A four-domain Kunitz-type proteinase inhibitor from Solen grandis is implicated in immune response

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A B S T R A C T

Serine proteinase inhibitor (SPI) serves as a negative regulator in immune signal pathway by restraining the activities of serine proteinase (SP) and plays an essential role in the innate immunity. In the present study, a Kunitz-type SPI was identified from the mollusk razor clam Solen grandis (designated as SgKunitz). The full-length cDNA of SgKunitz was of 1284 bp, containing an open reading frame (ORF) of 768 bp. The ORF encoded four Kunitz domains, and their amino acids were well conserved when compared with those in other Kunitz-type SPIs, especially the six cysteines involved in forming of three disulfide bridges in each domain. In addition, the tertiary structure of all the four domains adopted a typical model of Kunitz-type SPI family, indicating SgKunitz was a new member of Kunitz-type SPI superfamily. The mRNA transcripts of SgKunitz were detected in all tested tissues of razor clam, including muscle, mantle, gonad, gill, hepatopancreas and hemocytes, and with the highest expression level in gill. When the razor clams were stimulated by LPS, PGN or β-1, 3-glucan, the expression level of SgKunitz mRNA in hemocytes was significantly up-regulated (P < 0.01), suggesting SgKunitz might involved in the processes of inhibiting the activity of SPs during the immune responses triggered by various pathogens. Furthermore, the recombinant protein of SgKunitz could effectively inhibit the activities of SP trypsin and chymotrypsin in vitro. The present results suggested SgKunitz could serve as an inhibitor of SP involving in the immune response of S. grandis, and provided helpful evidences to understand the regulation mechanism of immune signal pathway in mollusk.

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1. Introduction

Serine proteinase inhibitors (SPIs) have been widely found in multicellular organisms, and play various and crucial roles in numerous biological processes, such as blood coagulation, inflammation, development, and activation of prophenoloxidase and complement system [1–6]. As suggested by their name, SPIs participate in these processes by limiting the excessive proteolytic activity of serine proteinases (SPs). Such precise regulation is quite important, for once the balance between SPs and SPIs is broken, the enzymes activity out of control might be fatal to the cells or organisms [7]. Based on the primary sequence, tertiary structure and mechanisms of binding motifs, SPIs have been classified into several families [8], and among of them, family of kazal, kunitz, serpin and α-macroglobulin have been well characterized [9,10].

Kunitz-type SPIs are present in a variety of animals, including sea anemone, snails, ectoparasites, snake venom, horse crab, fly, tick and bovine [11–15]. This family of SPIs is characterized by one or more Kunitz domain in their molecules. Each Kunitz domain consists of approximately 60 amino acids forming a compact α+β structure, stabilizing by three conserved disulfide bridges [16]. Kunitz-type proteinase inhibitors exhibit inhibitory activity against the common SPs, such as trypsin, chymotrypsin or thrombin [17]. The inhibitory activity toward SPs is closely associated with the P1 amino acid of the reactive site and amino acid sequence surrounding the region that interacts with the proteases [8]. For example, inhibitors with a lysine or arginine at their reactive sites can inhibit trypsin or trypsin-like enzymes, while inhibitors with tyrosine, phenylalanine, leucine or methionine at the relevant position tend to inhibit chymotrypsin and chymotrypsin-like enzymes [8].
Several families of SPIs including Kunitz-type proteinase inhibitors are believed to involve in the immune signal pathway by modulating the proteolytic activities of SPs, owing to their prominent inhibitory abilities [9]. For example, Serpin-4 and Serpin-5 from Manduca sexta function to regulate the proPO-activation pathway by inhibiting three hemolymph proteinases upstream of the pathway [6], while SPs in Drosophila hemolymph are believed to regulate the activity of the cytokine spatzle, which is associated with the activation of Toll signal pathway and the production of antimicrobial peptides “Drosomycin” against Gram-positive bacteria and fungi [9,18].

