Original Article

Novel serine proteinase inhibitor with inhibitory activity for metalloproteinase

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Abstract

[**OBJECTIVES**] Vitelline envelope (VE), an extracellular coat of non-mammalian eggs, plays important roles in fertilization including protection of eggs from bacterial attack. One of the candidate molecules for protection is proteinase inhibitor within VE. We previously purified and sequenced proteinase inhibitor against thermolysin (a metalloproteinase) in dace (*Tribolodon hakonensis*) egg envelope (O. Hosomi et al. (2004). *J Exp Zool* 301A, 756–766). In order to elucidate the structure-function relationship of proteinase inhibitor in VE, we overexpressed the C-terminal region of proteinase inhibitor with consensus sequences for serine proteinase inhibitor (rPI) in *E. coli*, and analyzed the proteinase inhibitory activity for serine proteinases and metalloproteinase.

[**METHODS**] The rPI protein tagged with glutathione-S-transferase was expressed in *E. coli*, and purified by glutathione-Sepharose and gel filtration. Inhibitory activities of the rPI for thermolysin, trypsin and elastase activities were measured using specific substrates and a spectrophotometer.

[**RESULTS**] The rPI protein had proteinase inhibitory activities for trypsin, elastase and thermolysin as 73.0%, 61.0% and 58.5%, respectively. These results suggest that the C-terminal region of TribSPI has inhibitory activities for serine proteinase and metalloproteinase.

[**CONCLUSION**] We speculate that the inhibitory activity for the two serine proteinases is caused by the C-terminal region of TribSPI which contains two WAP domains, which are also found in an inhibitor for elastase. Investigation of the relationship of inhibitory activity for metalloproteinase and the 23 amino acid residues in C-terminal region with unknown functions is now in progress. This study shows that the rPI, which includes the domains for serine proteinase inhibitors, has inhibitory activity for thermolysin, a metalloproteinase. This novel bifunctional proteinase inhibitor may be a useful tool for the protection of organisms and foods from bacterial attack.

Key words: Serine Proteinase, Metalloproteinase, Vitelline Envelope, Proteinase Inhibitor, Bacterial Infection

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1. Introduction

Mammalian and non-mammalian eggs are surrounded by an extracellular envelope called zona pellucida[9)18)20)] and a vitelline envelope (VE)[7)14)], respectively. VE plays important roles in fertilization such as species-specific sperm entry, blocking of polyspermic fertilization and protection of embryo (fertilized egg) until hatching[11)]. To establish the fertilization, several steps are required. Sperm, guided by egg chemicals, attach to the egg membrane from a cavity of VE called micropyle. Sperm entry triggers ''cortical reaction'', causing the exocytosis of the cortical alveoli contents. After sperm-egg fusion, cortical reaction induces the structural change of VE such as sealing of mycropyle, hardening of envelope by transglutaminase (a Gln-ε-Lys crossbridging enzyme), and formation of fertilization envelope (FE) by cortical alveoli contents between VE and egg plasma membrane. FE, reaction product of cortical alveoli exudate after cortical reaction, blocks additional polyspermic fertilization. Thereafter, FE protects the embryo from physical damage and bacterial infection until hatching.

