

Effects of Bovine β -Casein (1–28) and Its Chemically Synthesized Partial Fragments on Proliferative Responses and Immunoglobulin Production in Mouse Spleen Cell Cultures

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Effects of bovine β -casein (1–28) having a phosphoserine-rich region (Glu¹⁴-SerP-Leu-SerP-SerP-SerP-Glu-Glu²¹) and its chemically synthesized partial fragments on proliferation of lymphocytes and immunoglobulin production were investigated in mouse spleen cell cultures. The parent fragment 1–28 and all fragments containing SerP-Leu-SerP and/or SerP-SerP-SerP had a significant mitogenic effect, stimulated proliferation of lymphocytes induced by lipopolysaccharide, phytohemagglutinin, or concanavalin A, and increased immunoglobulin (IgG + IgM + IgA) or IgA levels in the cell cultures. In contrast, dephosphorylated β -casein (14–21) and SerP-SerP amide had hardly any immunoregulatory activity. On the other hand, SerP-Leu-SerP amide reacted little with antibodies specific to bovine β -casein (1–28), but β -casein (14–21), and SerP-SerP-SerP amide obviously reacted with the antibody. These results confirm that the immunoregulatory activity of casein phosphopeptides is attributable to SerP-X-SerP, which may well be available as a non-allergic food ingredient having an adjuvant activity for mucosal IgA responses.

Key words: casein phosphopeptide; SerP-X-SerP; mitogenic activity; lymphocytes; non-allergic IgA stimulator

Milk is normally the first food for mammalian newborns. Hence, milk contains not only essential nutrients but also biologically active components expressing important physiological functions for the newborns¹⁾. Whey proteins such as immunoglobulins, lactoferrin, and lysozyme are well known anti-infectious factors in milk.

Caseins, the major proteins in all milk, have been

traditionally considered to be random coil-type proteins and principally a source of amino acids for young mammals. In recent years, however, a number of biologically active peptide fragments have been demonstrated in digests of caseins from various species^{2,3)}. These demonstrations introduced a new criterion in evaluating the nutritive value of caseins. A partial sequence, which is hidden in an inactive state within the casein molecule, may be released by digestive processes in mammalian intestinal tracts and may act as potential modulators of the regulating process in the body of newborn mammals⁴⁾.

Among biologically active peptide fragments released from caseins, one of the most characterized peptides is casein phosphopeptide. Casein phosphopeptides are casein fragments having phosphoserine residues⁵⁾. Casein phosphopeptides can form complexes with calcium ion, and inhibit formation of precipitates between calcium and phosphate. Lee *et al.*⁶⁾ demonstrated that the intestinal absorption of calcium increased in rats given a diet containing casein because of the formation and accumulation of casein phosphopeptides. Moreover, oral ingestion of casein phosphopeptides was found to improve calcium absorption in the intestinal tracts of animals⁷⁾. Therefore, casein phosphopeptides have been industrially produced by the tryptic hydrolysis of bovine whole casein and are admitted in Japan as a food for specified health use having a capacity to increase bioavailability of calcium in the intestinal tract.

In 1996, Wong *et al.*⁸⁾ reported that the mitogen-induced proliferative response of ovine lymphocytes was significantly increased by bovine β -casein. Two years later, Hata *et al.*⁹⁾ identified bovine β -casein (1–25) and α s1-casein (59–79) as having phos-

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Abbreviations: β -casein (1–28), residues 1–28 of β -casein; SerP, phosphoserine; Ig, immunoglobulin; LPS, lipopolysaccharide; PHA, phytohemagglutinin; Con A, concanavalin A; PBS, 0.01 M-sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl; ELISA, enzyme-linked immunosorbent assay

phoserine-rich regions as immunomodulating factors in trypsin digests of bovine caseins. These fragments had mitogenic activity, and stimulated lipopolysaccharide (LPS)- or phytohemagglutinin (PHA)-induced proliferation and immunoglobulin production in mouse spleen and rabbit Peyer's patch cell cultures⁹. More recently, Otani *et al.*^{10,11} have observed that the oral administration of commercially available casein phosphopeptide preparations increases intestinal total and antigen-specific IgA levels in mice and piglets.

