Kasetsart J. (Nat. Sci.) 41 : 169 - 179 (2007)

Characterization of Antibacterial Protein from Hemolymph of Oyster *Crassostrea belcheri*

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ABSTRACT

The hemolymph of oyster *Crassostrea belcheri* was found to possess significant antibacterial activity against *Vibrio harveyi*, *V. vulnificus* and *V. cholerae*. Hemolymph was partially purified by gel filtration chromatography on Sephacryl S-200 and fractioned into four parts: P1, P2, P3 and P4. The P3 showed the highest antibacterial activity against *V. parahaemolyticus, V. cholerae, V. alginolyticus, V. harveyi* in the inhibition ranges from 95-62% and *V. vulnificus* was inhibited only 8% with 64 mg protein. The antibacterial activity of P3 protein was stabilized at pH 6-8 and 4-30°C. In addition, the activity required calcium ion. Molecular weight of the antibacterial protein P3 determined by gel filtration was approximately 18.7 kDa. Antibacterial protein gave two bands of 25.0 and 30.5 kDa in SDS-PAGE and pI was 3.0 and 5.0, respectively. Both bands were analyzed by LC-MS/MS and compared with nrFasta database. The 30.5 kDa band was homologous to hemocyte extracellular superoxide dismutase from *C. gigas* and 25.0 kDa band was homologous to sarcoplasmic calcium-binding protein.

Key words: antibacterial protein, *Vibrio* species, *Crassostrea belcheri*, superoxide dismutase, LC-MS/MS

INTRODUCTION

The host of defense system (immune system) of all modern animals has its roots in very ancient organisms (Alexander *et al*., 2004). The ability to distinguish self from non-self is a fundamental aspect of immunity. Although invertebrates lack antibody-based humoral immune systems, they are believed to have defense molecules that are similar in function to antibodies (Arason, 1999; Muller *et al*., 1999). Invertebrates must defend themselves from infection without antibodies and lymphocytes normally involved in the adaptive immunity of vertebrates. An innate immune response is the only way invertebrates have to combat microorganisms. In this regard, naturally occurring antibacterial peptides have been found in both vertebrates and invertebrates, reflecting the presence of a common immune response (Tasiemski *et al.*, 2000). Basic knowledge of biochemical and antimicrobial properties from hemolymph of oyster in Thailand is lacking. The oyster (*Crassostrea belcheri*) is an important commercial bivalve species along the

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Received date : 02/08/06 Accepted date : 10/11/06

Gulf of Thailand in southern Thailand. The development of a procedure to purify antibacterial protein from hemolymph of oysters will allow investigations of its potential role in the oyster host defense.

The objectives of this study were to (1) partially purify protein from hemolymph of oyster and to test of antibacterial activity, (2) estimate the molecular mass and isoelectric point of the purified antibacterial protein, (3) determine the optimal pH, temperature and protein concentration for activity of the partial purified antibacterial protein and (4) analyze partial amino acid sequences of antibacterial protein by liquid chromatography mass spectrometry (LC-MS/MS).

MATERIALS AND METHODS

1. Preparation of hemolymph and purification of antibacterial proteins

Two hundred adult oysters, 9-11 cm in shell length, were collected from the south coast of Thailand in Suratthani Province. Hemolymph was withdrawn from the adductor muscle sinus with a 10 ml syringe equipped with a 25 gauge needle through a notch on the dorsal side of the shell. Hemolymph was pooled and centrifuged at 9,000 rpm for 10 min at 4°C. Eight hundred ml of plasma were collected and stored at -80°C until use.

Six mg of crude protein from hemolymph were purified by gel filtration chromatography on a column of Sephacryl S-200 (Amersham Bioscience, 1.6 × 70 cm). The elution buffer was 0.15 M NaCl in 0.05 M Tris-HCl, pH 7.1 at the flow rate of 0.5 ml/min. Fractions (3 ml) were collected and measured by spectrophotometric absorbance at 280 nm. The activity of purified proteins was estimated by comparing its mobility with that of the following markers: blue dextran (MW 2,000 kDa), albumin (MW 67 kDa), ovalbumin (MW 45 kDa), chymotrypsinogen A (MW 25 kDa) and ribonuclease A (MW 13.7 kDa).