VE in fish is composed of major VE components (VEα, VEβ and VEγ)[3)] and other proteins[12)15)~17)]. Some of other proteins (phospholipase D (PLD), lysozyme, proteinases, DNases) exist in VE and FE, and may be effective for protection of unfertilized egg and embryo from bacterial infection in water environment[12)15)~17)]. In addition, VE contains proteinase inhibitory components for some bacterial proteinases in VE and FE[8)13)]. This suggests that envelopes could protect embryo from bacterial infection by inhibition of proteolysis. In dace (*Tribolodon hakonensis*), proteinase inhibitory component is for egg cortical alveolus sialoglycoproteins (a serine proteinase) and for thermolysin, a bacterial metalloproteinase from *Bacillus thermoproteolytics*[13)]. Hosomi et al.[8)] purified proteinase inhibitory protein for thermolysin from dace VE (TribSPI) by thermolysin-affinity column and deduced the protein sequence from the cDNA. The protein sequence of the TribSPI contains a Kazal-like motif, which is a consensus sequence for serine proteinase inhibitor but does not contain a consensus sequence for metalloproteinase inhibitor. Thus it is unclear whether this inhibitor has inhibitory activity for serine proteinases and which region has inhibitory activity for metalloproteinase. Our purpose was to elucidate functions as proteinase inhibitors on VE and to develop the application of safe medicines and food conservatives for protection of human and other animal bodies from pathogenic microbe attacks. In this study we analyzed the inhibitory activities of recombinant C-terminal peptide (rPI) for trypsin and elastase as serine proteinases, and for thermolysin as metalloproteinase.

2. Materials and Methods

2.1. Materials.

Superscript II reverse transcriptase and *Taq* polymerase were purchased from Invitrogen (CA, USA). Trypsin, elastase, thermolysin, benzoyl-L-arginine ethyl ester (BAEE) and *N*-succynyl-L-Ala-L-Ala-L-Ala-*p*-nitroanilide (Suc–Ala_3–pNA) were purchased from Sigma-Aldrich, Co. Ltd (MO, USA). Carboxybenzoyl–Gly–L–Phe–amide (Cbz–Gly–Phe–NH_2) was purchased from Bachem Bioscience Inc (PA, USA). All other reagents were obtained from commercial sources of the highest possible quality.

2.2 Expression of Recombinant rPI

The expression procedure of the recombinant *Tribolodon hakonensis* serine proteinase inhibitor (rPI) was performed according to standard method[21)] with some modification. A part of the cDNA sequence (Arg125–Tyr287) encoding the TribSPI protein[8)] was cloned into an expression vector, pGEX–4T–1 (GE Healthcare bioscience), and expressed in *E. coli*. The vector was prepared by digestion with *Bam*H I and *Eco*R I restriction enzymes. After extraction of the mRNA from the fish ovarian tissue[2)], the cDNA was synthesized and amplified by reverse transcription-polymerase chain reaction (RT-PCR) with Superscript II reverse transcriptase and *Taq* polymerase. Nucleotide sequences that set of two primers with *Bam*H I and *Eco*R I restriction sites were as follows: sense primer 5′–CGG GAT CCA

GGT GTA TCA TGA AGG ATA AAG AG-3′ containing the ATG starting codon and antisense primer 5′-GAA TTC TCA GTA GGA CAC ATA GTC TGG AGC-3′ corresponding to the AGG (Arg125)～GAG (Glu132) and GCT (Ala281)～TGA of the nucleotide sequences, respectively. After digestion of pGEX-4T-1 expression vector with *Bam*H I and *Eco*R I, amplified cDNA was ligated with the vector. Constructed plasmid was designated for expression of the rPI tagged with glutathione-S-transferase (GST). All constructs were confirmed by sequencing, and some clones having plasmid ligated with the rPI cDNA fragment were selected. Furthermore, the one colony (No. Ex1-17) of them was incubated in 1 liter of Luria-Bertani (LB) culture media at 37℃ for 48 h. After incubation of *E. coli* with 0.5 mM isopropylthio-β-galactoside (IPTG, Sigma), the bacteria were collected by centrifugation at 6,000 rpm for 10 min, and the pellet was repeatedly washed with phosphate-buffered saline (PBS, 137 mM NaCl, 12 mM Phosphate, 2.7 mM KCl, pH 7.0). The sample was suspended in about 30 mL of the same buffer and sonicated 1 s with 1 s intervals for 30 min. The sample was centrifuged at 10,000 rpm for 30 min and the supernatant was obtained.