Our investigation was done to discover the active center of casein phosphopeptides to stimulate lymphocyte proliferation and IgA production using β -casein (1–28) containing a phosphoserine-rich region and its chemically synthesized partial fragments in mouse spleen cell cultures. In addition, an antigenic reactivity of the smallest fragment having immunoregulatory activity with anti- β -casein (1–28) antibody was investigated.

Materials and Methods

Materials. This experiment was done in accordance with the guidelines for Regulation of Animal Experimentation in the Faculty of Agriculture of Shinshu University, and according to Law No. 105 and Notification No. 6 of the Japanese government.

The proliferation of B-lymphocytes in mouse spleen cells was induced by LPS stimulation while that of T-lymphocytes was done by PHA and concanavalin A (Con A)¹². LPS of *Salmonella typhimurium* was purchased from Difco Laboratories (Detroit, MI, USA). Con A and PHA were obtained from Wako Pure Chemical Industries (Osaka, Japan) and Seikagaku Kogyo (Tokyo, Japan), respectively. Celgrossor-P and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sumitomo Seiyaku (Tokyo, Japan) and Sigma Chemical Company (St Louis, MO, USA), respectively. Penicillin G, streptomycin Sulfate, and CPP-III were obtained from Meiji Seika (Tokyo, Japan). Goat affinity purified antibody to mouse immunoglobulin (IgG + IgM + IgA) or IgA (Fc) and horseradish peroxidase-conjugated goat affinity purified antibody to mouse immunoglobulin (IgG + IgM + IgA) were purchased from Organon Teknika Corporation (Durham, NC, USA). Horseradish peroxidase-conjugated goat affinity purified antibody to mouse IgA (Fc) was from O. E. M. Concepts, Inc. (Toms River, NJ, USA). Horseradish peroxidase-conjugated goat affinity purified antibody to rabbit IgG (F(ab)'2) was from Cooper Biomedical, Inc. (Malvern, PA, USA). The other chemicals were of the highest analytical grade commercially available.

Bovine β -casein (1–28) and its chemically synthe-

sized partial fragments. Bovine β -casein (1–28) was purified from a commercially available casein phosphopeptide preparation, CPP-III, mainly consisting of β -casein (1–28) and α s2-casein (1–32)¹³ by Pharmacia Standard Liquid Chromatography (P.S.L.C) system with a Q-Sepharose column (Pharmacia Biotech Co., Ltd., Tokyo, Japan). The purified peptide showed a single peak on reversed-phase high pressure liquid chromatography, and was identified as sequence 1–28 of bovine β -casein on amino acid analysis and TOF-MS analysis.

Eight kinds of chemically synthesized fragments, which had been synthesized according to the solid-phase FMOC strategy, were used in this study. The C-terminal α -carboxyl group of all the fragments having SerP at the C-terminal position was converted into an amide group. The chemically synthesized phosphoserine-rich region of bovine β -casein, Glu¹⁴-SerP-Leu-SerP-SerP-SerP-Glu-Glu²¹, was obtained from Bio-Synthesis, Inc. (Lewisville, TX, USA) and identified as β -casein (14–21) by amino acid analysis and on TOF-MS analysis. Chemically synthesized bovine β -casein (14–18) amide, β -casein (14–17)-Glu, β -casein (15–17) amide, β -casein (17–21), β -casein (17–19) amide, β -casein (17–18) amide and dephosphorylated β -casein (14–21) were purchased from Bex Co., Ltd. (Tokyo, Japan) and identified by amino acid analysis and TOF-MS analysis. The purity of all the chemically synthesized fragments was more than 80% on reversed-phase high pressure liquid chromatography.

Antibody specific to β -casein (1–28). Anti- β -casein (1–28) IgG antibody was obtained from Sawady Technology (Tokyo, Japan). The antibody had been prepared from antisera obtained from rabbits injected with β -casein (1–28)-conjugated ovalbumin by affinity chromatography using a bovine β -casein (1–28)-immobilized column.

