

Selective Hydrolysis of Milk Proteins To Facilitate the Elimination of the ABBOS Epitope of Bovine Serum Albumin and Other Immunoreactive Epitopes[†]

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ABSTRACT

Milk proteins are hydrolyzed to prevent immunological reactions, but immunoreactive epitopes, including the ABBOS epitope of bovine serum albumin (BSA), can still be detected in commercially available milk protein hydrolysates. We used lactococcal cell-envelope proteinase (CEP) for the hydrolysis of the individual milk proteins and of mixtures thereof, or for the hydrolysis of sodium caseinate (contaminated with whey proteins). CEP exclusively degraded casein, leaving the four major whey proteins intact. This property facilitated the removal of the intact whey proteins from the casein fragments by ultrafiltration. Depending on the molecular mass of the whey protein to be removed, membranes with cutoff values between 3 and 30 kDa were used, resulting in casein hydrolysates free of protein fragments with cross-reactive whey-protein-specific IgE (immunoglobulin E) or ABBOS antibody-binding sites. Even the casein itself was degraded in such a way by CEP that cross-reactive casein-specific IgE antibody-binding sites could be eliminated. The product could find application in infant formulas for therapeutic and preventive treatment of children with cow's milk allergy; in addition, the preventive use of such formulas in children genetically susceptible to the development of insulin-dependent diabetes mellitus (IDDM) should be considered if a relationship between the consumption of BSA and IDDM were to become more apparent. The method is also applicable for preparing casein-free whey protein preparations.

Cow's milk protein is the most common allergen during infancy and early childhood, and consumption may lead to allergic reactions (18, 19). In addition cow's milk protein may be an environmental trigger for the development of insulin-dependent diabetes mellitus (IDDM) in susceptible persons (16, 34). The residues 126 to 144 (ABBOS) of the whey protein bovine serum albumin (BSA) (21) have been reported to be possibly responsible for an autoimmune reaction directed against pancreatic islet cells. In addition, abnormal T-cell reactivity to β -casein (6) and to β -lactoglobulin (β Lg) (29, 35) has been demonstrated in patients with recent-onset IDDM.

We have shown that when pepsin digestion occurs in vitro under conditions that prevail in the stomach of infants (pH > 4), the degradation of the milk proteins is less efficient than under conditions present in the stomach of adults (pH 2). As a result immunoreactive epitopes, including the ABBOS epitope of BSA, are not completely eliminated (3, 31). This situation may cause an increased risk of developing immunological responses, since the immature gastrointestinal mucosal barrier of infants will allow the passage of antigenic fragments into the systemic circulation (23, 25).

In order to treat or prevent immunological reactions, infant formulas based on milk protein hydrolysates have

been developed. Nonspecific enzymatic hydrolysis of milk proteins followed by ultrafiltration (17, 38) is often used to reduce the antigenicity of cow's milk and to remove large, probably antigenic residues. However, we have recently shown that many of these so-called hypoallergenic infant foods still contain protein fragments which can cross-react with the milk proteins and the ABBOS peptide (36, 37).

The inability of the lactococcal cell-envelope proteinase (CEP) to degrade the four major whey proteins (including BSA) was first encountered with the initial efforts to prepare cheese from ultrafiltered milk (which includes the whey proteins). During ripening of this type of cheese the whey proteins were shown to be resistant to the proteolytic enzymes of rennet and of the starter bacteria (7), probably including the lactococcal CEP. This unexpected observation in the 1980s formed the basis for exploring the use of food-grade lactococcal CEP for another application, namely selective hydrolysis in combination with ultrafiltration as a tool for protein separation. In the present article we show that this method can be used for the preparation of a casein hydrolysate free of protein fragments with cross-reactive milk-protein-specific IgE and ABBOS antibody-binding sites.

MATERIALS AND METHODS

Materials. Total casein was prepared by isoelectric precipitation from skim milk with HCl at pH 4.6. The precipitated casein was thoroughly washed with distilled water, redissolved by the addition of NaOH to pH 7.0, and then lyophilized. The proteins

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α -lactalbumin (α La) and β Lg (genetic variants A and B) were prepared from cheese whey by using anion-exchange chromatography as described earlier (33). BSA (A 4378) and IgG (I 5506) were purchased from Sigma Chemical Co. (St Louis, Mo., USA) and sodium caseinate from DMV (Veghel, The Netherlands). The chromogenic substrate 3-carbomethoxypropionyl-L-arginyl-L-prolyl-L-tyrosine-*p*-nitroanilide hydrochloride (MeO-succinyl-Arg-Pro-Tyr-*p*NA; S-2586) was obtained from Chromogenix AD (Mölnådal, Sweden), the chromogenic substrate L-lysine-*p*-nitroanilide dihydrobromide (Lys-*p*NA) from Sigma, phenylmethylsulfonylfluoride (PMSF) from Boehringer Mannheim (Almere, The Netherlands) and dimethylsulfoxide (DMSO) from Baker (Deventer, The Netherlands).

