

Identification of a kazal-type serine protease inhibitor with potent anti-staphylococcal activity as part of Hydra's innate immune system

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ABSTRACT

In the absence of migratory phagocytic cells the basal metazoan Hydra has developed a very effective immune system. Previous work has shown that epithelial cells, both in the ectoderm and endoderm, recognize PAMPs by TLR and produce a number of antimicrobial peptides. In this study we demonstrate that not only epithelial cells but also gland cells are critically involved in Hydra's innate host defense by producing a kazal-type serine protease inhibitor, *kazal2*, that has potent *in vitro* bactericidal activity against *Staphylococcus aureus*. The discovery of an antimicrobial serine protease inhibitor in Hydra may shed new light on the mechanisms of host defense early in metazoan evolution, and promises to open new avenues for the development of potent anti-staphylococcal compounds.

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1. Introduction

The epithelium of phylogenetically old cnidarians has been reported to be remarkably well equipped to prevent infectious agents from entering the body even in the complete absence of migratory phagocytic cells [1–3]. In *Hydra*, the innate pathogen defense system is based on receptor-mediated recognition of pathogen-associated molecular patterns (PAMPs) such as flagellin and LPS [1]. Upon ligand binding, the HyTRR-1/HyLRR-2 receptor complex [1] is thought to initiate a conserved signal transduction network [2] that activates a multifaceted defense response.

Interestingly, in different species of *Hydra*, antimicrobial activity seems to be directed against different microbes. *Hydra magnipapillata*, for example, in contrast to *Hydra oligactis*, has a particularly strong activity against *Staphylococcus aureus* (Augustin, pers. observation). In this study, we purified and characterized this activity. We discovered that strong microbicidal activity against a human pathogenic strain of *S. aureus* is due to a kazal-type domain containing protein produced by endodermal gland cells. These kazal-proteins are known to inhibit serine proteases and are present throughout the animal kingdom from cnidarians to humans [4–11].

The kazal-type serine protease inhibitors (SPIs) contain one or more kazal-domains. Each domain has a characteristic three-dimensional structure stabilized by the formation of three

intradomain disulfide bridges by six well-conserved cysteine residues. Such a kazal SPI (SPIPm2) was recently analyzed from black tiger shrimp *Penaeus monodon* and shed new light on a potential function of SPIs [7]. As reported, the SPIPm2 is expressed in hemocytes of the shrimp and its kazal-domains inhibit the growth of the Gram positive bacteria *Bacillus subtilis*, indicating a potential role in pathogen defense. Here, similar to SPIPm2 a kazal-type SPI from early branching metazoan Hydra has a bactericidal activity. This supports the view by Donpuksa et al. [7] that these proteins are crucial components of pathogen defense.

In addition, our observations indicate that the innate immune system in *Hydra* is more complex. It involves not only antimicrobial peptides but also serine protease inhibitors; and besides epithelial cells [1], secretory glands cells appear to play a crucial role in the context of Hydra's defense against bacteria.

2. Materials and methods

2.1. Animals

For the experiments *H. magnipapillata* strain 105 was used and cultured under standard conditions at 18 °C. Prior to experiments the polyps were not feed for 2 d.

2.2. Purification of *kazal2* from Hydra tissue

20 g (wet weight) of *H. magnipapillata* strain 105 tissue was extracted in 50 vol. of 1 M HCl, 5% (v/v) formic acid, 1% (v/v) TFA

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and 1% (w/v) NaCl at 4 °C overnight. After centrifugation at $30,000 \times g$ for 1 h, the supernatants were applied on two tC_{18} 6 cm^3 (500 mg) SepPak cartridges (Waters), the columns were washed with 0.1% (v/v) TFA, and bound material was eluted stepwise with 0.1% TFA/10%, 20%, 30%, 40%, 50% and 80% (v/v) acetonitrile. The eluates were lyophilized, and redissolved in 0.01% (v/v) TFA. Each fraction was subjected to microdilution susceptibility assay to determine the minimal inhibitory concentration (MIC) for *S. aureus* ATCC12600 growth. The SepPak 30% fraction (MIC = 20 $\mu\text{g/ml}$), which had the lowest MIC was further separated by reversed phase (RP)-HPLC using a C8-column (HAlsil 300 C8 5 μm ; 250 mm \times 3.0 mm; Higgins Analytical Inc.) with gradient elution starting from 0.1% TFA/25% (v/v) acetonitrile to 0.1% TFA/49% (v/v) acetonitrile (slope 0.5% (v/v) acetonitrile/min). Again, after determining the MIC of each fraction, the one with lowest MIC and the two adjacent fractions were pooled and subjected to the final purification step using a RP-HPLC C4 column (VyDAC, C4 5 μm , 250 mm \times 3.2 mm, Grace) with gradient elution starting from 0.1% TFA/25% (v/v) acetonitrile to 0.1% TFA/59% (v/v) acetonitrile (slope 0.5% (v/v) acetonitrile). The fractions with the lowest MIC values were analyzed on SDS-PAGE and silver stained. The one fraction showing only a single band on the silver gel and having the lowest MIC value, was reduced with 0.1 M DTT for 15 min at 60 °C and acetylated with 0.15 M IAA for 1 h at room temperature. After SDS-PAGE and transfer to the PVDF membrane the peptide was stained with 40% (v/v) methanol, 1% (v/v) acetic acid and 0.1% (w/v) Serva R-250 Coomassie blue. The visible band was cut from the membrane and subjected to Edman degradation (at DKFZ, Dr. Hans Heid). By Edman degradation a 15 amino acid peptide fragment was identified, which mapped a translated Hydra EST (CB072659). Using Compagen [12], the full length gene could have been assembled out of *H. magnipapillata* EST database.