Cells and cell cultures. Male C3H/HeN mice, 6 weeks of age, were obtained from Shizuoka Laboratory Animal Center (Shizuoka, Japan). The spleen cell culture was made by the procedure described in our previous paper⁹. Briefly, mice were killed by vertebral dislocation and their spleens were removed aseptically. Single-cell suspensions were prepared by gentle manipulation of the tissues in a culture medium (Celgrossor-P containing 100 units penicillin/ml and 100 μ g streptomycin/ml). The cells were washed 3 times in Celgrossor-P and resuspended at a concentration of 6×10^6 viable cells/ml. Cell cultures were set up in quadruplicate in flat-bottomed microtiter plates (Falcon Labware, Oxnard, CA, USA). Into each well were placed 100 μ l of the cell suspension, 10 μ l of a test fragment solution dissolved in 0.01 M-sodium phosphate buffer, pH 7.2, containing 0.15 MNaCl (PBS) and 10 μ l of mitogen solution dis-

solved in PBS. Final concentrations were: spleen cells, 5×10^6 viable cells/ml; test fragment, 0 to 4 nm/well; LPS, 0 or 50 $\mu\text{g/ml}$; PHA, 0 or 10 $\mu\text{g/ml}$; ConA, 0 or 1 $\mu\text{g/ml}$; penicillin, 100 units/ml; streptomycin, 100 $\mu\text{g/ml}$. The mixtures were cultured at 37°C in a humidified 5% CO₂-95% air atmosphere for 24–144 h for measurement of cell proliferation or for 24–192 h to measure immunoglobulin or IgA.

At the end of the cultivation period, the proliferative response of lymphocytes was estimated by the colorimetric MTT (tetrazolium) assay¹⁴. The result was expressed as mean absorbance at 570 nm (MTT value). At the end of cultivation period, the culture supernatant was collected by centrifugation and the immunoglobulin (IgG + IgM + IgA) and IgA levels were measured by a sandwich enzyme-linked immunosorbent assay (ELISA).

Measurement of immunoglobulin levels in culture supernatant. A sandwich ELISA was done principally using an adaptation of the method of Williams *et al.*¹⁵ for measurement of immunoglobulin and IgA levels in the culture supernatant. Polystyrene ELISA plates (Nunc A/S, Roskilde, Denmark) were coated overnight at 4°C with 200 μl goat anti-mouse immunoglobulin (IgG + IgM + IgA) or IgA (Fc) in 0.05 M-carbonate buffer, pH 9.6 (10 $\mu\text{g/ml}$). After they were washed at 25°C with PBS containing 0.05% Tween 20, the plates were post-coated for 1.5 h at 25°C with 300 μl bovine serum albumin in 0.05 M-carbonate buffer, pH 9.6 (2 mg/ml), and washed again with PBS containing 0.05% Tween 20. Culture supernatant (100 μl) was added to each well, and the plates were then incubated for 1.5 h at 25°C. After the plates were washed as above, 100 μl of optimally diluted goat anti-mouse immunoglobulin (IgG + IgM + IgA) or sheep anti-mouse IgA conjugated to horseradish peroxidase was added to each well. The plate was incubated for 1.5 h at 25°C and washed again. Peroxidase substrate (100 μl , orthophenylenediamine dihydrochloride in 0.1 M-citrate-0.2 M-sodium phosphate buffer containing 0.03% hydrogen peroxide, pH 5.0, 4 mg/ml) was added to each well. After 20 min at 25°C, the reaction was stopped by addition of 25 μl of 4N-H₂SO₄. The plates were read at 490 nm on a Bio-Rad model 550 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The quantity of immunoglobulin or IgA in the culture supernatants was derived from a standard curve constructed by plotting known concentrations of mouse immunoglobulin (IgG + IgM + IgA) or IgA, respectively, versus absorbance at 490 nm.

Antigenic reactivity of partial fragments of bovine β -casein (1–28) with antibody to the fragment 1–28. Antigenic reactivity of partial fragments of bovine β -casein (1–28) with antibody to the fragment 1–28 was measured by a two-step ELISA inhibition test. In

brief, the polystyrene ELISA plate was coated overnight at 4°C with 200 μl of β -casein (1–28) in 0.05 M-carbonate buffer, pH 9.6 (10 $\mu\text{g/ml}$). After washing, the plate was post-coated with bovine serum albumin and washed again, as described above. A small volume of IgG antibody specific to β -casein (1–28) dissolved in PBS was mixed with the same volume of 4 concentrations of β -casein (1–28) or its chemically synthesized partial fragments dissolved in PBS and incubated for 1 h at 37°C. A 100- μl portion of the incubated mixture was added to the ELISA plate well that had been coated with β -casein (1–28). After incubation and washing of the plates as above, 100 μl of optimally diluted goat anti-rabbit IgG conjugated to horseradish peroxidase was added to each well. The plate was incubated for 1.5 h at 25°C and washed again. The peroxidase substrate mentioned above was then added to each well. After 20 min at 25°C, the reaction was stopped and the plates were read as described above.

