Physicochemical Treatment of Food Allergens: Application to Cow’s Milk Proteins

R. Jost

Nestlé Research Department, Nestec Ltd., 1000 Lausanne 26, Switzerland

The scope of the present chapter is to discuss technologic treatments leading to a substantial reduction in the allergenicity of food proteins. In the case of cow’s milk, general dairy technology comes into application. Thus, concerns about bacteriological safety and minimum heat damage to proteins obviously limit the operational range. In the particular field of infant formulas, CODEX principles have to be considered. With a relatively limited freedom of operation, we must consider in great detail the physicochemical properties of the proteins involved, in order to direct our treatments toward products of low allergenicity.

PHYSICAL PROPERTIES OF MILK PROTEINS RELATED TO THEIR ALLERGENIC POTENTIAL

According to mass balance in milk, caseins (CNs) might be considered as the major antigens of cow’s milk for humans. However, because of “adaptation” of the casein/whey-protein (CN/WP) ratio, the situation is changed in industrial formulas. It is therefore not surprising to find some WPs frequently cited as allergens in hypersensitive infants (1,2). Basically all cow’s milk proteins are potential allergens in view of a greater or lesser degree of sequence nonhomology (Table 1) between bovine and human proteins. Besides differences in primary structure, some physical properties affecting solubility, acid stability, or digestibility may be important factors. Thus beta-lactoglobulin (β-LG) is the most acid stable WP and will not be coagulated at the stomach pH. However, if heated in the presence of CNs (e.g., in UHT milks or condensed milks), it will be associated with the micelle and will consequently be co-precipitated with the CNs (3). Such a situation may be important in the low β-LG-related antigenicity of strongly heated milk.
### TABLE 1. Properties of the major cow's milk proteins related to their allergenic potential

<table>
<thead>
<tr>
<th>Property</th>
<th>Caseins</th>
<th>Whey proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha_s$-CN</td>
<td>$\alpha_s$-CN</td>
</tr>
<tr>
<td>Concentration (g/liter) in milk</td>
<td>12–15</td>
<td>3–4</td>
</tr>
<tr>
<td>Calculated molecular weight*</td>
<td>23,600</td>
<td>25,200</td>
</tr>
<tr>
<td>Association with casein micelle</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Solubility at pH &lt; 4, &gt; 2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sequence homology (%) bovine vs. human</td>
<td>nd*</td>
<td>nd*</td>
</tr>
</tbody>
</table>

*Calculated from sequence data and rounded to next 100.

Carbohydrate neglected.

Composed of two H chains (each ~ 53,000 molecular weight) and two L chains (each 22,000 molecular weight).

Partial dissociation from micelle to serum at low temperature.

Heat-induced complex with micelle in severely heated milk.

nd, not determined.

$\beta$-CN is the major casein of human milk (27).

*For the glycomacropeptide part only (28).

No equivalent in human milk. Human serum retinol binding protein is homologous to some extent with $\beta$-LG (29,30).

Refs. 18 and 31.

*Main immunoglobulin of human milk is secretory IgA.
HEAT STABILITY OF MILK PROTEINS AND EFFECT OF HEAT PROCESSING ON ANTIGENICITY

Caseins possess ordered structure to a relatively low extent (Table 2). They resist heating at very high temperature for prolonged time. In contrast, the globular WPs are easily heat coagulated in the absence of the CN micelle, such as in whey. Their transition temperatures, as measured by differential scanning calorimetry, are within a range of 60°C to 80°C (4). The corresponding transitions lead to irreversible denaturation in the case of β-LG, bovine serum albumin (BSA), and IgG. In contrast, alpha-lactalbumin (α-LA) renatures after cooling and in the presence of Ca ions (5). Despite its relatively low transition temperature, α-LA is the most heat-stable WP from a practical point of view (2).

Denaturation of BSA starts above 50°C with partial helix disruption. At 60°C, heat-induced aggregation takes place (6). BSA, which is partially or fully saturated with fatty acids, shows an increased heat stability (7). Biologic activity of immunoglobulins is very heat-labile and is rapidly destroyed above 70°C, but we have little information as to whether its antigenicity as a bovine protein is likewise heat-labile.

