Protein purification and gene isolation of chlamysin, a cold-active lysozyme-like enzyme with antibacterial activity

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Abstract An antibacterial ~11 kDa protein designated chlamysin was isolated from viscera of the marine bivalve *Chlamys islandica*. Chlamysin inhibited the growth of all Gram-positive and Gram-negative bacteria tested. The isolated protein was highly efficient in hydrolyzing *Micrococcus luteus* cells only at low pH (4.5–6.2) and at low temperature (4–35°C). No significant loss of enzyme activity was observed after 30 days storage at room temperature or after heating to 70°C for 15 min, suggesting relatively high protein structure stability. Sequence-analyzed fragments of the protein revealed data which guided the isolation of the cDNA gene, encoding a 137 amino acid chlamysin precursor in scallops. The deduced protein contains a high portion of cysteine, serine and histidine residues and has a predicted isoelectric point below 7. The chlamysin protein was found to have sequence homology to an isopeptidase and to a recently published bivalve lysozyme.

Key words: Antibacterial protein; Cold-active enzyme; Molecular characterization; Marine invertebrate; *Chlamys islandica*

1. Introduction

Antimicrobial peptides and proteins are important components of the innate defense in all species investigated [1]. Several peptides with antibacterial effect against various Gram-positive and Gram-negative bacteria have been isolated from marine invertebrates including horseshoe crab [2], shore crab [3] and mollusks [4,5], and antibacterial proteins have been isolated from sea hare [6,7] and from bivalve mollusks [8]. Marine bivalves are filter feeders and free-living bacteria are one of their major sources of nutrition. This implies that these animals require a digestive system capable of degrading or hydrolyzing prokaryotic cells in addition to the need of a defense system against pathogenic bacteria. Not surprisingly, lysozymes or lysozyme-like enzyme activities have been found in many marine invertebrates including bivalves [9–12], and these enzymes are believed to be involved in digestive processes [9] as well as in host defense [13]. Two classes of lysozyme, the chicken-type and the goose-type, have previously been described in the animal kingdom (see [14] for a review), and a new class was recently proposed to be contained by invertebrate lysozymes [15]. Other types of lysozymes are also seen in phages [16], bacteria [17] and plants [18]. Lysozymes hydrolyze β-1,4-linked glycoside bonds of bacterial cell wall peptidoglycans.

The Icelandic scallop *Chlamys islandica* is a marine species located 20 m or deeper in the southern part of the arctic region having sea temperatures rarely exceeding 4°C. A putative lysozyme with high catalytic activity at low temperatures was previously partly purified from an extract prepared from the viscera of the scallop [11], and the extract was also found to contain substances able to inhibit growth of fish-pathogenic bacteria in vitro [19]. This extract has now been further processed and here we report biochemical and molecular characteristics of the cold-active chlamysin, an antibacterial lysozyme-like protein from Icelandic scallops.

2. Materials and methods

2.1. Purification of chlamysin, amino acid analysis and amino-terminal sequencing

The material used was an extract prepared earlier from viscera of the marine bivalve *C. islandica* [11]. The scallop viscera extract was stored at −30°C until use. After thawing at 8°C, the extract was brought to pH 4.8 by adding 6 N hydrochloric acid and then left for settling of the produced floc. The resulting supernatant was used as crude extract for protein purification. All further steps in isolation of chlamysin were carried out at 6–8°C. The crude extract was mixed with 3.5 volumes of 10 mM ammonium acetate buffer, pH 5.0, and applied to a S Sepharose FF cation-exchange column (1 l; Pharmacia) equilibrated with the same buffer. Fractions containing lysozyme-like activity eluted at 0.4 M ammonium acetate, pH 6.0, were pooled and then fractionated by ultrafiltration on an Amicon CH2A ultrafiltration unit fitted with an Amicon Hollow Fiber cartridge H1P 100-20 (cut off 100 kDa). Permeates obtained were concentrated using an Amicon Hollow Fiber cartridge H1P 10-43 (cut off 10 kDa). The retained solution was further concentrated on a PM 10 ultrafilter (cut off 10 kDa), using an Amicon Diaflo stirred cell, and subsequently centrifuged at $14\,000 \times g$ for 20 min. The supernatant was dialyzed against 10 mM ammonium acetate, pH 5.0, and subjected to re-chromatography on a S Sepharose FF column (20 ml). Following extensive washing with 20 mM ammonium acetate, pH 5.0, absorbed proteins were eluted by stepwise increasing concentrations of ammonium acetate. Fractions with lysozyme-like activity eluted at 0.5 M ammonium acetate, pH 5.0, were dialyzed against 10 mM ammonium acetate buffer, pH 6.0, and applied to a Blue Sepharose CL-6B column (26 ml; Pharmacia). Active fractions released by 0.18 M ammonium acetate, pH 6.0, were concentrated by ultrafiltration on a PM 10 ultrafilter, followed by size-exclusion chromatography on a Sephacryl S 200 HR column (493 ml; Pharmacia) equilibrated with 0.1 M ammonium acetate buffer, pH 5.0. An aliquot from each fraction containing lysozyme-like activity was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in the Phast System (Pharmacia) using precasted PhastGel Homogeneous High Density gels. Sample fractions showing a single protein band of ~11 kDa were pooled and the isolated protein, denoted

