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Effects of leucine-enkephalin on alpha-naphthyl acetate esterase and myeloperoxidase activity in haemolymph of the scallop *Chlamys farreri*

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Abstract

The study was designed to investigate the effects of leucine-enkephalin (L-ENK) on the activity of alpha-naphthyl acetate esterase (ANAE) and myeloperoxidase (MPO) in haemolymph of the scallop *Chlamys farreri*. Our results indicate that MPO activity with DAB and DEA substrate solution was induced when the concentration of L-ENK was 5 and 50 μg/ml, and the intracellular and extracellular MPO activity with two substrate solutions was highest when the concentration of L-ENK was 50 μg/ml. However, both the intracellular and extracellular MPO activity of 1 μg/ml group was significantly lower than that of control group. It was also found that the intracellular and extracellular ANAE activity was induced by L-ENK. The supernatant ANAE activity was highest when the concentration of L-ENK was 50 μg/ml, but the resultant HLS ANAE activity was highest when the concentration of L-ENK was 5 μg/ml. Overall, the data suggests an involvement of L-ENK in the regulation of the defense systems of the scallop *Chlamys farreri*. L-ENK may function as messengers in the bidirectional interaction between the neuroendocrine and the immune systems.

Keywords: *Chlamys farreri*; Leucine-enkephalin; Alpha-naphthyl acetate esterase; Myeloperoxidase; Haemolymph

Introduction

Phagocytosis is an important function of invertebrate immunocytes (Ottaviani et al. 1992). In bivalve species, phagocytic cells can be activated by foreign particles or organisms and release oxidative chemicals. This response is often referred to as an "oxidative burst". The initial metabolite of the "oxidative burst" is superoxide anion. Furthermore, superoxide anion can be dismutated to hydrogen peroxide (H_2O_2), and be converted to other toxic reactive oxygen species (ROS). Hydrogen peroxide also interacts with myeloperoxidase (MPO) and halide to produce hypochlorous acid (HOCl), which is an even more potent microbicidal compound. In the "oxidative burst", all of metabolites play an important part in phagocyte-mediated killing of microorganisms (Adema et al. 1991; Volety and Chu 1995; Ordas et al. 2000).

It has been reported that myeloperoxidase (MPO) is present in some invertebrate phagocytes (Nakamura et al. 1985). The cellular immunity level is also related with Alpha-naphthyl acetate esterase (ANAE). ANAE is a lysosomal enzyme (Miyasaka et al. 1983; Mueller et al. 1975; Zicca et al. 1981), which has been considered to be a marker of T lymphocytes in peripheral blood and tissue sections (Beya et al. 1986; Miyasaka et al. 1983). It has been found that ANAE is present in the amebocytes of the fresh water snails, and the amebocyte enzyme histochemistry resembled that in human granuloma macrophages (McKerrow et al. 1985).

Mammalian-like opioid peptides have been found in invertebrates (Salzet and Stefano 1997; Breslin et al. 1993; Isaac et al. 1998; Stefano et al. 1998). It has been found that some opioid peptides function as endogenous messengers of the immune system, and participate in the regulation of the immune response (Hughes 1991; Stefano et al. 1995; 1990; 1993; 1991; Osman et al. 2003). Since the roles played by endogenous opioid peptides closely parallel those in vertebrates (Leung and Stefano 1987; Scharrer 1987; Stefano et al. 1989), the question arises whether the endogenous opioids affect the immune response of the scallop *Chlamys farreri*. The aims of the present study were to investigate the effects of leucine-enkephalin (L-ENK) on the activity of ANAE and MPO in the hemolymph of the scallop *Chlamys farreri*.

Material and methods

Experimental Animals

A batch of apparently healthy scallop *Chlamys farreri* was purchased from a commercial farm in Yantai, China. The scallops were acclimated in aerated seawater in 120 litre glass containers held at 21°C for at least three days prior to each experiment. Stocking densities were generally maintained at 20 molluscs per container.

