Protease associated with lectin produced by *Perkinsus olseni* infected Manila clams (*Ruditapes philippinarum*)

Y.-S. Kang¹, K.-S. Choi⁴, Y.-B. Chung³, S. Kim⁵ and M. Cho¹, ² *

¹Departments of Biochemistry, ²Institute of Medical Science; ³Department of Parasitology school of Medicine, ⁴School of Applied Marine Science, College of Ocean Science; ⁵Faculty of Biotechnology, College of Applied Life Sciences, Cheju National University, Jeju 690-756, Korea.

Abstract

A specific protease (MCLAP) associated with manila clam lectin (MCL) of molecular mass 43 kDa, was purified and characterized from the *Perkinsus olseni* infected manila clam *Ruditapes philippinarum*. It was purified by affinity chromatography followed by gel filtration. From all the protease inhibitors tested, MCLAP was only inhibited by PMSF indicating that it is a serine protease. Co-immunoprecipitation using antibody against MCL revealed that the protease was associated with MCL. MCLAP may be involved in penetration of connective tissue (matrix) but not in nutrient digestion as it degraded collagen but not BSA. The inducible protease associated with MCL might be a major component of innate immunity in *R. philippinarum*.

Introduction

*Perkinsus* species parasitize some commercially important marine molluscs with noteworthy infections occurring on the east coast of the United States (Soniet, 1996), Korea (Park & Choi, 2001), Australia (Lester & Davis, 1981), and Spain (Figueras et al., 1996). Special attention has been given to *Perkinsus* spp. infection of marine bivalves owing to its association with tissue damage and host mortality (Montes et al., 1995). Invertebrate animals lack an adaptive immune system and have developed various defence systems (so-called ‘innate immunity’) that respond to common antigens on the surface of potential pathogens (Medzhitov & Janeway, 2000). As in many invertebrates, host defense in bivalves is largely non-specific, based on the activity of hemocytes circulating in the soft tissues (Cheng, 1996). Our previous studies have demonstrated that one of the host defence systems of *R. philippinarum* is the secretion of lectins, which agglutinate pathogens (Alexander et al., 2004). The ability of these lectins to distinguish between self and non-self is one of the characteristics of innate immunity (Moller-Kristensen et al., 2003). One of the major recognition molecules is mannose-binding lectin (MBL), a glycoprotein of the collectin family (Malhotra et al., 1992). MBLs detect pathogen-associated molecular patterns (PAMPs) and promote destruction of pathogens by activating the effector mechanisms of the complement system.

* Corresponding author’s E-mail: moonjcho@cheju.ac.kr

(Holmskov et al., 2003). MBL-controlled complement activation depends upon associated serine proteases, referred to as MBL-associated serine proteases or MASPs: MASP-1 (Matsushita & Fujita, 1992), MASP-2 (Thiel et al., 1997), MASP-3 (Dahl et al., 2001). The binding of MBL-MASPs to carbohydrates forms a third complement pathway termed the ‘lectin pathway’ (Thiel, 1992). MBL-mediated pathways assist in the elimination of pathogens and are important in innate immunity.

Invertebrates do not express immunoglobulin antibodies, and many studies have indicated that their lectin-mediated complement immune systems may have evolved before antibody-based complement activation mechanisms (Matsushita & Fujita, 1996, Nair et al., 2000). Evolutionary homologues of complement C3 have already been identified in both sea urchins and tunicates (Al-Sharif et al., 1998, Nonaka et al., 1998). However, lectin-mediated complement pathways have not yet been investigated in bivalve molluscs such as R. philippinarum.

We were interested in whether the purified MCL associated with the protease might be involved in innate immunity. In the present study, we purified and partially characterized the protease from R. philippinarum infected with P. olseni.

**Materials and methods**

**Sample preparation**

Manila clams infected with P. olseni were purchased from a local market, while uninfected clams were collected from the Gymnyeng, north east of Jeju Island. Crude extract prepared from whole tissue was centrifuged at 2800Xg and the supernatant was collected. Clam remains, which contained gills, mantle, and the visceral mass, were incubated in fluid thioglycollate medium (FTM), according to Wilson-Ormond (1993). After incubation for one week, the tissues were digested in 2 M NaOH, as described previously (Choi et al., 1997), and the presence of Perkinsus hypnospores was examined using a light microscope.

**Purification of protease associated with MCL lectin (MCLAP)**

The supernatant extracted from infected manila clams, was partially purified by mucin-sepharose CL-6B matrix (Alexander et al., 2004) 50 mM GalNAc in TBS-Ca was also used as bound protein eluting buffer. The bound protein fraction was then loaded in sephacryl S-200 HR column (Amersham Pharmacia) fitted with HPLC (Shimadzu) system. Protein was eluted with PBS, pH 7.4. The molecular mass was determined from the relative elution volume of the protein standard.

**SDS PAGE**

The molecular weight and homogeneity of MCLAP were tested by 12% SDS PAGE according to the method of Lammeli (1970) by comparing the relative mobility of the protein mass marker (Bio-Rad). The proteolytic activity of MCLAP was shown by 12% SDS PAGE containing 0.2% gelatin (gelatin SDS-PAGE). The molecular weight of MCLAP was also determined using protein standards (Bio-Rad).
Co-immunoprecipitation and types of purified protease

Crude extract of infected manila clams was incubated with lectin antibody for 1 h at 4°C. Protein A agarose beads equilibrated with PBS were then added and the mixture was incubated for 2 h at 4°C. The bound protein was separated from the beads by 10 mM glycine-HCl buffer, pH 2.5. 100 mM TBS, pH 8.0 was added to the bound protein and 12 % gelatin SDS-PAGE was carried out.