The thermal denaturation of β-LG appears to follow either a second-order process (8) or, at least in milk, a reaction of the order 1.5 (9). The lesson from these data is that heat processing of milk will mainly reduce WP-related antigenicity, but it is unlikely that CN-dependent antigenicity will be greatly affected.

Early studies on the allergenicity of heated milks in guinea pigs demonstrated an inverse relationship between antigenicity and the intensity of heat treatment (10–12). While CN-dependent allergenicity could not be satisfactorily abolished, WP antigenicity was decreased to a very large extent or even completely. Accordingly, if one wants to rely on heat processing alone, the ideal substrates are the WPs in the form of whey or whey protein concentrate (WPC). This option was indeed adopted (13,14); and it was shown that heating at 100°C, or under steam pressure at 120°C, drastically reduced the oral sensitization capacity of whey in guinea pigs, whereas the reduction in eliciting capacity was not entirely satisfactory (14).

Simplicity and low costs seem to be attractive features of such an “all-whey hypoallergenic formula”; on the other hand, however, one can foresee some problems related to the low protein solubility in heated whey both during processing and in the final application of the product. It is not yet clear to what extent the achieved level of hypoallergenicity will provide sufficient safety for infants predisposed to hypersensitivity.

SENSITIVITY OF MILK PROTEINS TO PROTEOLYTIC CLEAVAGE

Limited cleavage of the polypeptide chains of milk proteins by specific endopeptidases such as trypsin, chymotrypsin, papain, pepsin, and others leads to a
TABLE 2. Properties of cow's milk caseins and whey protein related to their heat stability

<table>
<thead>
<tr>
<th>Property</th>
<th>Caseins</th>
<th>Whey proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>αs_{1}-CN</td>
<td>αs_{2}-CN</td>
</tr>
<tr>
<td>Number of S-S bridges/mole</td>
<td>—</td>
<td>1</td>
</tr>
<tr>
<td>Number of SH groups/mole</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Secondary structure content (% of total chain)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-helix</td>
<td>Low^a</td>
<td>nd^b</td>
</tr>
<tr>
<td>β-sheet</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Transition temperature (°C) by DSC^g</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Heat coagulability in serum at 80–100°C</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>Holding time (sec) for &gt; 90% denaturation in skim milk\ at 90°C</td>
<td>200</td>
<td>900</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^aRef. 32.
^bnd, not determined.
^cRef. 33.
^dRef. 34.
^eRef. 35.
^fRefs. 36 and 37.
^gRef. 38.
^hRef. 4.
^iRef. 9.
drastic reduction in antigenicity (15). Because of the exposing of new ionized terminal amino and carboxyl groups, conformation-dependent antigenic sites collapse. Progressive hydrolysis gradually eliminates sequential (conformation-independent) determinants, more recently recognized as protein regions inducing antipeptide responses (15). It is quite impossible to specify general and precise limits of molecular weights for peptide immunogenicity, but polypeptides of less than 5,000 molecular weight tend to be weakly immunogenic, and oligopeptides are considered nonimmunogenic (16,17).

If we analyze CNs and WPs for their number of trypsin-sensitive sites in the chain, we find between 7 and 14 sites per 100 total residues (Table 3). If these sites were all attacked and cleaved in an \textit{in vitro} process, the mean tryptic peptide would be about 10 residues long and would range between 800 and 1600 molecular weight. In terms of immunogenicity, such peptides would already be at or below the limit. Few of them would be expected to be plurivalent antigens capable of triggering anaphylactic reactions.

In practice, the number of cleaved sites is lower than the total number of theoretical sites. Such resistance to endopeptidase attack may result from disulfide bridges shielding segments of the chain. This is clearly the case for BSA. Trypsin and other proteases were shown to produce large antigenic fragments (18). Cleavage of bovine immunoglobulins leads to formation of Fab-type fragments with antigen-combining capacity (19).

