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RESEARCH ARTICLE

Immunostimulatory effect of ikan peda (fermented *Rastrelliger sp.*) water extract on IgM production by HB4C5 cells

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ABSTRACT

Ikan peda is one of Indonesia's traditional fermented food products. It uses mackerel fish (*Rastrelliger sp.*), also known as ikan kembung in Indonesia, as its main ingredient. It is made through fermentation by adding high concentration of salt in order to extend the shelf-life and bring out the desired flavor of the fish. Although fermented foods are known to be beneficial towards health, fewer studies have been reported regarding the actual health promoting potential of Indonesian fermented foods, especially ikan peda. Thus, the immunostimulatory effects of ikan peda extract were examined. Water-extract of ikan peda was tested to human-human hybridoma HB4C5 cells. It was found that ikan peda extract stimulates the production of IgM by HB4C5 cells according to ELISA results. Data from real-time RT-PCR result showed that ikan peda extract has the ability to increase IgM gene expression by 3-fold at the highest concentration treatment. Results from the heat and enzyme treatments showed that the active substance contained in the ikan peda extract was expected to be a heat-stable protein. Although, the active substance and the mechanism is yet to be determined, and can be considered for future experiments. Our findings suggest that consumption of ikan peda would contribute to the promotion of health.

Keywords: *Ikan peda; IgM; HB4C5 cells; immunostimulatory*

INTRODUCTION

Ikan peda is one of Indonesia's traditional fermented foods that uses mackerel fish (*Rastrelliger sp.)*, known as ikan kembung in Indonesia, as its main ingredient. It is made by adding large quantities or high concentration of salt in order to extend the shelf-life (preservation) and bring out the desired flavor and aroma of the fish. The fermentation process of ikan peda is categorized as spontaneous fermentation, where no starter culture is used. Thus, the microbes responsible for the fermentation grow spontaneously due to the condition of its environment, which is made suitable for its growth (Desniar *et al*., 2009). The type of fermentation used to make ikan peda is classified as high salt fermentation, which lasts for 1-2 weeks.

Fermented foods are known to be beneficial towards health (Astuti *et al*., 2000;

Parvez *et al*., 2006). However, not many researchers in Indonesia have actually studied this matter, especially in ikan peda. Therefore, this research was conducted to assess the immunostimulatory effects of ikan peda, specifically towards Immunoglobulin M (IgM) production by HB4C5 cells.

IgM is the largest antibody in human, which is produced by B-cells and belongs to the adaptive (specific) immune system. It is known as the first antibody to be produced as an initial response to antigen exposure (Alberts *et al.* 2002). The major site of production is in the spleen, where the plasmablasts responsible for IgM production are located. The cells used in this research was HB4C5 cells, which is a lung cancer specific monoclonal antibody producing human-human hybridoma cells. It is a fusion product of a human B lymphocyte from lung cancer patient and human fusion partner, NAT-30 (Sugahara *et al*., 2005).

MATERIAL AND METHODS

This research was conducted at the Laboratory of Animal Cell Technology, Ehime University, Japan. The Animal Cell Technology Laboratory provided all the equipment and materials (for example, Bio-Rad DC Protein Assay Kit; ELISA and PCR reagents; and HB4C5 cells). The sample used in this research was ikan peda (fermented mackerel) bought from local traditional markets in Jakarta, Indonesia.

An experimental approach was used as the method for this research, by inoculating ikan peda extract to HB4C5 cells culture *in vitro*. Afterwards, enzyme-linked immunosorbent assay (ELISA) and real-time reverse transcription polymerase chain reaction (RT-PCR) assay were used in this research to determine the effect of ikan peda extract on IgM production and to find out the gene expression level, respectively. In addition, heat treatment and enzyme treatment were performed on the sample to identify the characteristics of the active substances contained in ikan peda water extract.

