-Pro-Phe-4-nitroanilide (suAAPFna) (Del Mar *et al.*, 1979) and against bovine pancreatic trypsin (0.1 μg) using the substrate Benzoyl-Phe-Val-Arg-4-nitroanilide (bFVRna) (Somorin *et al.*, 1978). Comparable inhibition data was generated using either chymotrypsin or trypsin as representative serine proteinases. However since the methodology used for the detection of chymotrypsin offered the greatest sensitivity, and in an effort to improve the clarity and brevity of figures, only chymotrypsin inhibition data is presented for chromatographic separations (Figures 1 – 4).

Specific activities (units of inhibitory activity per mg of protein) of pooled SPI fractions from each stage in the purification scheme were determined as follows, one unit of inhibitory activity was defined as the amount of SPI required to give 50% inhibition of 0.2 μg of active site titrated chymotrypsin under standard assay conditions: using suAAPFna as substrate (0.5 mM) in 100 mM Tris-HCl with 100 mM NaCl, 10 mM $CaCl_2$, 0.1 mg/ml BSA, pH 8.2 and 4% DMSO. Assays were carried out in 96 well flat bottom micro-

titre plates at 25 °C in a final assay volume of 250 µl. Residual enzymatic activity was assessed from the increase in absorbance (A 405 nm) of the sample relative to enzyme control samples measured by automatic plate reader. The protein content of SPI pools was determined by the BCA procedure, see above.

The percentage inhibition of various active site titrated serine proteinases (0.2 µg) by one unit of purified inhibitor was assessed under the aforementioned assay conditions, residual proteinase activity was determined using nitroanilide peptide substrates as indicated in Table 3.

Chondrocyte Culture in Alginate Beads Cartilage (~10 g) was aseptically harvested from two stifle joints, diced, rinsed in PBS, and suspended in serum free (1:1) Hams F12/DME media, supplemented with insulin, transferrin and sodium selenite, gentamycin (80 Units/ml) and buffered with 20 mM HEPES pH 7.2. Chondrocytes were released from cartilage pieces by sequential digestion in pronase (0.1%) for 3 hours at 37 °C and overnight digestion in collagenase (4 mg/ml). The released cells were pelleted (2500*g* x 10 min), re-suspended in serum free medium and viable cell numbers determined by trypan blue exclusion using a haemocytometer. The cells were recovered by centrifugation (2500*g* x 10 min) and re-suspended in alginic acid solution (1.2% w/v in 0.15M NaCl) to provide a density of ~ 6 x 10^6 cells/ml. This mixture was extruded dropwise through a 26 gauge needle into a gently agitated solution of $CaCl_2$ (100 mM) to form calcium alginate beads containing ~ 60 000 cells per bead which were allowed to cure for 10 min. The beads were established in Hams F12 : DMEM, in an atmosphere of 5% CO_2 in 95% air with a humidity of 98%, at 37 °C. After 24 h the culture medium was supplemented with [^{14}C]-lysine (1 µCi/ml). The medium was changed daily for 5 days and used as a source of radiolabelled SPIs for analysis by SDS-PAGE, Western blotting and Phosphor-screen autoradiography.

Electrophoretic Characterisation of the Ovine Cartilage and Chondrocyte Media SPIs

(i) SDS-PAGE

SPI samples were diluted 1:1 with 0.0625 M Tris-HCl pH 6.8 containing 10% v/v glycerol 2% v/v SDS 0.0025% w/v bromophenol blue and incubated for 1h at 37°C to form SDS-protein complexes. Electrophoresis was then conducted in 10 – 20% polyacrylamide gradient Tris-tricine gels for 120 min at 125 V using the Tris-tricine buffer system (0.1M Tris, 0.1M tricine and 0.1% SDS pH 8.4) (Schägger and von Jagow, 1987).

(ii) Western blotting of SPI samples and their visualisation using biotinylated trypsin

SDS-PAGE gels were equilibrated in 12 mM Tris-HCl, 96 mM glycine pH 8.3, 20% v/v methanol transfer buffer and electroblotted to nitrocellulose membranes (2 h at 200 mA). Membranes were blocked overnight by incubation in 0.1% (v/v) Tween 20 in 20 mM Tris-HCl pH 7.5 containing 500mM NaCl (TBS). The SPIs were visualised using a biotinylated trypsin/avidin/alkaline phosphatase detection system as indicated earlier (Melrose *et al.*, 1994).