2.3 Purification of the rPI

The supernatant was dialyzed against 20 mM PBS (137 mM NaCl, 20 mM Phosphate, 2.7 mM KCl, pH 7.0) (buffer A) for 24 h at 4℃. The supernatant was applied on a glutathione-Sepharose 4B column (1.0×2.0 cm, GE Healthcare bioscience) equilibrated with buffer A. A column was thoroughly washed with buffer A until the absorbance at 280 nm was reduced to less than 0.01, and the materials retained in the column were eluted with 50 mM PBS (137 mM NaCl, 50 mM Phosphate, 2.7 mM KCl, pH 8.0) containing 10 mM glutathione (buffer B). After mixing the eluted fractions with the same volume of 10 mM ethylenediamine tetraacetate (EDTA), the solutions were concentrated to about 1 mL using a Diaflo ultrafiltration cell with a PM-10 membrane (Amicon). Furthermore, the sample solution was applied on Sephadex G-75 (1.0×50 cm, GE Healthcare bioscience) equilibrated with 20 mM Tris-HCl (pH 7.2) (buffer C) for purification of the rPI.

2.4 Measurement of Inhibitory Activities for Several Proteinases

Inhibitory activities of the purified rPI (0.2 nmol each) were measured by using three proteinases with their substrates. Inhibitory activities were measured by duplicate analysis.

2.4.1 Inhibitory Activity for Trypsin

Inhibitory activity for trypsin (2.0 U) was analyzed in 3.2 mL of substrate solution (0.23 mM BAEE/ 63 mM sodium phosphate buffer, pH 7.6) with/without 0.2 nmol of the rPI at 25℃ in a cuvette. Absorbance of the reaction mixture at 253 nm was measured for approximately 5 min[26].

2.4.2 Inhibitory Activity for Elastase

Inhibitory activity for elastase (1.4 U) was analyzed in 3 mL of the substrate solution (0.3 mM Suc-Ala_3-pNA /100 mM Tris-HCl, pH 8.0) with/without 0.2 nmol of the rPI at 25℃ in a cuvette. Absorbance of the reaction mixture at 410 nm was measured for approximately 5 min[1].

2.4.3 Inhibitory Activity for Thermolysin

Inhibitory activity for thermolysin (1.4 pU) was analyzed in 0.5 mL of the substrate solution (1 μM Cbz-Gly-Phe-NH_2/10 mM $CaCl_2$/10 mM Tris-HCl, pH 8.0) with/without 0.2 nmol of the rPI at 40 ℃ for 10 min in a tube. After 1 min, 0.5 mL of 0.1 M CH_3COOH was added to the reaction mixture for stopping the reaction. Next 1 mL of ninhydrin solution, reagent for detection of amino acids, was added and the mixture was placed in boiling water bath for 15 min. The tube was cooled on ice and the solution was diluted with 2 mL of 50% ethanol solution. The absorbance of the reaction product of ninhydrin and degraded substrate at 570 nm was measured[19].

3. Results

3.1 Expression and Purification of the rPI

Since they remain unclear whether this inhibitor has inhibitory activity for serine proteinases and which region has inhibitory activity for metalloproteinase, recombinant proteinase inhibitory protein with domain III～V in the C-terminal region (rPI) (Fig. 1) was expressed in a large quantity in *E. coli*, and purified for functional analysis.

The rPI protein tagged with GST was expressed in *E. coli* and the protein was applied to glutathione-sepharose (Fig. 2a). The eluted fractions (No. 5–11 in Fig. 2a) with a broad peak at 280 nm were applied on a gel-filtration column (Fig. 2b). Two main (fr. Nos. 9–13, T1, and 26–28, T2 in Fig. 2b) and a minor (fr. Nos. 19–24, T3 in Fig. 2b) peaks at 280 nm were obtained but they had no inhibitory activities against trypsin, elastase and thermolysin. These fractions (T1～T3) had proteinase activities (data not shown) but not inhibitory activities. These proteinase activities are probably derived from *E. coli* and eluted with the rPI by interaction between proteinases and the inhibitor. The rPI activities were detected in the fractions 15–17 (T4). Thus the rPI protein was subjected to Sephadex G–75 gel-filtration column successfully to remove proteinases (data not shown).

