

Effects of *In Vitro* Proteolysis on the Allergenicity of Major Whey Proteins

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ABSTRACT

The effects of *in vitro* proteolysis on the allergenicity of major whey proteins (α -lactalbumin and β -lactoglobulin) by simulating the human gastrointestinal conditions were evaluated. The proteolysis of demineralized whey with pepsin (pH 2.0 for 30 min) and/or various pancreatic enzymes (pH 7.5 for 60 and 240 min) was performed by the pH-stat technique at 37°C. The enzyme inactivation was performed by heating at 80°C for 20 min. Allergenicity of the hydrolyzates was evaluated by RAST inhibition using sera obtained from children allergic to whey proteins. Selective proteolysis of whey by pepsin and α -chymotrypsin was the most efficient combination of enzymes to reduce the allergenicity of both α -lactalbumin and β -lactoglobulin. The above hydrolyzate could be used to develop an ingredient for infant milk formula with a lower allergenicity.

INTRODUCTION

ALTHOUGH NUTRITIONAL, psychological and immunological advantages of breast-feeding have become well recognized, appropriate infant food formulas must be available when mother's milk cannot be provided. However, most infant formulas contain cow's milk or its constituent proteins which could cause allergic symptoms in about 0.3 to 7.5% of infants in the U.S.A. each year (Bahna, 1978). The allergic reactions to cow's milk are favored by rapid absorption of incompletely digested milk products. This partial digestion could be explained by the low gastric acidity during infancy along with the high buffering capacity of milk (Mason, 1962), and by the low activity of exocrine pancreatic function (Lebenthal and Lee, 1980).

Heppell et al. (1984) suggested that whey-based formulas could be prepared by heat treatment. In their process diafiltration has to be performed to avoid the formation of undesirable Maillard-type reaction products during heating. The immunological safety of the formula was not clinically demonstrated and extensive heating could result in lower solubility and poorer digestibility due to possible cross-links between different protein molecules (McLaughlan et al., 1981).

The effects of enzymatic digestion of milk products were assessed by many researchers. Peptic and peptic-tryptic hydrolysis yielded breakdown products recognized by IgE antibodies (Haddad et al., 1979 and Spies et al., 1972). Pahud et al. (1985) indicated that trypsin-hydrolyzed (4 hr) whey protein remained inactive in inducing oral sensitization in guinea pigs. However, such an extensive hydrolysis could result in the production of high concentrations of bitter peptides (Clegg, 1977). Jost et al. (1987) indicated that combining selective hydrolysis by specific proteases with preceding or subsequent heat treatment(s) was promising in developing a hypoallergenic infant formula.

Previous work done in our laboratory (Asselin et al., 1988) showed that the allergenicity associated with α -lactalbumin (α -

La) and β -lactoglobulin (β -Lg) could be reduced, although not completely, by hydrolysis with α -chymotrypsin covalently fixed on activated agarose gel to avoid heat treatment to inactivate the enzyme. This approach of controlling the extent of proteolysis could be feasible in industry. Since the previous work did not completely eliminate the allergenicity of major whey proteins, the present study aimed at evaluating the *in vitro* effects of proteases involved in the human digestive system on the whey proteins, followed by heat treatment.

MATERIALS & METHODS

Materials

Enzymatic hydrolysis was performed on a commercial whey protein concentrate (13% protein) which was electrodialed and used in commercial infant food formulas (Wisconsin Dairies, Appleton, WI).

The following products were purchased from Sigma Chemical Company (St. Louis, MO): pepsin from porcine stomach mucosa (1:60000), α -chymotrypsin from bovine pancreas (type II), trypsin from bovine pancreas (type III), pancreatin from porcine pancreas (grade VI), hemoglobin from bovine blood.

The immunoreagents for Phadezym RAST were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden).

Enzymatic activity of proteases

Enzymatic activities of α -chymotrypsin (154 μ moles ATEE/min/mg) and trypsin (12 549 U BAEE/min/mg) were measured using substrates N-acetyl-L-tyrosyl ethyl ester (ATEE) and the N-benzoyl-L-arginine ethyl ester (BAEE), respectively (Schwartz and Takenaka, 1955). Pepsin (2 761 U Hb/min/mg) activity was evaluated with hemoglobin as substrate (Anson and Mirsky, 1932). Chymotryptic (6 μ moles ATEE/min/mg) and tryptic (431 U BAEE/min/mg) activities in pancreatin were also tested using ATEE and BAEE as substrates, respectively (Schwartz and Takenaka, 1955).