Statistical analysis. Results were expressed as means $\pm$ SD for the MTT value (A570 nm) and immunoglobulin or IgA level (ng/ml). The significance of the difference between the fragment-free culture and fragment-added culture was tested by Student's *t*-test.

Results and Discussion

Effects of bovine β -casein (1–28) on proliferation and immunoglobulin production of mouse spleen cells

Mouse spleen cells were cultured with bovine β -casein (1–28) at concentrations of 0–4 nm/well in the presence and absence of lymphocyte mitogens such as LPS, PHA, and Con A¹². The effect of the fragment on proliferative responses and production of immunoglobulin (IgG + IgM + IgA) and IgA was examined. β -Casein (1–28) displayed mitogenic activity towards spleen cells by itself in the absence of the commercial mitogen and significantly increased proliferations of spleen B- and T-lymphocytes induced by LPS, PHA, and Con A when cultured for 24–120 h, and the activity was maximum for 48 h of cultivation (data not shown). LPS-, PHA- and Con A-induced proliferations of mouse spleen cells were increased by 1.42, 1.23, and 1.30-fold, respectively, compared with those of the fragment-free culture when incubated for 48 h with β -casein (1–28) at a concentration of 2 nm/well (Fig. 1). The fragment 1–28 also significantly increased levels of immunoglobulin (IgG + IgM + IgA) and IgA when cultured for 72–192 h in the case of immunoglobulin and for 72–120 h in the case of IgA. Both immunoglobulin and IgA levels increased more than 2-fold compared with those of the fragment-free culture when incubated for 72–120 h in the case of

immunoglobulin and for 72 h in the case of IgA with β -casein (1–28) at a concentration of 2 nM/well (Fig. 2). It seemed unlikely that the fragment 1–28 was contaminated with LPS, a B-lymphocyte mitogen¹², from Gram-negative organisms since the mitogenic activity of the fragment 1–28 was not suppressed by addition of polymyxin B (result not shown), which inactivates the activity of LPS¹⁶. These facts confirm that the bovine β -casein (1–28) having a phosphoserine-rich region prepared in this study had a stimulatory effect on B- and T-lymphocyte proliferations and immunoglobulin production as well as bovine α 1-casein (59–79) and bovine β -casein (1–25) as reported earlier⁹. In addition, the

strongest stimulatory effect on the proliferation and immunoglobulin production was observed when bovine β -casein (1–28) was added at a concentration of 2 nM/well. Thus, we added chemically synthesized partial fragments of bovine β -casein (1–28) at a concentration of 2 nM/well in the following experiment.

Effects of chemically synthesized partial fragments of bovine β -casein (1–28) on proliferation and immunoglobulin production of mouse spleen cells

Mouse spleen cells were cultured with 8 kinds of chemically synthesized partial fragments of bovine β -casein (1–28) at a concentration of 2 nM/well in the presence and absence of LPS, PHA, and Con A. The effects of fragments on the proliferative response and production of immunoglobulin (IgG + IgM + IgA) or IgA were examined. Six kinds of partial fragments, not including dephosphorylated β -casein (14–21) and β -casein (17–18) amide, significantly increased the proliferation of spleen lymphocytes induced by the B- and T-lymphocyte mitogens such as LPS, PHA, and Con A, and had mitogenic activity by themselves in the absence of the mitogen (Table 1). Moreover, the 6 fragments significantly increased the level of immunoglobulin (IgG + IgM + IgA) and IgA (Tables 2 and 3). These results confirm that the stimulatory effect of bovine β -casein (1–28) on lymphocyte proliferation and immunoglobulin production is mainly attributable to the phosphoserine-rich region. We obtained no chemically synthesized fragment with SerP having a free α -carboxyl group at the C-terminal position. Hence, we used phosphopeptides as SerP-SerP amide, SerP-Leu-SerP amide, and SerP-SerP-SerP amide in this study. Both tripeptides, SerP-Leu-SerP amide and SerP-SerP-SerP amide, stimulated lymphocyte proliferation and immunoglobulin production, and had mitogenic activity towards spleen cells, but the dipeptide was inert.