Growth and harvesting of the organism. The organism *Lactococcus lactis* subsp. *cremoris* WG2 (collection Netherlands Institute for Dairy Research, B1021) was grown overnight at 30°C to a final pH of 4.6 to 4.8 in milk; stationary-phase cells were subsequently harvested as described by Exterkate (10).

Preparation of a crude and a partially purified lactococcal cell-envelope proteinase fraction. Essentially according to Mills and Thomas (26) the lactococcal CEP was autolytically released by incubating the cells in a calcium-free buffer (20 mM imidazole-HCl buffer, pH 6.4) at 30°C. After incubation, the cells were sedimented by centrifugation for 15 min at $15,000 \times g$. Supernatant fractions were pooled, extensively washed with the same buffer supplemented with 10 mM CaCl_2 , and concentrated with a 200-ml ultrafiltration cell (Amicon Corporation, Danvers, Mass., USA) equipped with a 30-kDa-cutoff Omega membrane (Filtron, Northborough, Mass., USA).

For further purification the crude CEP concentrate was centrifuged for 30 min at $48,000 \times g$, filtered (0.45- μm -pore-size filter) and applied onto a MonoQ HR 10/10 (Pharmacia, Uppsala, Sweden) anion-exchange column, equilibrated with 20 mM imidazole-HCl buffer (pH 6.4) supplemented with 10 mM CaCl_2 . After elution of nonbinding material, proteins were eluted at 2 ml/min with a 0 to 0.3 M NaCl gradient in the same buffer (27). Fractions containing the enzyme activity, and without activity of the intracellular exopeptidase PepN, were pooled, concentrated, divided into aliquots, and stored at -20°C . Before use, the enzyme activity was quantified as described below.

Enzyme assays. In order to quantify the enzyme activity of the lactococcal CEP and to test contamination with the intracellular exopeptidase PepN two different chromogenic substrates were used, S-2586 and Lys-*p*NA, respectively; both methods have been described by Exterkate et al. (11, 15). One proteinase unit represents the formation of 1 nmol of *p*-nitroanilide per minute at 30°C.

Hydrolysis experiments. Two different series of hydrolysis experiments were performed. In the first series the various milk proteins, total casein, α La, β Lg, BSA, or IgG were separately dissolved in 50 mM imidazole-HCl buffer (pH 6.5) for the incubation with the lactococcal CEP. The final protein concentration was 10 mg/ml, the enzyme activity 15 or 90 units per mg of protein, and the temperature 30°C. Aliquots of 20 μl were taken out of the incubation vessels at 0, 1, 3, 5, and 22 h after the addition of CEP.

In the second series of hydrolysis experiments commercially available sodium caseinate and a mixture of equal amounts of total casein, α La, and β Lg (on a protein basis) were dissolved in 50 mM imidazole-HCl buffer (pH 6.5) and offered as a substrate for the lactococcal CEP. The total protein concentration was 6 mg/ml and the enzyme activity 90 units per mg of protein. After hydrolysis for

22 h at 30°C, the hydrolysates were ultrafiltered through membranes (Omega-type, Filtron) with cutoff values ranging from 3 to 30 kDa.

Because BSA became insoluble in the imidazole-HCl buffer after heating, which is necessary to expose the ABBOS epitope for immunological detection (see below), a mixture of equal amounts of total casein and BSA was dissolved in distilled water and hydrolyzed using the pH-stat technique (1). During the hydrolysis with CEP the pH was kept constant at pH 7.0 by the addition of 0.05 M NaOH. The total protein concentration was 4 mg/ml and the enzyme activity 90 units per mg of protein. After hydrolysis for 22 h at 30°C, the hydrolysates were ultrafiltered through membranes (Omega-type, Filtron) with cutoff values ranging from 3 to 30 kDa.

All hydrolysate samples were immediately frozen and stored at -20°C until further analysis. In the case of reversed-phase high-performance liquid chromatography (RP-HPLC) analysis, the enzyme was inactivated by diluting the thawed enzyme-substrate mixtures with RP-HPLC buffer (see below). For the *in vitro* determination of the antigenic properties the samples were used immediately without freezing to avoid possible formation of protein aggregates, and PMSF (100 mM in DMSO) was added to a final concentration of 1 mM to inactivate the enzyme.