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chlamysin, was concentrated using a YM2 membrane disc (Amicon). In total, 1.45 mg purified chlamysin was recovered from 30 l of scallop viscera extract. Amino acid analysis was performed by compositional analysis after vacuum hydrolysis. The hydrolysates were analyzed on an automatic amino acid analyzer Model 421 (Applied Biosystems, Perkin Elmer). The N-terminal amino acid sequence of chlamysin was analyzed by automatic Edman degradation using a protein sequencer Model 477A (Applied Biosystems, Perkin Elmer) and a G1005A N-terminal sequencer (Hewlett-Packard). The protein was also reduced and alkylated with 4-vinyl-pyridine [20], and this alkylated chlamysin was cleaved by cyanogen bromide (CNBr) and the resulting polypeptides were separated by reversed-phase high performance liquid chromatography (RP-HPLC) using a Pep-S C2/C18 column (Pharmacia) [21]. Peaks were collected and taken for Edman degradation.

2.2. Enzyme assays

Activities of chlamysin and hen egg white lysozyme (HEWL) towards lyophilized *Micrococcus luteus* cells (0.2 mg/ml) were measured as previously described [11]. The activity was determined from the first minute of linear decrease in absorbance at 450 nm. One unit of enzyme activity is defined as the amount of enzyme that catalyzes a decrease in absorbance of 0.001 min^{-1}. For determination of pH for optimal activity, assay buffers of 10 or 50 mM sodium acetate and 5 or 20 mM sodium phosphate were used for pH ranges 3.6–5.6 and 6.0–8.0, respectively, after adjusting the buffer ionic strength using NaCl.

Chitinolytic activity was assayed using chitin-RBV (0.1 mg/ml) as substrate in 50 mM sodium acetate buffer, pH 5.2, as described [22].

2.3. M. luteus cell wall digestion for product analysis

Suspensions of 2 mg *M. luteus* in 1 ml 0.1 M sodium acetate buffer, pH 5.2, were incubated with 10 μg of chlamysin or HEWL for 2 h at 24°C. After incubation, the suspensions were centrifuged at $12\,000 \times g$ for 10 min to remove undigested debris and the supernatants were assayed for reducing groups [23] and for *N*-acetylamino sugars by the acidic Morgan–Elson assay as described [24].

2.4. Antibacterial assays

In vitro antibacterial assays were performed following principles of the microdilution technique [25]. All media used for growth inhibition studies were prepared in 0.1 M sodium phosphate buffer, pH 6.2. *Listeria monocytogenes* (CCMG 1452), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Proteus mirabilis* (ATCC 35659), *Staphylococcus epidermidis* (ATCC 12228) and *Enterococcus faecalis* (ATCC 29212) were grown in tryptic soy broth, *Bacillus cereus* (ATCC 10987) was grown in brain heart infusion medium (Difco), and marine broth (Difco) was used to grow *Vibrio salmonicida* (NCIMB 2245). Overnight bacterial cultures were diluted to give 10^3–10^5 colony forming units per ml. 100 μl of diluted bacterial suspensions was added to each microtiter plate well and combined with 11 μl of chlamysin at concentrations obtained by 2-fold serial dilutions made in 0.01% acetic acid and 0.2% bovine serum albumin. The plates were incubated 24–48 h at 22°C, or at 12°C for *V. salmonicida*, using a mixing device upon incubation. Cell growth was monitored by measuring the absorbance at 620 nm using a SPECTRAmax Plus Microplate Spectrophotometer (Molecular Devices). The minimal inhibitory concentration (MIC) was defined as the lowest concentration for which no bacterial growth was optically detectable.