3.2 Measurement of Inhibitory Activity

Inhibitory activity of the purified rPI (0.2 nmol each) for the three proteinases was measured by using the three proteinases with their substrates. As shown in Table 1, the rPI protein showed proteinase

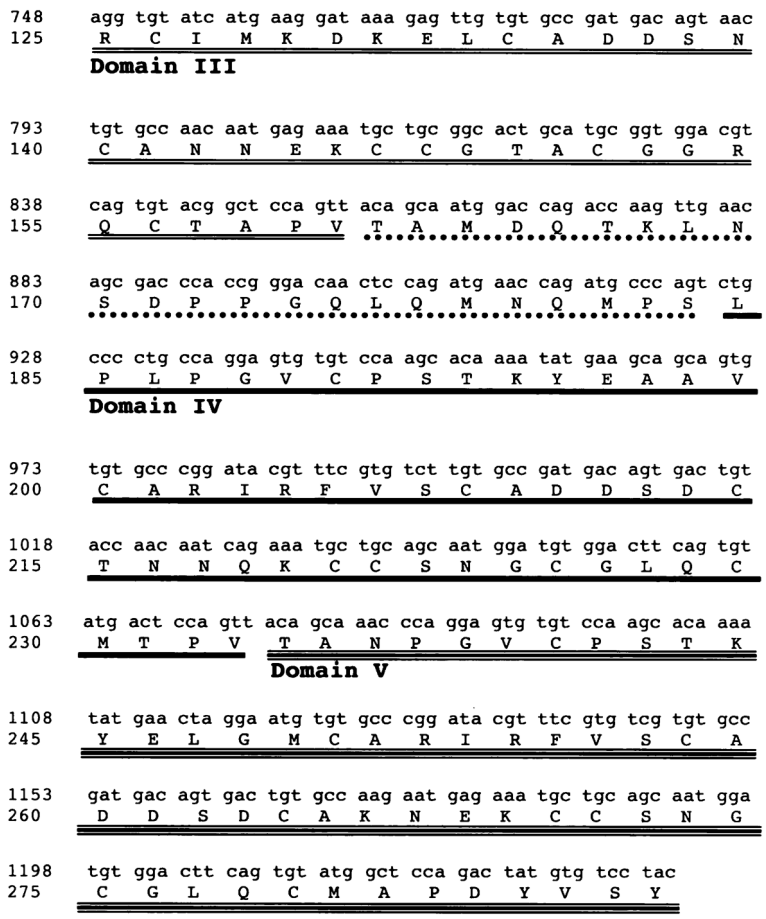

Fig. 1 Nucleotide sequences and deduced amino acid sequences of the rPI. Underlines indicate domain III, IV and V. Function of a unique peptide (dotted line) is unknown.

