Proteose Peptide Fraction of Bovine Milk Depressed IgE Production

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Proteose peptide (PP) is a heat-stable and acid-soluble protein in milk whey. We reveal in this study the IgE production-suppressing activity of the PP fraction in bovine milk. The PP fraction suppressed IgE production by human myeloma cell line U266 cells by depressing the IgE mRNA expression. The suppressive activity of the PP fraction was facilitated by trypsin digestion. An oral administration of the PP fraction significantly decreased the levels of total and ovalbumin (OVA)-specific IgE in the serum collected from OVA-sensitized mice. Moreover, the serum levels of other Ig classes in OVA-sensitized mice were not affected by the intake of the PP fraction. The PP fraction suppressed the mRNA expression level of IgE in mice splenocytes collected from OVA-sensitized mice. Moreover, the B cell population in the spleen was decreased, while the T cell population was increased by administering the PP fraction. These results suggest that the PP fraction modified the B/T cell balance.

Key words: allergy; bovine milk; IgE production-suppressing factor; ovalbumin; proteose peptone

Bovine milk contains many kinds of functional substance, and some of them possess an immunostimulation effect. Casein, lactoferrin, and β-lactoglobulin stimulate immunoglobulin production by lymphocytes and hybridoma cells in vitro. Bovine milk fermented by some types of fermentation bacteria also contains bioactive substances originated by fermentation. Hosoda et al. have reported that bovine milk cultured with lactic bacteria possessed anti-carcinogenic or anti-mutagenic activity. LeBlanc et al. have also reported that peptides from fermented milk stimulated humoral immune responses. We have also revealed that a 19.0-kDa protein in fermented bovine milk stimulated immunoglobulin production in human hybridoma cells and human peripheral blood lymphocytes. This protein was originally derived from protease peptone component 3 (PP3) in bovine milk, and degraded by fermentation by 12 kinds of microorganisms. PP is the heat-stable and acid-soluble fraction in milk whey. Although there are such PP proteins as components 3, 5, and 8, the biological functions of each PP component have not yet been fully elucidated.

Homologous proteins have been characterized in the milk of such other species as the camel, llama, ewe, and caprine. However, PP3 has not been found in human milk. A synthetic peptide of 23 residues corresponding to the carboxyterminal 113 to 135 region of PP3 has been investigated with regard to its antibacterial properties. We have reported that PP3 had immunostimulating activity toward human hybridomas and peripheral blood lymphocytes.

Allergies have become a serious problem, especially for infants and children. IgE is an immunoglobulin concerned with the allergic response. Binding of the allergen to IgE captured on mast cells and basophiles induces the release of such chemical mediators as histamine and leukotriene from these cells. IgE is an initial factor of the allergic reaction, so we have screened many factors suppressing the IgE production of immune cells to assist in the development of anti-allergy products such as functional foods. This screening has revealed that a white sorghum (Sorghum bicolor (L.) Moench) bran extract suppressed IgE production by human myeloma U266 cells. We also found that the PP fraction of bovine milk suppressed IgE production by U266 cells. This study is focused on the allergy suppression activity of the PP fraction of bovine milk in vitro and in vivo.

Materials and Methods

Reagents. The anti-human IgE antibody and biotin-conjugated anti-human IgE antibody were purchased from Biosource International (Camarillo, CA, USA). Goat anti-mouse IgG, biotinylated goat anti-mouse IgG, goat anti-mouse IgA, and the horseradish peroxidase-streptavidin conjugate were from Invitrogen (Calsbad, CA, USA). BALB/c mice were from Japan SLC (Shizuoka, Japan), and the RPMI 1640 medium was purchased from Sigma (St. Louis, MO, USA).

Sample preparation. Fat-free bovine milk was heated at 90°C for 30 min. Following this heat treatment, the pH value was adjusted to 4.6 with 3 m HCl, precipitating the majority of caseins and denatured whey proteins. The precipitate was removed by centrifugation at 100,000 × g for 30 min. The resulting supernatant was dialyzed against a 10 mM sodium phosphate buffer (NaPB at pH 7.4), sterilized by passing through a 0.22-μm filter (Millipore, Billerica, MA, USA), and used as the protease peptone (PP) fraction.
Cells and cell culture. The U266 IgE-producing human myeloma cell line was used to assay the IgE production-suppression activity. U266 was obtained from ATCC. The U266 cells were pre-cultured in the RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS) at 37°C in a humidified atmosphere of 5% CO₂-95% air.