2. Antibacterial activity

Antibacterial activities of crude and purified proteins were performed using *V. alginolyticus*, *V. harveyi*, *V. vulnificus*, *V. cholerae* and *V. parahaemolyticus*. Cultures were grown in 10 ml tryptic soy broth added with 1.5% NaCl at 30°C, 16-18 hr with continuous shaking. Twenty µl of cultures were added to 80 µl of samples (crude hemolymph and all of purified fractions, 64 µg), mixed well and incubated at 30°C for 1 hr. For growth inhibition test, samples from the incubated culture were diluted 10^{-2} to 10^{-6} fold. Aliquots were uniformly spread on tryptic soy agar added with 1.5% NaCl and incubated at 30°C overnight followed by colony counting according to Tunkijjanukij and Olafsen (1998).

Bacterial inhibition (%) =

$$\left(\frac{CFU_{\text{control}} - CFU_{\text{sample}}}{CFU_{\text{control}}}\right) \times 100$$

3. Molecular mass and isoelectric point determination

Molecular mass of protein fraction P3 was determined using SDS-PAGE and native PAGE. For SDS-PAGE, samples were mixed in a 1:1 ratio with buffer (0.06M Tris-HCl pH 6.8, 5% b-mercaptoethanol, 10% glycerol, 0.025% bromophenol blue and 2% SDS). Samples were denatured by heating for 5 min at 95°C before loading onto gels, as described by Laemmli (1970). Native PAGE was performed using the same buffer without SDS and β-mercaptoethanol and heating. Isoelectric point (pI) was determined by two-dimensional electrophoresis (2-DE) in IPG buffer pH 3-10. Forty µg of the purified proteins were loaded on to strips. The voltage program for the first-dimension isoelectric focusing was 12 hr of re-hydration, followed by 250 Vh 30 min, 500 Vh 30 min and 7,500 Vh 1 hr 30 min, respectively (Ettan IPGphor, Amersham Bioscience). The second dimension corresponding to SDS-PAGE was performed using a Hoefer miniVE with 12%

acrylamide gel (8×8 cm). Gels were stained with Coomassie Brilliant Blue R-250. Spot of interest were estimated molecular weight using MALDI-TOF (Bruker Daltonics) for confirmed with molecular weight of SDS-PAGE and 2D-PAGE.

4. Amino acid sequencing by liquid chromatography mass spectrometry

The Coomassie blue-stained protein spots of protein P3 were excised from the SDS-PAGE, and analyzed using LC-MS/MS (model Finnigen LTQ Linear Ion Trap Mass Spectrometer, Thermo Electron Corporation) at Bioservice unit, National Center for Genetic Engineering and Biotechnology, Thailand. The mass spectrophotometry spectra of the most intense peaks were obtained following each full-scan mass-spectrum. The peptide mass lists were submitted to the nrFasta search for protein identification using Biowork 3.1 softwere.

5. Effects of temperature, pH and metal ion on antibacterial activity

The effects of temperature, pH and metal ions on antibacterial activity of purified proteins P3 were examined. Aliquots of the proteins were incubated at 4°, 25°, 35°, 40°, 50°, 60°, 70°, 80°, 90° and 100°C for 20 min. For pH stability test, aliquots of the proteins were dialyzed with buffers adjusted to various pH levels (0.01M acetate buffer for pH 3, 4, 5 and 6; 0.01M Tris-HCl buffer pH 7, 8, 9 and 10) and stirred at 4°C for 24 h. The effects of Ca^{2+}, Mg^{2+} and Mn^{2+} ions on antibacterial activity were evaluated in the presence of EDTA as a metal ion chelator to secure the total absence of these ions in solution of protein. The P3 protein was dialyzed with 10 mM (final concentration) of $CaCl_2$, $MgCl_2$ and $MnCl_2$ buffers. For all factors tested, aliquots of the proteins were incubated with an equal volume of *V. parahaemolyticus* at 30°C for 1 hr. Samples from the incubated culture were diluted 10^{-2} to 10^{-6} CFU/ml, spreaded on tryptic soy agar (TSA plus 1.5% NaCl), incubated at 30°C overnight followed by colony counting.

RESULTS

1. Purification of antibacterial protein

Four fractions of proteins, P1, P2, P3, and P4, were separated from *C. belcheri* hemolymph by gel filtration chromatography (Figure 1a). The native molecular weights of P1, P2, P3 and P4 were 75.4, 58.4, 18.7 and 15.2 kDa, respectively as calculated from the relative fraction of the standard curve from gel filtration (Figure 1b).

