A porcine lung SLPI WAP 2 fragment inhibits trypsin by sharing the preserved antielastase reactive site P1' Met 73 and P2' Leu 74

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Abstract: - One acid stable trypsin inhibitor (around 16 kDa) was isolated from the porcine lung extract after clarification, acid precipitation and trypsin affinity chromatography. Its N-terminal sequence is MLNP. The polyclonal antibody against recombinant mouse secretory leukoproteinase inhibitor (SLPI) well recognized the inhibitor. The obtained N-terminal sequence corresponds to the antielastase reactive site P1' Met 73 and P2' Leu 74 and presents in SLPI WAP 2 domain. The reactive site was preserved active even after the inhibitor fragmentation that could occur during the trypsin affinity chromatography. The probable association of the 3.0 kDa SLPI WAP 2 fragment with GAG heparin (molecular mass around 14 kDa) responds to the molecular mass of the active inhibitor.

Key-Words: - proteinase inhibitor, antileukoprotease family, SLPI, WAP domain, lung protection

1 Introduction

We developed protocols to isolate the porcine aprotinin (ABY82797) based on the extreme resistance to acidic conditions of bovine aprotinin [1, 2]. After the porcine aprotinin purification, we focus to characterize the other porcine lung acid stable serine protease inhibitor, which we had also detected [2]. It is a band around 14 kDa what made us considering the secretory leukocyte proteinase inhibitor (SLPI). This inhibitor was historically described in lung secretions [3, 4]. SLPI belongs to the chelonianin family and was also classified in antileukoprotease family (ALP) as well as the other well-known member of the family: elafin and its precursor, pre-elafin (trappin-2) [5]. Together with them, they are very important endogenous protease inhibitors [5, 6, 7, 8]. SLPI has different names due to its ubiquity, which means its presence in almost all mucous fluids. SLPI preserves the local tissue against deleterious effects of inflammation inhibiting the release of leukocyte proteases [9]. Neutrophil elastases (NE) and mast cell proteases are its targets. SLPI is a 107 amino acids, non-glycosylated, basic protein comprising two domains: C and N terminals that share around 35% amino acid homology and have distinct enzymatic activities. Each of these domains presents eight cysteine residues that form four disulfide bridges stabilizing them. This type of structure domain was called WAP (name derived from the whey acidic protein where the structure was first observed). SLPI C terminal domain or WAP 2 is responsible for the antiprotease activity and the reactive site are the residues Leu

72 - Met 73 [5, 8, 10]. SLPI suppresses proteases in the upper respiratory tract and together with the pulmonary surfactant they serve as important defenses against viral infection in the airway [11]. SLPI has also anti-bacterial and anti-fungal activities probably determined by the N terminal domain and not directly related with its anti-proteolytic activity against trypsin, elastases and cathepsin G [8]. SLPI immuno-modulatory activity as "alarm molecules" in response to inflammatory mediators (cytokines or LPS) seemed to be associated with its anti-protease activity [9]. About biochemical characteristics of pre-elafin and elafin, the first one is the full-length protein with two domains whereas elafin comprises only the mature C-terminal domain (amino acids 39–95), which is the WAP domain with 40% sequence identity to the SLPI WAP domains [5, 12]. Opposed to A1-PI (α1-proteinase inhibitor), the prototypic serpin, which is present in large amounts in the circulation, SLPI and Pre-elafin/Elafin are good tissue-bound inhibitors due to their molecular characteristics [7].

The unquestionably importance of the ALP protease inhibitors impelled us to characterize the 14 kDa band inhibitor which was co-isolated with porcine aprotinin and prove to be a trypsin inhibitor by reverse zymography [2].

2 Materials and Methods

2.1 Isolation of the inhibitors

The porcine lung serine proteinase inhibitors were isolated as previously described in [2]. The active TCA clarified supernatant was concentrated by tangential flow filtration using the QuixStand Benchtop System® with Hollow fiber cartridge 100 NMWC (GE Healthcare). The protein concentration and the antitryptic activity of the 100 NMWC concentrate were determined.

