

Immunogenicity of Casein Phosphopeptides Derived from Tryptic Hydrolysis of β -Casein¹

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ABSTRACT

The immunogenicity of phosphopeptides derived from tryptic hydrolysis of β -casein (CN) was investigated in a rat model system. The titers of specific immunoglobulin (Ig)G and IgE antibodies made in response to intraperitoneal sensitization to β -CN, casein phosphopeptides, and skim milk proteins were examined using indirect and amplified indirect ELISA, respectively. Serum IgG antibodies from rats injected with β -CN were significantly more reactive to β -CN, casein phosphopeptides, and skim milk proteins coated on microtiter plate wells than were the IgG antibodies generated in rats that had been subjected to other treatments. A significant difference in titers because of the time of sampling (14 or 21 d postinjection) was noted for IgE but not for IgG. Rats that were injected with casein phosphopeptides did not produce IgG antibodies that crossreacted with either skim milk proteins or β -CN. Specific antibody levels for the IgE class rarely exceeded those of unimmunized controls. The findings suggest that immunogenicity of the phosphopeptides was reduced compared with that of native β -CN and skim milk proteins.

(**Key words:** β -casein, casein phosphopeptides, immunogenicity)

Abbreviation key: CPP = casein phosphopeptide, PBS-T = PBS with Tween 20.

INTRODUCTION

Allergies to bovine milk occur most frequently in infant populations; 1 to 3% of bottle-fed infants are affected by milk protein allergies (23). Casein and

whey hydrolysates are widely used for infant formulas because of their high nutritional value; high solubility over a wide range of pH, temperature, and ionic conditions (16); and lower immunogenicity (4). Within the US, casein hydrolysates are the most common ingredients used for the production of hypoallergenic formulas (24). Despite the extensive degree of hydrolysis of the protein to amino acids and small peptides, instances of anaphylaxis and other adverse reactions in a small population of highly sensitive infants have been reported (21, 23).

Clinical challenges frequently reveal that patients who are allergic to milk react to the multiple protein fractions found in bovine milk (22). Caseins have been shown to be the major allergens in bovine milk (1, 22, 24, 27), although no single major allergen is evident. Caseins constitute approximately 80% of the total protein in milk and are a heterogeneous group of phosphoproteins with molecular masses ranging from approximately 11,500 to 25,000 (25). Baldo (1) found that all of the caseins, including the glycomacropptide from κ -CN, were capable of eliciting IgE responses and of reacting with IgE antibodies. Otani (18) stated that the minimum number of antigenic determinant sites for α_{s1} -CN, β -CN, and κ -CN were 6, 6, and 4, respectively.

Tryptic phosphopeptides derived from casein have recently generated interest because of their unique functional and biological properties. Phosphopeptides are derived from proteolysis of α_{s1} -CN, α_{s2} -CN, β -CN, or κ -CN. The phosphorylated residues are commonly grouped in sequences of three or more (28). Amino acid sequencing of the caseins has shown that phosphate groups were present as monoesters of serine and threonine. One of the primary consequences of the high phosphoserine content of caseins is their ability to sequester divalent ions, notably Ca^{2+} , Zn^{2+} , Mn^{2+} , and Fe^{2+} . Although casein phosphopeptides (CPP) may adversely affect Zn^{2+} bioavailability, absorption of the Ca^{2+} and Fe^{2+} that are bound by CPP may be stimulated (28). The CPP have been shown to increase calcium bioavailability in rats (14) and to alter temporal systolic blood pressure in spontaneously hypertensive rats (13). Increases in bone cal-

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cification in rats fed CPP were attributed to the prevention of precipitation of insoluble calcium phosphate salt (12). Because CPP have the potential to serve as a functional ingredient for delivering increased concentrations of bioavailable calcium (12), it is important to examine their immunological activity further.

Because of the inherent danger of testing immunological activity using challenge studies in humans, animal model systems have traditionally been used to examine immunogenicity. If protein antigens are proven not to induce immune responses in model systems, the costs and dangers associated with clinical testing may then be justified. Sprague-Dawley and Hooded Lister rats have been used to examine the IgE response to enzymatic hydrolysates (6) and to the whey protein β -LG (7, 8). The primary objective of this study was to employ a rat model system to examine the immunogenicity of CPP that were derived from tryptic hydrolysis of β -CN. Relative immunoactivity of specific IgG and IgE antibodies to the CPP, native β -CN, and skim milk proteins was investigated.