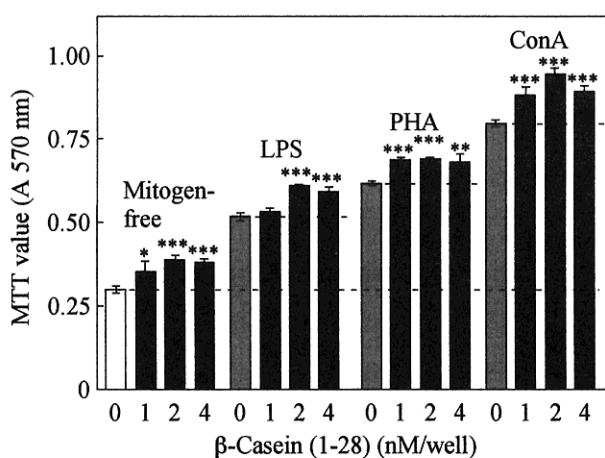


Fig. 1. Effects of Bovine β -Casein (1–28) on Proliferative Responses of Mouse Spleen Lymphocytes Induced by LPS, PHA, and Con A in Cell Cultures.

Experimental conditions are given in Materials and Methods. Values are means with their SD represented by vertical bar ($n = 4$). The proliferative response between each fragment-free and fragment-added culture was significantly different at * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

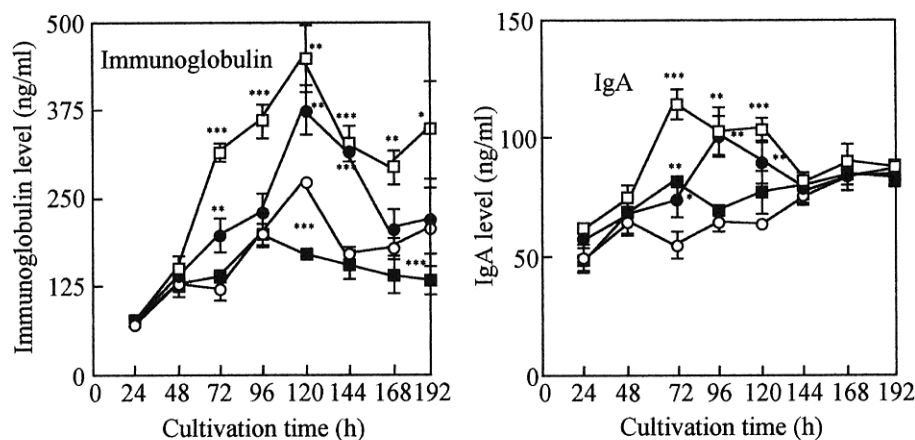


Fig. 2. Effects of Bovine β -Casein (1–28) on Levels of Immunoglobulin (IgG + IgM + IgA) and IgA in Mouse Spleen Cell Cultures.

Experimental conditions are given in Materials and Methods. Concentration of the fragment 1–28: $\circ$, 0 nM/well; $\bullet$, 1 nM/well; $\square$, 2 nM/well; $\blacksquare$, 4 nM/well. Values are means with their SD represented by a vertical bar ($n = 4$). The level of immunoglobulin or IgA between each fragment-free and fragment-added culture was significantly different at * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

Table 1. Effects of β -Casein (1–28) and Its Chemically Synthesized Partial Fragments on Proliferative Responses of Mouse Spleen Cells Induced by Mitogens

Fragment	MTT value (A 570 nm)			
	Mitogen-free	LPS-induced	PHA-induced	ConA-induced
Free	0.300 ± 0.008	0.458 ± 0.003	0.516 ± 0.006	0.748 ± 0.015
1–28 (Native)	0.391 ± 0.007***	0.510 ± 0.003***	0.691 ± 0.009***	0.920 ± 0.018***
14–21	0.361 ± 0.010***	0.505 ± 0.010***	0.613 ± 0.005***	0.885 ± 0.011***
Dephosphorylated 14–21	0.306 ± 0.003	0.463 ± 0.008	0.512 ± 0.027	0.772 ± 0.021
14–18 amide	0.368 ± 0.007***	0.509 ± 0.005***	0.620 ± 0.011***	0.920 ± 0.012***
17–21	0.331 ± 0.007**	0.487 ± 0.007**	0.641 ± 0.005***	0.861 ± 0.004***
14–17-Glu	0.328 ± 0.007**	0.489 ± 0.005***	0.552 ± 0.014**	0.886 ± 0.014***
15–17 amide	0.327 ± 0.007**	0.493 ± 0.005***	0.598 ± 0.005***	0.870 ± 0.013**
17–19 amide	0.356 ± 0.015***	0.481 ± 0.006***	0.561 ± 0.004***	0.907 ± 0.012***
17–18 amide	0.319 ± 0.015	0.459 ± 0.022	0.512 ± 0.017	0.772 ± 0.012