2.2 Analytical and enzymatic assay

Protein concentrations were determined with a bicinchoninic acid protein assay kit (BCA; Pierce Chemical). The trypsin inhibition assay was carried out according to the [1]. The antitryptic activity was monitored at 405 nm following the tryptic hydrolysis of N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA) in the presence of the inhibitor. The assay was performed in 300 µl held in 96-microwell plates. Inhibitory activity was expressed in terms of inhibitory unit (IU), where 1 IU was arbitrarily defined as the amount of protein sample required to inhibit 1 µg trypsin.

2.3 Gel electrophoresis

Protein samples were separated by 15% (v/v) SDS-PAGE and stained with silver nitrate.

2.4 Western blot

For immunoblot analysis, the protein samples were resolved by SDS/PAGE under reducing conditions. The gels were transferred electrophoretically to PVDF membranes in Towbin buffer [13] at 300 mA for 2 h. Immunodetection was performed by incubation at 25ºC for 4 h with rabbit polyclonal antibody against recombinant mouse SLPI (Calbiochem). The anti-rabbit IgG (whole molecule)-Peroxidase from goat (Sigma) was used as secondary antibody and 3,3'-Diaminobenzidine tetrahydrochloride as substrate.

2.5 Indirect enzyme-linked immunosorbent assay

Microplates 96 wells (Corning) were coated with protein 1 µg / well (50 µL) at 4°C overnight. After this time, microplates were washed with PBS-Tween 20 0,05% and then blocked with 10% fetal calf serum (Sigma) in BPS-T at 37°C for 2 h. After the blockage, microplates were incubated at 37ºC for 2 h with the rabbit polyclonal antibody against recombinant mouse SLPI (Calbiochem) in PBS-T (50 µl/well). The microplates were washed and then incubated with anti-rabbit IgG (whole molecule)-Peroxidase from goat (Sigma) diluted in PBS-T (50 µl/well) for one hour at 37ºC. The microplates were washed and incubated with O-phenylenediamine (OPD) in sodium acetate buffer containing 0.05% H_2O_2 for 15 minutes, then color development was stopped with 4.5 M H_2SO_4. The optical density was read at 492 nm using a microtiter plate spectrophotometer (Multiskam).

2.6 Trypsin-resin

The trypsin-affinity column was carried out using an XK 16 column (16 x 200 mm) (GE Healthcare) with 8 mL of trypsin-sepharose resin. Bovine trypsin (Sigma) was immobilized on the CNBR-activated Sepharose 4B (GE HealthCare) according to the resin supplier's instructions (GE HealthCare). The column was equilibrated with 50 mM Tris/HCl pH 8.0 (5 volumes). After the sample was loaded (6 mL/min), the column was washed with the 50 mM Tris/HCl pH 8.0 plus 0.3 M NaCl and then with Tris/HCl pH 8.0. The elution step (4 mL/min) was done with 10 mM HCl pH 2.0. The pH of the eluted fractions (8.0 mL) was adjusted with 1 M NaOH to 7.8.

2.7 Reverse phase chromatography

The last step of the protein purification was a reversed-phase chromatography performed in a HPLC system (Shimadzu LC-20AT series). Samples with trypsin inhibitory action and molecular mass bands around 14 kDa (230 μg of total protein) were applied to a C-18 column (Sephasil Peptide C_{18} 4.6 mm × 250mm, Amersham Pharmacia Biotech), and the protein elution was carried out by a linear gradient of trifluoroacetic acid (solvent A: 0.1% TFA in water) and acetonitrile (solvent B: 90% acetonitrile + 10% of solvent A). The gradient was established between 5% and 80% of solvent B in 50 min. The protein peaks were eluted with a flow rate 0.5 ml/min, and were manually collected according to the optical density in 214 nm.

2.8 Amino acid sequences

The N-terminal amino acid sequence of peak 19, which was the best recognized peak by the rabbit polyclonal antibody against recombinant mouse SLPI (Calbiochem), was determined by automated Edman degradation using a Shimadzu Protein Sequencer (PPSQ-21).