MATERIALS AND METHODS

Tryptic Hydrolysis of β -CN

Fresh skim milk (North Carolina State University Dairy Farms, Raleigh) was the starting material used for obtaining β -CN, which was purified by stepwise elution on a Macro-Prep Q anion-exchange column (19). The method of Janolino and Swaisgood (9) was used to activate trypsin sequentially and to immobilize trypsin on succinamidopropyl-glass, using controlled pore glass beads (CPG-2000A; 120/200 mesh size) (20). Assayed by the method of Taylor and Swaisgood (26), the activity of the immobilized trypsin was 49.4 U/g of beads, measured at 25°C. Limited proteolysis of purified casein was carried out in 50 mM Tris, pH 8.0, containing 0.02% NaN₃, at refrigeration temperatures. A fluidized bed reactor containing 2 ml of immobilized trypsin beads was used to recirculate 8 ml of a β -CN solution (10 mg/ml) for 1.5 h. After digestion, the hydrolysate was separated via gel filtration chromatography using a Sephadex G10-120 column (Pharmacia, Piscataway, NJ) for desalting. β -Casein hydrolysate fractions from this column were loaded onto a QAE-Sephadex A-25 column and equilibrated with 50 mM Tris, pH 8.0, with a flow rate of 3 ml/min. Individual peptide fractions were collected after stepwise elution with 0.1, 0.2, 0.4, and 0.5 M KCl containing 50 mM

Tris-HCl at pH 8.0. The phosphopeptide fraction used for immunological studies had a protein content of 0.330 mg/ml, as measured using the BCA assay (Pierce, Rockford, IL) with a β -CN standard. The phosphate content was determined to be 2.683 μ g/ml as determined by colorimetric assay. Molecular masses of peptides within the hydrolysate ranged from 4000 to 4500 as determined by size exclusion chromatography and SDS-PAGE.

Immunization Protocol

Female Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN) were housed individually in stainless steel cages and provided with water and a diet free of bovine milk protein (Prolab Rat, Mouse, Hamster 3000 formula; Agway Country Foods Inc., Syracuse, NY) for ad libitum intake. Five groups of 8 rats were divided into treatment groups. Body weights (group mean $\pm$ SEM) were 248 $\pm$ 9.1 g for unimmunized controls, 218 $\pm$ 6.3 g for rats immunized with adjuvant only, 223 $\pm$ 6.7 g for rats immunized with β -CN, 214 $\pm$ 5.8 g for rats immunized with CPP, and 215 $\pm$ 4.9 g for rats immunized with skim milk. Rats were immunized by i.p. injection of *Bordetella pertussis* vaccine (Michigan Department of Public Health, Lansing) containing 4×10^{10} cfu of *B. pertussis* cells, mixed with 170 μ g of protein antigen (β -CN, CPP, or skim milk proteins). For each of the five treatment groups, 4 rats were anesthetized with 90 mg of ketamine mixed with 10 mg of xylazine/kg of body weight; rats were killed by exsanguination (cardiac puncture) at 2 wk postimmunization and 3 wk postimmunization. Blood was allowed to clot at 4°C overnight and was then centrifuged; serum was recovered and stored at -80°C.

Determination of Specific IgE by ELISA

An amplified indirect ELISA was developed for the detection of IgE that were specific to the antigens being investigated. Microtiter plates (Maxisorb™; Nunc, Naperville, IL) were coated 18 h at 4°C with 100 μ l/well of 10 μ g/ml of β -CN, CPP, or skim milk proteins dissolved in 0.05 M sodium carbonate-bicarbonate buffer, pH 9.6. Following adsorption of antigen, wells were washed (EL-40108 8-well washer; Bio-Tek, Winooski, VT) three times with 0.01 M sodium phosphate buffer, pH 7.2, containing 0.15 M NaCl and 0.05% Tween 20 (**PBS-T**), followed by an incubation period of 5 min to block nonspecific adsorption. The wash and incubation cycles were repeated three times. Test antisera were serially diluted in PBS, and 100 μ l were added to each well for incuba-

TABLE 1. The ANOVA for specific IgG and IgE titers against β -LG in rats injected with either casein phosphopeptides, β -CN, skim milk mixed with adjuvant, or adjuvant alone or in untreated controls at 14 and 21 d postinjection.