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Preparation of Haemocytes and Haemocyte Lysate Supernatant

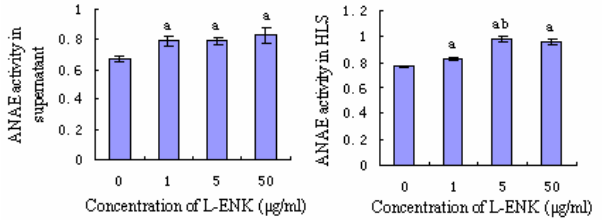
A haemolymph sample about 10 ml was drawn from the posterior adductor muscle of 10 molluscs with a 25-gauge hypodermic needle. L-ENK (Sigma Co, USA) was added into the haemolymph at three different concentrations (1, 5, and 50 μg/ml), and the control group was not received any L-ENK (0 μg/ml). Following incubation at room temperature for 1 h, the haemolymph were centrifuged at 3000 rpm for 10 min. According to a protocol described by Sung et al (2004), the supernatants were collected in order to determine the extracellular enzymatic activity, and the cell pellets were used in preparing the haemocyte lysate supernatant (HLS) which was used to determine the intracellular enzymatic activity. The haemocyte lysate supernatant (HLS) was prepared according to procedures modified by Sung et al (1998). Briefly, cell pellets were resuspended in diluted water, and then homogenized with a sonicator equipped with a microtip and centrifuged at 6000 rpm for 10 min at 4°C.

Assay of alpha-naphthyl acetate esterase (ANAE) and myeloperoxidase (MPO) activity

The MPO activity in the haemocyte lysate supernatant (HLS) and supernatant was separately determined with 3, 3′-diaminobenzidine (DAB, Sigma Co, USA) and diethanolamine (DEA, Sigma Co, USA) substrate solution according to procedures described by Coles et al (1994) and Schlenk D et al (1991). The ANAE activity in the resultant HLS and supernatant was determined with alpha-naphthyl acetic acid (Sigma Co, USA) substrate solution.

Statistics

Data are expressed as mean ± SD, and statistical analysis was performed by One-way analysis of variance (ANOVA) followed with comparison test. $P<0.05$ were considered significant and $P<0.01$ were considered very significant. The protein concentration of the resultant HLS and supernatant was determined according to procedures described by Lowry et al (1951), using bovine serum albumin as a standard.



(A) Extracellular ANAE Activity (B) Intracellular ANAE Activity

Figure 1: Extracellular and intracellular ANAE activity after haemolymph were added into different concentrations of L-ENK (n=6, 6 different measurements from the same extract). [a] P<0.01 compared with control group, [b] P<0.01 compared with 1 μg/ml group. The error bars represent the SD.

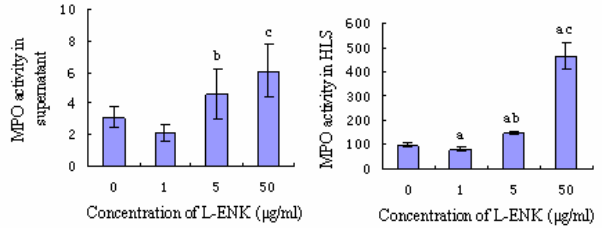
Results

The influence of L-ENK on ANAE activity with alpha-naphthyl acetic acid substrate solution

The results showed that the resultant HLS and supernatant ANAE activity of 1, 5 and 50 μg/ml group was very significantly higher than that of control group (P<0.01) (Fig.1.A, B). However, there was no significant difference between ANAE activity of 1, 5 and 50 μg/ml group (Fig.1.A). The resultant HLS ANAE activity was highest at the concentration of 5 μg/ml, which was 0.98±0.02 (U) (Fig.1.B). Moreover, intracellular ANAE activity of 5 μg/ml group was very significantly higher than that of 1 μg/ml group (P<0.01) (Fig.1.B).

The influence of L-ENK on MPO activity with DEA substrate solution

Using DEA as substrate solution, the supernatant MPO activity of 5 and 50 μg/ml group was not significantly higher than that of control group (P 0.05), and the supernatant MPO activity of 1 μg/ml group was not significantly lower than that of control group (P 0.05) (Fig.2.A). The resultant HLS MPO activity of 5 and 50 μg/ml group was very significantly higher than that of control group (P<0.01), but the resultant HLS MPO activity of 1 μg/ml group was very sign-



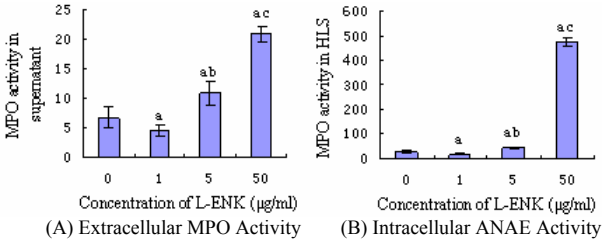
(A) Extracellular MPO Activity (B) Intracellular ANAE Activity

Figure 2: Extracellular and intracellular MPO activity with DEA substrate solution after haemolymph were added into different concentrations of L-ENK (n=6, 6 different measurements from the same extract). [a]P<0.01 compared with control group, [b]P<0.01 compared with 1 μg/ml group; [c]P<0.01 compared with 5 μg/ml group. The error bars represent the SD.