2. Antibacterial activity

Antibacterial activities of the crude and purified proteins against five species of *Vibrio* were shown in Table 1. Crude protein strongly inhibited the growth of *V. cholerae*. All of purified proteins except crude protein showed strong inhibition against *V. parahaemolyticus*. Only proteins P2 and P3 were able to inhibit the growth of all *Vibrio* spp.

3. Molecular mass and isoelectric point determination

Only proteins from fraction P3 which showed strong antibacterial activities against all *Vibrio spp*. was subjected to SDS-PAGE and 2-D gel electrophoresis (Figures 2 and 3). Two bands, 30.5 and 25.0 kDa were observed on SDS-PAGE.

Three protein spots of protein fraction P3 were observed on 2D-PAGE at pI 5.0, 5.2, and 3.0, respectively. Molecular masses of protein spot 1 and spot 2 were 30.5 kDa. Protein spot 3 had molecular mass of 25.0 kDa.

The spot 1, 2 and 3 were all estimated molecular mass using MALDI-TOF, that show in Figures 4-6. The molecular mass of spot 1, 2 and 3 were 27258, 29715 and 25451 Da, respectively.

4. Amino acid sequences of antibacterial protein subunits

Two peptides could be identified from

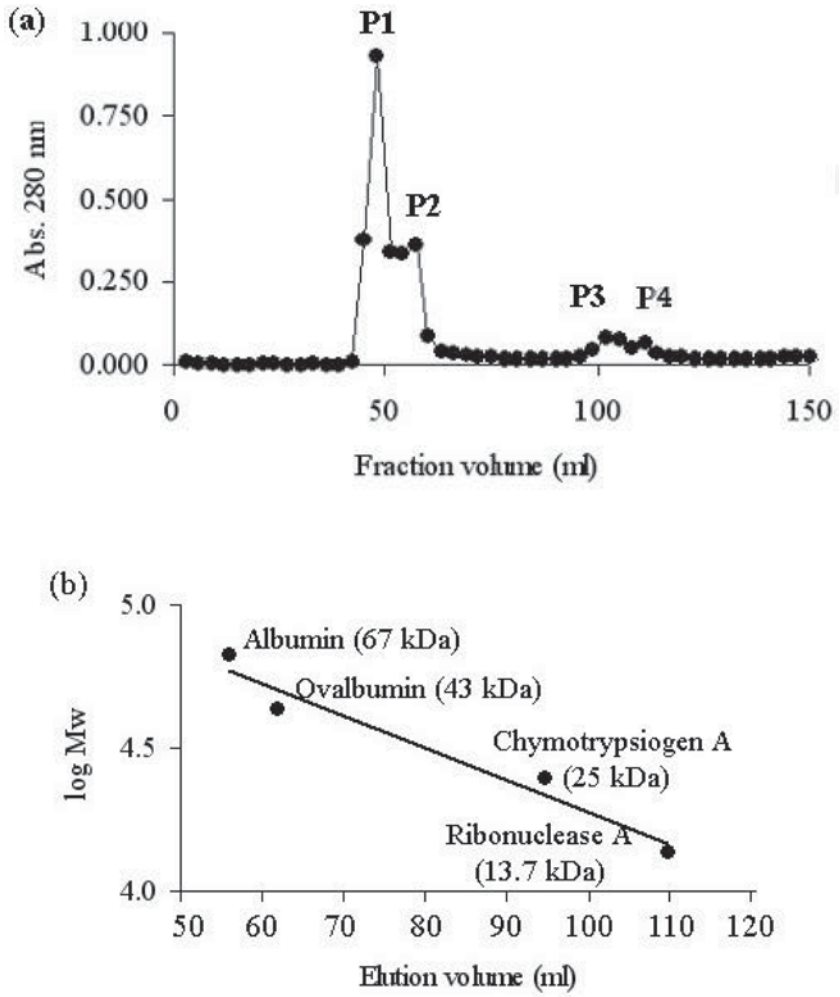

Figure 1 (a) Gel filtration chromatography and determination of the molecular size of P1, P2, P3 and P4. (b) The standard protein used were albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa) and ribonuclease A (13.7 kDa).

Table 1 Antibacterial spectrum of crude and purified proteins from hemolymph.