Assay of the IgE production-suppressing activity. The assay of the IgE production-suppressing effect of PP was performed in a 96-well culture plate. U266 cells were inoculated at 5.0 × 10⁴ cells/ml in the RPMI 1640 medium supplemented with 10 mg/ml of insulin, 20 μg/ml of transferrin, 20 μg ethanolamine, and 25 μg sodium selenite (ITIES-RPMI 1640) supplemented with PP. After 24 h of cultivation, IgE produced in the culture medium was measured by an enzyme-linked immunosorbent assay (ELISA), using the anti-human IgE antibody. Briefly, 1.0 μg/ml of the goat anti-human IgE antibody in a 50 mM carbonate-bicarbonate buffer (pH 9.6) was added to a 96-well ELISA plate (Nunc, Roskilde, Denmark) at 100 μl/well, and the plate incubated for 2 h at 37°C. After washing three times with 0.05% Tween 20-PBS (T-PBS), each well was blocked with 1.0% bovine serum albumin (BSA)-PBS for 2 h at 37°C. After this blocking reaction, each well was washed three times with T-PBS and then treated with 50 μl of the culture supernatant for 1 h at 37°C. After washing three times with T-PBS, 100 μl of the biotinylated goat anti-human IgE antibody diluted 1,000 times with 1% BSA-PBS was added to each well, and the plate incubated for 1 h at 37°C. After washing again three times with T-PBS, 100 μg/ml of the streptavidin-HRP conjugate (Invitrogen, Calsbad, CA, USA) diluted 4,000 times with 1% BSA-PBS was added to each well, and the plate incubated for 1 h more. The plate was again washed three times with T-PBS, 0.6 mg/ml of 2.2-azino-bis(ethylbenzothiazoline-sulfonic acid) diammonium salt (ABTS) dissolved in a 0.03% H₂O₂-0.05 M citrate buffer (pH 4.0) was added to each well at 100 μl/well for enzyme reaction, and the absorbance at 415 nm was measured after adding 100 μl of T-PBS and then reaction performed with MMLV-reverse transcriptase (Promega, Madison, WI, USA) and an oligo-(dT)₁₃ primer (Toyobo, Osaka, Japan). Denaturation at 95°C for 1 min was followed by primer annealing at 60°C for 1 min, extension at 72°C for 1 min, and a final extension phase of 7 min. The CDNA was subjected to real-time PCR with the Step One Plas system (Applied Biosystems, Foster City, CA, USA). The amount of mRNA for human IgE was normalized to that for GAPDH, and mRNA levels of IL-4 was normalized to that for β-actin. The specific primer sequences for each gene were as follow. Human IgE: sense, 5'-ATGACCTTACGACCCAC-3' and antisense, 5'-GGTTGTGTGTGCTGACCCAG-3'; human GAPDH: sense, 5'-CCATGACAACTTTGGCATCGTG-3' and antisense, 5'-GTGTGCTGTTGAATGCACAGAGAC-3'; mouse β-actin: sense, 5'-CATCCGGTAAGACCTTATGCCCAC-3' and antisense, 5'-ATGAGGCCACCGTAGAC-3'; mouse GAPDH: sense, 5'-AGACCTTACGACCCAC-3' and antisense, 5'-GGTTGTTGTGTGCTGACCCAG-3'; mouse IL-4: sense, 5'-TCTCGAATGTACCAGCCACAG-3' and antisense, 5'-AGACCTTACGACCCAC-3'.

Flow cytometric analysis. Splenocytes prepared from OVA-immunized mice were washed with cold PBS. The cell surface markers on the splenocytes were stained on ice with FITC-conjugated anti-mouse B220 and PE-conjugated anti-mouse CD3 (Biolegend, San Diego, CA, USA) antibodies for 30 min. After washing with cold PBS, the cells were analyzed by using a FACS Calibur flow cytometer (BD Biosciences).