2.3. Bioinformatics

Nucleotide, protein and translated BLAST engines at the NCBI server [13], Compagen [12] and at <http://hydrascope.metazome.net/cgi-bin/gbrowse/hydra/> were used for homology searches. ClustalW was used for sequence alignments [14]. SMART [15] and SignalP [16] were used for domain analysis. To compare and to analyse the conserved sequence elements within the promoter of the five *kazal* genes, the GATA alignment tool was conducted [17]. Comparisons comprised 1.100 bp 5' flanking region upstream ATG and 42 bp of coding sequence.

2.4. Assay for the anti-*S. aureus* ATCC12600 activity

The determination of the minimal (growth) inhibitory concentration for *S. aureus* ATCC12600 was made as described previously by a microdilution susceptibility assay [18]. In brief, fractions were twofold serial diluted in 10 mM sodium phosphate pH 7.4. 100 colony forming units of *S. aureus* ATCC12600 were added to these peptide dilutions and incubated at 37 °C until sediments of grown bacteria were clearly visible (24–35 h). The MIC is defined as the peptide dilution where no bacterial sediments could be detected after incubation. To discriminate between growth inhibition and killing of the bacteria by the peptides, the microtiter plate for determining the MIC was used subsequently. The well at the minimal inhibitory concentration and the two neighboring higher concentrated ($2\times$ and $4\times$) dilutions (the whole liquid, 100 μl) were plated on to LB-agar plates and checked for bacterial growth to determine the minimal bactericidal concentration (MBC). The values were expressed as the median of at least two experiments, each performed in duplicates, with a divergence of not more than one dilution step. For internal control three additional peptides were tested all the

time in same assay: aprotinin (bovine, Sigma), a kunitz type SPI, the honey bee poison melittin (Sigma) and recombinantly expressed hydramacin-1, an antimicrobial peptide from *Hydra*. Aprotinin and hydramacin-1 served as negative controls that showed no activity against *S. aureus* ATCC12600 [1,19]. Melittin was used as positive control.

2.5. In situ hybridization

In situ hybridization was performed with *H. magnipapillata* strain 105 as described previously [20]. The digoxigenin labeled probe used, covers the whole open reading frame of the *kazal2* gene. Therefore, the probe will cross-hybridize to the other members of the *kazal*-gene family. The ability of the *in situ* probe to penetrate the tissue sometimes compromises the interpretation of the results. To demonstrate that, we cut head and foot region and wounded the gastric tissue also after fixation with 4% paraformaldehyde.

2.6. Interference with *kazal2* gene expression

Polyps were treated in three different ways to interfere with transcription of *kazal2* gene: (I) 100 *H. magnipapillata* strain 105 polyps were incubated in a heat inactivated *S. aureus* ATCC12600 bacterial suspension (1.5×10^7 cfu's/ml) for 24 h. 100 control polyps were kept in hydra medium instead. (II) 50 animals starved for 7 d, whereas another 50 polyps were fed continuously. (III) The head and foot region of 50 polyps were cut off, followed by regeneration for 4 h. 50 control polyps were treated in the same manner, but were not allowed to regenerate.

2.7. Semiquantitative RT-PCR

For RT-PCR, total RNA was isolated from polyps using TRIzol reagent (Invitrogen) followed by a DNase I digest (Fermentas) for 35 min at 37 °C and inactivation for 10 min at 70 °C. 5 μg of total RNA were reverse transcribed as previously reported [21,22]. cDNA samples were equilibrated with primer (AK Actin 34 5' AAG CTC TTC CCT CGA GAA ATC 3'; AK Actin 35 5' CCA AAA TAG ATC CTC CGA TCC 3') against β -actin (15 cycles) and *glyceraldehyde-3-phosphate dehydrogenase* (GAPDH forward 5' GCC TTA TGA CAA CCA TTC AT 3', GAPDH reverse 5' TCA ACA ACA GAA ACA TCT GC 3') (20 cycles) as described previously. To amplify the *kazal2* gene, specific primers were used (for (I) forward 5' ATG AAG TGT ATT GCT GTA ATT ATG 3', reverse 5' TTA TTT ATC TTT GCA TGG TCC ATT G 3'; for (II–III) forward 5'ACA GCG ATT GTG CCT TAA AAT 3', reverse 5' CAA TCA TCT TTT TTA AGA GCT CG 3') (20 cycles). Water control and equilibration controls were included into every reaction. PCRs were performed according to standard profiles. Annealing temperatures were set due to T_m of the specific primer pair. Only data obtained reproducibly in three to five independent experiments were further analyzed.

2.8. Production of recombinant *kazal2* domains 1–3 in *Escherichia coli*

All domains were overexpressed using *E. coli* Rosetta (Novagen). DNA according to amino acid residue 19–67 for D1, 68–119 for D2 and 120–166 for D3 were ligated into pET 28a vector (Novagen). The resulting proteins were fusion proteins that contain a His-tag at the N-terminus. After transformation, clones were grown overnight on LB plates containing chloramphenicol (34 $\mu\text{g/ml}$) and ampicillin (100 $\mu\text{g/ml}$). One clone for each domain was picked and grown in 5 ml LB containing ampicillin (100 $\mu\text{g/ml}$) overnight. A day culture with an OD_{600} of 0.6 was obtained by diluting the overnight culture 1:100 and was incubated at 37 °C for 3 h. The protein expression was induced by the application of 2 mM IPTG.