2.5. mRNA isolation, cDNA synthesis and isolation of the chlamysin gene

Six crystalline styles from freshly collected Islandic scallops were dissected and mRNA was subsequently isolated using the PolyATract System® 1000 (Promega). cDNA was synthesized by the use of the Smart™ PCR cDNA Synthesis kit (Clontech). Degenerated oligonucleotides chlaN4 (5′-GTWCCAGGMGCYTAYYTVGA-3′) and chlaCT (5′-CCDGGGGGSCCSCCATTRTG-3′), identical to the DNA sequences derived from the amino acid sequences obtained from the purified chlamysin protein (see Fig. 1A), were utilized as primers in amplification of a part of the chlamysin gene applying the Advantage® cDNA PCR kit (Clontech). The resulting 290 bp PCR product of chlamysin was sequenced following the instructions for the Thermo Sequenase™ Radiolabeled Terminator Cycle Sequencing kit (Amersham), exploiting the PCR primers as primers for sequencing. The sequence information was the basis for the design of two intragenic oligonucleotides. These oligonucleotides CmarF (5′-TTGACGTATACTCCGACTCGTGTGG-3′) and CmarR (5′-AGGTTCGTGAACACGAAGTATGGCC-3′) were applied as primers in 3′- and 5′-random amplification of cDNA ends (RACE) reactions, respectively, in combinations with the AP1 primer as described for the Marathon™ cDNA Amplification kit (Clontech). The RACE products were sequenced and the cDNA sequence informations of the 3′- and 5′-ends were included for the design of two new oligonucleotide primers ChlaBF (5′-GCAGACCAGTACGACATAATGATG-3′) and ChlaBR (5′-TATAAAACAATAATGACTGCGACCTTC-3′) that were used in PCR amplification of the entire chlamysin cDNA gene using the high-fidelity *Pfu* DNA polymerase (Stratagene). PCR products were cloned in a plasmid vector of the pCR-Script™ Amp SK(+) Cloning kit (Stratagene), and both strands of the cloned PCR products were sequenced using primers for vector-contained sequences as well as gene-specific primers.

2.6. Computer analysis

DNA and translation product sequence analysis were performed mainly by programs in the Wisconsin Package Version 10.0, Genetics Computer Group, Madison, WI, USA. Protein homology searches and protein sequence alignments were done by the programs BLAST [26] and ClustalW [27], respectively. Predictions of a possible protein signal for transport and a putative cleavage site were produced by SignalP [28] and by PSORT II [29]. Finally, the GeneDoc program [30] was used to prepare the multiple sequence alignments for presentation.

3. Results

3.1. Isolation and analysis of chlamysin

The purified chlamysin protein obtained after size-exclusion chromatography appeared as a single band of M_r ~11 kDa after SDS-PAGE and Coomassie-staining (not shown). The amino acid composition analysis (not shown) of chlamysin indicated a protein containing about 100 amino acids including one methionine and a high portion of cysteines (11%), serines (10%) and histidines (7%). N-terminal sequence analysis of the purified chlamysin revealed a sequence of the first 30 residues. Later, and due to the presence of one methionine, CNBr was used to cleave the reduced and alkylated protein. RP-HPLC revealed that this produced three protein fragments of which two were shown to be sequence homologs of the initial N-terminal part of the protein. The third fragment of chlamysin produced a sequence of 29 residues representing the C-terminus of the chlamysin protein. The protein sequences shown in Fig. 1A demonstrate a few ambiguities observed in the three separately performed N-terminal sequencing analyses. The isolated chlamysin lacks a methionine in the start of the protein which strongly suggests that the protein has been processed by the removal of a signal peptide starting with a methionine residue.