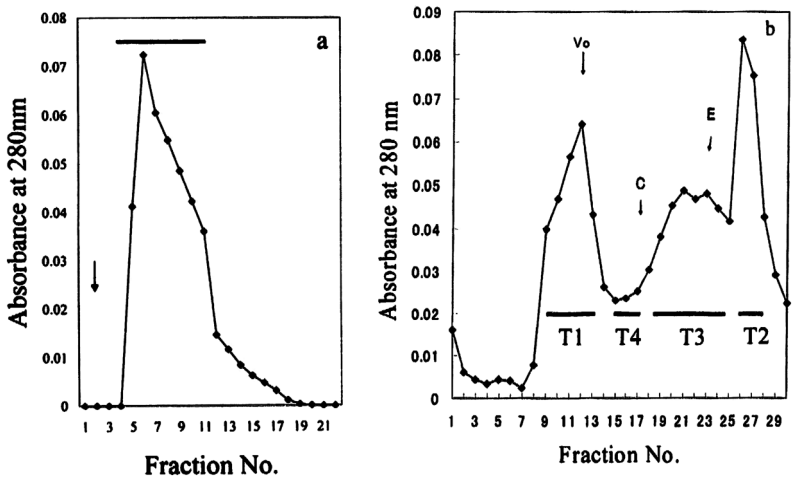

Fig. 2 Purification of the rPI. a. Affinity chromatography for isolation of the rPI protein by a glutathione-Sepharose column. The materials bound to the column were eluted with 10 mM glutathione at the arrow. Each fraction (1.0 mL) was measured for the absorbance at 280 nm and the fractions indicated by the bar were collected. b. Purification of the rPI protein by Sephadex G–75 gel filtration. Fraction T1, T2 and T3 were concentrated and analyzed for the proteinase activities and proteinase inhibitory activities. V_0 indicates void volume. Elution position of standard proteins (Bio-Rad) indicates C (chicken ovalbumin, MW = 44 kDa) and E (equine myoglobin, MW = 17 kDa).

Table 1 Inhibitory activity of the rPI protein to trypsin, elastase and thermolysin.

Proteinase	Substrate	Inhibition rate (%)
Trypsin	BAEE	73.0
Elastase	Suc–Ala_3–pNA	61.0
Thermolysin	Cbz–Gly–Phe–NH_2	58.5

The rPI protein (0.2 nmol) was used for each inhibition experiment.

inhibitory activities against trypsin, elastase and thermolysin were 73.0%, 61.0% and 58.5%, respectively.

4. Discussion

This study showed that C-terminal peptide of the TribSPI (rPI) has inhibitory activities for trypsin, elastase and thermolysin. We have previously showed that the TribSPI serine proteinase inhibitor protein consisted of five Kazal-like motifs and each of them had eight cysteine residues[8]. Furthermore in this experiment, we demonstrated that the rPI protein, a recombinant C-terminal peptide of the TribSPI constructed with 163 amino acids, had three Kazal-like motifs and one unique (T161～S183) sequence. The Kazal consensus sequence (CxxxxxPxCGTD) has been known as a sequence containing potential trypsin binding sites of the secretory proteinase inhibitor from bovine pancreas and human leukocyte[6)24)]. As shown in Fig. 1, three Kazal-like sequences which contained CxxxxxxxCxxD sequence were found in every domain of the rPI protein. Moreover, two R–I sequences were detected in two CxxxxxxxCxxD sequences (domain IV and V) at the corresponding positions of the trypsin-binding site (Arg-Ile, R–I) in the Kazal consensus sequence[6)]. Thus we supposed that these two R-I sequences in the rPI protein were trypsin-binding sites.

In the present work, we have shown that the rPI has an inhibitory activity for elastase and another serine proteinase. Elastase-binding sites, L–M and M–L (Leu–Met and Met–Leu), have been reported in human and porcine antileukoproteinases, respectively[4)23)]. The binding sites for elastase in domain II

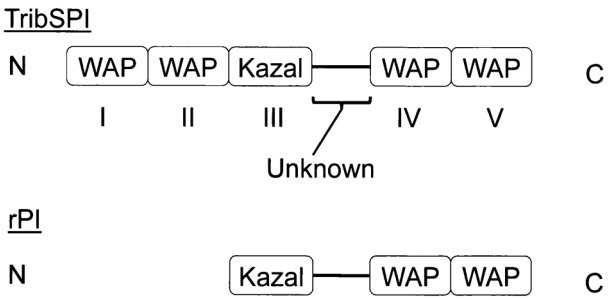

Fig. 3 Illustration of domain structure for the TribSPI. "Kazal" and "WAP" indicate Kazal-like motif and WAP domain, respectively. I～V indicate domains in the TribSPI that described previously (Hosomi et al. 2004) (13). "Unknown" indicates the region between domains III and IV with unknown functions. In the present study, we analyzed the rPI protein, C-terminal peptide of TribSPI tagged with GST.

and III did not exist in Kazal-like motif. Database search for the TribSPI showed that domains I, II, IV and V, including Kazal-like sequence, resemble WAP domain, whey acidic protein-type four-disulfide core domains[22)27)] (Fig. 1, 3). This domain is a member of the family that includes whey acidic protein, elafin (elastase-specific inhibitor)[22)25)27)28)], caltrin-like protein (a calcium transport inhibitor)[5)] and other extracellular proteinase inhibitors[22)]. We supposed that elastase inhibitory activity of the rPI is derived from WAP domains.