Experimental conditions are given in Material and Methods. The concentration of fragment was 2 nM/well. Each value is mean ± SD (n = 4). The proliferative response between fragment-free culture and fragment-added culture was significantly different at **P < 0.01 and ***P < 0.001.

Table 2. Effects of β -Casein (1–28) and Its Chemically Synthesized Partial Fragments on Production of Immunoglobulin (IgG + IgM + IgA) in Mouse Spleen Cell Cultures

Fragment	Immunoglobulin level (ng/ml), Mean ± SD		
	72 h	96 h	120 h
Free	305.758 ± 1.040	334.975 ± 0.959	373.942 ± 0.952
1–28 (Native)	370.658 ± 0.885***	391.267 ± 1.240***	434.117 ± 0.996***
14–21	364.725 ± 1.536***	408.233 ± 0.742***	430.067 ± 0.607***
Dephosphorylated 14–21	306.567 ± 1.027	336.625 ± 1.064	381.183 ± 1.051
14–18 amide	369.658 ± 0.842***	403.692 ± 0.916***	426.108 ± 1.463***
17–21	372.467 ± 1.647***	399.950 ± 0.575***	428.433 ± 1.188***
14–17-Glu	349.350 ± 1.419***	390.075 ± 1.559***	424.000 ± 1.036***
15–17 amide	348.825 ± 1.853***	392.017 ± 1.188***	429.775 ± 1.925***
17–19 amide	360.608 ± 1.047***	393.567 ± 1.014***	429.950 ± 1.107***
17–18 amide	306.158 ± 1.387	338.067 ± 1.171	376.675 ± 1.083

Experimental conditions are given in Material and Methods. The concentration of fragment was 2 nM/well. Each value is mean ± SD (n = 4). The level of immunoglobulin between fragment-free culture and fragment-added culture was significantly different at ***P < 0.001.

Table 3. Effects of β -Casein (1–28) and Its Chemically Synthesized Partial Fragments on Production of IgA in Mouse Spleen Cell Cultures

Fragment	IgA level (ng/ml), Mean ± SD		
	72 h	96 h	120 h
Free	36.317 ± 2.922	50.973 ± 0.713	52.966 ± 2.539
1–28 (Native)	62.209 ± 3.209***	74.103 ± 3.026***	74.823 ± 3.231***
14–21	61.389 ± 2.511***	74.108 ± 2.548***	74.581 ± 2.182***
Dephosphorylated 14–21	42.324 ± 1.612	53.912 ± 2.702	57.359 ± 2.839
14–18 amide	59.691 ± 3.145***	73.891 ± 3.149***	75.950 ± 5.041***
17–21	58.802 ± 3.310***	72.261 ± 2.867***	73.725 ± 3.573***
14–17-Glu	58.395 ± 3.360***	71.934 ± 4.279***	72.651 ± 1.574***
15–17 amide	57.313 ± 1.838***	71.734 ± 3.996***	72.159 ± 5.602***
17–19 amide	56.562 ± 0.322***	71.708 ± 4.677***	71.330 ± 3.836***
17–18 amide	42.581 ± 3.659	53.267 ± 5.21	54.861 ± 1.917

Experimental conditions are given in Material and Methods. The concentration of fragment was 2 nM/well. Each value is mean ± SD (n = 4). The level of IgA between fragment-free culture and fragment-added culture was significantly different at ***P < 0.001.

These results suggest that the immunomodulatory active center of casein phosphopeptide may be tripeptides having 2 SerP residues at N- and C-terminal positions.