3 Results and Discussion

During the isolation of aprotinin from porcine lungs (ABY82797) we detected another porcine lung acid stable trypsin inhibitor with molecular mass around 14 kDa. This other inhibitor was also obtained from the trypsin active supernatant, which originates aprotinin [2]. In both case, the active sample was obtained after clarification and acid precipitation of the porcine lung extract. In this study, before the affinity chromatographic step the active supernatant was concentrated by tangential flow filtration. The eletrophoretic profile of the 100 NMWC concentrate sample showed two bands around 67 kDa and two bands below 20 kDa (Figure 1B, lane 2). The sample was then submitted to the affinity trypsin-column. Some affinity fractions: the fraction 5 and the pool of fractions 4 and 6 are the most active fractions (Figure 1A) and presented similar profiles of predominantly 3 bands below 20 and around 14 kDa (Figure 1B, lanes 4 and 3, respectively). The pool of affinity fractions 4 and 6 (Figure 1B, lane 3) was used to load the C-18 reverse phase chromatography column to finish the separation.

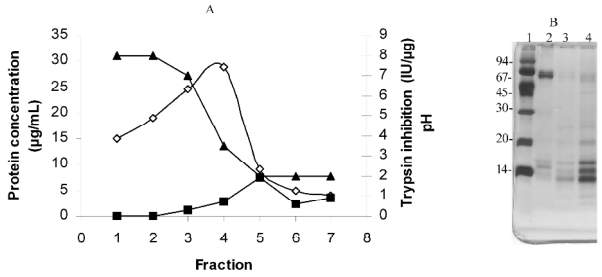

Figure 1 (A) – 10 mM HCl pH 2.0 elution fractions from the trypsin-sepharose loaded with 100 NMWC concentrate, showing the protein concentration, pH and trypsin inhibitory specific activity as determined by BAPNA assay. ▲ = pH, ◊ = protein concentration, ■ = trypsin inhibition. (B) SDS-PAGE (15%) of the samples from the trypsin-column eluted fractions. 5 μg of protein was loaded on each line. Lane 1: Molecular marker. Lane 2: 100 NMWC concentrate. Lane 3: Pool of fractions 4 and 6. Lane 4: fraction 5.

Twenty-four protein elution peaks were obtained and their trypsin inhibition ability was first analyzed (Figure 2A). In the used assay conditions (1 μg of total protein of each peak) the peaks 20 to 22 did not show trypsin inhibitory ability. The peaks with this ability were selected, submitted to SDS/PAGE (Figure 2B) and to ELISA using the polyclonal antibody against recombinant mouse SLPI. One band around 7 kDa (porcine aprotinin) was the unique band of peak 16 and presented the second best inhibitory activity (Figure 2B, lane 4). The peak

17 showed three very faint bands: one of 14, one of 7 and one below 7 kDa (Figure 2B, lane 5) and it is the most active peak, probably because of the inhibitors association including porcine aprotinin (Figure 2A). The peak 18 showed a single band around 14 kDa (Figure 2B, lane 6) and showed similar inhibitory activity as peak 19, which, presented a major band around 16 kDa (Figure 2B, lane 7). Besides peak 19 was the best-recognized peak by the polyclonal antibody against recombinant mouse SLPI (Figure 3). The ELISA results convinced us to sequence the N-terminal of the 16 kDa major band. The obtained sequence was MLNP (Figure 4A). After search in the NCBI bank data, we could not associate any protein with this N-terminal sequence, except to two N-terminal protein fragments. One of them was not active fragment and it was obtained during the elastase specific inhibitor (ESI) purification; currently known as elafin [14] (Figure 4 B). The other one was active and it was obtained during the investigation of SLPI fragmentation normally occurred in saliva [15]. Thus this particular sequence (MLNP) is either present in the elafin WAP domain or in the SLPI WAP 2 (Figure 4C) [8]. As we had performed an affinity chromatography using a trypsin-agarose column to raise the sample, which loaded the reverse phase column, we excluded elafin as this inhibitor because elafin does not inhibit trypsin [5]. And besides, it is present in lesser amounts than SLPI in the healthy lung [5]. Moreover, we have mentioned above that two similar domains form SLPI and the reactive site in the WAP 2 is located between Leu 72 and Met 73 [5, 12]. When SLPI is isolated by trypsin or chymotrypsin immobilized in an insoluble matrix, SLPI could be cleaved in the reactive site (Leu 72) and still remained active [16]. It again supports the obtained sequence and the preservation of the inhibitory activity of the reactive site by the presences of Met 73 and also Leu 74 [15, 16]. The molecular mass of our fragment must be around 3.0 kDa, but our SDS results present a band of molecular mass around 16 kDa (Figure 2B, lane 7). The binding of heparin to the basic amino acids (arginines and lysines) of the SLPI WAP 1 and 2 domains, [17] could respond by our data and it could also support the homology between the two SLPI domains [5, 12]. Heparin isolated and purified from animal tissue is 90% GAG heparin (free of core protein) with the average molecular mass of 14 kDa [17, 18].