In addition, some SPIs also serve as an inhibitor to the microbial proteinase and protect the animal from infection by pathogens [19]. In recent years, increasing SPIs have been identified from marine invertebrates, and most of them are proved to involve in the immune response. For instance, the mRNA expression of many kazal-type and serpin-type SPIs from crab, shrimp or scallop are significantly up-regulated by infection of virus or bacteria [7,20–23], suggesting their crucial role in the immune defense against invaders. However, the knowledge about the functions molluscan Kunitz-type SPIs performed in innate immunity is still limited. In present study, a full-length cDNA of Kunitz-type SPIs was identified from mollusk S. grandis, and the main objectives were (1) to investigate its tissue distribution and the temporal expression after stimulation of pathogen-associated molecular patterns (PAMPs), (2) to examine the inhibitory activity of the recombinant protein toward trypsin and chymotrypsin, and (3) to better understand the role Kunitz-type SPIs played in the immune system of S. grandis.

Table 1

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′–3′)</th>
<th>Primers used in the present study.</th>
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</thead>
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<tr>
<td>Oligo(dT)-adapter</td>
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</tr>
<tr>
<td>M13-F (forward)</td>
<td>GTAAAGACAGCCCGAC</td>
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</tr>
<tr>
<td>M13-R (reverse)</td>
<td>CAGGAACACCTTATGACC</td>
<td></td>
</tr>
<tr>
<td>SgKunitz RTF (forward)</td>
<td>CGAAAGGGGGTTTCCCTGAGT</td>
<td></td>
</tr>
<tr>
<td>SgKunitz RTR (reverse)</td>
<td>CTCTTTAGATATCAAGGGTGG</td>
<td></td>
</tr>
<tr>
<td>β-actin AF (forward)</td>
<td>TGTACCCGAACATCTGCTTC</td>
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</tr>
<tr>
<td>β-actin AR (reverse)</td>
<td>CATCGTATCTGTTCTGATC</td>
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</tr>
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<td>CAGTCTTGTACGTGTAAGATGA</td>
<td></td>
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<tr>
<td>SgKunitz RER (reverse)</td>
<td>TGCGCTCTTACATTCTTTCACAA</td>
<td></td>
</tr>
<tr>
<td>T7 Primer</td>
<td>TAAATACGACTCACTATAGGG</td>
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</tbody>
</table>

2. Materials and methods

2.1. Razor clam

Natural captured healthy razor clams S. grandis with average shell length of 85 mm, from Yantai, Shandong Province, China, were collected and maintained in the aerated seawater at 20–22 °C for a week before processing.

2.2. EST analysis and cloning of the full-length SgKunitz cDNA

A cDNA library was constructed with the whole body of a razor clam, using the SMART cDNA Library Construction Kit (Clontech). Random sequencing of the library using M13 primer yielded 2038 successful sequencing reactions. BLAST analysis of the EST sequences revealed that an EST of 788 bp was highly similar to the corresponding clone was selected and completely sequenced using primer M13-F and M13-R (Table 1).

2.3. Sequence analysis

The cDNA sequence and deduced amino acid sequence of SgKunitz was analyzed using the BLAST algorithm (http://www.ncbi.nlm.nih.gov/blast) and the Expert Protein Analysis System (http://www.expasy.org/). The protein domains were predicted with the simple modular architecture research tool (SMART) version 4.0 (http://smart.embl-heidelberg.de/). Homology analysis was conducted using the Ident and Sim Analysis provided at http://www.bioinformatics.org/sms/. Multiple sequence alignment of SgKunitz was performed with the ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw/). The presumed tertiary structures of SgKunitz were established using the SWISS-MODEL prediction algorithm (http://swissmodel.expasy.org/) [24,25] and displayed by PyMOL version 0.97. A phylogenetic tree was constructed based on the deduced amino acid sequences of SgKunitz and other Kunitz-type SPIs from invertebrates and vertebrates by the neighbor-joining (N) algorithm using the MEGA4.1 software. To derive confidence value for the phylogeny analysis, bootstrap trials were replicated 1000 times.