Statistical analysis. Each result is expressed as the mean ± SD. Student’s t test was used to assess the statistical significance of the difference, p < 0.05, *p < 0.01**, and **p < 0.001*** being considered statistically significant.

Results and Discussion

Effect of the PP fraction on IgE production by U266 cells

We had already screened the substances in food that suppress IgE production to ease allergy. We evaluated the effect of the PP fraction in bovine milk. As shown in Fig. 1, the PP fraction reduced IgE production by U266 cells in a dose-dependent manner. The viability of the U266 cells was therefore not affected by the PP fraction at any dose (data not shown), and the PP fraction suppressed IgE productivity in the cells. Moreover, dialysis of the PP fraction showed that the IgE production-suppressing activity of this fraction was facilitated. This means that eliminating the low-molecular-weight substances (approx. <10 kDa) in the PP fraction facilitated the activity, and that the active substance was not of low molecular weight.

Preparation and culture of the spleen lymphocytes. Splenocytes were isolated from the spleen immediately after their excision for an ex vivo analysis. The splenocytes were strained through nylon mesh (40 mm pore size), and the collected cells were treated with a hemolysis buffer (155 mM NH₄Cl, 15 mM NaHCO₃, and 1 mM EDTA at pH 7.3). The splenocytes were obtained after washing twice with PBS and then inoculated in the RPMI 1640 medium supplemented with 5% FBS at 1 × 10⁶ cells/ml into a 96-well culture plate. Aftercultivating for 48 h, the culture supernatant was collected, and the Ig level determined by ELISA.

Measurement of immunoglobulin levels in sera. Sandwich ELISA was performed as previously described to measure the Ig levels in the sera and culture media. The OVA-specific IgE level was determined by using a mouse OVA-specific IgE detection kit (Dainippon Sumitomo Pharma, Osaka, Japan).

Determination of the gene expression levels. Total RNA was isolated from splenocytes by using Sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan) according to the manufacturer’s instructions. Total RNA (1 mg) was used as a template for a cDNA synthesis reaction performed with MMLV-reverse transcriptase (Promega, Madison, WI, USA) and an oligo-(dT)₁₃ primer (Toyobo, Osaka, Japan). Denaturation at 95°C for 1 min was followed by primer annealing at 60°C for 1 min, extension at 72°C for 1 min, and a final extension phase of 7 min. The cDNA was subjected to real-time PCR with the Step One Plas system (Applied Biosystems, Foster City, CA, USA). The amount of mRNA for human IgE was normalized to that for GAPDH, and mRNA levels of IL-4 was normalized to that for β-actin. The specific primer sequences for each gene were as follow. Human IgE: sense, 5'-ATGACCTTACGACCCAC-3' and antisense, 5'-GGTTGTGTGTGCTGACCCAG-3'; human GAPDH: sense, 5'-CCATGACAACTTTGGCATCGTG-3' and antisense, 5'-GTGTGCTGTTGAATGCACAGAGAC-3'; mouse β-actin: sense, 5'-CATCCGGTAAGACCTTATGCCCAC-3' and antisense, 5'-ATGAGGCCACCGTAGAC-3'; mouse GAPDH: sense, 5'-AGACCTTACGACCCAC-3' and antisense, 5'-GGTTGTTGTGTGCTGACCCAG-3'; and mouse IL-4: sense, 5'-TCTCGAATGTACCAGCCACAG-3' and antisense, 5'-AGACCTTACGACCCAC-3'.

