Effects of β-1,3-glucan on the Activation of Prophenoloxidase Cascade in Penaeus japonicus Hemocyte

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Phenoloxidase (PO) in crustaceans is believed to be derived from its precursor prophenoloxidase (proPO), however, the conversion mechanism remains unclear. Possible roles of β-1,3-glucan, laminarin, of hemocyte lysate supernatant (HLS) from Penaeus japonicus in proPO activation were studied. PO activity was induced in HLS from Penaeus japonicus by treatment of laminarin, although laminarin had no influence on purified PO and proPO. p-APMSF and leupeptin, typical serine protease inhibitors, completely hampered this activation. Laminarin also enhanced activity of protease(s) in HLS, which specifically hydrolyzes synthetic peptides containing Arg in their P1 sites, while the activity was significantly suppressed by the serine protease inhibitors. These results indicate that the activation of proPO in hemocyte of Penaeus japonicus may be achieved after at least three steps: that is the recognition of β-1,3-glucan, activation of serine protease, and processing of proPO.

Key words: phenoloxidase, prophenoloxidase, β-1,3-glucan, laminarin, hemocyte, Penaeus japonicus, protease inhibitor

Phenoloxidase (PO) is a key enzyme in the reactions related to biological melanin formation, which catalyzes the hydroxylation of phenols to o-diphenols and deprotonation of o-diphenols to o-quinones. The discoloration of marine crustaceans during storage is attributed to melanogenesis resulting from the oxidation of phenols and their derivatives by this enzyme. In invertebrates, PO normally exists as its precursor, prophenoloxidase (proPO), and works in various aspects of life such as sclerotization, pigmentation, wound healing, and defense reactions.1-3) PO has been detected in hemolymph,1 cuticle,2 egg,4 and other tissues, while its gene expression is confirmed only in hemocyte1,5 where it plays a crucial role in invader(s). The activation of proPO in hemocyte or hemolymph is initiated by minute amounts of bacterial or fungal cell wall components (β-1,3-glucans, lipopolysaccharides, and peptidoglycans),1,2,6 and intermediated by several factors, including protease cascade reactions.1,3) The cascade reactions called "proPO System" are present in most invertebrates. Studies on proPO System have been mostly focused on the initial and final stages of the cascade: the recognition of the polysaccharides and the processing of the proenzyme.1,3) The specific proteins recognize each polysaccharides, triggering the activation of proPO System.1,3) The stimulation of proPO is achieved by limited proteolysis and removal of propeptide in proPO by serine protease.1,3) The properties of other factors in proPO System and the interactions among them still remain unclear. Moreover, the knowledge on proPO and its activating system has been accumulated mainly in insects5-10 and freshwater crayfish.11-13) Very little is known about proPO System in marine crustaceans.

Limited knowledge about proPO System in marine crustaceans is ascribed to instability of the precursor proteins involved, which are spontaneously activated during the process of experiments. The objectives of this work are to prepare these factors of marine crustacean Penaeus japonicus in their latent form in vitro and to show the involvement of β-1,3-glucan inducible factors in proPO activation.

Materials and Methods

Sample Animal
Kuruma prawn Penaeus japonicus (total weight: 20-30 g, total length: 15-18 cm) were purchased from a wholesale market in Kyoto, Japan. They were kept in an artificial sea water at 20°C until hemolymph was withdrawn.

Chemicals
Laminarin, L-3-(3,4-dihydroxyphenyl) alanine (L-DOPA), and p-amidinophenyl methanesulfonyl fluoride hydrochloride (p-APMSF) were purchased from Wako Pure Chemicals. Leupeptin, E-64, pepstatin, and all synthetic peptides were obtained from Peptide Institute Inc. o-Phenanethroline was purchased from Nacalai Tesque. All other reagents were of analytical grade.

Preparation of Hemocyte Lysate Supernatant (HLS)
HLS was prepared as described previously14 except that the hemocyte was sonicated in 50 mM HEPES, pH 7.8 including 5 mM MgCl₂ and 5 mM CaCl₂.