Sample	Bacterial inhibition (%)				
	V. parahaemolyticus	*V. cholerae*	*V. alginolyticus*	*V. harveyi*	*V. vulnificus*
Crude	0	83.39±0.42	0	27.59±2.59	18.98±6.94
P1	94.54±0.95	0	60.19±1.05	40.86±3.16	66.28±2.04
P2	93.26±1.18	16.90±0.94	71.93±2.24	39.30±1.05	55.37±4.25
P3	95.03±0.47	91.13±0.85	86.06±1.13	62.31±0.46	8.77±3.82
P4	80.05±1.16	0	0	25.26±1.25	0

Means of bacterial inhibition (%) ± S.D.

Means with different superscripts are significantly different ($p<0.05$).

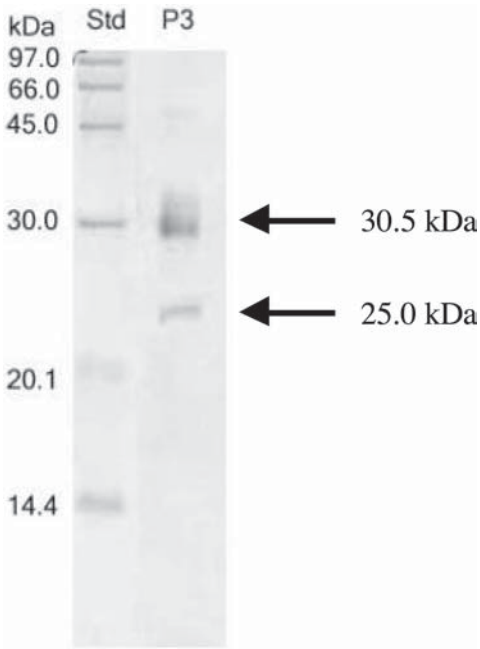

Figure 2 Purified P3 proteins and protein molecular marker in PAGE. Denaturing conditions, SDS gel stained with Coomassie Brilliant Blue R-250.

the hemolymph of oysters, hemocyte extracellular superoxide dismutase (E-value 1.9) and sarcoplasmic calcium-binding protein (E-value 0.007) (Figures 7 and 8).

5. Effects of pH, temperature and metal ion on antibacterial activity

The antibacterial activity of the P3 protein was stable between pH 6 and 9 and was optimal at pH 7.1 (Figure 9a). The optimal temperature of the P3 protein ranged from 4° to 30°C and declined at higher temperature (Figure 9b). The activity was completely destroyed at 50°C

The antibacterial activity of P3 protein was decreased by demetalisation. This effect was reversible, as the addition of $CaCl_2$ to metal-free P3 protein restored their activity. The minimal Ca^{2+} concentration for optimal antibacterial activity was 10 mM. Other metal ions, such as Mg^{2+} and Mn^{2+}, did not promote antibacterial activity in the absence of Ca^{2+}.