Source	IgG titers					IgE titers				
	SS	df	MS	F-Ratio	P	SS	df	MS	F-Ratio	P
Plate ¹ (P)	0.110	2	0.055	19.527	0.0001	4.008	2	2.004	52.977	0.001
Treatment ² (T)	0.586	4	0.146	51.906	0.0001	1.763	4	0.441	11.653	0.001
Week ³ (W)	0.004	1	0.004	1.399	0.2400	0.295	1	0.295	7.790	0.006
P $\times$ T	0.268	8	0.034	11.87	0.0001	1.194	8	0.149	3.946	0.001
T $\times$ W	0.008	4	0.002	0.706	0.5902	1.057	4	0.264	6.986	0.001
W $\times$ P	0.075	2	0.037	13.233	0.0001	0.102	2	0.051	1.347	0.265
T $\times$ W $\times$ P	0.358	8	0.045	15.855	0.0001	0.462	8	0.058	1.527	0.159
Error	0.254	90	0.003			3.405	90	0.038		

¹Plates were coated with 10 mg/ml of either purified β -CN, casein phosphopeptides derived from tryptic hydrolysis of β -CN, or skim milk.

²Sprague-Dawley rats ($n = 8$) were subjected to one of five treatments: injected i.p. with 170 mg of either β -CN, casein phosphopeptides, or skim milk proteins mixed with adjuvant (4×10^{10} cfu of *Bordetella pertussis* cells), or adjuvant alone or untreated.

³Blood samples were taken at 2 and 3 wk postinjection.

tion for 2 h at 37°C. The plates were then washed three times with PBS-T, and 100 μ l of a 1:2000 (vol/vol) dilution of biotinylated mouse anti-rat IgE (Zymed Laboratories, Inc., San Francisco, CA) were added for 2 h at 37°C. The plates were again washed three times with PBS-T, and 100 μ l of a 1:5000 (vol/vol) dilution of peroxidase-avidin (Zymed Laboratories, San Francisco, CA) were added to wells for 1 h at 37°C. After incubation, plates were washed three times with PBS-T. A substrate buffer containing 16 mM o-phenylenediamine and 16 mM hydrogen peroxide (Sigma Chemical Co., St. Louis, MO) in 0.15 M citrate buffer was added to wells (100 μ l per well). Plates were then incubated for 8 min at room temperature, the reaction was stopped by the addition of 25 μ l of 2 M H₂SO₄, and absorbances were read at a wavelength of 490 nm (Dynatech MR 7000 plate reader; Dynatech, Chantilly, VA).

Determination of Specific IgG by ELISA

The indirect ELISA that was developed for detection of antigen-specific IgG was the same as that for detection of specific IgE, with the following alterations. After antigen was adsorbed and incubated with diluted antisera, 100 μ l per well of a 1:65,000 (vol/vol) dilution of peroxidase-conjugated goat anti-rat IgG (Zymed Laboratories, San Francisco, CA) were added. After incubation for 2 h at 37°C, substrate buffer was added, and absorbances were read at a wavelength of 490 nm.

Statistical Analysis

Mean absorbances of quadruplicate wells for each antibody dilution were determined for individual rats.

For individual measurements of dilution absorbance, ELISA precisions were typically $\pm 10\%$. Differences among treatments were determined by ANOVA. Multiple mean comparisons were analyzed using Tukey's highly significant difference test.

RESULTS

Specific Antibody Responses to β -CN

The IgG response to β -CN was greater at 2 wk than at 3 wk postinjection (Figure 1A). Overall, different specific titers from different times of sampling (2 wk vs. 3 wk postinjection) ($P < 0.05$) were observed for IgE titers but not for IgG titers (Table 1). The apparent lack of an IgG response against β -CN in rats injected with CPP (Figure 1A) indicates that the antigenic determinants present on β -CN are not preserved through tryptic hydrolysis. Concentrations of IgG that are specific to β -CN generally decreased at 3 wk postinjection until they were no longer significantly greater in rats originally injected with β -CN than in rats in other treatments. For IgE concentrations specific to β -CN at 2 or 3 wk postinjection, no treatment mean exceeded that of the uninjected control, suggesting the lack of an allergic response to these antigens in this experiment.

Specific Antibody Responses to CPP

At 2 wk postinjection, IgG concentrations specific to CPP were greater ($P < 0.05$) than those of uninjected controls only in rats injected with β -CN (Figure 1B). Specific IgG concentrations for all treatments had decreased nearly to that of the untreated