3 h after induction the bacteria were harvested by centrifugation for 10 min at $8000 \times g$ and stored at -80°C . The bacteria pellet was resuspended in 50 mM sodium phosphate buffer pH 8.0; 300 mM NaCl. The suspension was sonicated on ice using regime of 10×15 s burst with a 15 s cooling period between each burst. After centrifugation for 30 min at $15,000 \times g$ the supernatants were applied to Ni-TED columns (Macherey & Nagel). The columns were washed and the bound proteins were eluted with 250 mM levanisole in 50 mM sodium phosphate pH 8.0, 300 mM NaCl. Protein concentration was measured using the MicroBCA Kit (Pierce). Refolding was obtained by rapid dilution (drop by drop) of elution fractions into 50 mM sodium phosphate pH 8.0, 4 mM glutathion (reduced), 0.4 mM glutathion (oxidized) to a final protein concentration of $50 \mu\text{g/ml}$. After incubation at room temperature for 3 d the refolded protein was desalted by dialyses and concentrated by $t\text{C}_{18}$ 6 cm^3 (500 mg) SepPak cartridges (Waters).

2.9. Inhibitory assay

The inhibitory activity of each domain towards the serine proteinases trypsin (bovine pancreas, TPCK treated, Sigma) and subtilisin A, type VIII (*Bacillus licheniformis*, Sigma), was assayed using a procedure of Hergenbahn et al. [23]. The reaction mixture consisted of 0.1 mM Tris-HCl, pH 8.0; 0.55 mM of N-benzoyl-Phe-Val-Arg-p-nitroanilide (Sigma) for trypsin and subtilisin; and $0.2 \mu\text{M}$ of subtilisin and trypsin in a total volume of $80 \mu\text{l}$. Proteinases were separately pre-incubated with increasing concentration of inhibitor (1-, 2-, 4-, 8-, 16-, 32-, 64-fold to the enzyme) in the buffer for 5 min at 30°C . Then the remaining activity was tested by the addition of N-benzoyl-Phe-Val-Arg-p-nitroanilide (Sigma) solution diluted in the buffer (0.1 mM Tris-HCl, pH 8.0). The reaction was incubated at 30°C for 15 min and then terminated by adding $20 \mu\text{l}$ of 50% acetic acid. The extinction of p-nitroanilide formed, was measured at 405 nm. The percentages of remaining activity were calculated and plotted against the molar ratios of inhibitor domain to proteinase. Each reaction was performed in triplicates and the median was calculated.

2.10. Accession numbers

kazal1 (FJ496649), *kazal2* (FJ496650), *kazal3* (FJ496651), *kazal4* (FJ496652), *kazal5* (FJ496653); *promoter kazal1* (FJ496656), *promoter kazal2* (FJ496655), *promoter kazal3* (FJ496658), *promoter kazal4* (FJ496657), *promoter kazal5* (FJ496654). AS_EI (P16895), MC_API (P82968), NV_Kazal (XP_001634346), and PM_Kazal (AAP92780).

3. Results

3.1. Purification of a kazal-type serine protease inhibitor active against *S. aureus*

Homogenates of *H. magnipapillata* inhibit the growth of *S. aureus* (Augustin, pers. observation). To characterize this *S. aureus* directed activity, extract of homogenized polyps was subjected to several rounds of hydrophobic adsorption chromatography using different columns with different matrices. After each step the minimal inhibitory concentration value for *S. aureus* was determined by a microdilution susceptibility assay. Extract was first fractionated using SepPak $t\text{C}_{18}$ cartridges. Stepwise elution with acetonitrile revealed two fractions (SepPak 30% and SepPak 40%) with high activity against *S. aureus*. Proteins in the SepPak 30% fraction were further separated by preparative C8-reversed phase-HPLC (Fig. 1A). Using the MIC antibacterial assay system, the fraction showing highest bactericidal activity against *S. aureus*