Enzymatic hydrolysis of whey proteins

Proteolysis of 5% commercial demineralized whey (13% protein) in water was performed according to the pH-stat technique (pH 7.5, 37°C) with various pancreatic enzymes, preceded or not by pepsin treatment (pH 2, 30 min). The degree of hydrolysis (DH), which is defined as the percentage of peptide bonds cleaved by the pancreatic enzymes, was determined according to the method of Adler-Nissen et al. (1983). The enzymes used for hydrolysis were α -chymotrypsin (96% protein), trypsin (100% protein), pancreatin (48% protein) and pepsin (88% protein). The enzyme to substrate ratio was 1 to 25 on protein basis. Aliquots were taken after 60 and 240 min of hydrolysis and heated at 80°C for 20 min to inactivate the enzyme and to reduce the more heat-labile but enzyme-resistant whey antigens, after which the hydrolyzate was freeze dried. Each enzymatic hydrolysis was performed in triplicate with three different lots of demineralized whey which were prepared by the same process. The calculation of the DH was done with the following formula:

$$DH = B \times N_b \times 1/\alpha \times 1/MP \times 1/h_{tot} \times 100\%$$

where B = Base consumption in ml (NaOH); N_b = Normality of the base (0.1N); α = Average degree of dissociation of the α -NH₂ groups; MP = Mass of protein in g; h_{tot} = Total number of peptide bonds in the protein substrate (meqv/g protein).

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Allergenic analysis of hydrolyzates

Six children (less than 2 years of age; 3 boys and 3 girls) with history of immediate allergic reaction to cow's milk (gastrointestinal, cutaneous and/or respiratory symptoms) were evaluated at the Allergy Clinic of Centre Hospitalier de l'Université Laval. The diagnosis was confirmed by skin test with cow's milk (Omega, Mtl) using the prick technique and by serum specific IgE antibody levels against cow's milk, α -La and β -Lg as measured by radioallergosorbent test (RAST) according to the procedure provided by Pharmacia Fine Chemical (Uppsala, Sweden). In spite of the fact that skin test and RAST are very specific and very sensitive (predictive values of 100%) for detecting subjects allergic to cow's milk (Bédard and Hébert, 1987), an opened milk challenge (including placebo) was also performed on 4 of 6 patients and the test was positive when eliciting immediate gastrointestinal, cutaneous and/or respiratory symptoms. These patients were evaluated by the Allergy Consultant at the Centre Hospitalier de l'Université Laval (Québec, Canada).

According to the procedure provided by Pharmacia, the allergenicity of the various hydrolyzates was evaluated by their ability to inhibit the binding of human serum specific IgE antibodies to β -Lg or α -La coated discs. In this procedure, nitrocellulose discs coated with either α -La or β -Lg were incubated overnight at room temperature with a pooled sera (25 μ L) from the above six patients along with 25 μ L of the different hydrolyzates (10 mg/mL) for 3 hr. The reaction was developed and the optical density (O.D.) read with an automatic spectrophotometer (Titertek) at 420 nm. The percentage inhibition of specific IgE to coated discs was then calculated according to the following formula:

$$\text{Inhibition \%} = \left[1 - \left(\frac{\text{O.D. with hydrolyzate}}{\text{O.D. without hydrolyzate}} \right) \right] \times 100$$

RAST inhibition analysis was performed in duplicate for each hydrolyzate. The results were subjected to analysis of variance by Waller-Duncan Method (SAS, 1985). In reporting the results of this investigation, only differences statistically significant at $P < 0.05$ are discussed.

RESULTS & DISCUSSION

THE CHARACTERISTICS of the patients with immediate hyperreactivity reactions to cow's milk are given in Table 1.

The degree of hydrolysis (DH) obtained for the hydrolyzates produced by different enzymatic systems is presented in Table 2. DH is related to the specific activity of each protease used: α -chymotrypsin hydrolyzes the bonds involving aromatic amino acids and many others (Pélissier, 1984), while trypsin is more specific for basic amino acids. The combination of both proteases gave a slight increase in DH when compared with that of hydrolyzates obtained by α -chymotrypsin alone. The DH obtained with pancreatin was much higher since this enzyme is essentially a mixture of α -chymotrypsin, trypsin and carboxypeptidase B (an exopeptidase). However, pretreatment with pepsin before hydrolysis with pancreatic enzymes changed the DH only slightly after 60 and 240 min.

Binding inhibition of IgE antibodies to α -La or β -Lg by various hydrolyzates is given in Table 3. Compared to the untreated proteins, allergenicity was significantly reduced by all

Table 2—Degree of hydrolysis (DH) measured by pH-stat method for each hydrolyzate of whey proteins after 60 and 240 min of reaction

Enzyme(s) used for hydrolysis	Degree of hydrolysis (%)	
	After 60 min	After 240 min
Control	0.4	0.9
Chymotrypsin	8.3	11.2
Trypsin	6.4	8.2
Chymotrypsin + trypsin	10.5	13.9
Pancreatin	15.4	20.5
Pepsin	1.0	1.7
Pepsin and chymotrypsin	9.1	11.8
Pepsin and trypsin	7.1	9.0
Pepsin and chymotrypsin + trypsin	10.1	12.2
Pepsin and pancreatin	15.8	20.1

Table 3—Percentage of inhibition of IgE binding to α -lactalbumin or β -lactoglobulin by various hydrolyzates obtained after 60 and 240 min of reaction