Sample preparation

The whole fish (flesh, bones, and head) was chopped into little pieces and then boiled in 300 mL of distilled water using autoclave for 2 hours at 105°C. After boiled, the sample was let to cool at room temperature. The sample solution was transferred into centrifuge tubes and then centrifuged for 20 minutes with a speed of 550 × *g* at 4°C. The supernatant was then filtrated using a 3 µm cellulose acetate membrane and a syringe. To reduce the salt content, the filtrate was dialyzed using a 500 Da mw-cut dialysis membrane submerged in distilled water for approximately 2 days at 4°C. After dialysis, the pH of the sample was adjusted to ± 7.4. The sample was sterilized using a 0.22 µm filter and stored in several 1.5 mL microtubes at −35°C until it was used for further analysis.

Enzyme treatment

Ikan peda water extract was mixed with 100 µL of proteinase K enzyme solution (Fujifilm Wako Pure Chemical, Osaka, Japan). The enzyme used had a concentration of 5 mg/mL and was already sterilized using a 0.45 µm filter. The mixture was then incubated for 20 hours at 37°C. After incubation, the enzyme was deactivated by heat treatment at 100°C for 5 minutes, then cooled until it reached room temperature. The sample was then centrifuged for 10 minutes with a speed of 160 × *g* at 4°C. The supernatant was transferred into 1.5 mL microtubes and stored again in −35°C until used.

Heat treatment

Ikan peda extract was heated at 100°C for 30 minutes, then cooled until it reached room temperature. The sample was then centrifuged for 10 minutes with a speed of 160 × *g* at 4°C. The supernatant was transferred into 1.5 mL microtubes and stored in −35°C until used.

Determination of sample concentration (protein assay)

The protein concentration in the sample was measured using Bio-Rad (Hercules, California, USA) DC Protein Assay Kit. Standard solution for protein assay was prepared by mixing Bovine Serum Albumin (BSA) dan 2 mg/mL Phosphate Buffer Saline (PBS), making several concentrations (0, 200, 400, 600, 800, 1000, 1200, 1400, 1600, 1800, 2000 µg/mL). After that, 5 µL of each standard concentration and the sample solution were added into each well on the 96-well plate. Each well was added with 25 µL of reagent A, followed by 200 µL of reagent B. Then, the plate was stirred using a plate mixer for 15 minutes with a speed of 25 × *g* to ensure that all the solutions were mixed thoroughly. After 15 minutes, the absorbance was measured using a microplate reader at 655 nm wavelength with 415 nm as the reference.

Determination of IgM concentration using ELISA

a. HB4C5 cell culture condition

Ikan peda extract was diluted using a 100 mM sterile NaPB (sodium phosphate buffer) into several dilution factors (5^0, 5^1, and 5^2). Then, 100 µL sample from each dilution factor was inserted into the 96-well plate. One hundred microliter of control sample, which was 10 mM NaPB, was also inserted into the same 96-well plate. After that, the medium for HB4C5 cells was prepared. The medium used was 20 mL of 2× ERDF mixed with 10 µg/mL insulin, 20 µg/mL transferrin, 20 µM ethanolamine, and 25 µM sodium selenite (ERDF-ITES), each for 200 µL, following the methods performed by Sugahara *et al.* (2005). The HB4C5 cells that was previously cultured with 1x ERDF-ITES was centrifuged, then the supernatant was discarded. The remaining pellet was suspended with 2x ERDF-ITES medium that was previously prepared and the cell concentration was adjusted to 1.0 × 10^5 cells/mL. Afterwards, 100 µL of the cell suspension was inserted into the 96-well plate that had been filled with the samples, then incubated for 6 hours in an incubator at 37°C with 5% CO_2 level prior to conducting ELISA.

b. ELISA

ELISA was performed according to following steps. (1) **Coating** (first antibody): 100 µL of anti-human IgM antibody (CAPEL 550732) that was diluted 1,000 folds using 50 mM carbonate buffer (pH 9.6), was added into each well in the 96-well ELISA plate (only the standard and sample wells). Plate was then covered with plastic sticker and incubated for 2 hours at 37^OC. (2) **Blocking**: After coating, the 96-well plate was washed 3 times using 0.05% T-PBS. All the wells were blocked with 300 µL of 5% skim milk-PBS and incubated for 2 hours at 37^OC. (3) **Sampling**: Standard solution which consisted of 5% skim milk-PBS and 100 ng/mL IgM was prepared in several concentrations (0, 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, and 70 ng/mL). After the 96-well plate was washed 3 times using 0.05% T-PBS solution, the standards and culture