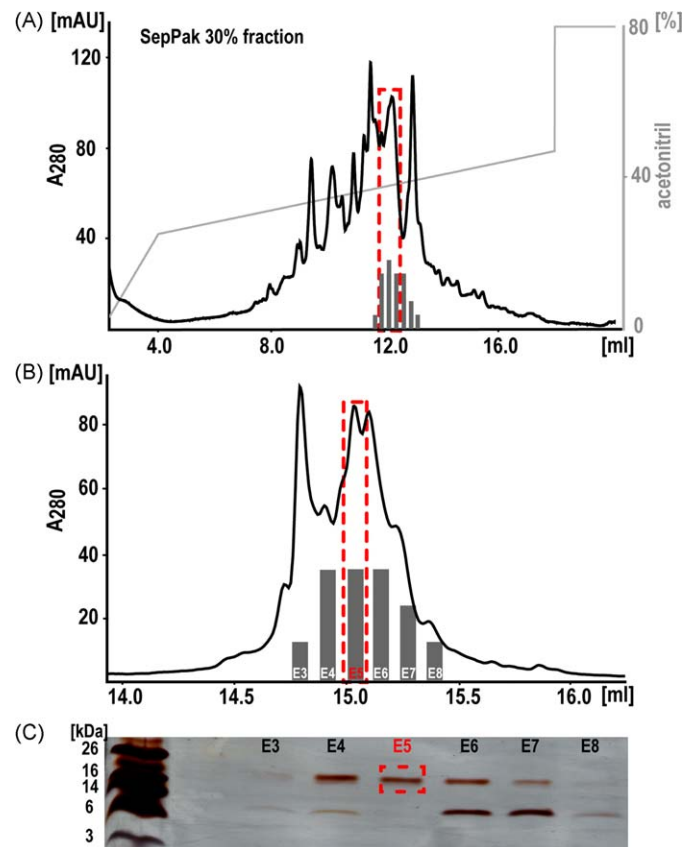


Fig. 1. Purification of kazal2. (A) Proteins, which bound to SepPak $t\text{C}_{18}$ column and were eluted with 30% acetonitrile, have been separated by RP-C8-HPLC. To elute proteins from RP-C8-HPLC a gradient elution starting from 0.1% TFA/25% (v/v) acetonitrile to 0.1% TFA/49% (v/v) acetonitrile (slope 0.5% (v/v) acetonitrile/min) (light grey curve) was performed. Elution was monitored by measuring the extinction at 280 nm. (B) Fraction with highest activity against *S. aureus* ATCC12600 (grey bars) were collected, combined (red dotted square) and subjected to further separation on RP-C4-HPLC column. (C) Fractions detected to be active against *S. aureus* ATCC12600 were separated on a SDS-PAGE under reducing conditions and silver stained. Fraction E5 was submitted to Edman degradation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

together with the two adjacent fractions were pooled and purified to homogeneity using a RP-HPLC C4 column (Fig. 1B). As shown in Fig. 1C, fraction E5 contains only a single band but is strongly active against *S. aureus*. Attempts to identify the amino acid sequence by MALDI-ToF “peptide mass fingerprint” which requires a trypsin digest failed, because no small fragments could be detected. Is this due to the inhibition of trypsin by the isolated protein? By Edman degradation we identified a 15 amino acid peptide fragment (indicated by red letters in Fig. 2A) which mapped to a translated Hydra EST (CB072659). Using the Compagen platform [12] and the *H. magnipapillata* EST database, the full length sequence was assembled. BLASTP searches revealed a significant match to kazal1 (71% identity), a serine protease inhibitor that has been reported to serve cytoprotective functions during regeneration in *Hydra* [5]. Since our sequence was similar but not identical to kazal1, we termed it kazal2. Analysis of the full length sequence of kazal2 showed that the predicted SPI has a calculated mass of 16 kDa, consists of 168 amino acids and has a relatively high pI of 8.5 indicating that the predicted protein under physiological conditions is a positively charged molecule. The protein contains a putative signal sequence directly followed by three kazal-type domains (Fig. 2B). Each of the kazal-type domains contain the characteristic six conserved cysteine residues (Fig. 2C) which are identical to those of the non-classical SPI kazal-protein family

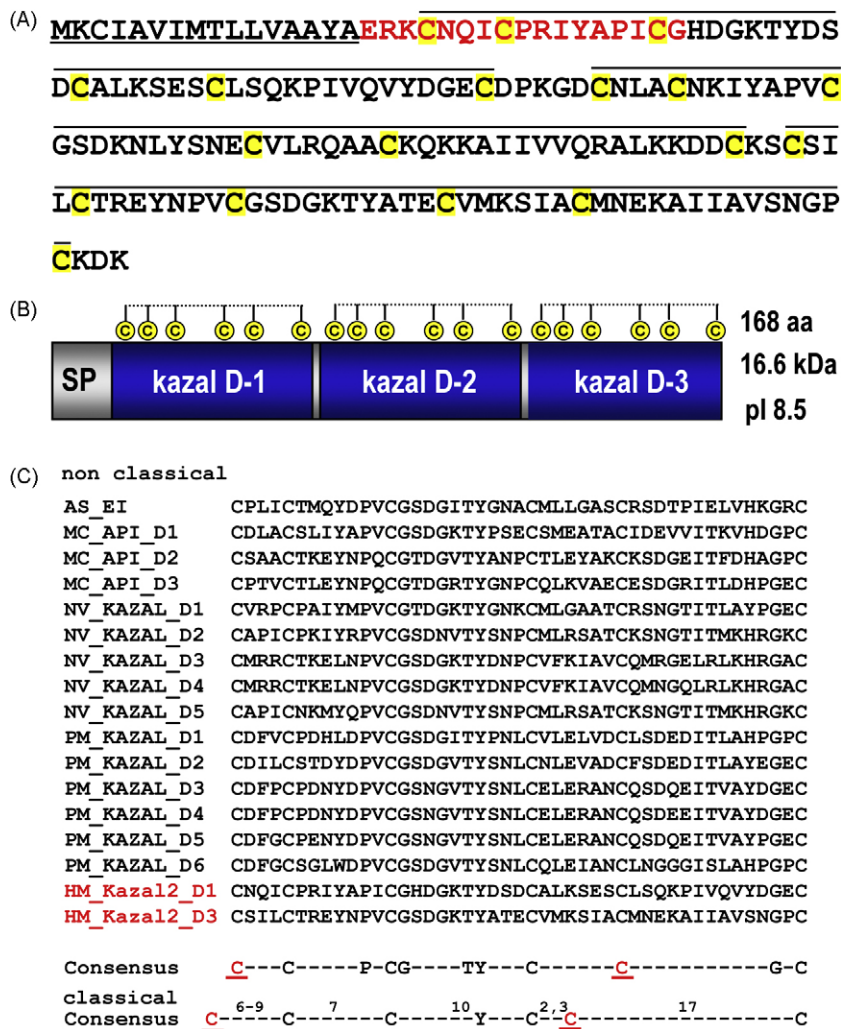


Fig. 2. Amino acid sequence of kazal2. (A) Amino acid sequence. Underlined signal peptide, overlined kazal-domains, amino acids in red indicate Edman sequenced fragment. Cysteines are highlighted in yellow. (B) Predicted protein structure with signal peptide (SP) and the three kazal-domains. (C) Domain alignment with other non-classical kazal-domains. Note, the cysteine pattern is different between non-classical and classical kazal-domain. Cys1 and cys5 from the consensus sequences are shifted to the N-terminus (marked in red underlined letter). The alignment clearly shows that kazal2 belongs to the non-classical kazal-proteins. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