Antigenic reactivity of chemically synthesized partial fragments of bovine β -casein (1–28) with antibody specific to fragment 1–28

Figure 3 shows a typical inhibition pattern of the reaction between immobilized bovine β -casein (1–28) and antibody specific to β -casein (1–28) by free β -

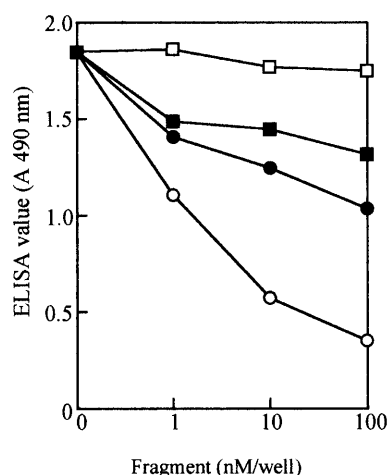


Fig. 3. An Inhibition Analysis of Binding between Bovine β -Casein (1-28) with Its Antibody by the Fragment and Its Chemically Synthesized Partial Fragments.

Experimental conditions are given in Materials and Methods.

○, bovine β -casein (1-28); ●, chemically synthesized β -casein (14-21); □, chemically synthesized β -casein (15-17) amide (SerP-Leu-SerP amide); ■, chemically synthesized β -casein (17-19) amide (SerP-SerP-SerP amide).

casein (1-28), phosphoserine-rich region of the fragment (residues 14-21), SerP-Leu-SerP amide (residues 15-17 amide) and SerP-SerP-SerP amide (residues 17-19 amide). The antigenic reactivity of the immobilized fragment 1-28 with the antibody was well inhibited by the free phosphoserine-rich region (residues 14-21) as well as the free β -casein (1-28), although the curve for β -casein (14-21) was shifted to the right in comparison to that of β -casein (1-28). This result indicates that the anti- β -casein (1-28) antibody had a higher affinity with β -casein (1-28) than β -casein (14-21), but confirms that the antibody recognizes the phosphoserine-rich region as an intact epitope or a part of an epitope of β -casein (1-28). SerP-SerP-SerP amide also had an affinity to the anti- β -casein (1-28) antibody, although less than that of the residues 14-21. In contrast, SerP-Leu-SerP amide had little affinity to the antibody. These results suggest that the residues 17-19, or SerP-SerP-SerP, may be a part of an epitope of the phosphoserine-rich region, but the residues 15-17, or SerP-Leu-SerP, may be out of the epitope.

Allergies to bovine milk occur in 1~3% of bottle-fed infant populations¹⁷⁾. The major allergens of casein fractions are calcium-sensitive α - and β -caseins¹⁸⁾. Bernard *et al.*¹⁹⁾ proposed that the phosphoserine-rich region of calcium-sensitive caseins may be recognized as an epitope in some milk allergy patients because IgE antibodies in sera from some patients with clinical symptoms of cow's milk allergy strongly bound to bovine β -casein (1-25) but little to dephosphorylated β -casein (1-25). In this work, we demonstrated that the anti-bovine β -casein (1-28) antibody raised in mice reacted strongly with phos-

phoserine-rich regions of the fragment but had little reactivity with SerP-Leu-SerP amide. In addition, we demonstrated in this work that SerP-Leu-SerP amide stimulated the IgA production as well as β -casein (1-28). We have observed previously that the oral administrations of commercially available casein phosphopeptide preparations consisting of β -casein (1-28) and α s2-casein (1-32) increase intestinal antigen-specific and total IgA levels in mice¹⁰⁾ and piglets¹¹⁾. Thus, SerP-Leu-SerP may be an effective food ingredient having a capacity to stimulate intestinal IgA responses.

In this work, we investigated the effects of bovine β -casein (1-28) having a phosphoserine-rich region and its chemically synthesized partial fragments on proliferation of lymphocytes and immunoglobulin production in mouse spleen cell cultures. The antigenic reactivity of chemically synthesized partial fragments of bovine β -casein (1-28) with the antibody specific to the fragment 1-28 was also examined. As mentioned above, we found that the phosphopeptides containing SerP-X-SerP increased the lymphocyte proliferation and immunoglobulin, in particular IgA, production, and confirmed that SerP-Leu-SerP amide retained little antigenic reactivity with the antibody specific to bovine β -casein (1-28). In conclusion, we propose that some tripeptides consisting of SerP-X-SerP may be available as non-allergic food ingredients having an adjuvant activity for mucosal IgA responses.

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