Both \(\alpha\)-LA and \(\beta\)-LG are extensively hydrolyzed by trypsin, chymotrypsin, or pancreatin. Antigenic properties of tryptic fragments of \(\beta\)-LG were recently studied (20). Hydrolysis of undenatured \(\beta\)-LG by tosyl-phenylalanine chloromethyl ketone (TPCK)-treated trypsin led to the fragmentation pattern shown in Fig. 1. Of 18 T-sites, 11 sites were cleaved as theoretically expected, whereas seven were cleaved at a very low rate or were not attacked at all. Some of the fragments were active in binding anti-\(\beta\)-LG rabbit antibody, but a single fragment gave a precipitation line in agar gel diffusion with anti-\(\beta\)-LG serum (Table 4). This disulfide-linked two-chain fragment (molecular weight 2,700) did not trigger passive cutaneous anaphylaxis (PCA) at a maximum concentration of 2 mg/ml in passively sensitized guinea pigs in which 10 \(\mu\)g/ml of \(\beta\)-LG repeatedly gave a positive response. The total digest was active at 20 mg/ml but was inactive at 2 mg/ml. The triggering activity of the unfractionated digest might be to residual \(\beta\)-LG or, alternatively, to cooperation between nontriggering peptides.

Caseins, because of their open and strongly hydrated structure, should \textit{a priori} be easier substrates than WPs. This is what is also observed in practice, with the limitation that the highly acidic regions containing the phosphoserine clusters are rather protease-resistant.

Cyanogen bromide cleavage of \(\beta\)-CN (which contains six methionine residues) yielded large fragments which, in mixture, reconstituted close to 100% of the total antibody-precipitating activity of \(\beta\)-CN (21). This observation is of interest because it presents an experimental possibility of determining the chain-length limit for precipitating antigenic peptides. Punctual hydrolysis of \(\alpha_s\)-CN with TPCK-
<table>
<thead>
<tr>
<th>Property</th>
<th>Caseins</th>
<th>Whey proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>αs1-CN</td>
<td>αs2-CN</td>
</tr>
<tr>
<td>Σ residues/mole</td>
<td>199</td>
<td>207</td>
</tr>
<tr>
<td>Mean residue weight*</td>
<td>119</td>
<td>122</td>
</tr>
<tr>
<td>Σ lysine, arginine/mole</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>Mean molecular weight, tryptic peptide**</td>
<td>1,184</td>
<td>592</td>
</tr>
<tr>
<td>Mean molecular weight, tryptic/chymotryptic peptide**</td>
<td>592</td>
<td>505</td>
</tr>
<tr>
<td>Hydration (g H₂O/g protein)</td>
<td>&gt; 3</td>
<td>&gt; 6</td>
</tr>
<tr>
<td>Observed in vitro digestibility*</td>
<td>High</td>
<td>nd</td>
</tr>
<tr>
<td>Formation of plurivalent antigens during tryptic in vitro digestion</td>
<td>+ f</td>
<td>nd</td>
</tr>
</tbody>
</table>

* Molecular weight/residue per mole.
* nd, not determined.
** Σ [residual per mole/Σ (Lys, Arg)] × mean residue weight.
*** Σ [residual per mole/Σ (Lys, Arg, Trp, Tyr, Phe)] × mean residue weight.
** Tryptsin pH 8, 40°C, 4 hr.
* Precipitating antiprotein antibodies in a quantitative immunoprecipitation test (21, 22).
* Precipitation arc in agar gel; see also Table 4.
* Pancreatin instead of trypsin (39).
* High-molecular-weight antigenic fragments obtained (40).
* Fragments corresponding to Fab, Fab', and Fc (19).
H2N

H-Leu-Ser-Phe-Asn-Pro-Thr-Leu-Gln-Glu-Glu-Gln-Cys-His-Ile-OH

H-Trp-Glu-Asn-Asp-Glu-Cys-Ala-Gln-Lys-OH

FIG. 1. Observed cleavage of undenatured β-lactoglobulin by TPCK-treated trypsin at 1% E/S ratio, pH 7.8, 4 hr at 40°C (20). ↑, Lys-Lys sequences giving rise to free lysine; •, sites which were not cleaved or at a very low rate only. (Bottom) Disulfide-bridged tryptic fragment which represents a major antigen of the total tryptic digest.