supernatant were added to the well for 50 µL each. Plate was then incubated again for 1 hour at 37^{O}C. (4) **Second Antibody**: Anti-human IgM antibody (Biosource International, AHI0604) was diluted by 20,000 folds using 5% skim milk-PBS to create secondary antibody. After the 96-well plate was washed 3 times using 0.05% T-PBS, 100 µL of secondary antibody was added into the standard and sample wells. The plate was incubated again for 1 hour at 37^{O}C. (5) **Coloring reaction**: Coloring reagent was prepared by mixing 0.05 M citrate buffer (pH 4.0), distilled water, and 6 mg/mL ABTS with a ratio of 10:9:1, respectively. After the 96-well plate was washed 3 times using 0.05% T-PBS, 100 µL of coloring reagent was added into the standard and sample wells. After coloring reaction had occurred (around 10-20 minutes), 100 µL of 1.5% oxalic acid was added to each well in order to terminate the coloring reaction. Lastly, the absorbance was measured using a microplate reader at 415 nm wavelength with 655 nm as the reference wavelength.

Determination of gene expression level using Real-Time RT-PCR

a. HB4C5 cell culture

Ikan peda extract and control sample (10 mM NaPB) were added into mini petri dishes for 1 mL each. HB4C5 cell cultured with serum-free medium was centrifuged for 5 minutes with a speed of 160 × *g*. After centrifuged, the pellet was suspended in 2× ERDF-ITES medium and the concentration was adjusted to 5.0 × 10^5 cells/mL. Afterwards, 1 mL of cell suspension was added into each petri dish containing the ikan peda extract and control sample, then incubated in an incubator for 6 hours at 37°C. After incubation, each sample was transferred into 2 mL microtubes and centrifuged for 5 minutes with a speed of 160 × *g*. The supernatant was aspirated and each tube was washed with 1 mL sterilized PBS. After washing, the cells were centrifuged again and the supernatant was discarded.

b. RNA extraction

One milliliter of Sepasol RNA I Super G (Nacalai Tesque) was added into each previously prepared cells pellet in the microtube and then left at room temperature for 5 minutes. After that, 200 µL of chloroform was added to each tube, mixed well, and then centrifuged for 15 minutes with a speed of 12,000 × *g* at 4°C. After centrifuged, the mixture will form 3 layers (RNA, protein, and DNA layer). The RNA water layer (upper layer) was carefully transferred into a new 1.5 mL microtube. 500 µL of 2-propanol was added into each tube containing RNA, mixed, and then left at room temperature for 10 minutes. The tubes were then centrifuged again for 15 minutes with a speed of 12,000 × *g* at 4°C and the supernatant was discarded. Afterwards, 1 mL of 75% ethanol was added into each tube, gently mixed, and then centrifuged for 15 minutes with a speed of 12,000 × *g* at 4°C. The supernatant was carefully removed using a micropipette and the microtube lids were kept open for approximately 30 minutes to dry out the ethanol. After dried, the isolated RNA was dissolved in 15 µL DEPC water. One microliter of RNA solution was diluted in 99 µL of TE buffer, and then the RNA concentration and purity were measured

using UV spectroscopy machine. The ratio between the absorbance at 260 nm and 280 nm was used to evaluate the purity of the nucleic acid. A ratio around 2.0 is expected to show a pure RNA.

c. Reverse transcription

RNA suspension with a known concentration (µg/µL) was mixed with DEPC water and 0.5 µL of 10 µM Oligo dT primer to reach the RNA concentration of 1 µg (total volume = 9 µL). The RNA mixture was then heated for 5 minutes at 70^{O}C using a thermal cycler. After that, a mixture of reagent which consisted of 5 µL of 10 mM dNTP, 5 µL of 5× buffer, 0.5 µL of RNase inhibitor, 0.2 µL of MMLV-reverse transcriptase (Promega), and 0.3 µL of DEPC water were added into the RNA mixture (to reach final volume = 20 µL). The mixture was then heated (reacted) using the thermal cycler for 1 hour at 42°C to create cDNA molecule.