The rPI protein showed the thermolysin inhibitory activity as shown in Table 1 but the binding site (s) could not be determined in this study. T161～S183 of the rPI protein has unknown functions. This region has a unique peptide sequence consisting of about 23 amino acids between domain III and IV (Fig. 3). In order to determine the precise binding site in the rPI protein, another recombinant protein derived from the TribSPI which has only one unique sequence or domain(s) without the unique sequence must be studied.

A function of VE is protection of eggs from bacterial attack. Possible molecules for protection of eggs are phospholipase D, lysozyme, proteinases and DNases. These are minor components of the VE. Proteinase inhibitor is also a possible molecule for protection from bacterial infection. Kudo et al. showed that VE extracts from dace eggs have inhibitory activities for proteinases from plants and bacteria[13)]. Iwamori et al.[10)] showed that WAP domain has bacteriostatic activity. We speculate that TribSPI could be an antibacterial proteinase inhibitor. Proteinase inhibitors are thought to protect organisms from bacterial attack. The study of antibacterial activities of the rPI protein is now in progress.

5. Conclusion

Novel serine proteinase inhibitor found in vitelline envelope of Japanese dace egg (TribSPI) has dual inhibitory effect on serine proteinase and also metalloproteinase. Recombinant C-terminal region of the inhibitor, which contains domains of serine proteinase inhibitor, inhibited activities of trypsin and elastase. The same C-terminal region of the inhibitor also has inhibitory effect on thermolysin, while the region does not include consensus sequence of metalloproteinase inhibitor. This novel bifunctional proteinase inhibitor may be a useful tool for protection of organisms and foods from bacterial attack.

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7. References

1) Bieth, J., Spiess, B. & Wermuth, C. G. (1974). The synthesis and analytical use of a highly sensitive and convenient sub-