2.4. Real-time PCR analysis of SgKunitz mRNA expression in different tissues

The total RNA from hemocytes, gill, gonad, muscle, hepatopancreas and mantle were extracted from five adult razor clams as parallel samples using TRIzol reagent (Invitrogen). The first-strand synthesis was carried out based on Promega M-MLV RT Usage information using the DNase I (Promega)-treated total RNA as template and oligo(dT)-adapter as primer (Table 1). The reaction were performed at 42 °C for 1 h, and terminated by heating at 95 °C for 5 min. The cDNA mix was diluted to 1:100 and stored at −80 °C for subsequent SYBR Green fluorescent quantitative real-time PCR (RT-PCR).

Two gene-specific primers for SgKunitz, RTF and RTR (Table 1), were used to amplify a product of 106 bp from cDNA, and the PCR product was sequenced to verify the specificity of RT-PCR. And β-actin gene was selected to normalize relative gene expression, for its convincible and statistically repeatable results. Two β-actin primers, AF and AR (Table 1), were used to amplify a 213 bp fragment as an internal control to verify the successful transcription and calibrate the cDNA template for correspond samples.

Real-time PCR amplification was carried out in an ABI 7300 Realtime Thermal Cycler according to the manual (Applied Biosystems). Dissociation curve analysis of amplification products was performed at the end of each PCR to confirm that only one PCR product was amplified and detected. After the PCR program, data were analyzed using ABI 7300 SDS software V2.0 (Applied Biosystems). To maintain consistency, the baseline was set automatically by the software. The 2−ΔΔCT method was used to analyze the expression level of SgKunitz [26]. All data were given in terms of relative mRNA expressed as mean ± SE (N = 4).

2.5. Expression analysis of SgKunitz after PAMPs stimulation

Two hundred razor clams were employed for the PAMPs stimulation experiment. The razor clams were randomly divided into 5 groups and each group contained 40 individuals. Four groups received an injection of 50 μL phosphate buffered saline (PBS, 0.14 M sodium chloride, 3 mM potassium chloride, 8 mM disodium hydrogenphosphate dodecahydrate, 1.5 mM potassium phosphate monobasic, pH 7.4), LPS from Escherichia coli 0111:B4 (Sigma–Aldrich, 0.5 mg ml−1 in PBS), PGN from Staphylococcus aureus (Sigma–Aldrich, 0.8 mg ml−1 in PBS), glucan from Saccharomyces
The concentration of purified protein was detected by reducing 15% SDS-polyacrylamide gel electrophoresis containing 1 m urea. The purified protein was refolded in gradient urea-TBS glycerol buffer (50 mmol L\(^{-1}\) imidazole under denatured condition (8 mol L\(^{-1}\) urea). The purified protein was incubated in LB medium (containing 50 mg ml\(^{-1}\) ampicillin) at 37 °C with shaking at 220 rpm. The culture medium reached OD\(_{600}\) of 0.5–0.7, the cells were incubated for 4 additional hours with the induction of IPTG at the final concentration of 1 mmol L\(^{-1}\). The recombinant protein SgKunitz was purified by a Ni\(^{2+}\) chelating Sepharose column, pooled by elution with 400 mmol L\(^{-1}\) imidazole under denatured condition (8 mol L\(^{-1}\) urea). The purified protein was refolded in gradient urea-TBS glycerol buffer (50 mmol L\(^{-1}\) Tris–HCl, 50 mmol L\(^{-1}\) NaCl, 10% glycerol, 2 mmol L\(^{-1}\) reduced glutathione, 0.2 mmol L\(^{-1}\) oxidized glutathione, a gradient urea concentration of 6, 5, 4, 3, 2, 1, 0 mol L\(^{-1}\) urea in each gradient, pH 8.0; each gradient at 4 °C for 12 h). Then the resultant proteins were separated by reducing 15% SDS-polyacrylamide gel electrophoresis (SDS–PAGE), and visualized with Coomassie bright blue R250. The concentration of purified rSgKunitz was quantified by BCA method [28].