3.2. cDNA gene isolation and sequencing analysis

Degenerated primer sequences derived from back-translation of the amino acid sequence of the chlamysin protein (see Fig. 1A) defined a PCR product of 290 bp when applied in the amplification of the scallop cDNA template (not shown). Based on the DNA sequence of the PCR product, 5′- and 3′-cDNA ends were subsequently produced in separate RACE reactions, and a final PCR amplification of the entire gene and its flanking cDNA ends was performed. The DNA sequence presented in Fig. 1B includes a 411 bp open reading frame of a 137 amino acid polypeptide which comprises sequences determined for the chlamysin protein fragments. Six nucleotide positions were found to carry two alter-

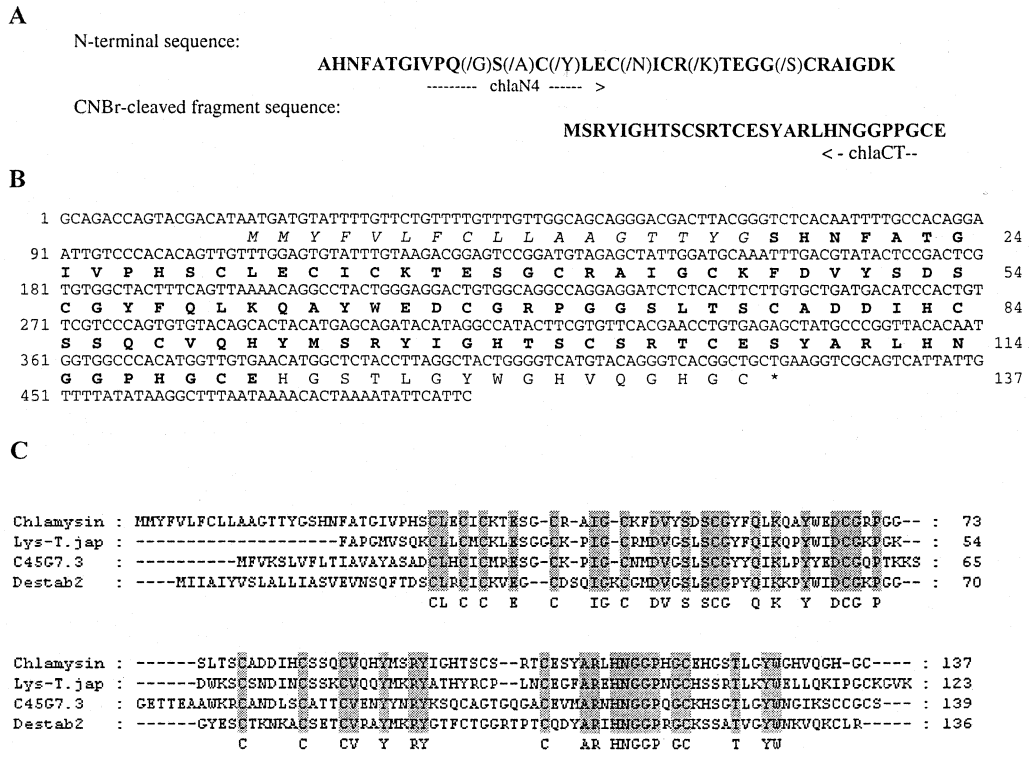

Fig. 1. Protein and cDNA sequences of chlamysin and multisequence alignment of homologs. A: N-terminal amino acid sequence of the purified protein and of the fragment obtained by CNBr splitting. The N-terminus was sequenced three times and ambiguous residues are subsequently presented in brackets. Denotions below the sequences mark the amino acids applied in back-translations to DNA for design of two degenerated oligonucleotides chlaN4 and chlaCT used to prime the initial PCR amplification of a part of the chlamysin cDNA gene. B: The cDNA sequence and the deduced polypeptide encoded by the gene. A predicted 17 amino acid signal peptide is written in italics and the part of the protein believed to constitute the active protein is shown in bold. Stop in translation is marked by an asterisk. The following nucleotide position ambiguities were observed: T or C in position 37, G or A in position 52, G or C in position 69, T or C in position 70, and C or G in position 102. C: Multisequence alignment of chlamysin and its homologs. The chlamysin protein sequence deduced from the cDNA gene of *C. islandica* and the top three sequence homologs were aligned using ClustalW [27]. The homologs are the bivalve lysozyme from *T. japonica* [15], destabilase 2 (GenBank accession U24122 under the name of destabilase I) from *H. medicinalis* [31] and the hypothetical gene product C45G7.3 (SwissProt entry O76357) from *C. elegans* [32]. Chlamysin may carry one or more of the following amino acid substitutions: Leu-7, Thr-12, Ala-18, Gln or Gly-28, Ala-29, Tyr-30, Arg-36, Gly-39 or Ser-40. The chlamysin nucleotide sequence has been assigned the EMBL database accession number AJ250028.

nating nucleotides in each of the six positions in the cDNA. These variations, interpreted to be allelic forms present in the scallops, produced four amino acid changes (positions 7, 12, 18 and 28) in the deduced protein. The sequenced chlamysin protein, purified from extracts of a huge number of scallops, indicates the existence of a few additional amino acid variations that possibly are not encoded by the six scallop individuals used as source in the gene isolation.