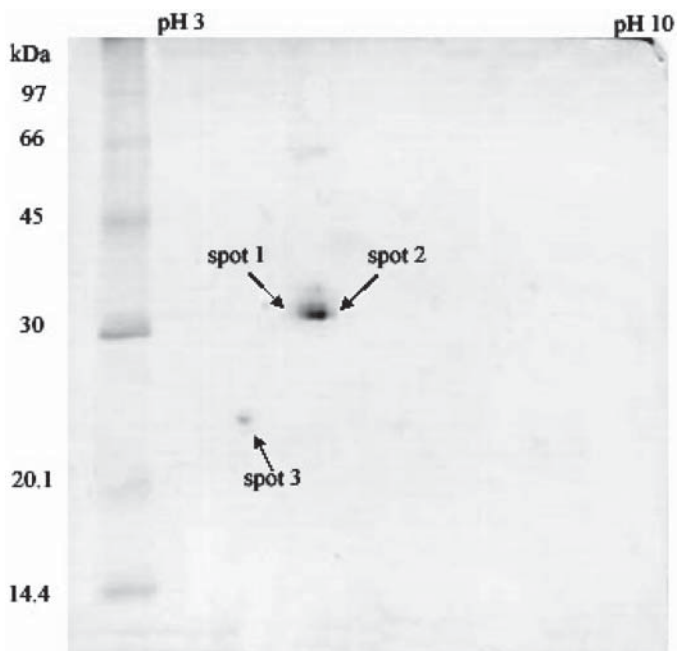

Figure 3 2D-PAGE of the P3 protein from hemolymph of *C. belcheri.*

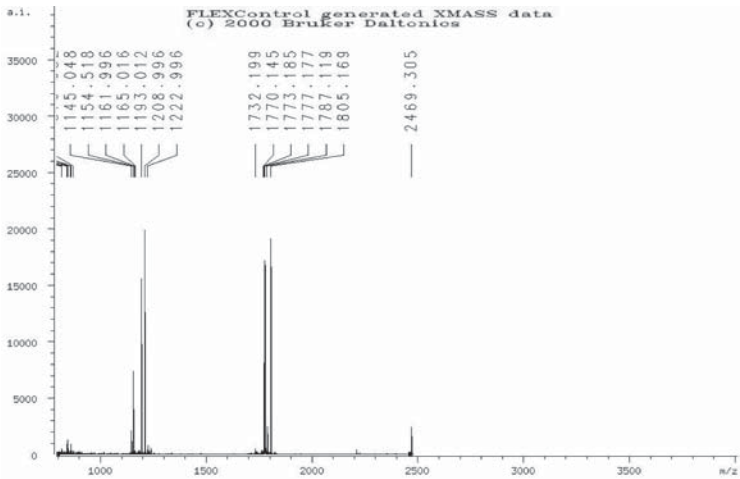

Figure 4 Mass spectrum for spot 1 (30.5 kDa) by MALDI-TOF.

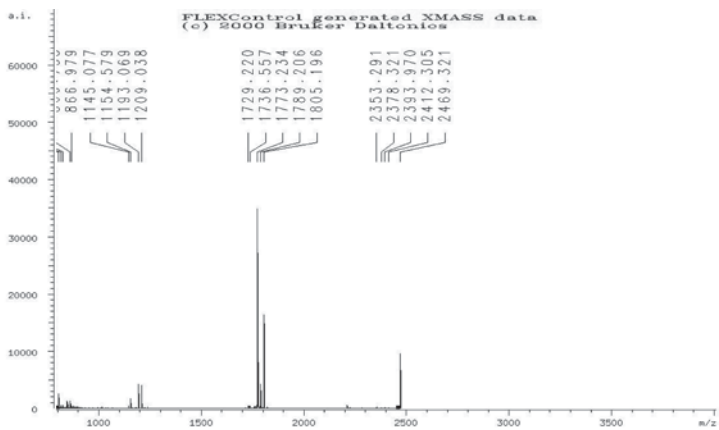

Figure 5 Mass spectrum for spot 2 (30.5 kDa) by MALDI-TOF.

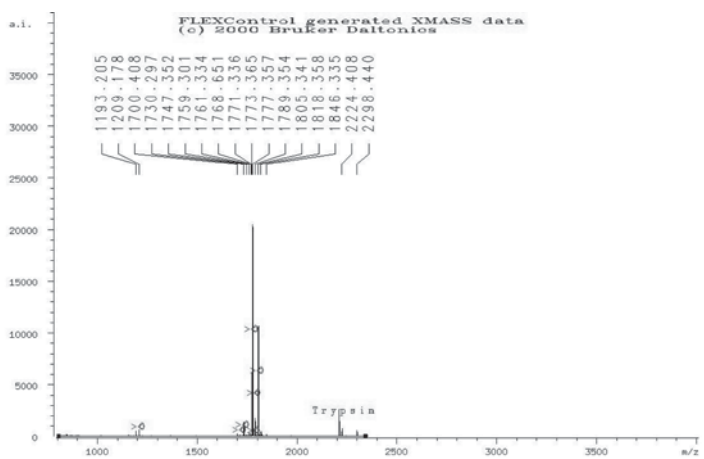

Figure 6 Mass spectrum for spot 3 (25.0 kDa) by MALDI-TOF.