strate of elastase. Biochem Med 11, 350–357.
2) Chomczyski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162, 156–159.
3) Darie, C. C., Biniossek, M. L., Jovine, L., Litscher, E. S. & Wassarman, P. M. (2004) Structural characterization of fish egg vitelline envelope proteins by mass spectrometry. Biochemistry 43, 7459–7478.
4) Farmer, S. J. Fliss, A. E. & Simmen, R. C. M. (1990). Complementary DNA cloning and regulation of expression of the messenger RNA encoding a pregnancy-associated porcine uterine protein related to human antileukoproteinase. Mol Endocrinol 4, 1095–1104.
5) Furutani, Y., Kato, A., Kawai, R., Fibriani, A., Kojima, S., & Hirose, S. (2004). Androgen-dependent expression, gene structure, and molecular evolution of guinea pig caltrin II, a WAP-motif protein. Biol Reprod 71, 1583–1590.
6) Greene, L. J. & Bartelt, D. C. (1969). The structure of the bovine pancreatic secretory trypsin inhibitor-Kazal's inhibitor. II. The order of the tryptic peptides. J Biol Chem 244, 2646–2657.
7) Hedrick, J. L. (1996). Comparative structural and antigenic properties of zona pellucida glycoproteins. J Reprod Fertil Suppl 50, 9–17.
8) Hosomi, O., Ohe, Y., Takeya, A., Hosaka, K., Okubo, T., Iyobe, S. & Kudo, S. (2004) Nucleotide and protein sequences of a proteinase inhibitor from the vitelline envelope of dace (*Tribolodon hakonensis*) eggs. J Exp Zool 301A, 756–766.
9) Ikeda, K., Yonezawa, N., Naoi, K., Katsumata, T., Hamano, S. & Nakano, M. (2002). Localization of N-linked carbohydrate chains in glycoprotein ZPA of the bovine egg zona pellucida. Eur J Biochem 269, 4257–4266.
10) Iwamori, T., Nukumi, N., Itoh, K., Kano, K., Naito, K., Kurohmaru, M., et al. (2010). Bacteriostatic activity of whey acidic protein (WAP).
J Vet Med Sci 72, 621–625.
11) Kudo, S. (1991). Fertilization, cortical reaction, polyspermy-preventing and anti-microbial mechanisms in fish eggs. Bull Inst, Academia Sinica, Monograph 16, 313–340.
12) Kudo, S. (1992). Enzymatic basis for protection of fish embryos by fertilization envelope. Experimentia 48, 277–281.
13) Kudo, S. (1998). Presence of a proteinase inhibitor in the vitelline envelope and its role in eggs of the fish *Tribolodon hakonensis*. J Exp Zool 281, 626–635.
14) Kudo, S., Hosomi, O. & Teshima C. (2000) Polymerization of a vitelline envelope (VE)–like structure produced by using fractionated VE extracts from carp eggs. J Exp Zool 286, 755–766.
15) Kudo, S. & Inoue, M. (1986). A bactericidal effect of fertilization envelope extracts from fish eggs. Zool Sci 3, 323–329.
16) Kudo, S. & Inoue, M. (1991). Enzyme activities responsible for the defense mechanism of the fertilization envelope. Naturwissenchaften 78, 172–173.
17) Kudo, S. & Teshima, C. (1991). Enzyme activities and antifungal action of fertilization envelope extracts from fish eggs. J Exp Zool 259, 392–398.
18) Litscher, E. S., Williams, Z. & Wassarman, P. M. (2009). Zona pellucida glycoprotein ZP3 and fertilization in mammals. Mol Reprod Dev 76, 933–941
19) Matsubara, H. (1970). Purification and assay of thermolysin. Methods Enzymol 19, 642–651.
20) Nakano, M., & Yonezawa, N. (2001). Localization of sperm ligand carbohydrate chains in pig zona pellucida glycoproteins. Cells Tissues Organs 168, 65–75.
21) Sambrook, J., Fritsh, E. F. & Maniatis, T. (1989) Molecular cloning: A laboratory manual. 2nd Ed., Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory Press.
22) Schalkwijk, J., Wiedow, O., & Hirose, S. (1999). The trappin gene family: Proteins defined by an N-terminal transglutaminase substrate domain and a C-terminal four-disulphide core. Biochem J 340, 569–577.
23) Stetler, G., Brewer, M. T. & Thompson, R. C. (1986). Isolation and sequence of a human gene encoding a potent inhibitor of leukocyte proteases. Nucleic Acids Res 14, 7883–7896.
24) Thompson, R. & Ohlsson, K. (1986). Isolation, properties, and complete amino acid sequence of human secretory leukocyte protease inhibitor, a potent inhibitor of leukocyte elastase. Proc Natl Acad Sci USA 83,6692–6696.
25) Trexler, M., Banyai, L. & Patthy, L. (2001). A human protein containing multiple types of protease-inhibitory modules. Proc Natl Acad Sci USA 98, 3705–3709.
26) Walsh, K. A. & Wilcox, P. E. (1970). Serine protease. Methods Enzymol 19, 31–41.
27) Wiedow, O., Schroder, J. M., Gregory, H., Young, J. A. & Christophers, E. (1990). Elafin: an elastase-specific inhibitor of human skin. J Biol Chem 265, 14791–14795.
28) Zeeuwen, P. L., Hendriks, W., de Jong, W. W. & Schalkwijk, J. (1997). Identification and sequence analysis of two new members of the SKALP/elafin and SPAI–2 gene family. Biochemical properties of the transglutaminase substrate motif and suggestions for a new nomenclature. J Biol Chem, 272, 20471–20478.

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