Assay for Phenoloxidase Activity
PO activity in HLS was measured spectrophotometricaly using L-DOPA as its substrate.15) A 50 µl aliquot of HLS (6 mg protein/ml) was added to a mixture of 50 µl of L-
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DOPA (15 mM/ml), 20 µl of laminarin (1 mg/ml), and 75 µl of 50 mM HEPES, pH 7.8, including 5 mM MgCl₂ and 5 mM CaCl₂. Then the mixture was incubated at 37°C and the absorbance at 490 nm was continuously monitored to quantify the concentration of dopachrome formed by PO.

Preparation for Purified proPO and PO

Purified proPO and PO were obtained by the method described previously. 14) In short, proPO was purified from HLS using Blue-Sepharose, and DEAE-cellulose, Hydroxypatite, and Butyl-Sepharose column chromatographies and proPO was converted into PO by SDS treatment.

Assay for Peptidase Activity and Protein Concentration

Assay of peptidase activity was performed using peptidyl-MCA as the substrate according to Morita et al. 16) A 50 µl portion of HLS (2 mg protein/ml) was mixed with 25 µl of laminarin (1 mg/ml) and 720 µl of 50 mM HEPES, pH 7.8, including 5 mM MgCl₂ and 5 mM CaCl₂, then the mixture was preincubated at room temperature for 10 min. The reaction was initiated by adding of 5 µl of 10 mM synthetic peptides to the reaction mixture and terminated after the incubation at 37°C for 30 min by adding 200 µl of 50% acetic acid. Fluorescent assays were performed at λex = 380 nm and λem = 460 nm, using fluorescence spectrophotometer (HITACHI 650-10LC). The amount of the substrate hydrolyzed was calculated from the value of AMC standard solutions. Protein concentration was determined by the dye-binding method of Bradford, 17) using a Bio-Rad protein assay kit, with bovine serum albumin as the standard.

Results and Discussion

Induction of PO Activity in HLS by Laminarin

As shown in Fig. 1, PO activity in laminarin-treated HLS increased about 7 times as high as that of control after incubation for 90 min. The activity after 45 min incubation increases much steeper than that of the earlier phase, suggesting that an induction phase is required for the activation of proPO. Possibly, several components, such as a recognition factor of β-1,3-glucan or several proteases involved in proPO System interact each other to activate proPO in this phase. We also tested the effects of lipopolysaccharide, peptidoglycan and bacterial cell wall on the activation as well but no meaningful values have been obtained in each case so far (data not shown).

Effect of Laminarin on PO Activity of Purified proPO and PO

Figure 2 shows the influence of laminarin on PO activities of purified proPO and PO. No induction of PO activity and no conversion of proPO into PO were observed by adding laminarin to the reaction mixtures, indicating hemocyte from Penaeus japonicus contains all the components indispensable for the proPO activation by β-1,3-glucan, since the interaction between laminarin and proPO or PO is indirect.

Inhibition of proPO System by Various Protease Inhibitors

Figure 3 shows that the activation of proPO in laminarin-treated HLS was almost completely inhibited by p-APMSF, leupeptin, and E-64. Little effects were observed in the reaction mixtures treated with a metalloprotease inhibitor (o-phenanethroline) and an aspartic acid protease inhibitor (pepstatin). Furthermore purified proPO was not activated by the laminarin treatment (Fig. 2). These results suggest that the activation of proPO in hemocyte can be intermediated by serine or cysteine type of proteases which may be, directly or indirectly, activated by β-1,3-glucan in HLS. Leupeptin that combines a cysteine protease inhibitor and a serine protease inhibitor completely inhibited the activation of proPO in HLS, while the inhibitory effect of E-64 was not well reproducible, indicating a serine protease must play a key role in proPO System in hemocyte.

Induction of Peptidase Activity in HLS by Laminarin

As shown in Fig. 4, laminarin improved hydrolytic efficiency of synthetic peptides. The activities toward Boc-Leu-Thr-Arg-MCA (5), Boc-Phe-Ser-Arg-MCA (6), Boc-Val-Pro-Arg-MCA (11), and Boc-Ile-Glu-Gly-Arg-MCA (12) in the presence of laminarin were twice the control. It is noteworthy that PI sites in these peptides were Arg. The same experiments were carried out under the reaction mixture containing 1 mM p-APMSF (Fig. 5). The stimulation of cleavage by laminarin was suppressed in the cases of the
peptides described above. The remarkable decrease of hydrolytic rate in Boc-Val-Pro-Arg-MCA (11) was particularly noteworthy in both laminarin treated and untreated HLS.