2.7. Protease inhibition assay

The protease inhibition assay was performed according to the previous method [29]. In brief, 10 μl (50 mM Tris buffer, pH 8.0) containing 1 μg of rSgKunitz was incubated with 10 μl containing 1 μg of trypsin (Sigma) or chymotrypsin (Sigma) at room temperature for 10 min in a flat bottomed microtiter plate. Then 180 μl of an appropriate chromogenic substrate solution in 50 mM Tris buffer (pH 8.0) was added, and the resultant proteolytic activity was measured at A\(_{405}\) after 1 min of incubation. For positive controls, rSgKunitz was substituted with the same quantity of aprotinin (Sigma) from the bovine lung, which was a well-known inhibitor against trypsin and chymotrypsin. Negative control data were obtained without inhibitor. 100 μM of N-Benzoyl-Phe-Val-Arg-p-nitroanilide hydrochloride (Sigma) and N-Succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (Sigma) were used for the substrates of trypsin and chymotrypsin, respectively. Each data from a single experiment was performed in triplicate for statistical analysis.

To determine the dose-dependent inhibition activity of SgKunitz, 10 μl buffer containing 0.25, 0.5, 1.0, 2.0 or 4.0 μg of rSgKunitz was incubated with protease, respectively. And protease inhibition assay was performed as described above.

3. Results

3.1. Characteristic of the SgKunitz full-length cDNA

One EST from S. grandis cDNA library was found homologous to the previously identified Kunitz genes. The clone corresponding to the EST was re-sequenced, and yielded a cDNA sequence of 1284 bp. The cDNA sequence of SgKunitz was deposited in GenBank under accession number JQ730814. The complete nucleotide sequence of SgKunitz cDNA consisted of a 5′ terminal untranslated region (UTR) of 52 bp, a long 3′ UTR of 464 bp with a poly (A) tail, and an open reading frame (ORF) of 768 bp (Fig. 1). The ORF encoded a polypeptide of 255 amino acids with a theoretical isoelectric point of 8.13 and predicted molecular weight of 28.6 kDa. SMART analysis showed that the amino acids 1 (M) ~ 17 (A) encoded a signal peptide, and amino acids 26 (R) ~ 79 (G), 84 (P) ~ 137 (G), 142 (P) ~ 195 (K) and 200 (P) ~ 253 (K) encoded a Kunitz domain, respectively (Fig. 1).

3.2. Homologous, structure and phylogenetic character of SgKunitz

BLAST analysis revealed that SgKunitz shared significant sequence similarities with other Kunitz-type proteinase inhibitors, such as 46% identity with Sciaenops ocellatus tissue factor pathway inhibitor 2 (ADM64310) and 46% with Sabellastarte magnaica carboxypeptidase inhibitor (CAK55547). Based on the multiple sequences alignment between SgKunitz and other five Kunitz-type proteinase inhibitors with three or four Kunitz domains, it was found that the six cysteine residues involved in the formation of the three internal disulfide bridges were well conserved in each domain (Fig. 2), while the putative reactive site P1 were diversity in each Kunitz-type SPIs except for P1 residues in the second Kunitz domain (Fig. 2).

The potential tertiary structures of four Kunitz domains in SgKunitz were established using the SWISS-MODEL prediction algorithm, respectively (Fig. 3). The tertiary structures were quite similar to each other: all of them were consisted of two \(\alpha\)-helices (\(\alpha_{1} \rightarrow \alpha_{2}\)) and two \(\beta\)-strands (\(\beta_{1} \rightarrow \beta_{2}\)), and the six cysteines which formed three disulfide bridges were well conserved among the four domains.

A phylogenetic tree was constructed using neighbor joining method with 1000 bootstrap test based on the multiple alignments of SgKunitz and Kunitz-type SPIs from other animal, including invertebrate Kunitz-type SPIs and vertebrate tissue factor pathway inhibitor 2. In the phylogenetic tree, SgKunitz clustered with Kunitz-type SPIs from Ancylostoma caninum firstly, and then formed a sister group with the same molecules from Latriodectus hesperus, suggesting there was a close relationship between SgKunitz and other invertebrate Kunitz-type SPIs (Fig. 4).