Several protease inhibitors (1 mM EDTA, cystatin and PMSF; 10 μM pepstatin and leupeptin) were separately added to purified protease and incubated for 10 min at room temperature. The samples were then loaded on 12 % gelatin SDS-PAGE.

Results and discussions

Previously, MCL was purified and its binding capacity with the hypnospore of Perkinsus olseni was reported (Alexander et al., 2004). MCL was found only in Perkinsus infected clams and that synthesis was triggered in hemocytes upon infection (Kim et al., 2006). We observed that body lysates from infected animals seemed to be more proteolytic than those derived from uninfected animals. Many studies have shown that pathogens secrete extracellular materials in response to host defence mechanisms. Some of the most interesting of these pathogen-derived secretions are proteases, which are capable of degrading proteins and are important in the host's immune defence for facilitating pathogen invasion (Mckerrow et al., 1985). In this study we aimed to quantify the differences in proteolytic activity between body lysates of infected and uninfected clams.

First we investigated whether the increased proteolytic activity was due to proteases inherent in Perkinsus cells, by analyzing purified lysates derived from P. olseni extracted from manila clams. Figure 1 shows that there are more protease bands in the lysate of Perkinsus infected manila clams than uninfected specimens and again such bands were not obtained in Perkinsus itself. In previous studies we used mucin-sepharose CL-6B to purify MCL from infected manila clams, and found that the purified MCL was contaminated with protease. In the present study, after affinity purification both eluting buffer TBS-EDTA and TBS-Ca containing GalNAc gave rise to a 43 kDa protein band with others. MCLAP was further purified by gel filtration. The molecular weight of MCLAP was 43 kDa suggested by gel filtration (Figure 2), SDS-PAGE (Figure 3) and gelatin SDS-PAGE (Figure 1). Purified protease was not affected by various protease-inhibiting agents, such as EDTA (metallo-), leupeptin (trypsin-
like serine and some cysteine-), pepstatin (aspartic-), and cystatin (cysteine-) (Figure 4). The purified protease was inhibited by PMSF (Figure 4), which indicated that MCLAP was a serine protease. MCLAP only degraded collagen but not BSA (data not shown) that is the protease may be involved in penetration of connective tissue (matrix) but not in nutrient digestion. From the above result it can be concluded that clams also produce proteases with lectin in response to infection, presumably to destroy pathogenic cells and structures. Significant increases in lysozyme activity have been reported in the hemolymph of manila clams challenged with the pathogen *Vibrio tapetis* (Allam et al., 2000).

There are two possible explanations for the results, either the protease contained a lectin domain, or the protease was associated with MCL. Detection of the protease in the affinity fractions by GalNAc pointing out that the protease might have a receptor for GalNAc or be associated with MCL (Figure 5) In western blot and gelatin SDS-PAGE analysis, purified MCLAP and MCL behaved independently during gel filtration (Figure 2, 3), which indicated that their binding avidity may not be sufficient to survive gel filtration. The association of protease and MCL was not affected by other additional factors, such as Ca\(^{2+}\) and EDTA. The co-immunoprecipitation assay showed that MCLAP can be purified by MCL antibody from crude extract of infected species (Figure 6). These results suggested that MCLAP was physically associated with MCL and had no carbohydrate-domain. Therefore we used the name MCL-associated protease (MCLAP) for this protease.

Association with lectin is a common feature of MBL-associated serine protease (MASP). Such relationships of MBL and MASP are
likely to be useful during pathogenic infection. Once MBL recognizes a carbohydrate on the pathogen surface, the proenzyme MASP is converted to the active form, resulting in proteolytic activation of the complement components C4, C2, and C3 (Ji et al., 1993; Matsushita & Fujita, 1995; Endo et al., 2003). Turner (1996) reported that MBL binds to carbohydrates on the surfaces of microorganisms and particulate materials through its C-terminal Ca\(^{2+}\)-dependent lectin domain.
Despite the important differences between vertebrate and invertebrate immune systems, there are functional similarities in the defence molecules used in innate immunity. Homologues of C3 (Al-Sharif et al., 1998; Nonaka et al., 1998), and MASP proteins (Ji et al., 1997) have been identified in the sea urchin Strongylocentrotus purpuratus and the tunicate Halocynthia roretzi. These recent discoveries demonstrate that the lectin-mediated pathway plays a pivotal role in invertebrate innate immune systems. However, few examples are known of lectin-associated immune reaction in marine molluscs. Our previous experiments provided a few clues about lectin-mediated pathways in manila clams. Synthesis of Ca²⁺ dependent MCL was triggered by Perkinsus infection and the lectin bound to the surface of Perkinsus hypnospores (Alexander et al., 2004; Kim et al., 2006). This results implicate that MCL might be a functional homologue of MBL, and part of the lectin-mediated innate immunity of manila clams. MCL could serve as a recognition molecule, while the associated protease might play a role in defence mechanism against penetration by Perkinsus spp.

Acknowledgements

This work was supported by a grant (R01-2006-000-11316-0) from the Basic Research Program of the Korea Science & Engineering Foundation.

References