These results indicate that these peptides were mainly hydrolyzed by β-1,3-glucan-inducible serine protease(s) in HLS. In addition, P1 sites of all these peptides showed Arg residue. We cannot conclude here that proPO is activated by β-1,3-glucan-inducible protease(s) in HLS either directly or indirectly. HLS from crayfish, however, contains only a few components recognized by 3H-labeled serine protease inhibitor, and the number of the candidates for proPO activator is limited by the fact that their activities should be laminarin inducible. Prophenoloxidase activating enzymes (PPAE), which directly activates proPO with removal of propeptide, have been purified and characterized in several sources. All of them show similar properties to our results, in that they specifically cleave synthetic peptides wherein P1 site is Arg and their activity is severely hampered by serine protease inhibitors. Therefore the estimated protease is a highly potent candidate to activate proPO in HLS. p-APMSF allowed to decrease markedly both the rates of activation of proPO (Fig. 3) and hydrolysis of Boc-Val-Pro-Arg-MCA (11) (Fig. 5), indicating the possibility that PPAE of Penaeus japonicus acts on this peptide specifically. If these results reflect the properties of PPAE, the activities in laminarin-untreated samples (Fig. 4: 5, 6, 11, 12) and residual activities toward these peptides after p-APMSF treatment (Fig. 5) can be ascribed to other constitutive protease(s), spontaneously activated PPAE or other component(s) of proPO System, of which activity is not enough to activate proPO (Fig. 1).

As shown in Fig. 5, Boc-Phe-Ser-Arg-MCA (6), Z-Phe-Arg-MCA (9), and Suc-Leu-Leu-Val-Tyr-MCA (14) were cleaved by the laminarin inducible protease in HLS containing p-APMSF. These results show the existence of laminarin inducible protease(s) other than PPAE in HLS. On the other hand, Z-Phe-Arg-MCA (9) and Suc-Leu-Leu-Val-Tyr-MCA (14), whose rates of hydrolysis were declined by the laminarin treatment in the reaction mixture without p-APMSF (Fig. 4), were up-regulated by adding 1 mM p-APMSF. Boc-Val-Leu-Lys-MCA (15) and Suc-Gly-Pro-Leu-Gly-Pro-MCA (14) were also not significantly affected by the laminarin treatment. These results may reflect the complicated network of proPO System.

Our current view of the activation scheme of proPO from Penaeus japonicus is summarized in Fig. 6. proPO and its activation factors are located in hemocyte under normal conditions. They are released from hemocytes through an unknown mechanism, which is controversial even in other species, because the interaction between the hemocyte and β-1,3-glucan remains unclear (step 1). β-1,3-glucan recognition factor, a member of proPO System,
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Fig. 6. Activation scheme of proPO.
Explanation is described in Results and Discussion.

binds to glucoc chain and transmits the signal to other factors, including some kinds of serine proteases (step 2). proPO, a heterotetramer protein, is converted to PO through the processing by serine protease(s). However, this proenzyme can be also activated by SDS and methanol treatment, indicating that removal of the propeptide is not indispensable for its activation (step 3). PO activity is regulated by Ca²⁺ or Mg²⁺, of which optimum concentration is around 50 mM in both cases. The relations among these cations and activating factors remain unclear (step 4).

In this study, we successfully prepared proPO System of hemoocyte lysate supernatant (HLS) from crustacean, Penaeus japonicus in vitro and investigated the properties of the factors involved in proPO System. As a result, the existence of some laminarin inducible proteases involved in the system was detected. The clarification of the mechanism of this system is extremely significant not only in physiological fields such as defence reaction and sclerotization but also in food science since the activation of proPO triggers the melanogenesis in prawn during the storage. Further investigation is necessary to purify these factors as "latent form" and analyze the interactions among the molecules.

References