Effect of PP on allergic model mice. Female 6-week-old BALB/c mice were kept under specific pathogen-free facilities. They were given free access to food and water, and the animal room was maintained under the following controlled conditions: temperature, 24°C; humidity, 55%; and 12-h light/12-h dark cycle. A specific allergic reaction against OVA was induced in the mice. After preliminary breeding for 1 week, the mice were orally given the dialyzed PP fraction or 10 mM NaPB as the control vehicle for 28 d. The protein concentration of the dialyzed PP fraction was 1,500 μg/ml, and each mouse (the average body weight was 20 g) was given 20 μl of the PP fraction/body/d. This means that the amount of the PP fraction administered was 1.5 mg/kg/d. In other words, a human (60 kg body weight) can take this amount of the PP fraction by drinking 400 ml of bovine milk every day. The mice received the first dose of OVA (100 μg/body) with an alum adjuvant (LSL, Japan) by an intra-peritoneal (i.p.) injection on days 9 and 23 to elicit a specific IgE response. Blood and splenocytes were collected 5 d after the second OVA challenge on day 23. The mice in the negative control (NC) group were orally administered the same amount of the dialyzed PP fraction without immunization with OVA. The body weight was measured on alternate days for 28 d. All animal experiments were carried out in accordance with the protocol approved by Ehime University Animal Care and Use Committee and the applicable guidelines and regulations.
Fractionation of the active substance in the PP fraction by ultrafiltration

The active substance in the PP fraction did not pass through the dialysis membrane. To estimate the molecular weight of the active substance in this fraction, PP was fractionated by ultrafiltration. The filtrate passed through a 30-kDa ultrafiltration membrane did not suppress IgE production by U266 cells, and the active substance remained in the concentrate (Fig. 2). This means that the molecular weight of the active substance or complex in the PP fraction was more than 30 kDa.

Effect of treating the PP fraction with trypsin on the IgE production-suppressing activity

To characterize the active substance in the PP fraction, the trypsin sensitivity of this substance was examined. Trypsin did not affect IgE production by U266 cells (data not shown). The area expressed as the relative suppression activity of the trypsinized PP fraction against the intact fraction (Fig. 3). As indicated in Fig. 3, the IgE production suppressing activity of the PP fraction was accelerated by trypsin digestion. The activity was enhanced approximately 2-fold by the trypsin treatment at 375°C compared with the intact PP fraction. The active substance in the PP fraction was modified and activated by the trypsin treatment in a dose-dependent manner. This result suggests that the substance in the PP fraction was a trypsin-sensitive protein, and partial modification of its molecular conformation resulted in activation of the substance.

IgE mRNA expression level in U266 cells treated with the PP fraction

The effect of the PP fraction on the expression level of mRNA for the IgE ε chain in U266 cells was examined by quantitative real-time PCR. U266 cells were treated with the PP fraction for 24 h, and the ε chain mRNA level was examined. As indicated in Fig. 4, the ε chain mRNA expression level in the U266 cells was obviously depressed by treating with 220 µg/ml of the PP fraction. It is presumed from this result that the decrease in IgE production was due to suppression of the expression level of ε chain mRNA in the U266 cells.
Effect of the PP fraction on the serum IgE level of allergic model mice

To examine the in vivo effect of an oral administration of the PP fraction on the specific allergy reaction, the mice were immunized with OVA. The serum was harvested on day 28, and the total and OVA-specific IgE levels were measured by ELISA. As indicated in Fig. 5A, the total IgE level in serum of the PP fraction-administered group was significantly lower than that of the control vehicle group. In addition, the OVA-specific IgE level in serum was also clearly decreased by PP administration (Fig. 5B).

Fig. 4. Effect of the PP Fraction on the IgE mRNA Expression in U266 Cells.
U266 cells were inoculated in the ITES-RPMI 1640 medium supplemented 10 mM NaPB or the PP fraction at 1.0 × 10^5 cells/ml, and then cultured for 24 h. Total mRNA was prepared and analyzed for GAPDH and the IgE ε chain in the U266 cells. GAPDH mRNA was analyzed as an internal control. Data are presented as the relative IgE mRNA expression level ± SD against the expression level in U266 cells cultured in the medium supplemented with 10 mM NaPB (n = 3). Asterisks indicate that the data are significantly different from the control data. *p < 0.01.