d. Real-time PCR

The RT-PCR reagent used for 1 sample was consisted of 10 µL THUNDERBIRD SYBR qPCR Mix (Toyobo), 1 µL forward primer, 1 µL reverse primer, and 6 µL ultrapure water. The amount of reagent prepared was adjusted with the number of samples to be tested. After that, 18 µL of the reagent were mixed with 2 µL of each previously prepared cDNA. A total of 20 µL of the reagent and sample mixture were inserted into a 96-well plate special for PCR use, and then spin for 5 minutes. Afterwards, Real-Time RT-PCR was performed. The PCR result was analyzed using *StepOnePlus Real-time PCR System* (Applied Biosystem) and the relative gene expression was analyzed using the *StepOne Software* v2.1 (Applied Biosystem).

Statistical analysis

Data obtained from the determination of IgM concentration and data from the gene expression levels were analyzed using Microsoft Excel. The significance of these data were statistically analyzed using Tukey's t-test at * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

RESULT AND DISCUSSION

HB4C5 cells were inoculated at a concentration of 1.0×10^5 cells/mL in ERDF-ITES medium that was supplemented with various concentrations of ikan peda extract. After 6 hours of incubation, the amount of IgM in each culture medium was measured using ELISA.

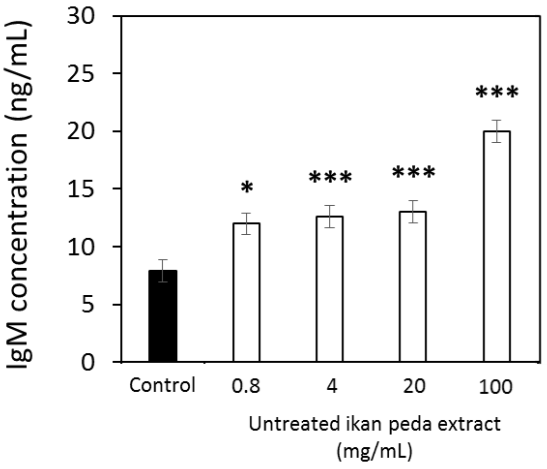

Figure 1. The effect of ikan peda extract towards the IgM concentration in HB4C5 cells. Data shown are representative of two independent experiments with similar results. Each result is expressed as mean ± standard deviation (SD). Tukey's t-test was used to assess the statistical significance of the difference. Each value of *$p < 0.05$ or ***$p < 0.001$ is considered to be statistically significant.

As shown in Figure 1, the production of IgM by HB4C5 cells increased significantly ($p < 0.05$ and $p < 0.001$) as the concentration also increased.

The IgM production by HB4C5 cells increased by 12.1 ng/mL at the highest concentration compared to that of control.

In this research, it can be seen that the ikan peda extract possess an immunostimulatory effect. Based on the results of heat treatment and enzyme treatment (proteinase K), it was assumed that the active substance contained in ikan peda extract was a heat-stable protein that can withstand high temperature treatment (100^{O}C).

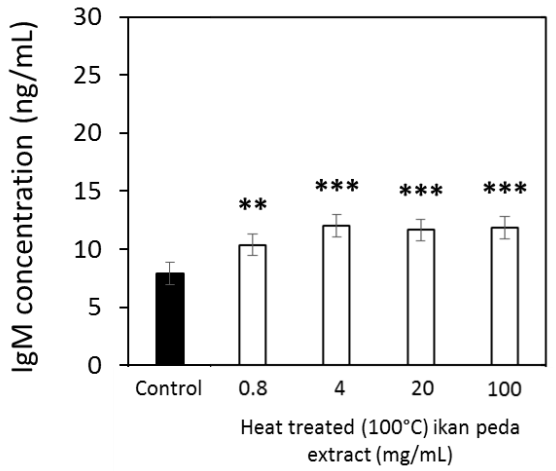

Figure 2. The effect of heat treated ikan peda extract towards the IgM concentration in HB4C5 cells. Data shown are representative of two independent experiments with similar results. Each result is expressed as mean ± standard deviation (SD). Tukey's t-test was used to assess the statistical significance of the difference. Each value of $^{**}p < 0.01$ or $^{***}p < 0.001$ is considered to be statistically significant.