3.3. The mRNA expression profile of SgKunitz in different tissues

The SYBR Green real-time PCR analysis was employed to study the mRNA expression of SgKunitz with \(\beta\)-actin as internal control. In the healthy razor clams, the mRNA transcript of SgKunitz was found to be constitutively expressed in a wide range of tissues with different levels, including mantle, gill, gonad, hemocyte, muscle, and hepatopancreas (Fig. 5). The SgKunitz mRNA was expressed with higher level in gill and hepatopancreas, which was 8303.9 and 3916.5-fold compared with that in muscle, while the expression level in hemocyte, mantle and gonad was lower, and was lowest expressed in muscle (Fig. 5).
3.4. Temporal expression of SgKunitz mRNA post PAMPs stimulation

The temporal expression of SgKunitz mRNA was monitored after the razor clams were stimulated by three PAMPs. In the LPS stimulation group, expression level of SgKunitz mRNA was significantly up-regulated \((P < 0.01)\), and it reached the peak at 6 h post-stimulation, which was 3.1-fold compared with the blank group, and as time progressed, the expression level decreased gradually to 0.4-fold of the origin level at 48 h post-injection (Fig. 6, A).

Compared with LPS, PGN stimulation could induce much higher expression level of SgKunitz, which were 7.3 and 127.6-fold \((P < 0.01)\) compared with that in blank group at 3 h and 6 h post-injection, respectively (Fig. 6, B). After razor clams were stimulated by \(\beta\)-1, 3-glucan, transcript level of SgKunitz was kept on a consecutive high level from 6 h to 48 h, and it reached the maximal level at 48 h after stimulation (6.5-fold compared with blank group, \(P < 0.05)\) (Fig. 6, C). There was no significant change of SgKunitz expression in the control group treated with PBS (Fig. 6, A–C).
3.5. Recombination and purification of SgKunitz protein

After IPTG induction, the whole cell lysate of E. coli BL21 (DE3)-Transetta with pEASY-E1-SgKunitz was analyzed by SDS-PAGE, and a distinct band with molecular weight of \( 28 \) kDa was revealed, which was consistent with the predicted molecular mass (Fig. 7, line 3). After purification by a Ni\(^{2+}\) chelating Sepharose column, a unique band of \( 28 \) kDa representing the protein of rSgKunitz was revealed (Fig. 7, line 4). The concentration of the purified rSgKunitz was \( 395 \) mg ml\(^{-1}\) according to the BCA assay.

3.6. Inhibitory effect of SgKunitz on proteolytic activity of protease

The inhibitory activities of rSgKunitz against trypsin and chymotrypsin were tested. As a consequence, rSgKunitz was capable of inhibiting the proteolytic activity of both proteases. When 1 \( \mu \)g of rSgKunitz was incubated with 1 \( \mu \)g of protease, it could inhibit about 57.2% and 43.1% of the activity of trypsin and chymotrypsin, respectively (Fig. 8, A). As a positive control, bovine aprotinin could inhibit 88.1% and 86.3% of the activities, accordingly (Fig. 8, A).

The dose-dependent inhibition activity of SgKunitz against trypsin and chymotrypsin was also determined. The protease activities were decreased corresponding with the increasing of rSgKunitz concentration (Fig. 8, B), suggesting the inhibition activity of SgKunitz exhibited dose-dependent effect. When 4 \( \mu \)g of rSgKunitz was incubated with 1 \( \mu \)g of protease, the proteolytic activities of trypsin and chymotrypsin were inhibited 76.0% and 56.9%, respectively (Fig. 8, B).

4. Discussion

Protease inhibitors play important roles in the innate immunity, either by regulating the host defense reaction, or aiming against the proteinases from the invading microorganisms [8]. Four families include...
of serine proteinase inhibitors including kazal, kunitz, serpin and α-macroglobulin are proved to involve in the immune response [9], and have been well characterized in mammals. However, little is known about the Kunitz-type SPIs in mollusk, especially their functions and regulative mechanisms in innate immunity.