members, and which were shown previously to be essential for stabilization and protection of the proteinase inhibitor against digestion by specific serine proteases [24]. Kazal2 D2 has four additional amino acid residues in front of cys6 that will extend the

space between cys5 and cys6 and may have implications on the structural level. Strictly speaking, kazal2 domain D2, therefore, does not belong into the same subgroup of non-classical kazal-SPI as kazal2 D1 and kazal2 D3.

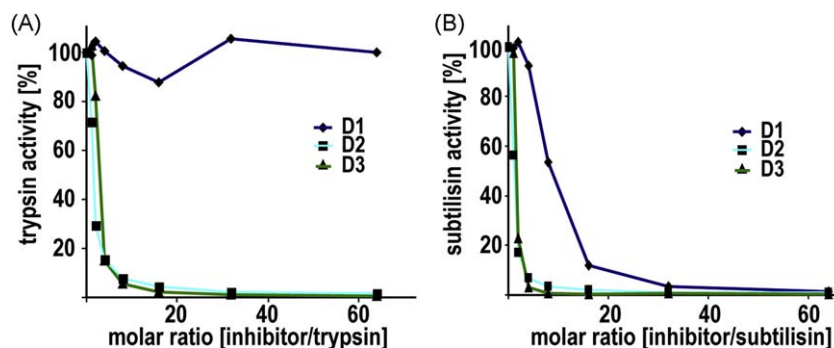


Fig. 3. Trypsin and subtilisin are inhibited by recombinant kazal2 domains. (A) Kazal2 domain 2 (cyan line, black square) and domain 3 (green line, triangle) were found to inhibit trypsin in a molar ratio of 16:1 (inhibitor:trypsin). Kazal2 domain 1 (blue line, diamond) showed no trypsin directed inhibitory activity. (B) All three domains show inhibitory activity against subtilisin. Domain 2 (cyan line, black square) and domain 3 (green line, triangle) display a significantly higher subtilisin inhibition (4:1; inhibitor:subtilisin) than kazal2 domain 1 (blue line, diamond; 32:1; inhibitor:subtilisin). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 1
Antimicrobial activity of native kazal2 and recombinant kazal2 domains 1–3.

	<i>S. aureus</i> ATCC12600 MIC pH 7.4	<i>S. aureus</i> ATCC12600 MBC pH 7.4
Native kazal2 (D1, D2, D3)	0.7–0.8 μ M	n.d.
Recom. kazal2 D1	33 μ M	33 μ M
Recom. kazal2 D2	36 μ M	36 μ M
Recom. kazal2 D3	38 μ M	38 μ M

As shown in Fig. 2 the cysteine pattern in these non-classical kazal-SPI is different from the one found in classical kazal-SPI such as LEKTI [10], SPINK1 [25] or SPINK3 [26]. We note, therefore, that the previous assumed close relationship between kazal1 and SPINK [5] is not supported by the data shown in Fig. 2C.

3.2. Recombinant kazal2 protein has serine protease inhibitor activity

To functionally analyse kazal2, we produced recombinant kazal2 protein. The high cysteine content of the 16 kDa protein (Fig. 2) makes it unlikely that the recombinant protein will have the correct and functionally active folding pattern, when over-expressed in *E. coli*. We, therefore, decided to produce recombinant protein of each of the three kazal-type domains of kazal2. Each domain was subjected to an assay testing trypsin and subtilisin

inhibition. While kazal2 domains 2 and 3 were found to inhibit trypsin in a molar ratio of 16:1 (inhibitor:trypsin), kazal2 domain 1 showed no trypsin directed inhibitory activity (Fig. 3A). Interestingly, and in contrast to trypsin, all three domains showed inhibitory activity against subtilisin (Fig. 3B) with domains 2 and 3 displaying a significantly higher subtilisin inhibition (4:1; inhibitor:subtilisin) activity than kazal2 domain 1 (32:1; inhibitor:subtilisin). Since subtilisin is a microbial protease, this may indicate that the Hydra kazal2-type serine protease inhibitor is directed against microbial proteases.