Two sequence analysis programs gave statistical support of an N-terminal 17 amino acid residue transport signal in chlamysin. According to these predictions, the secretion signal is cleaved off regardless of which of two amino acids (alanine or serine) is present as residue 18. This assumably secreted chlamysin protein has a calculated molecular mass of 13 kDa, a net charge of -2 and an isoelectric point of 6.9. The protein has a particular high number of cysteines, serines and histidines (14, 14 and 9, respectively) that in sum make up almost 30% of the total amino acid content of chlamysin. No 'signatures' or 'motifs' of any known classes of enzymes are recognized in the chlamysin primary structure.

Searches in accessible databases revealed no published sequence homologous of the chlamysin gene. In contrast, the protein displays partial identity to a 123 residues bivalve lysozyme (58% in 119 residues overlap) recently reported to belong to a novel class of lysozymes [15], to destabilase 2 (60% in 109 residues overlap) which is a 136 residue endo-ε(γ-Glu)-Lys isopeptidase from the leech *Hirudo medicinalis* [31], and to several hypothetical proteins of similar size from *Caenorhabditis elegans* (46% in 129 amino acid residues overlap to the C45G7.3 protein) [32]. A multisequence alignment of chlamysin and the three homologous proteins is shown in Fig. 1C. Except for a gapping of seven residues caused by a short non-homologue part of the *C. elegans* protein, a remarkable high degree of amino acid conservations is seen in these four invertebrate proteins. Less significant homology was found to various lysozymes like the pigeon egg white lysozyme (32% in 70 residues overlap) [33].

3.3. Enzymatic properties and protein stability

The specific activity of the isolated scallop chlamysin against *M. luteus* cells was 5×10^5 U/mg protein at pH 5.2 and ionic strength 0.1. At a low concentration of enzyme in assay (0.1–5 ng/ml), chlamysin resembles conventional lysozymes in degrading *M. luteus* cells at a linear rate. At higher concentrations of chlamysin in assay, however, the activity decreased with time, indicating a product inhibitory effect (Fig. 2A). In contrast, HEWL degraded the bacterial suspension at a constant rate. Chlamysin hydrolyzes *M. luteus* cells

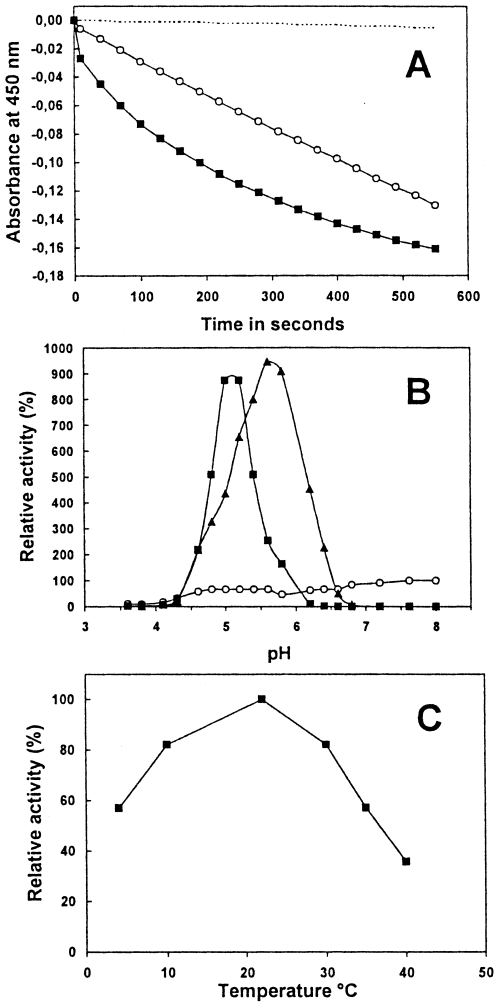

Fig. 2. *M. luteus*-hydrolyzing activities. A: Time course of activities at pH 5.2 and ionic strength of 0.1 using 0.5 μg of chlamysin (■) and HEWL (○). B: pH-dependent lysis by chlamysin at ionic strength 0.1 (■) and 0.03 (▲) compared to HEWL at ionic strength 0.1 (○). All values are normalized to the activity of HEWL at pH 7.2 (100%) and indicate relative specific activities. C: Effect of temperature on chlamysin activity. Assays were performed at pH 6.5 and ionic strength of 0.03.