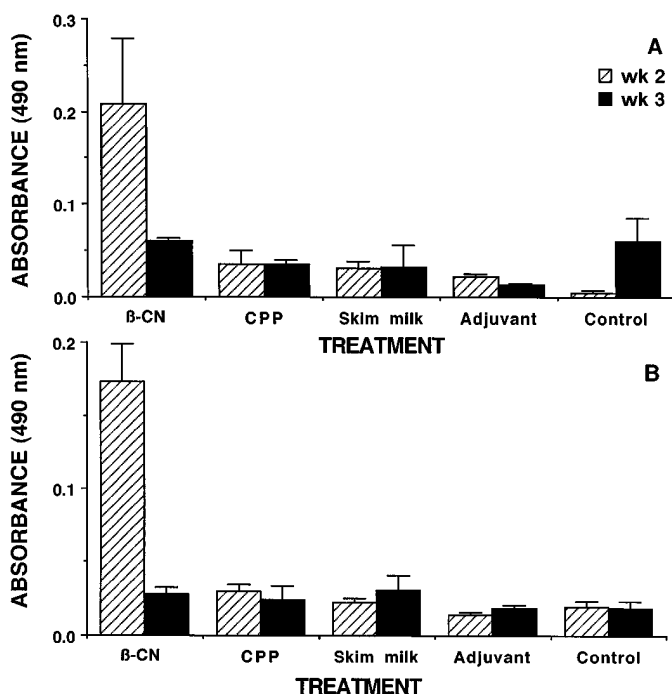


Figure 1. A. Levels of serum IgG antibodies that were specific to β -CN (A) and casein phosphopeptide (B) at 2 and 3 wk postinjection in Sprague-Dawley rats injected i.p. with either β -CN, casein phosphopeptides (CPP), skim milk mixed with adjuvant, or adjuvant (*Bordetella pertussis*) alone or untreated. Rats were injected with the material indicated on the x-axis, and ELISA plates were coated with either β -CN (A) or CPP (B) from β -CN. Error bars represent the standard error of the mean ($n = 4$).

control by 3 wk postinjection. Concentrations of IgE that were specific to CPP were greater ($P \leq 0.05$) in rats injected with β -CN, CPP, or skim milk proteins than in the untreated control group.

Specific Antibody Responses to Skim Milk Proteins

Rats that were injected with β -CN exhibited specific IgG response toward skim milk components (Figure 2A) that were greater than responses of rats on all other treatments. In contrast to the general trend for IgG responses, IgG titers that were specific to milk protein increased between 2 and 3 wk postinjection for rats injected with β -CN. At 2 wk postinjection, only the rats that had been injected with skim milk had concentrations of Ig specific for skim milk protein that were higher than those of controls (Figure 2B). In contrast to the trend observed for IgE antibodies against β -CN or CPP, significant differences were noted in specific IgE concentrations at 3 wk postinjection. Rats that were injected with either

CPP or adjuvant only were the two treatment groups to exhibit specific IgE concentrations that were greater than those of the untreated control group. Rats that were injected with β -CN had no detectable IgE toward skim milk proteins at either sampling time.

DISCUSSION

Bovine β -CN is a 24,000-Da, 209-amino acid residue phosphoprotein. A corresponding casein is present in human milk. Although human β -CN differs in length from bovine β -CN by only 3 amino acid residues, quantitative precipitin reactions indicated that the antigenicity of the two was substantially different (18). The concentration (9 to 11 g/ml) of β -CN in bovine milk is second only to that of α_{s1} -CN, and the high proline content (35 residues) prevents β -CN from assuming a regular secondary structure. Of the minimum of six antigenic determinants in β -CN, the majority appear to be located within the N-terminal region. The quantitative precipitin reaction