3.3. Native and recombinant kazal2 has antimicrobial activity against *S. aureus*

To explore the antimicrobial activity of native kazal2, we used the HPLC-purified fraction E5 shown in Fig. 1B and C. The minimal inhibitory concentration value of E5 for *S. aureus* was determined by a microdilution susceptibility assay. Native kazal2 was found to have highly potent activity against *S. aureus* ATCC12600 in a MIC range of 0.7–0.8 μ M (Table 1). Since the amount of native kazal2 protein available is very limited, further functional assays had to be performed using recombinant protein. As also shown in Table 1, recombinant kazal2 domains 1–3 all are active against *S. aureus* ATCC12600. Activity of individual domains, however, is lower than activity of the native protein. This observed discrepancy is possibly

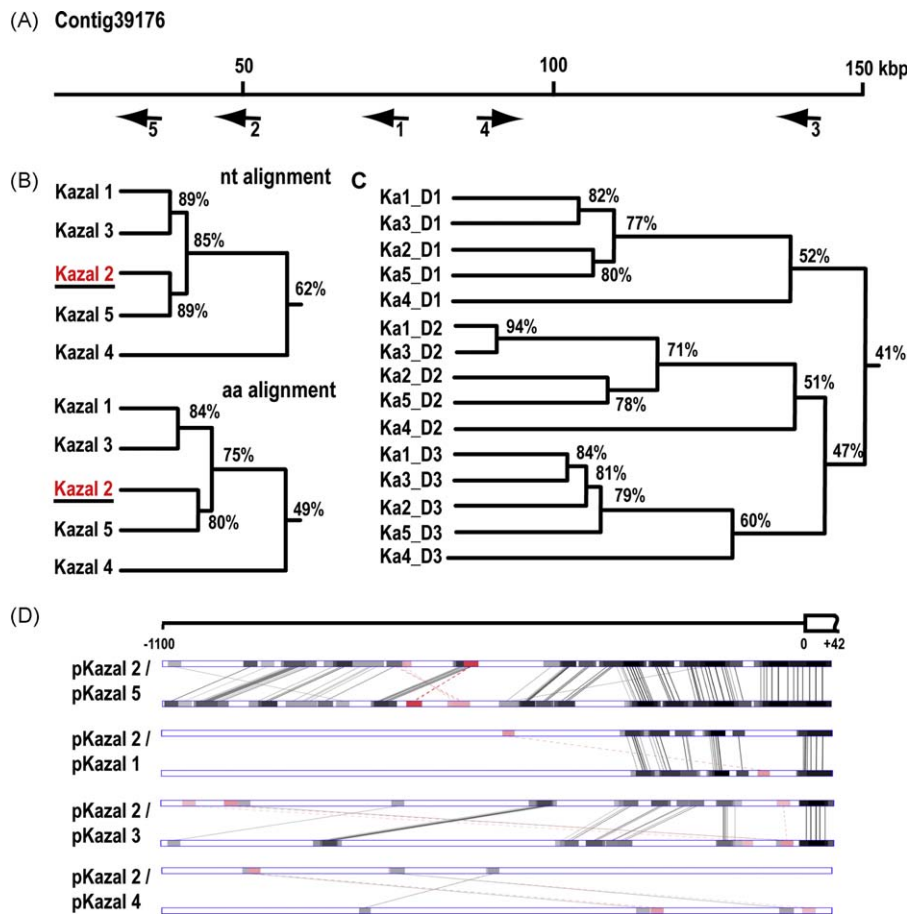


Fig. 4. Kazal2, one member of the *kazal*-gene family in *H. magnipapillata*. (A) All five members of the *kazal*-gene family are clustered in the genomic contig 39176 of *H. magnipapillata*. While *kazal1*, *kazal2*, *kazal3* and *kazal5* had been detected on one strand of the DNA, *kazal4* is located on the complementary strand. (B) The nucleotide (nt) and the amino acid alignment (aa) show the close homology of kazal genes/proteins, although *kazal1* and *kazal3* as well as *kazal2* and *kazal5* are more similar to each other than to the other pair, but all four are more distantly related to *kazal4*. (C) The domain amino acid alignment reflects the overall similarity, which indicates that the whole gene was duplicated rather than single domains. (D) The 5' flanking region of *kazal2* with the four other members of the gene family is compared using GATA algorithms. *Kazal5* which is most closely related to *kazal2* shares a large number of sequence elements with *kazal2*. The more distantly related genes *kazal1* and *kazal3* share some but fewer elements than *kazal5* with *kazal2*. The 5' flanking region of gene *kazal4* seems to be significantly different from *kazal2*.

due to a synergistic effect, when all domains are within molecule, so that *kazal2* is more than the sum of its parts. Nevertheless, what we can demonstrate with help of these recombinant domains is that the minimal bactericidal concentration of each of the *kazal2* domains is identical to the MIC value (Table 1). This provides direct evidence that the recombinant *kazal2* domains not only inhibit growth of *S. aureus* but also kill the microbes. This bactericidal activity seems to be specifically directed against *S. aureus* since *E. coli* K12 DH5 α growth was not affected (data not shown). Thus, Hydra *kazal2* SPI appears to inhibit a protease which is essential for growth of *S. aureus* ATCC12600 and which may not be present in *E. coli*.

3.4. *Kazal2*, one member of the *kazal*-gene family in *H. magnipapillata*

Analysis of the *H. magnipapillata* ESTs as well the genome (<http://hydrazome.metazome.net/>) indicated that in addition to *kazal2* and *kazal1* [5] there are three more genes present in *Hydra* encoding *kazal*-type SPIs (Fig. 4A). All five genes have three introns and four exons (Supplementary Table 1) and are clustered in the genome within about 150 kbp. While *kazal1*, *kazal2*, *kazal3* and *kazal5* were detected on one strand of the DNA, the gene encoding *kazal4* is located on the complementary strand (Fig. 4A).

The inferred homology for all five *kazal*-type genes showed (Fig. 4B) that *kazal2* and *kazal5* as well as *kazal1* and *kazal3* are more closely related to each other than to the other members of the gene family and to *kazal4*. Interestingly, the most closely related genes are also located in the genome in closest proximity, suggesting recent gene duplication events. This assumption is supported by the fact that similarity between the *kazal*-type genes is higher at nucleotide level than at the amino acid level (Fig. 4B). Since the domain homology tree (Fig. 4C) mirrors the homology of the genes shown in Fig. 4B, indicating that the gene duplication event covered the complete gene, as a functional unit, rather than individual domains.