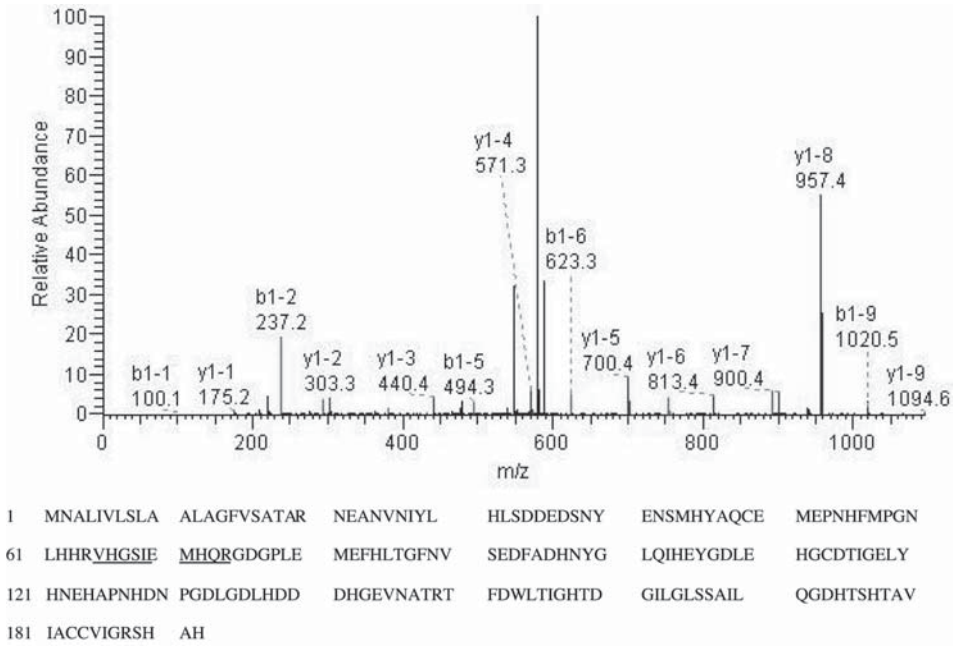

Figure 7 LC-MS/MS mass spectrometry spectrum of 30.5 kDa matching with hemocyte extracellular superoxide dismutase from *C. gigas*. Mass and sequences of tryptic peptide mapping to the protein sequence are underlined.

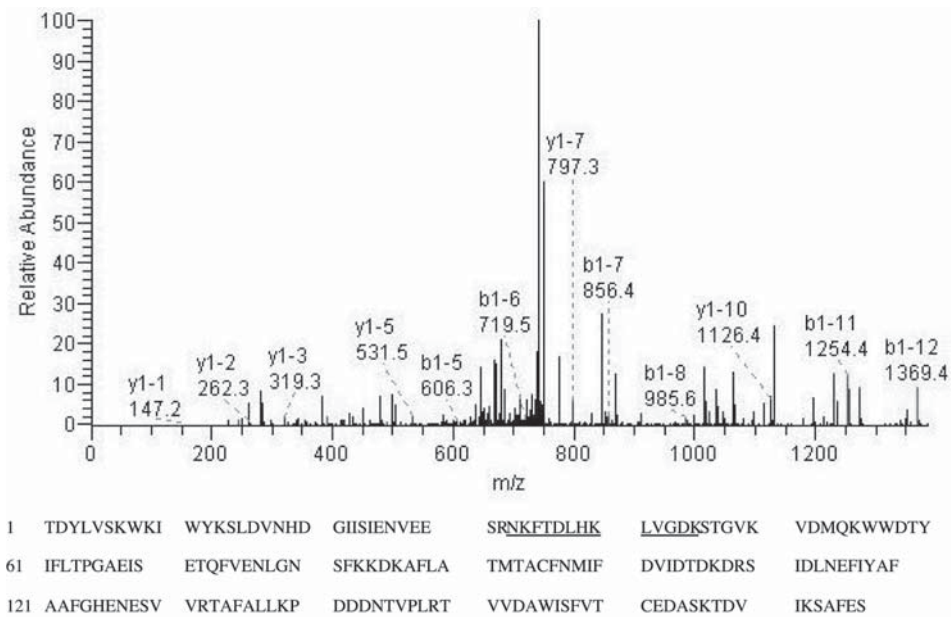

Figure 8 LC-MS/MS mass spectrometry spectrum of 25.0 kDa matching with sarcoplasmic calcium-binding protein. Mass and sequences of tryptic peptide mapping to the protein sequence are underlined.