(iii) Characterisation of the carbohydrate content of the SPIs using biotinylated lectins

Following SDS-PAGE and electrotransfer to nitrocellulose membranes as described previously (ii), SPI samples were examined using a biotinylated lectin kit (Pierce) following the manufacturers instructions.

Amino Terminal Sequencing of Ovine SPI Species Following electrophoretic separation by SDS-PAGE on 10 – 20% Tris-tricine gradient gels, proteins were transferred to PVDF (Immobilon) membranes which were stained with Amido black (0.1% in 1% acetic acid, 20% methanol) and the bands of interest excised for sequencing. Purified SPI samples from chromatography were spotted directly on PVDF inserts prior to further analysis. Amino terminal sequencing was conducted by sequential Edman degradation on an Applied Biosystems Model 470 gas-phase protein sequencer fitted with an on-line phenylthiohydantoin detection system. The samples were not reduced and alkylated prior to hydrolysis thus cysteine was not detected.

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References

Anderson, S., and Kingston, B.I. (1983). Isolation of a genomic clone for bovine pancreatic trypsin inhibitor by using a unique-sequence synthetic DNA probe. Proc. Natl. Acad. Sci. USA *80*, 6838 – 6842.

Andrews, J.L., and Ghosh, P. (1990). Low molecular weight serine proteinase inhibitors of human articular cartilage. Arthritis Rheum. *33*, 1384 – 1393.

Arsenis, C., Thonar, E.J.-M.A., and Kuettner, K.E. (1986). Degradation of cartilage matrix by purified elastase and its control by an endogenous purified specific elastase inhibitor. Agents and Actions Suppl. *18*, 63 – 68.

Creighton, T.E., and Charles, I.G. (1987a). Biosynthesis, processing and evolution of bovine pancreatic trypsin inhibitor. Cold Spring Harbor Symp. Quant. Biol. *52*, 511 – 519.

Creighton, T.E., and Charles, I.G. (1987b). Sequences of the genes and polypeptide precursors for two bovine protease inhibitors. J. Mol. Biol. *194*, 11 – 22.

Creighton, T.E., Bagley, J.B., Cooper, L., Darby, N.J., Freedman, R.B., Kemmink, J., and Sheikh, A. (1993). On the biosynthesis of bovine pancreatic trypsin inhibitor (BPTI). Structure, processing, folding, disulphide bond formation of the precursor *in vitro* and in microsomes. J. Mol. Biol. *232*, 1176 – 1196.

Del Mar, E.G., Largman, C., Brodrick, J.W., and Geokas, M.C. (1979). A sensitive new substrate for chymotrypsin. Anal. Biochem. *99*, 316 – 320.

DiMuzio, M.T., Walsh, S., Tripier, D., and Kuettner, K.E. (1987). The isolation, purification and partial sequence of an elastase inhibitor derived from cartilage. Trans Orthop. Res. Soc. *12*, 252.

Fioretti, E., Angeletti, M., Fiorucci, L., Barra, D., Bossa, F., and Ascoli, F. (1988). Aprotinin-like isoinhibitors in bovine organs. Biol. Chem. Hoppe-Seyler *369 (Suppl)*: 37 – 42.

Fioretti, E., Angeletti, M., Barra, D., and Ascoli, F. (1990). Kunitz-type proteinase inhibitors in sheep and ox. Comp. Biochem. Physiol. *96(3)*, 445 – 449.

Fioretti, E., Binotti, I., Barra, D., Citro, G., Ascoli, F., and Antonini, E. (1983). Heterogeneity of the basic pancreatic inhibitor (Kunitz) of various bovine organs. Eur. J. Biochem. *130*, 13 – 18.

Ghosh, P., Andrews, J.L., Osborne, R.A., and Lesjak, M.S. (1986). Variation with ageing and degeneration of the serine and cysteine proteinase inhibitors of human articular cartilage. Agents and Actions Suppl. *18*, 69 – 81.

Guo, J.F., Jourdian, G.W., and MacCallum, D.K. (1989). Culture and growth characteristics of chondrocytes encapsulated in alginate beads. Connect. Tissue Res. *19*, 277 – 297.

Häuselmann, H.J., Fernandes, R.J., Mok, S.S., Schmid, T.M., Block, J.A., Aydelotte, M.B., Kuettner, K.E., and Thonar,

E.J.-M.A. (1994). Phenotypic stability of bovine articular chondrocytes after long-term culture in alginate beads. J. Cell Sci. *107*, 17 – 27.