Comparing the 5' flanking region of *kazal2* with the four other members of the gene family using GATA algorithms (Fig. 4D) revealed that *kazal5* which is most closely related to *kazal2* (Fig. 4B) shares a large number of sequence elements with *kazal2*. This points to a coregulation of both genes and provides additional support for the idea that both genes originated via gene duplication. Fig. 4D also indicates that the more distantly related genes *kazal1* and *kazal3* share some but less elements than *kazal5* with *kazal2*. The 5' flanking region of gene *kazal4*, which is located at the opposite strand of the DNA (Fig. 4A) seems to be significantly different from *kazal2*. Taken together, *kazal2* is a member of a family of five *kazal*-type SPI encoding genes which are clustered in the hydra genome and most likely coregulated.

3.5. *Kazal2* is constitutively expressed in endodermal secretory cells

In situ hybridization revealed that *kazal2* is expressed exclusively in gland cells located in the endodermal epithelium along the entire body column (Fig. 5A and B), but not in secretory gland cells at the very oral and aboral end. Due to high sequence similarity (Fig. 4B), the *kazal2* specific probe is expected to cross-hybridize also to *kazal1*, *kazal3* and *kazal5*. If the *kazal2* specific probe also binds to *kazal4* transcripts remains to be shown. Since *kazal1* was reported earlier [5] to be expressed in an identical manner as *kazal2* shown in Fig. 5 of this study and the high probability of cross-hybridization, we conclude that the genes encoding *kazal*-type SPIs in *Hydra* are all expressed in secretory gland cells in the endodermal epithelium.

To determine whether *kazal2* is constitutively expressed or inducible by microbial products, we exposed *H. magnipapillata* to pathogenic *S. aureus* ATCC12600 prior to RNA isolation, since *kazal2* was shown to inhibit the growth of *S. aureus*. Since feeding

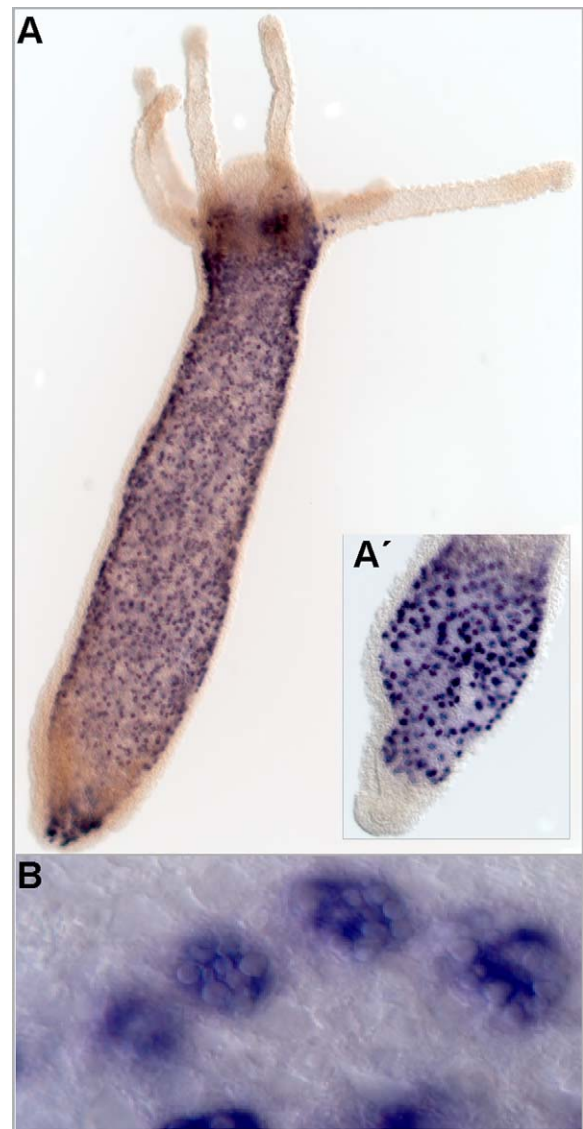


Fig. 5. Expression pattern of *kazal2*. (A) *Kazal2* is expressed in gland cells along the body column. Note, there are no *kazal2* expressing gland cells at the oral (A) and aboral end (A'). (B) *Kazal2* expressing gland cells in the endoderm at higher magnification.

results in rapid release of the content of gland cell vesicles in the gastric lumen [27], we also investigated whether uptake of non-sterile food causes induction of *kazal*-type protease gene expression. As shown in Fig. 6A and B, neither exposure to *S. aureus* nor food-uptake does upregulate *kazal*-type serine proteinase inhibitor expression.