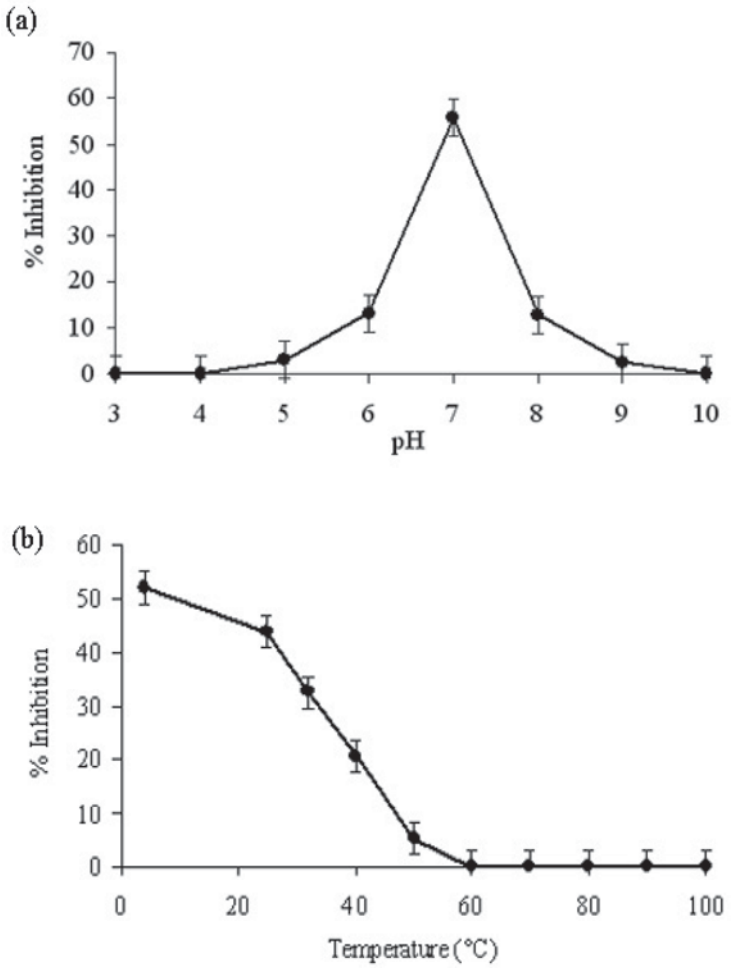

Figure 9 Effects of pH (a) and temperature (b) on antibacterial test against *V. parahaemolyticus* of purified protein P3.

Table 2 Effects of metal ions on antibacterial activity of protein P3.

Buffer	Percentage of inhibition
0.01 M Tris-HCl, 0.15 M NaCl, pH 7.1	85.30
0.01 M disodium-EDTA	17.82
0.01 M $CaCl_2$	73.00
0.01 M $MgCl_2$	17.26
0.01 M $MnCl_2$	19.38

DISCUSSION

Antibacterial activity has previously been described in a wide range of mollusc species such as oyster (*C. virginica*), mussel (*Mytilus edulis* and *Geukensia demissa*), muricid mollusks (*Dicathais orbita*) and sea hare (*Dolabella auricularia*) (Anderson and Beaven, 2001; Benkendorff *et al*., 2001; Iijima *et al*., 2003). In most of the species studied, the hemolymph, egg

mass or the whole body have been tested for activity. The antibacterial protein might act as an important function in the first step of defense against pathogenic microorganisms. The present study demonstrates the presence of antibacterial protein in hemolymph. A preference for recognition of *Vibrio* spp. was also observed in hemolymph of the Eastern oyster, *C. virginiga* (Timplin and Fisher, 1989) and the horse mussel (Tunkijjanukij and Olafsen, 1998).

The P3 protein purified from hemolymph of oysters (*C. belcheri*) by a gel filtration chromatography exhibited a wide range of antibacterial activity against Gram-negative bacteria, *Vibrio* spp. The antibacterial protein as indicated by SDS-PAGE, 2D-PAGE and LC-MS/MS, consisted of two bands with molecular weight of 30.5 and 25.0 kDa (Figure 2) and with pI 3.0 and 5.0, respectively. (Figure 3) Results from MALDI-TOF supported the mass of SDS-PAGE. In gel filtration, the purified P3 protein showed a smaller molecular mass than in SDS-PAGE, which reflects that the native P3 protein behaves as a smaller molecule in the native conformation. Similarly, the subunit of lectin from manila clam under reducing conditions were larger than molecular mass in gel filtration (Bulgakov *et al*., 2004).

Amino acid analysis of two subunits of the P3 protein by LC-MS/MS compared against nrFasta database revealed that the 30.5 kDa protein was homologous to hemocyte extracellular superoxide dismutase from *C. gigas* and the 25.0 kDa protein was homologous to sarcoplasmic calcium-binding protein.