trypsin led to a variety of fragments, some of which retained rather limited precipitating capacity (22). Extended hydrolysis of total CN with pancreatin and fungal proteases led to hydrolysates devoid of sensitizing or eliciting capacity (23). Casein hydrolysates (such as those present in therapeutic formulas) have been shown to be safe in hypersensitive infants. Their major disadvantages are a low palatability and bitterness or off-flavors. Bitterness is the result of bitter peptide formation during proteolysis of CN (24). Debittering procedures exist, but they lead to even higher costs and disequilibration of the amino acid composition of the hydrolysate (25).

ANTIGENICITY OF WHEY PROTEIN HYDROLYSATES

WPCs such as those produced by ultrafiltration of whey are very potent allergens to guinea pigs (oral route). Such protein concentrates contain close to 30%
TABLE 4. Immunoreactivity of tryptic fragments of β-LG in vitro and in vivo

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Calculated molecular weight</th>
<th>Concentration mg/ml</th>
<th>Activity RIA¹</th>
<th>Precipitation arc²</th>
<th>Eliciting capacity³</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-LG</td>
<td>15-20</td>
<td>696</td>
<td>2.0</td>
<td>$2.9 \times 10^{-3}$</td>
<td>&lt; &lt;</td>
</tr>
<tr>
<td>β-LG</td>
<td>21-40</td>
<td>2,030</td>
<td>&lt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-LG</td>
<td>41-60</td>
<td>2,314</td>
<td>~ 2</td>
<td>~ $10^{-3}$</td>
<td>6 $\times 10^{-6}$</td>
</tr>
<tr>
<td>β-LG</td>
<td>61-69s (140-162s)</td>
<td>2,761</td>
<td>2.0</td>
<td>$0.7 \times 10^{-3}$</td>
<td>4 $\times 10^{-4}$</td>
</tr>
<tr>
<td>β-LG</td>
<td>92-101</td>
<td>1,065</td>
<td>2.0</td>
<td>$1.9 \times 10^{-3}$</td>
<td>1.7 $\times 10^{-6}$</td>
</tr>
<tr>
<td>β-LG</td>
<td>125-135</td>
<td>1,245</td>
<td>2.0</td>
<td>$1.6 \times 10^{-3}$</td>
<td>0.8 $\times 10^{-6}$</td>
</tr>
<tr>
<td>Total digest¹</td>
<td></td>
<td></td>
<td>2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-LG</td>
<td></td>
<td></td>
<td>20.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-LG</td>
<td></td>
<td></td>
<td>$2 \times 10^{-3}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-LG</td>
<td></td>
<td></td>
<td>$2 \times 10^{-2}$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹Data are from ref. 20.
²Solid-phase RIA with rabbit anti-LG serum. Relative reactivity of pure β-LG is 1.
³Ouchterlony double-diffusion test.
⁴PCA: donor sera from whey protein sensitized animals.
⁵TPCK-treated trypsin (2% weight/weight), pH 7.8, 4 hr at 40°C. The digest was separated by reverse-phase LC on C-18 column (20).

of immunoreactive β-LG, estimated by RIA. Table 5 shows that trypsin-hydrolyzed WP was nonsensitizing (oral route) and had no eliciting capacity (intravenous route) in guinea pigs sensitized to either WP or milk (26). The hydrolysate showed about a 300-fold decrease in β-LG specific antigenicity and gave (at 50 mg/ml) a weak precipitation line in the Ouchterlony diffusion test. It also showed (at an antigen dilution up to 1:8) a precipitation arc with anti-BSA serum. Accordingly, trypsic hydrolysis had reduced this antigen by not more than a factor of about 8 (titer in WPC 1:64). Electrophoresis (SDS-PAGE) revealed that the antigen(s) consisted of intact BSA. In addition, BSA fragments of 20,000 to 24,000 had accumulated. Heating of the hydrolysate at 80°C or higher led to the inactivation of these antigens (Table 5).