Jacoby, A.S., Melrose, J., Robinson, B.G., Hyland, V.J., and Ghosh, P. (1993). Secretory leukocyte proteinase inhibitor is produced by human articular cartilage chondrocytes and intervertebral disc fibrochondrocytes. Eur. J. Biochem. *218*, 951 – 957.

Kingston, I.B., and Anderson, S. (1986). Sequences encoding two trypsin inhibitors occur in strikingly similar genomic environments. Biochem. J. *233*, 443 – 450.

Knight, J.A., Stephens, R.W., Bushell, G.R., Ghosh, P., and Taylor, T.K.F. (1979). Neutral proteinase inhibitors from human intervertebral disc and femoral head cartilage. Biochem. Biophys. Acta *584*, 304 – 310.

Melrose, J., Rodgers, K.J., and Ghosh, P. (1994). The preparation and use of biotinylated trypsin in Western blotting for the detection of trypsin inhibitory proteins. Anal. Biochem. *222*, 34 – 43.

Melrose, J., Ghosh, P., and Patel, M. (1995). Biotinylated aprotinin: a versatile probe for the detection of serine proteinases on Western blots. Int. J. Biochem. Cell Biol. *27*, 891 – 904.

Mistry, R., Snashall, P.D., Totty, N., Guz, A., and Tetley, T.D. (1991). Isolation and characterisation of sheep α_1-proteinase inhibitor. Biochem. J. *273*, 685 – 690.

Murphy, G., Ward, R., Gavrilovic, J., and Atkinson, S. (1992). Physiological mechanisms for metalloproteinase activation. Matrix Suppl. *1*, 224 – 230.

Nagase, H., Suzuki, K., Morodomi, T., Enghild, J.J., and Salvesen, G. (1992). Activation mechanisms of the precursors of matrix metalloproteinases 1, 2 and 3. Matrix Suppl. *1*, 237 – 243.

Niemann, M.A., Narkates, A.J., and Miller, E.J. (1992). Isolation and serine protease inhibitory activity of the 44 residue, C terminal fragment of alpha-I-antitrypsin from human placenta. Matrix *12*, 233 – 241.

Rasp, G., Hochstrasser, K., Wachter, E., and Reisinger, W.M. (1987). The amino-acid sequence of the trypsin-released inhibitor from sheep inter-α-trypsin inhibitor. Biol. Chem. Hoppe-Seyler *368*, 727 – 731.

Rifkin, D.B., and Crowe, R.M. (1977). Isolation of a proteinase inhibitor from tissues resistant to tumour invasion. Hoppe-Seylers Z. Physiol. Chem. *358*, 1525 – 1531.

Rodgers, K.J., Melrose, J., and Ghosh, P. (1995). Biotin-labelled potato chymotrypsin inhibitor-1: A useful probe for the detection of chymotrypsin-like serine proteinases on Western blots and its application in the detection of a serine proteinase synthesised by articular chondrocytes. Anal. Biochem. *227*, 129 – 134.

Rodgers, K.J., Melrose, J., and Ghosh, P. (1996). Biotinylated trypsin and its application as a sensitive, versatile probe for the detection and characterisation of an ovine chondrocyte serine proteinase inhibitor using Western blotting. Electrophoresis *17*, 213 – 218.

Roughley, P.J., Murphy, G., and Barrett, A.J. (1978). Proteinase inhibitors from bovine nasal cartilage. Biochem. J. *169*, 721 – 724.

Schägger, H. and von Jagow, G. (1987). Tricine-sodium sulphate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. *166*, 368 – 379.

Sinha, U., Sinha, S., and Janoff, A. (1988). Characterisation of alpha-1-proteinase inhibitor. Am. J. Respir. Dis. *137*, 558 – 563.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. (1985). Measurement of protein using bicinchoninic acid. Anal. Biochem. *150*, 76 – 85.

Somorin, O., Tokura, S., Nishi, N., and Noguchi, J. (1978). The action of trypsin on synthetic chromogenic substrates. J. Biochem. *85*, 157 – 162.

Sorgente, N., Kuettner, K.E., and Eisenstein, R. (1976). Purification and partial characterisation of proteinase inhibitors from bovine cartilage and aorta. In: Proc. 23rd Colloquium on Protides of Biological Fluids. H. Peters, ed. (Oxford, UK.: Pergamon Press, pp. 227 – 230.)

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