During regeneration the tissue of the polyp is very vulnerable by bacterial pathogens. Thus, a higher expression level of *kazal2* gene for protection would be favourable. However, as shown in Fig. 6C, by RT-PCR we could not obtain any evidence that *kazal2* transcription is upregulated by regeneration or wounding. Previously, the expression of *kazal1*, a closely related gene to *kazal2* (see Fig. 4B), was reported to be strongly upregulated immediately following tissue wounding, as demonstrated by *in situ* hybridization [5]. We, therefore, evaluated the *kazal2* gene expression pattern during regeneration by whole mount *in situ* hybridization as described by Chera et al. [5]. As shown in Supplement Fig. 4A–C, there appeared a strong accumulation of *kazal2* transcripts at the site of wounding which, if true, would be in sharp contrast to the PCR data shown in Fig. 6C. However, the

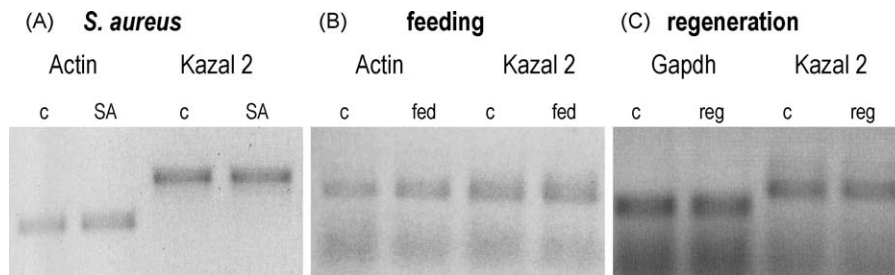


Fig. 6. *Kazal2* is a constitutively expressed gene. The expression level is not increased by exposure to *S. aureus* ATCC12600 (A), feeding (B) or regeneration (C).

polyps shown in Supplement Fig. 4A–C were fixed in paraformaldehyde prior to wounding. Thus, the strong *in situ* hybridization signal cannot indicate induction of *kazal* transcription. It appears that the wounded tissue simply is more accessible to the hybridization probe and, therefore, the binding to the target mRNA is much more effective compared to non-injured tissue. In conclusion, by *in situ* hybridization as performed by Chera et al. it is not possible to judge whether the transcription of the *kazal* genes is upregulated by regeneration/wounding or not. Based on the RT-PCR data shown in Fig. 6, *kazal*-type gene transcripts in *Hydra* appear to be constitutively expressed in endodermal gland cells at a high level.

4. Discussion

Our study showed that *kazal2*, a protein consisting of three *kazal*-domains, is responsible for the anti-*S. aureus* activity observed earlier in *H. magnipapillata* tissue. Furthermore, we could demonstrate that the separated *kazal2* domains exhibit an inhibitory activity for the serine proteases trypsin and subtilisin. It is therefore reasonable to assume that this holds true for the native, all three domains containing protein. In that aspect, the stronger inhibitory activity against a bacterial protease, subtilisin, may indicate that *kazal2*'s activity directed against *S. aureus* is up to the inhibition of a bacterial specific serine protease. Serine proteases are widely spread in many pathogenic bacteria, where they have critical functions related to colonization of host tissue and evasion of host immune defenses, acquisition of nutrients for growth and proliferation, facilitation of dissemination, or tissue damage during infection [28]. Inhibiting one or more of these described processes will lead to growth inhibition or reduced pathogenesis, if not to death of the bacteria. With the help of *kazal2*, in the future it will be interesting to identify the specific serine protease from *S. aureus*. With this knowledge it may be possible to design potent inhibitors against this specific protease, which than may become promising molecules and very specific agents to destroy even multiresistant *S. aureus*.

While previous efforts were focused on epithelial cells as major effector cells of *Hydra*'s TLR-mediated antibacterial immune responses [1] the role of non-epithelial cells remained to be shown. *Kazal2* and all the members of the *kazal*-gene/protein family are expressed in secretory gland cells all over the gastric column in *Hydra*. During feeding, the food and a large number of bacteria are taken up into the gastric cavity [1]. Stimulated by the uptake of food the secretory gland cells release their content into gastric cavity [27]. By that the *kazal* SPIs and a high amount of other serine protease inhibitors such as, for example antistasin [29], are also released and may inhibit there the overgrowth of bacteria until they are taken up or killed by endodermal epithelial cells [1].

This passive and broad-spectrum response mediated by gland cells via releasing protease inhibitors into the gastric cavity is

based on two criteria. First every component has to be made constitutively at a relatively large amount and second, the protease inhibitors need to have a degree of diversity to inhibit a large variety of proteases. As we could show, *kazal2* is made constitutively in relatively large amounts. It has a comparable expression to the housekeeping metabolic gene GAPDH, which was assumed from the number of cycles used in PCR amplification. How is the inhibitory diversity obtained? To gain diversity, gene duplication is a very common feature of proteinase inhibitors. Many inhibitors are present as small gene families with altered specificities among the paralogues [30]. In *Hydra*, the high similarity (>80%) between the DNA sequences of the five *kazal*-type genes indicating gene duplication events. This notion is supported by the fact that all five genes are located on one genomic region of <150 kbp. The observed divergence between members of the *kazal*-family obtained by particular substitutions of nucleotides at the first and second position of the codon triplets might have been favourable in evolutionary terms. With a higher probability these nucleotide substitution lead to an amino acid substitution; which due to minor structural changes in the protein, may change the specificity of the *kazal* SPIs after duplication. However there is no evidence that genes encoding inhibitory proteins duplicate at higher rates than any others. Their maintenance in the genome is more driven by selective advantages [30,31], which could result in the case of *kazal*-type SPI in the coevolution with serine proteases from bacteria.

Taken together, our findings have two major implications. First, they may indicate that the role of protease inhibitors in epithelial defense, which was hitherto unknown, is at least as significant as the function of antimicrobial peptides. Second, in light of the increasing prevalence of antibiotic-resistant microbes and the undisputed need for new antibiotics [32]; *kazal2*-type protease inhibitors might constitute a new class of highly effective antibiotics, suitable for optimization into a new anti-staphylococcal compound.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.dci.2009.01.009.

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