-ificantly lower than that of control group (P<0.01) (Fig.2.B). Both the resultant HLS and supernatant MPO activity was highest at the concentration of 50 μg/ml, which was 6.09±1.72 (U) and 464.99±53.69 (U), respectively (Fig.2.A, B). However, intracellular and extracellular MPO activity of 5 μg/ml group was very significantly higher than that of 1 μg/ml group (P<0.01), and MPO activity of 50 μg/ml group was very significantly higher than that of 5 μg/ml group (P<0.01) (Fig.2.A, B).

The influence of L-ENK on MPO activity with DAB substrate solution

We also examined intracellular and extracellular MPO activity using DAB as substrate solution. The results showed that both the resultant HLS and supernatant MPO activity of 5 and 50 μg/ml group was very significantly higher than that of control group (P<0.01). However, both the resultant HLS and supernatant MPO activity of 1 μg/ml group was very significantly lower than that of



(A) Extracellular MPO Activity (B) Intracellular ANAE Activity

Figure 3: Extracellular and intracellular MPO activity with DAB substrate solution after haemolymph were added into different concentrations of L-ENK (n=6, 6 different measurements from the same extract). [a]P<0.01 compared with control group, [b]P<0.01 compared with 1 μg/ml group; [c]P<0.01 compared with 5 μg/ml group. The error bars represent the SD.

control group (Fig.3.A, B). Both the resultant HLS and supernatant MPO activity was highest at the concentration of 50 μg/ml, which was 20.95±1.33 (U) and 475.32±17.90 (U), respectively (Fig.3.A, B). Moreover, intracellular and extracellular MPO activity of 5 μg/ml group was very significantly higher than that of 1 μg/ml group ($P<0.01$), and MPO activity of 50 μg/ml group was very significantly higher than that of 5 μg/ml group ($P<0.01$) (Fig.3.A, B).

Discussion

It has been found that the nervous and immune systems of invertebrates can exchange information through opioid peptides (Stefano et al. 1996; 1998; 1991). In our previous research, L-ENK participates in the regulation of nitric oxide synthase and catalase activity in the haemolymph of *Chlamys farreri* (Liu 2008; Liu and sun 2008). The present studies demonstrated that the MPO activity with DAB and DEA substrate solution was induced when the concentration of L-ENK was 5 and 50 μg/ml. However, the MPO activity was decreased when the concentration of L-ENK was 1 μg/ml. Both the intracellular and extracellular MPO activity with two substrate solutions was highest when the concentration of L-ENK was 50 μg/ml. The intracellular and extracellular ANAE activity was also induced by L-ENK. The intracellular ANAE activity was highest when the concentration of L-ENK was 5 μg/ml.

In phorbol myristate acetate-stimulated rat peritoneal macrophages, M-ENK increased H_2O_2 and NO production mainly through δ1 opioid receptor (Vujic et al. 2004). The haemocytes of invertebrate contain the μ3 subtype opioid receptor, which mediates the inhibition of cytokine-induced activation and chemotaxis by morphine and other opiates (Makman et al. 1996). In our previous experiments, we have found delta receptors and L-ENK on haemocytes of the scallop *Chlamys farreri* (Liu 2008). Thus we surmise that L-ENK may affect the activitiy of ANAE and MPO by binding with different opioid receptors on haemocytes. At 1 μg/ml L-ENK, L-ENK maybe binded with mu opioid receptors and MPO activitiy was inhibited. Although intracellular ANAE activity was highest when the concentration of L-ENK was 5 μg/ml, there is not significant difference with 50 μg/ml group. However, it is a complex network between the nervous and immune systems. The reason why different concentration of L-ENK has different function on the defense systems of the scallop *Chlamys farreri* needs to be further researched.

In summary, our results indicate that L-ENK participates in the regulation of the defense systems of the scallop *Chlamys farreri*. L-ENK may function as messengers in the bidirectional interaction between the neuroendocrine and the immune systems.

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