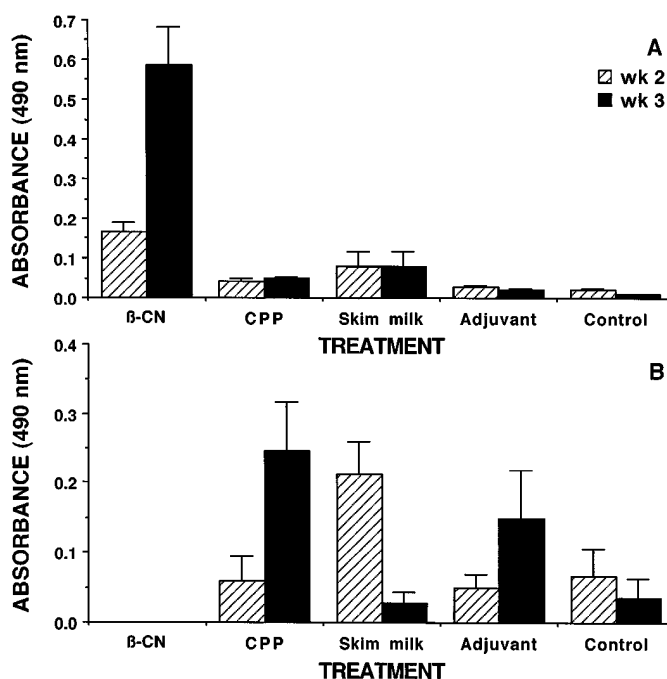


Figure 2. The ELISA absorbance from IgG (A) and IgE (B) serum antibodies that were specific to skim milk protein at 2 and 3 wk postinjection in Sprague-Dawley rats injected i.p. with β -CN, casein phosphopeptides (CPP), skim milk mixed with adjuvant, or adjuvant (*Bordetella pertussis*) alone or untreated. Rats were injected with the material indicated on the x-axis, and ELISA plates were coated with skim milk proteins. No IgE response was detected in rats that were injected with β -CN. Error bars represent the standard error of the mean ($n = 4$).

applied to peptic digests of β -CN revealed that 83% of the antigenic activity of native β -CN was present in the first 139 or 140 residues of the protein (18).

In this study, β -CN proved to be more immunogenic than CPP and skim milk proteins, consistently evoking significantly greater titers of specific IgG than either CPP or skim milk proteins. Absorbance values (Figure 1A) indicated that, at 2 wk postinjection, IgG titers in response to β -CN were approximately seven times greater than those induced by either the phosphopeptides or skim milk proteins. The reduced response in the rats that were injected with skim milk may be due to the lower dose of β -CN given to those rats; all rats in this study were injected with the same quantity of total protein. The finding that concentrations of IgE that are specific to β -CN commonly did not exceed those of untreated controls does not mean that the level of specific IgE present cannot provoke IgE-mediated allergic reactions. Allergens are by definition immunogenic (3); thus, proteins or peptides eliciting strong IgG immune responses would likely not be considered to be candidates for hypoallergenic protein sources.

The tryptic phosphopeptides that were isolated in this study by the method of Park et al. (20) had an apparent molecular mass of 4000 to 4500 Da; for peptides isolated by the method of Juillerat et al. (10), the reported molecular mass was calculated to be 3123 to 3469 Da. Tryptic hydrolysis of β -CN results in the generation of a 25-amino acid phosphopeptide containing four phosphoserine groups (10, 17) and the possible generation of a 28-amino acid phosphopeptide containing the same phosphoserine cluster (10). The hydrolysate produced in the present study falls between the moderate and extensive classifications of the degree of hydrolysis range (16). Usually peptides <2500 Da are not immunogenic (without modification), and proteins <10,000 Da are weakly immunogenic (3). The CPP used here had approximately 14 to 17% of the immunogenicity of native β -CN based on ELISA absorbance. Although the IgG antibodies that were generated in response to β -CN significantly ($P \leq 0.05$) crossreacted with the CPP derived from β -CN, the rats that were injected with CPP did not produce more IgG antibodies capable of crossreacting with β -CN or skim milk proteins than did control rats. Products based on this ingredient might be better at preventing milk allergies than at eliminating symptoms in allergic consumers.

This study focused on β -CN because of the high yield and purity of β -CN with our separation method (19). Other investigations (5, 11) of casein immunogenicity have primarily focused on α_{s1} -CN, in

part because of its status as the most abundant protein in bovine milk (12 to 15 g/L). In addition, no counterpart to bovine α_{s1} -CN exists in human milk, making this bovine protein immunologically foreign to humans (18) and a likely immunogen. Enomoto et al. (5) found that peptides derived from α_{s1} -CN and containing both T- and B-cell determinants could elicit specific antibody production as efficiently as the original peptide itself. The humoral response of mice that had been orally sensitized to α_{s1} -CN was later found to be directed against a small number of antigenic determinants (11). Otani (18) concluded that antigenic determinants on β -CN were based on primary sequence, rather than on conformation. Antigenic determinants present on α_{s1} -CN are also thought to be sequential rather than conformational (11) because the protein is thought to be lacking an ordered three-dimensional conformation (25). These collective results suggest that the CPP that were derived from hydrolysis of α_{s1} -CN might be even less antigenic than those derived from β -CN if the peptide corresponding to amino acids 61–80 can be isolated.

Commercial casein hydrolysates are commonly produced with the addition of a mixture of endo- and exopeptidases (15). Mixtures of proteases are necessary to achieve a nutritionally optimal range of free amino acids, peptides, and tripeptides. As a generality, hypoallergenic formulas cannot be produced by the use of a single protease unless an ultrafiltration step is also included in the process. The findings of the present study indicate that CPP derived from β -CN are significantly less immunogenic than β -CN and should be studied further for their application as a functional nutritional additive possessing reduced immunogenicity. Future research will address the immunogenicity of phosphopeptides derived from α_{s1} -CN.

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