Physicochemical characterization of the protein hydrolysates. To follow the degradation of the milk proteins during hydrolysis, we applied RP-HPLC as described by Visser et al. (40). Separation is based on a difference in hydrophobicity of the protein fragments. Before injection (50 μl), the hydrolysates were diluted with RP-HPLC buffer to a final concentration of 1 mg/ml for each individual milk protein. To follow the degradation of the intact individual milk proteins during hydrolysis, we quantitated the decrease of the intact-protein peak areas by integration (Turbochrom, Perkin Elmer Co., Cupertino, Calif., USA).

Immunological characterization of the protein hydrolysates. The occurrence of cross-reactivity between the ABBOS peptide and the various CEP hydrolysates was determined by competitive enzyme-linked immunosorbent assay (ELISA), using rabbit anti-ABBOS serum. In order to expose the ABBOS epitope, hydrolysates were heated for 5 min at 95°C (3). The procedure and preparation of the rabbit anti-ABBOS serum have been previously described (3, 36).

The same method was applied to determine the presence of cross-reactive milk-protein-specific IgE antibody-binding sites in the various CEP hydrolysates (37, 31). We used a human antiserum with high IgE antibody titers to all individual milk proteins consisting of pooled sera from five children with clinically diagnosed cow's milk allergy. Sera collected from eight subjects not allergic to cow's milk proteins were pooled and served as a normal control human serum.

Reliability of results. All hydrolysis and analytical experiments were performed at least in duplicate. The precision of the ELISA, expressed as the repeatability standard deviation (S_r), was determined from separate experiments. S_r was 1.77% ($df = 19$) including between-well and between-plate variation.

RESULTS

Purification of CEP. The CEP concentrate obtained after release from the washed lactococcal cells appeared to be contaminated with intracellular proteolytic enzymes as measured with the chromogenic substrate Lys-*p*NA. After anion-exchange chromatography, the CEP-containing fractions were clearly separated from the fraction containing the intracellular exopeptidase PepN activity.

Physicochemical characterization: Degradation of the individual milk proteins. The specificity of the lactococcal CEP for the various milk proteins is shown in Fig. 1. Degradation of protein under the chosen conditions was only detectable when total casein was used as a substrate; the whey proteins α La, β Lg, BSA, and IgG remained intact. And in addition degradation products of the whey proteins could not be detected (data not shown). The increase in RP-HPLC peak area for IgG (Fig. 1) cannot be explained, but may be connected with the observed insolubilization of IgG after thawing of the hydrolysate samples. With an increase in the enzyme concentration to 90 units per mg of protein, total casein was degraded within 5 h (Fig. 1).

Separation of casein and whey protein(s). The discriminating behavior of CEP leading to the specific degradation of casein in the presence of the whey proteins (α La, β Lg, BSA, and IgG) caused an increased difference in molecular weight between the smaller casein fragments and the still-intact whey proteins. Depending on the cutoff value of the membrane, specific removal of intact whey proteins from the casein fragments was possible. Membranes with cutoff values up to 30 kDa selectively removed BSA from a mixture of total casein and BSA or from a commercially available sodium caseinate (generally contaminated with whey proteins) after treatment with CEP. Fig. 2 shows the specific degradation of total casein by CEP in the presence of BSA with subsequent removal of the intact BSA using a 30-kDa membrane. It also shows the slower degradation of α _{S1}-casein than of β -casein.

Immunological characterization of the casein hydrolysates: Residual ABBOS antigenicity. RP-HPLC analysis could not detect BSA or fragments thereof in permeate fractions obtained after hydrolysis of a mixture of total casein and BSA or sodium caseinate with CEP followed by ultrafiltration (Fig. 2). Using the more sensitive immunological method these results were confirmed. We showed that the