In present study, a Kunitz-type SPI with four tandem Kunitz domains, which were frequently observed in the Kunitz-type SPI family, was identified from marine mollusk *S. grandis*. There was a canonical signal peptide at the N-terminus of deduced amino acid sequence, revealing that SgKunitz was a secreted protein, which was consistent with other Kunitz-type SPIs [30]. The amino acid sequence of SgKunitz shared highly similarities with Kunitz-type SPIs from other animals. In the phylogenetic tree, SgKunitz kept a close evolutionary relationship with Kunitz-type SPIs from other invertebrate. In addition, the tertiary structure of SgKunitz adopted the typical model of this family, and all the three disulfide bridges were well conserved in the four Kunitz domains. The existence of the Kunitz domain and the conserved sequence and structure motifs, together with the high sequence identity, strongly suggested that SgKunitz should be a member of Kunitz-type SPI family. Meanwhile, the similar hallmarks both in sequence and structure also suggesting SgKunitz might perform the same function as other Kunitz-type SPIs [29,31].

![Phylogenetic tree of SgKunitz with other Kunitz-type SPIs.](image)

**Fig. 4.** Phylogenetic tree of SgKunitz with other Kunitz-type SPIs. The tree is constructed by the neighbor-joining (NJ) algorithm using the Mega 4.1 program based on the multiple sequence alignment by ClustalW. Bootstrap values of 1000 replicates (%) are indicated for the branches.

![Temporal expression of SgKunitz mRNA relative to β-actin was analyzed by real-time PCR in hemocytes after LPS, PGN, glucan and PBS challenge for 3, 6, 12, 24 and 48 h. The values are shown as mean ± SE (N = 4).](image)

**Fig. 5.** SgKunitz mRNA expression level in different tissues of *S. grandis* detected by real-time PCR. SgKunitz transcript level in mantle, gill, gonad, hemocyte, muscle, and hepatopancreas were normalized to that of muscle. Vertical bars represent the mean ± SE (N = 4).

![Temporal expression of SgKunitz mRNA relative to β-actin was analyzed by real-time PCR in hemocytes after LPS, PGN, glucan and PBS challenge for 3, 6, 12, 24 and 48 h. The values are shown as mean ± SE (N = 4).](image)

**Fig. 6.** Temporal expression of SgKunitz mRNA relative to β-actin was analyzed by real-time PCR in hemocytes after LPS, PGN, glucan and PBS challenge for 3, 6, 12, 24 and 48 h. The values are shown as mean ± SE (N = 4). (*: P < 0.05, **: P < 0.01). A: LPS group; B: PGN group; C: glucan group.
Detecting the tissue specific expression pattern of SgKunitz gene would benefit understanding its potential role in inhibitory behavior and the functions in immune response. The Kunitz-type SPI HMKI from *Haemaphysalis longicornis* was expressed uniquely in midgut of the adult tick [32], while *Rhipicephalus (Boophilus) microplus* Kunitz-type SPI BmCI was expressed in hemocytes, ovary and salivary glands, but not fat body [30]. Unlike the Kunitz-type SPIs in other invertebrates, SgKunitz exhibited a wide range of tissues distribution with different levels. Considering razor clams completely expose themselves to the aquatic environment with large number of pathogenic microorganism, the wide distribution of SgKunitz also suggested their important roles in limiting the excessive proteolytic activity of serine proteases causing by the continuing microbial invaders. For mollusk, hemocyte and hepatopancreas were two important immune-related tissues [33–35], while gill was the tissue that took the responsibility to seize oxygen and alga from the seawater, which frequently contact with pathogens [33]. Therefore, the fact that SgKunitz was specifically expressed in these tissues indicated SgKunitz might contribute to the immune system as a crucial molecule against the infection by microbes.