Hemocyte extracellular superoxide dismutase, originally discovered by McCord and Fridovich (1969), occurs as different metalloproteins with different cellular distributions. Invertebrates maintained in anoxic conditions also increase SOD activities to withstand the transition from anoxia to normoxia and the subsequent burst in the production of reactive oxygen species (ROS). Many marine invertebrates produce ROS. Bivalve molluscs produce ROS in response to xenobiotics and also important in the cell-mediated immune response of mollusks to both prokaryotic and eukaryotic pathogens (Michael, 2006).

The Antarctic scallop, *Adamussium colbecki*, has high activities of SOD in its gills and also exhibits seasonally invariant antioxidant capacities (Regoli *et al*., 2002). The purified SOD showed optimum temperature of 35°C and was temperature stable up to 45°C within 30 min. The effect of temperature on some SODs activity showed an increase in activity with increase in temperature up to 37°C (Cui Luan *et al*., 2004). Huvet *et al*. (2004) reported the transcripts clustered into categories corresponding to energy generation or immune function were fully sequenced and found to encode homologues of the following proteins: cavortin (AY551094), no significant homology of sequence was found in databases (E value >0). Furthermore, cavortin, recently characterized as a self-aggregating haemolymph glycoprotein in mussel (called Pernin) (Scotti *et al*., 2001) and oyster (*C. gigas*) (Huvet *et. al*., 2004) has a superoxide dismutase (SOD) domain. Cavortin which are suggested to act in cellular host protection against ROIs displayed a similar reduction in mRNA levels in R and S progeny in response to *Vibrio* inoculation.

Sarcoplasmic calcium-binding protein (SCP) are acidic cytosolic proteins found in invertebrate nerves and muscle. All invertebrate SCP's has a low isoelectric point. Recently, the amino acid sequence of four kinds of SCPs of two classes (crustacea, mollusc) from their sequence homology (Tomoyoshi *et al*., 1984). Calcium-binding protein are presumed to play an important role in calcium mediated signal transduction (Leonard *et al.*, 1997).

Renesto *et al.* (2005) reported the major challenge in the post-genomic era will be to determine whether these predicted proteins are

expressed. Proteomic approach, which relies on two-dimensional gel electrophoresis (2D-PAGE) coupled with liquid chromatography mass spectrometry (LC-MS/MS), is a powerful tool. Although widely used, it is time-consuming, laborious and has some limitations in dealing with very large or small proteins, proteins having extreme pI, and membrane or low-abundance proteins.

The antibacterial activity of P3 protein against *V. parahaemolyticus*, which composed of SOD and SCP was stabilized at pH 6-8 and temperature between 4° to 30°C. In addition, the activity of P3 was required calcium ion. Calcium is a second messenger in many different cell types with fluctuations in intercellular free Ca^{2+}, triggering a vast array for physiological and biological responses in the cell (Leonard *et al.*, 1997). The major metal ion in the seawater consisted of calcium, magnesium, litium and manganese. They plays important roles in the catalysis of a large number of enzyme and protein in immune-like response of invertebrate (Xiao *et al*., 2002). Bulgakov *et al*. (2004) demonstrated that the lectin antibacterial protein of the manila clam hemolymph were fully active at normal seawater temperatures and activity decreased dramatically above 30°C. The incubation of the lectin at 90°C led to irreversible denaturation was pH-dependent. The activity of protein was stable between pH 6 to 9 which is in agreement with this activity of oyster antibacterial protein.

The promising results from other investigators showed involvement of these proteins related to toxic alleviation or antibacterial activity that need further investigation in oyster *C. belcheri*. Further purification of the P3 protein is necessary in order to separate SOD and SCP to elucidate their antibacterial activity.

CONCLUSION

This study showed that protein purified from hemolymph of *C. belcheri* could inhibit certain *Vibrio* species commonly cause disease in marine animals. The amount of purified P3 protein at 64 μg exhibited strong antibacterial effect against *V. parahaemolyticus*, *V. cholerae*, *V. alginolyticus* and *V. harveyi* into 95%, 91%, 86% and 62%, respectively. The P3 protein shows two bands in SDS-PAGE with molecular weights of 30.5 and 25.0 kDa. The amino acid sequence was analyzed by LC-MS/MS and comparison with nrFasta database gave hemocyte extracellular superoxide dismutase and sarcoplasmic calcium-binding protein corresponding to immune response of molluscs to both prokaryotic and eukaryotic pathogens.

ACKNOWLEDGEMENTS

The research is supported by the Center for Agricultural Biotechnology through the fund from Subproject Graduate Study and partially supported by the Graduate School, Kasetsart University, Thailand.

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