This occurrence is shown in Figure 2, where a significant increase of the IgM concentration ($p < 0.1$ and $p < 0.001$) in ikan peda extract can be observed compared to that of the control sample, despite the high temperature treatment given.

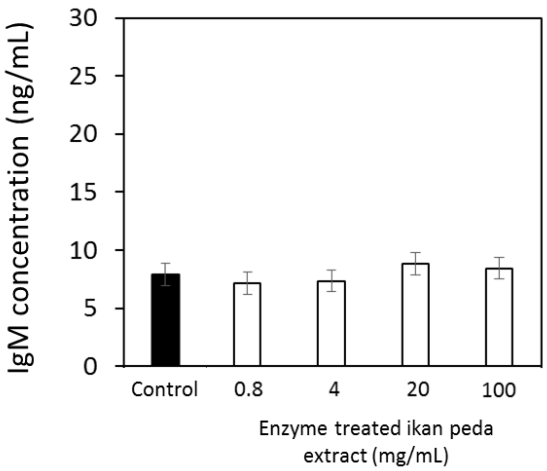

Figure 3. The effect of proteinase K enzyme treatment on ikan peda extract towards the IgM concentration in HB4C5 cells. Data shown are representative of two independent experiments with similar results. Each result is expressed as mean ± standard deviation (SD). Tukey's t-test was used to assess the statistical significance of the difference. Data did not show any significant differences.

Moreover, Figure 3 shows that enzyme treatment did not stimulate the IgM production significantly. These findings indicated that the active substance contained in ikan peda was some kind of a heat-stable protein.

It has been proven before (Figure 1) that ikan peda extract stimulates IgM production. Therefore, the effect of ikan peda extract towards the IgM gene transcription activity was also assessed in this research using the real-time RT-PCR method.

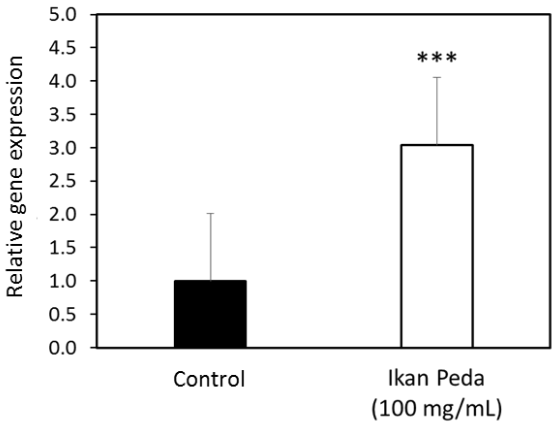

Figure 4. The relative gene expression of IgM in HB4C5 cells. Data shown are representative of two independent experiments with similar results. Each result is expressed as mean ± standard deviation (SD). Tukey's t-test was used to assess the statistical significance of the difference. The value of ***$p < 0.001$ is considered to be statistically significant.

Figure 4 shows that the relative gene expression of IgM in HB4C5 cells was significantly higher ($p < 0.001$) compared to that of the control sample. From both results, it can be said that ikan peda extract stimulates the IgM production in HB4C5 cells by increasing the relative gene expression of IgM. These results, are in accordance with the findings reported by Racine *et al*. (2011), also indicate that ikan peda extract has the potential to stimulate the adaptive (specific) immune system.

CONCLUSION

Ikan peda not only significantly stimulated IgM production by HB4C5 cells, but it also increased the relative gene expression of IgM itself. Based on the results of heat treatment and enzyme treatment, it can be concluded that the active substance contained in ikan peda extract is a heat-stable protein. Taken together, Ikan peda extract has great potential to improve the immune system. This potential is very useful because it can act as a stimulator for the body's defense system and may help protect against infection caused by foreign macromolecules and pathogens. However, detail identification of the active substance and its underlying mechanism in increasing IgM production by HB4C5 cells need to be studied further.

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