The physiological function of Kunitz-type SPIs was setting to inhibit the SPs, and protecting tissues from the proteolysis by excessive activities of SPs. Several isolated or recombinant Kunitz-type SPIs from invertebrates could effectively inhibit the activities of SPs [29,31,36]. For instance, Kunitz-type SPI purified from hemolymph of *Galleria mellonella* larvae could inhibit the activities of trypsin, plasmin and kallikrein [29], while recombinant Kunitz-type SPIs of silkworm exhibit effective inhibitory activity toward chymotrypsin [36]. In present study, the recombinant SgKunitz was proved to inhibit 57.2% and 43.1% of the activity of trypsin and chymotrypsin, respectively. Since the three disulfide bridges forming by six conserved cysteines in each Kunitz domain were crucial for the stability and activity of the molecule, and not all the cysteines could form disulfide bridges correctly on the processes of refolding, the fact that inhibitory activity of SgKunitz was relative lower when compared with commercial inhibitor aprotinin would be reasonable. Conventionally, the inhibitory specificity of Kunitz domain could be speculated from the P1 residue in the reactive site, which was the second residue after the second conserved cysteine residue. If P1 residue was lysine or arginine, it could inhibit trypsin, while if with tyrosine, phenylalanine, leucine or methionine at the relevant position, it tend to inhibit chymotrypsin [8]. In present study, there were four Kunitz domains in SgKunitz, among of them the P1 residue in Kunitz domain 1 was leucine, while the relevant residues in Kunitz domain 2 and 3 were arginine and lysine, respectively, which indicated SgKunitz might inhibit the activity of trypsin and chymotrypsin synchronously. Such deduction was confirmed by our subsequent result, which manifested the recombinant SgKunitz could inhibit the activities of both aforementioned enzymes, and this result was consisted with the Kunitz-type SPI *P*-mulgins-2 in *Pseudechis australis* [31]. However, except for the aforementioned residues, the P1 position in Kunitz domain 4 was glutamic acid that had not been reported before, whether it could increase the inhibitory spectrum or not was still an unknown question that needed further illustration.

In arthropod, SPIs served as a negative regulator in the innate immune signal pathway and eliminated the excessive activity of...
SPs at the appropriate time. The invading pathogens were detected by the immune receptors targeting on the PAMPs, as a result several kinds of SPs were activated, which would subsequently triggered the activation of prophenoloxidase cascade and Toll signal pathway \[6,18,37\]. Both aforementioned signal pathways were precisely regulated by the SPs, especially the serpin-type SPs, and once the pathogens were finally eliminated, SPs would deprive the excessive activity of SPs in the first time. To preliminarily unravel the regulation mechanism of Kunitz-type SPs in the innate immunity, the temporal expression of SgKunitz mRNA in hemocytes, was observed in our study, all the three PAMPs including LPS from Gram-negative bacteria, PGN from Gram-positive bacteria and β-1, 3-glucan from fungi could significantly up-regulated the expression of SgKunitz, suggesting SgKunitz might involved in the processes of inhibiting the activity of SPs during the immune responses triggered by various pathogens. The expression of SgKunitz exhibited a higher level after stimulation of PGN or β-1, 3-glucan than that in LPS group, while the up-regulative respondence toward β-1, 3-glucan was later than other two PAMPs. This obvious difference might be a consequence of the activation of different immune signaling pathway responding to diverse PAMPs stimulation. In Drosophila, two kinds of antimicrobial peptides were regulated by SPs via different immune signaling pathways. Both Gram-positive bacteria and fungi were recognized by peptidoglycan recognition protein (PGRP-L) or Gram-negative bacteria binding protein (GNBP), and anti-fungi antimicrobial peptides “Drosomycin” was produced via the activation of Toll immune signaling pathway, while Gram-negative bacteria was sensed by PGRP-S and subsequently triggered IMD immune signaling pathway to produce Gram-negative bacteria specific antimicrobial peptides “Diptericin” \[9,18,37\]. Therefore, the diverse signaling pathways SPs and SPs-involved in might be the main reason responsible for the different expression pattern of SgKunitz toward three PAMPs. In mollusk, though it was far from well understanding the regulation of SPs- and SPs-involved immune signaling pathway, a primitive Toll pathway had been proved to be existence in scallop and oyster \[38,39\]. In Zhikong scallop, all the facts that expression of pattern recognition receptors (CfPGRP-S1 and CfLGBP), SP (CfSP), SPI (CfKZSPI) and TLR (CfTLR) were significantly up-regulated after the stimulation of PAMPs or pathogens \[7,33,39–41\], indicated that molluscan SPs and SPs might also serve as modulators involving in the immune signaling pathway like that in Drosophila. Considering there was still no regulation mechanism illuminating this pathway in mollusk, our results which revealed the expression patterns of Kunitz-type SPI toward PAMPs stimulation in S. grandis would provide helpful evidence to understanding the regulation mechanism of the immune signal pathway in marine mollusk.

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