with the resultant liberation of reducing groups and *N*-acetylamino sugars (Table 1), indicating a cleavage of the β-1,4 linkage between *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG). The reaction catalyzed by chlamysin produced only 25% and 50% of reducing groups and released acetylamino sugars, respectively, compared to the amounts produced by HEWL. The scallop enzyme at a concentration of 15 μg/ml did not exert detectable chitinase activity in the assay systems used in which the same amounts of HEWL were measured to have a chitinolytic activity (results not shown).

Table 1
Liberation of reducing groups and *N*-acetylamino sugars from *M. luteus* cell walls

	Reducing groups (pmol NAM)	*N*-acetylamino sugars (pmol NAG)
Chlamysin	10.2	6.7
HEWL	41.8	12.9

Table 2
Antibacterial effect of chlamysin

Bacterial strains	MIC (μM)
Gram-negatives:	
V. salmonicida	>0.6–<1.25
E. coli	10
P. aeruginosa	10
P. mirabilis	10
Gram-positives:	
L. monocytogenes	>2.5–<10
B. cereus	>2.5–<10
S. epidermidis	10
E. faecalis	10

The pH optimum conditions at ionic strength of 0.03–0.1 for the hydrolyzing activity of chlamysin are shown in Fig. 2B. No hydrolysis of the *M. luteus* suspension was produced by the protein at neutral or basic pH. The enzyme activity of chlamysin was studied at temperatures ranging from 4 to 40°C by adding 1 μl samples to pre-heated *M. luteus* suspensions (Fig. 2C). Chlamysin showed a remarkable high initial catalytic activity at low temperature and the activity declined above 25°C. The temperature-dependent enzyme activity of chlamysin is not a reflection of thermal instability since preincubation of chlamysin at 70°C for 15 min has no effect on the hydrolyzing activity of the enzyme (not shown). We observed only 20% loss of activity in a dilute chlamysin preparation (2.6 μg/ml) after storage at 23°C for 1 month (not shown), a feature which suggests a high structure stability of the protein.

3.4. Antibacterial activity of chlamysin

The extract prepared from viscera of scallops had previously been shown to contain in vitro antibacterial activities to a few fish-pathogens tested [19]. We therefore wanted to verify potential growth-inhibiting effects of the isolated chlamysin against various bacteria with preference to some that mainly are associated with infection diseases in humans and animals. Thus, seven strains of clinically relevant bacteria and one fish-pathogen were selected by convenience, representing both Gram-positives and Gram-negatives. Moderate concentrations (2–10 μM) of chlamysin totally inhibited growth of all bacteria (Table 2). No convincing differences in response to chlamysin are seen between the two Gram-staining groups of bacteria, but the Gram-positives are possibly slightly more susceptible than the Gram-negatives. We have not yet established the mechanism of antibacterial activity mediated by chlamysin.

4. Discussion

The data presented in this report show that the crystalline style, a rod-like structure located in association with the digestive gland in marine bivalves [34], contains a novel 11 kDa lysozyme-like chlamysin protein with antibacterial activities. Chlamysin isolated from the viscera of *C. islandica* inhibits the growth of several bacteria causing infections in humans and animals. The isolated antibacterial protein displays low-temperature lysozyme-like *M. luteus*-hydrolyzing activity only at acidic pH, but the enzyme lacks chitinase activity. A partly purified 18 kDa lysozyme from the mussel *Mytilus edulis* [35]

was previously shown to hydrolyze *M. luteus* in acidic or neutral environments at moderate temperatures [36]. The recently reported 13 kDa marine bivalve lysozyme from *T. japonica* demonstrates many features that resemble those of HEWL, including chitinase activity and high optimal temperature for lysozyme activity [15]. Chlamysin and the *T. japonica* enzymes have *M. luteus*-hydrolyzing activities several fold higher than HEWL. Our studies also revealed that the reaction product of the *M. luteus* degradation inhibits the lysozyme-like activity of chlamysin.