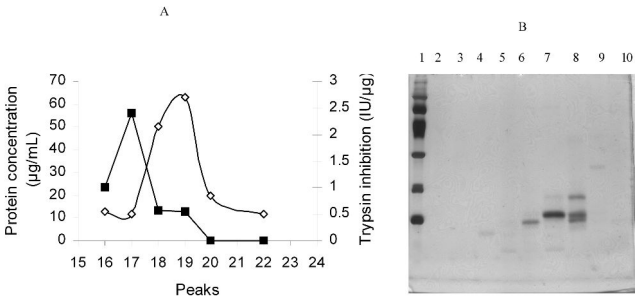

Figure 2 (A) Protein concentration and trypsin inhibitory specific activity as determined by BAPNA assay (◊ = protein concentration, ■ = trypsin inhibition) of 24 protein elution peaks from the reversed-phase chromatography column C-18 Sephasil Peptide performed in a HPLC system, which was loaded with the sample pool of fractions 4 and 6 from trypsin-sepharose. (B) SDS-PAGE (15%) of the same peaks (2µg protein / lane). Lane 1: Molecular marker. Lane 2: Flow-through. Lane 3: Peak 10. Lane 4: Peak 16. Lane 5: Peak 17. Lane 6: Peak 18. Lane 7: Peak 19. Lane 8: Peak 20. Lane 9: Peak 22. Lane 10: Peak 24.

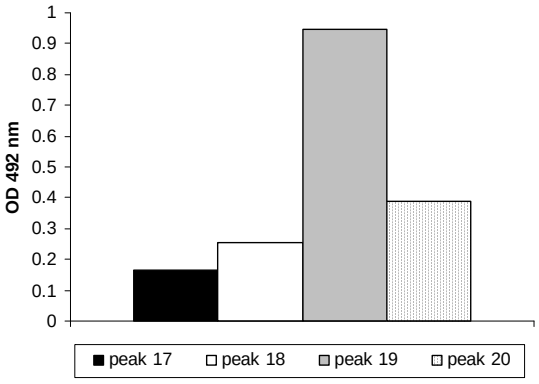

Figure 3 Indirect ELISA of the elution peaks from the reversed-phase chromatography column C-18 Sephasil Peptide performed in a HPLC system and loaded with the sample “pool of fractions 4 and 6 from trypsin-sepharose”. Microplates 96 wells (Corning) were coated with protein 1 µg / well (50 µL) at 4°C overnight. After the blockage, microplates were incubated

at 37 ºC for 2 h with the rabbit polyclonal antibody against recombinant mouse SLPI (Calbiochem) in PBS-T (50 µl/well) diluted 1:2000.

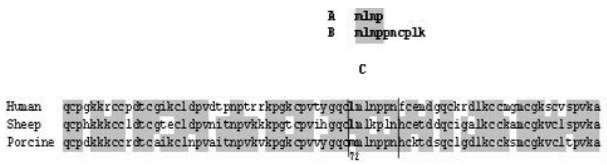

Figure 4 **A –** N-terminal sequence of reverse phase chromatography/HPLC peak 19 **B**- N-terminal sequence of the peak p1b from ESI described by Sallenave and Ryle, 1991 **C –** Comparison of the SLPI WAP 2 protein sequence among species (human, sheep, pig). The region delimited by bars represents the antielastase reactive site. Amino acid residues conserved between the species are shown on a grey background. The human sequence shows 61% homology with the sheep and 52% with the pig sequence. The pig sequence shows 62.5% homology with120

4 Conclusion

Therefore, we purify SLPI WAP 2 fragment with the N-terminal sequence MLNP which preserved the inhibitor reactive site (P1' Met 73 and P2' Leu 74) probably complexed with GAG heparin.

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