Hydrolyzates (10 mg/mL) produced with	INHIBITION (%) ^a			
	After 60 min		After 240 min	
	α -La	β -Lg	α -La	β -Lg
Control	87.6	77.2	85.0	77.0
Chymotrypsin	37.7	64.9	32.0	63.9
Trypsin	76.7*	79.7*	61.4	76.1*
Chymotrypsin + trypsin	40.4	64.1	38.0	55.0
Pancreatin	62.0	74.9*	32.3	52.7
Pepsin	89.1*	20.4	89.6*	11.5
Pepsin and chymotrypsin	31.6	3.7	45.1	8.9
Pepsin and trypsin	75.7*	14.5	56.4	25.0
Pepsin and chymotrypsin + trypsin	35.0	10.9	29.8	12.6
Pepsin and pancreatin	56.2	4.9	29.3	14.5

^a Phosphate-buffered saline (PBS) was used to determine the 0% of inhibition.

* These values are not significantly different compared to that of the control ($P \geq 0.05$).

pancreatic enzymes except trypsin (on α -La and β -Lg) and pancreatin (on β -Lg only), after 60 min. After 240 min, only the trypsin treatment on β -Lg was not significantly different from the control. The combination of α -chymotrypsin and trypsin significantly reduced the allergenicity of α -La and β -Lg. However, the percentage of inhibition was not different from the one obtained with α -chymotrypsin alone. Pancreatin showed significant decrease in the allergenicity of α -La after 60 min. The effects of enzymatic treatment were more important with time.

Treatment with pepsin alone was effective in reducing the allergenicity of β -Lg but had no effect on the α -La, even after 240 min of reaction. The most effective reduction in the allergenicity of both whey proteins of interest was obtained by treatment with pepsin and chymotrypsin successively. The specificity of both enzymes was complementary since pepsin and α -chymotrypsin are very active on allergenic determinants from β -Lg and α -La, respectively.

The degree of hydrolysis of α -La and β -Lg is not the only factor influencing the allergenicity, as shown in Tables 2 and 3. For example, pancreatin gave a DH value of 15.4 at 60 min compared to chymotrypsin hydrolyzate with a value of 8.3. However chymotrypsin hydrolyzate was less allergenic than pancreatin hydrolyzate. This suggests that the relative susceptibilities of the peptide bonds involved in allergenic site(s) of β -Lg and α -La depend on the specific activities of the enzymes. Similarly, Pahud et al. (1985) observed that most of the degradation of β -Lg determinants occurred during the first hour of hydrolysis, although polyvalent peptide antigens were still present in the controlled tryptic hydrolysis of whey protein.

Surprisingly, the allergenicity of β -Lg in whey protein hydrolyzates produced with pepsin and pancreatic enzyme, was increased after 240 min hydrolysis compared to that obtained after 60 min. This could be explained in part by peptide-peptide cross-linking and/or by the plastein reaction. When a protein is hydrolyzed enzymatically and the hydrolyzate then incubated with certain proteolytic enzymes under appropriate conditions, the hydrolysis is reversed and a high molecular

Table 1—Immunological evaluation of patients with history of reaction to cow's milk

Patients	Skin tests CM ^a (mm)	Specific IgE antibodies levels ^b			Opened milk challenge
		CM	α -La	β -Lg	
TMJP	4	3	3	3	ND ^c
CV	7	4	4	4	+
TC	1	3	2	2	ND
GJ	7	3	3	3	+
LS	8	4	3	4	+
MCF	4	2	3	2	+

^a Skin test by prick technique with cow's milk (CM): diameter of flare reaction in millimeter.

^b Phadezym RAST class: 4 = very high level of allergen specific IgE antibody; 0 = Absent or undetectable.

^c Not determined.

weight protein-like substance, plastein, is formed whose properties are somewhat different from the original protein (Eriksen and Fagerson, 1976).

In this study the allergenicity of the minor whey proteins such as immunoglobulins and bovine serum albumin was not evaluated. However, the application of heat treatment on whey might reduce the antigenicity of these minor proteins which contain heat-labile but enzyme-resistant antigens (Jost et al., 1987).

Thus, it is possible to prepare an infant formula by selective hydrolysis with specific proteases (pepsin and α -chymotrypsin) and a subsequent heat treatment. Further work is needed to control the reaction with these enzymes in order to obtain peptides of appropriate molecular size and to limit bitterness for a palatable milk formula.

CONCLUSION

THIS STUDY clearly indicates the importance of peptic enzyme and probably the acid secretion of the gastric digestive system for appropriate denaturation and/or degradation of whey proteins. The hydrolysis with pepsin followed by α -chymotrypsin was the most efficient combination of enzymes to reduce the allergenicity of α -La and β -Lg. The results also suggest that hydrolyzed whey proteins obtained by pepsin and α -chymotrypsin might be a promising ingredient in adapted cow's milk formulas of low allergenicity.

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