The full-length cDNA gene of chlamysin encodes 137 amino acids, and after removal of the signal peptide, the translation product is predicted to have a molecular weight of 13 kDa. Analysis of the total amino acid composition of the purified protein, however, shows that the enzyme including the antibacterial activities is contained by a polypeptide of approximately 100 amino acid residues. Results from sequencing the C-terminal CNBr fragment suggested that the active protein ends with a glutamate residue corresponding to position 121 of the cDNA translation product. The N-terminus of the purified protein was found to start with an alanine and this is in agreement with the start of the allelic cDNA translation products after the predicted removal of a 17 amino acid signal sequence. A polypeptide like the cDNA-encoded protein from position 18 to 121 has a theoretical M_r of 11.4 kDa which is compatible with the ~11 kDa migration of the purified chlamysin in SDS-PAGE and with the approximately 100 amino acids estimated from the composition analysis. Thus, the combined protein and cDNA sequence data indicate that the mature and active chlamysin is a secreted protein of 104 amino acids with a primary structure identical to the cDNA-derived polypeptide sequence starting at residue 18 and ending with residue 121. At present, we have no specific proposition for a potential protease involved in the removal of a 16 amino acid residues C-terminal fragment from the translation product.

Several mollusk lysozymes [37] have been shown to have high sequence homology to a lysozyme in the starfish *Asteria rubens* [38] although the available sequence information was limited to the N-terminal part of the proteins. These invertebrate enzymes were said to belong to a new lysozyme family. Recently, three new invertebrate lysozymes were reported [15] and all have partial sequence homology to the N-terminal fragment of the starfish lysozyme [38]. A surprisingly high identity in primary structures was found between the *T. japonica* lysozyme [15] and two other invertebrate proteins which have no previous records of lysozyme protein relationships; the destabilase 2 from *H. medicinalis* [31] and the hypothetical gene product of CELF22A3.6 in *C. elegans* [32]. The authors concluded that their results demonstrate a novel invertebrate class of the lysozyme family. Chlamysin scores high homology to the *T. japonica* lysozyme, to destabilase 2 and to several putative *C. elegans* proteins. The group of homologous proteins has conserved structurally important amino acid residues and has with a few exceptions a fixed distance between the conserved residues as also discussed by Ito et al. [15]. In particular, the high content of cysteines and a consequently potential of forming several intramolecular disulfide bridges in these proteins point to a class of structurally stable molecules. Cold-active enzymes are believed to need a high degree of structural flexibility to retain their activities at low temperatures in contrast to the expected rigidity of thermophilic enzymes (reviewed in [39]). In this perspective, the cold-active and highly stable chlamysin seems like a paradox.

Sequence homologies between the Japanese bivalve lysozyme [15] and the arctic scallop protein in this study could indicate homologous activities as well. There are, however, several distinct enzymatic characteristics that distinguish these two enzymes. The *T. japonica* lysozyme and HEWL have several common features such as high lysozyme activity at neutral pH, high optimal temperature (about 70°C) and a contained chitinase activity [15]. In contrast, chlamysin lacks chitinase activity, has no bacteriolytic activity at neutral or higher pH and the enzyme displays low-temperature hydrolyzing activity. The observed product inhibition of chlamysin has not been reported for HEWL or the *T. japonica* lysozyme. An apparent contradiction in the finding of a high initial rate of *M. luteus* hydrolysis and a low efficient generation of lysozyme-specific reaction products by chlamysin could be explained by product inhibition due to a much higher concentration of chlamysin used in the product analysis assay compared to the amount used in the kinetic study experiments. An alternative explanation is that chlamysin possesses enzyme activities additional to the β-1,4 NAM-NAG hydrolase activity. The primary structure of chlamysin is similarly homologous to the *T. japonica* lysozyme and to destabilase 2. Although not convincing to Ito et al. [15], destabilase 2 was shown to have an endo-ε(γ-Glu)-Lys isopeptidase activity [31]. Thus, primary structure data relate chlamysin to a lysozyme, as well as to an isopeptidase. Taking in consideration several described non-lysozyme features of chlamysin, we hesitate to classify chlamysin among lysozymes until future studies have examined the hydrolyzing activities of chlamysin.

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