Table 1. Effect of PP on Total Ig Levels in Serum from OVA-Immunized Mice

<table>
<thead>
<tr>
<th>Ig Production (mg/ml)</th>
<th>Control</th>
<th>PP</th>
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<tbody>
<tr>
<td>IgA</td>
<td>1.5 ± 0.2</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>IgG</td>
<td>6.4 ± 1.3</td>
<td>6.1 ± 1.7</td>
</tr>
<tr>
<td>IgM</td>
<td>2.3 ± 0.1</td>
<td>2.3 ± 0.1</td>
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Effect of the PP fraction on IgE and IL-4 mRNA expression levels in splenocytes from the allergic model mice

Administration of the PP fraction depressed the serum IgE level in the allergic model mice. We therefore evaluated the expression levels of IgE and IL-4 mRNA in splenocytes derived from the OVA-immunized mice. IL-4 activates the humoral immune response and induces B cells to produce IgE. As shown in Fig. 6, the IgE ε chain mRNA expression level in splenocytes was slightly suppressed by administering the PP fraction. It is presumed from this result that the decrease in IgE level in the serum was due to depression of the mRNA level in splenocytes. On the other hand, the IL-4 gene expression level in splenocytes was disposed to

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suppression by the PP intake, although the effect was not significant.

**Effect of the PP fraction on the proportion of lymphocytes in the spleen**

We next examined the effect of the PP fraction on the B/T cell proportion in the spleen. As shown in Fig. 7, B cells in spleen were decreased by administering the PP fraction, and T cells were slightly increased. This result suggests that intake of the PP fraction relatively activated the cellular immune response compared with the humoral immune response in OVA-immunized mice. As indicated in Fig. 6, the intake of PP was slightly disposed to suppression of the IL-4 expression level in splenocytes. It is presumed from these results that the intake of PP may have slightly suppressed the humoral immune response, and inactivated the allergy response.

We have presented in this study the anti-allergy effect of the PP fraction in bovine milk. IgE is one of the major mediators of a type 1 immediate hypersensitivity reaction such as seasonal allergy, food allergy, asthma, and anaphylaxis.20) The level of serum IgE in many individuals is roughly correlated with the severity of these allergic diseases.21) We had previously investigated the biological function of the PP fraction in bovine milk,6) so we examined in this study the effect of the PP fraction on IgE production by U266 cells. The results show that the PP fraction suppressed IgE production by depressing IgE mRNA expression in U266 cells. The activity of the PP fraction was accelerated by eliminating low-molecular-weight substances by dialysis. We are now purifying the active substance in the PP fraction. Although purification has not yet been completed, but we have estimated the molecular weight of the active substance in the PP fraction as 89 kDa by gel filtration (data not shown). Trypsin digestion activated the IgE production suppressing activity of the substance. It is presumed from these findings that the active substance was this 89-kDa protein, and that the activity of the degraded fragment would be stronger than that of the intact substance.

We orally administered the PP fraction to OVA-challenged allergy model mice. The PP fraction decreased the OVA-specific IgE level in the serum. The IgE expression level in splenocytes from OVA-immunized mice was down-regulated by the PP fraction, and IL-4 gene expression was slightly decreased. Moreover, intake of the PP fraction increased the T cell population and decreased the B cell population of splenocytes. It is presumed from these results that an intake of PP may inactivate the allergy response by suppressing the humoral immune response.

**References**


**Fig. 6.** Effect of an Oral Administration of the PP Fraction on mRNA Expression in Splenocytes from Allergy Model Mice.

Mice splenocytes were collected from the PP fraction-administered OVA-immunized mice. Total mRNA was prepared, and a quantitative real-time PCR assay was performed by using mRNA β-actin (internal control), IgE, and IL-4. Data are presented as the relative expression levels of mRNA for the IgE ε chain and IL-4 in splenocytes from the PP fraction-administered allergy model mice against those from control vehicle allergy model mice (n = 8). Asterisks indicate that the data are significantly different from the control data. **p < 0.01.

**Fig. 7.** Effect of the PP Fraction on the Cell Population of Splenocytes.

A cell population analysis of splenocytes from the PP fraction-administered OVA-challenged mice was performed. The cell surface markers on the splenocytes were stained on ice with FITC-conjugated anti-mouse B220 and PE-conjugated anti-mouse CD3 antibodies for 30 min. After washing with cold PBS, the cells were analyzed by using a FACSCalibur flow cytometer.