Hydrolysates fractionated by ultrafiltration and diafiltration gave permeates, essentially composed of peptides. The permeate fraction, obtained in yields ranging from 50% to 60% of the parent hydrolysates, showed no precipitation lines in immunodiffusion with anti-β-LG, anti-α-LA, or anti-BSA sera. Their β-LG-specific binding activity was about one order of magnitude lower than that of the original hydrolysates (Table 5). The same observations were made with pancreatic hydrolysates, fractionated by heat coagulation and desludging of insolubles.

Despite clear-cut differences in residual antigenicity in vitro, the lower level of antigenicity of fractionated hydrolysates was not reflected in the guinea pig tests because the unfractionated hydrolysates were already unreactive. It is possible that such a difference in allergenicity becomes manifest if, instead of oral sensitization, another route would be used to sensitize the animals.
### Table 5. Immunoreactivity of whey protein hydrolysates in vitro and in vivo

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Activity RIA* (μg β-LG/mg)</th>
<th>Precipitation arc (Ouchterlon)†</th>
<th>Allergenicity (guinea pigs)*</th>
<th>Oral sensitization (positive/total)</th>
<th>Eliciting capacity (PCA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whey protein concentrate*</td>
<td>280 ± 50</td>
<td>+ (1/1,024)</td>
<td>+ (1/256)</td>
<td>+ (9/9)</td>
<td>+ +</td>
</tr>
<tr>
<td>Trypsin-hydrolyzed WPC*</td>
<td>~ 1.0</td>
<td>± (1/1)</td>
<td>–</td>
<td>– (0/11)</td>
<td>– –</td>
</tr>
<tr>
<td>Heated (20 min/80°C) hydrolysate†</td>
<td>0.4–1.0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>nd*</td>
</tr>
<tr>
<td>Heated (120 sec/125°C) hydrolysate†</td>
<td>0.4–1.0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>(0/8)</td>
</tr>
<tr>
<td>Ultrafiltrated hydrolysate (permeate)</td>
<td>0.03–0.15</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>– –</td>
</tr>
<tr>
<td>Pancreatic hydrolysate (soluble fraction†)</td>
<td>0.02–0.10</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>(0/8)</td>
</tr>
<tr>
<td>For comparison: human milk whey*</td>
<td>0.06–0.10</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>nd* nd* nd*</td>
</tr>
</tbody>
</table>

*Solid-phase RIA (26).
*Initial antigen concentration 50 mg/ml.
*Guinea pig model described in ref. 26. Donor sera from whey-sensitized animals (A) and from skim-milk-sensitized animals (B).
*Essentially undenatured WP produced by ultrafiltration of sweet whey. Protein content 84% total dry solids.
*Porcine trypsin (2% weight/weight), pH 7.5, 55°C, 4 hr.
*Hydrolysates heated batchwise.
*nd, not determined.
*Hydrolysate heated continuously.
*Hydrolysate ultrafiltrated and diafiltrated on membranes with nominal cut-off 10,000; permeate fraction recovered.
*Pancreatin (5% weight/weight), pH 7.5, 55°C, 4 hr. Heat coagulation (20 min/80°C), separation of insolubles by desludger and filtration.
*Pool of several wheys dialyzed to remove lactose and salts. Protein: 75% on total dry solids.

### CONCLUSIONS

Limited or extensive hydrolysis with proteases followed by heat treatment, as well as hydrolysis followed by fractionation, is a practical way of reducing milk protein antigenicity. Purified CN hydrolysates, as well as hydrolysates of WP, are on the market and were found to be extremely well tolerated in allergic infants.

Technologically, the route from a heat-modified whey formula to the purified
hydrolysates used in therapeutic oligopeptide diets is long. En route we lose palatability and functionality and we increase costs.

In the near future we hope, by an appropriate combination of enzyme and heat treatments, to achieve more palatable and less expensive hypoallergenic formulas. Their more general availability and better organoleptic properties could make an important contribution to preventing hypersensitivity to cow’s milk proteins in infants.

REFERENCES