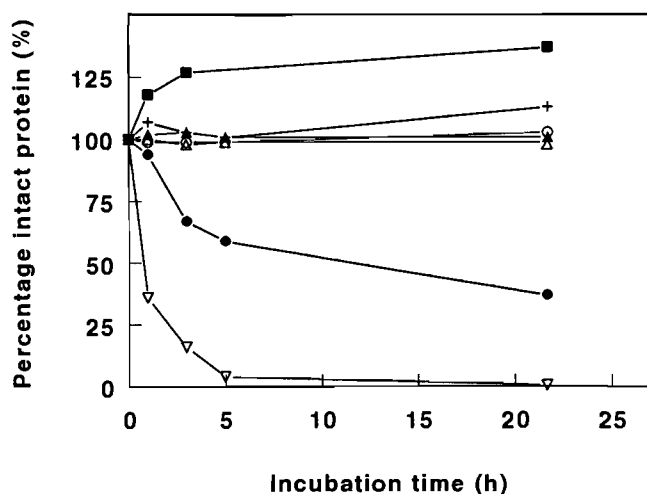


FIGURE 1. Degradation of the various milk proteins during incubation with CEP (15 units per mg protein) expressed as percentage intact protein. (+) BSA; (Δ) BSA (90 units per mg protein); (○) β Lg; (■) IgG; (▲) α La; (●) total casein; (∇) total casein (90 units per mg protein).

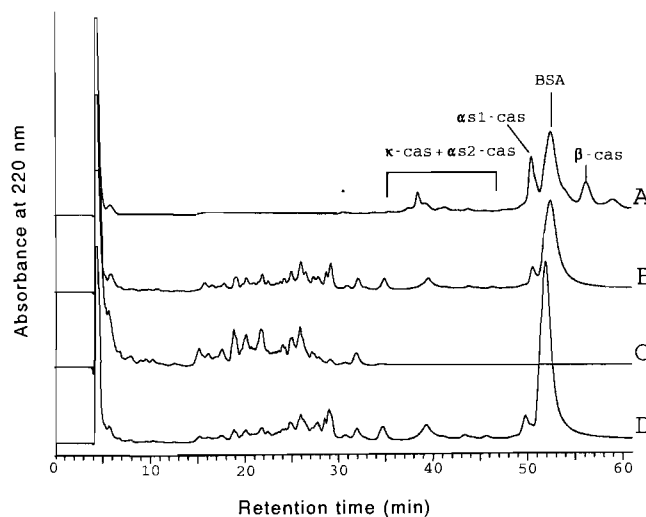


FIGURE 2. RP-HPLC analysis of hydrolysates in which a mixture of total casein and BSA was hydrolyzed with CEP (90 units per mg protein). Intact BSA and casein fragments were subsequently separated by means of ultrafiltration (cutoff value 30 kDa). (A) Mixture of casein and BSA, $t = 0$ h; (B) Mixture of casein and BSA, $t = 22$ h; (C) 30 kDa permeate fraction of the $t = 22$ h hydrolysate; (D) 30 kDa retentate fraction of the $t = 22$ hours hydrolysate.

rabbit anti-ABBOS serum did not show any binding inhibition with the permeate fractions up to an inhibitor concentration of 1 g/liter (Fig. 3). In contrast, the retentate fractions and the control (ABBOS peptide) showed 100% inhibition at that protein concentration.

Residual IgE antigenicity. Treatment of a mixture of total casein, β Lg, and α La or sodium caseinate with CEP followed by ultrafiltration through membranes with lower cutoff values (5 or 3 kDa) additionally removed the lower molecular weight antigenic whey proteins. Fig. 4 shows typical examples of the inhibition ELISA for β Lg, α La, and total casein for these hydrolysates. The human antiserum did

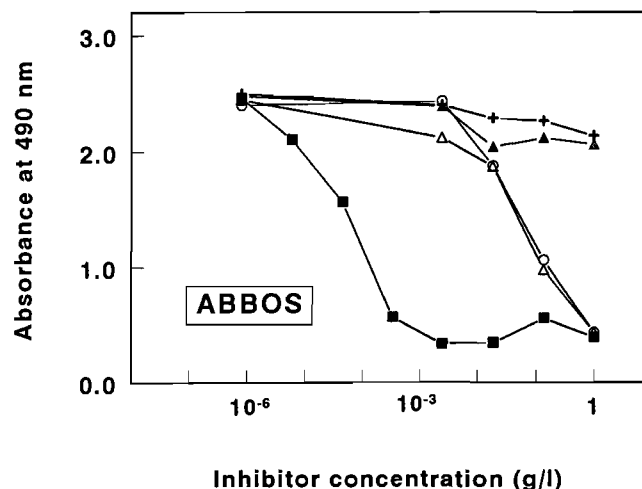


FIGURE 3. Inhibition of the binding to rabbit IgG anti-ABBOS antibodies of retentate and permeate fractions obtained after ultrafiltration of a mixture of total casein and BSA hydrolyzed with CEP (90 units per mg protein). (■) synthesized ABBOS peptide; (Δ) 10-kDa retentate; (○) 30-kDa retentate; (+) 10-kDa permeate; (▲) 30-kDa permeate.

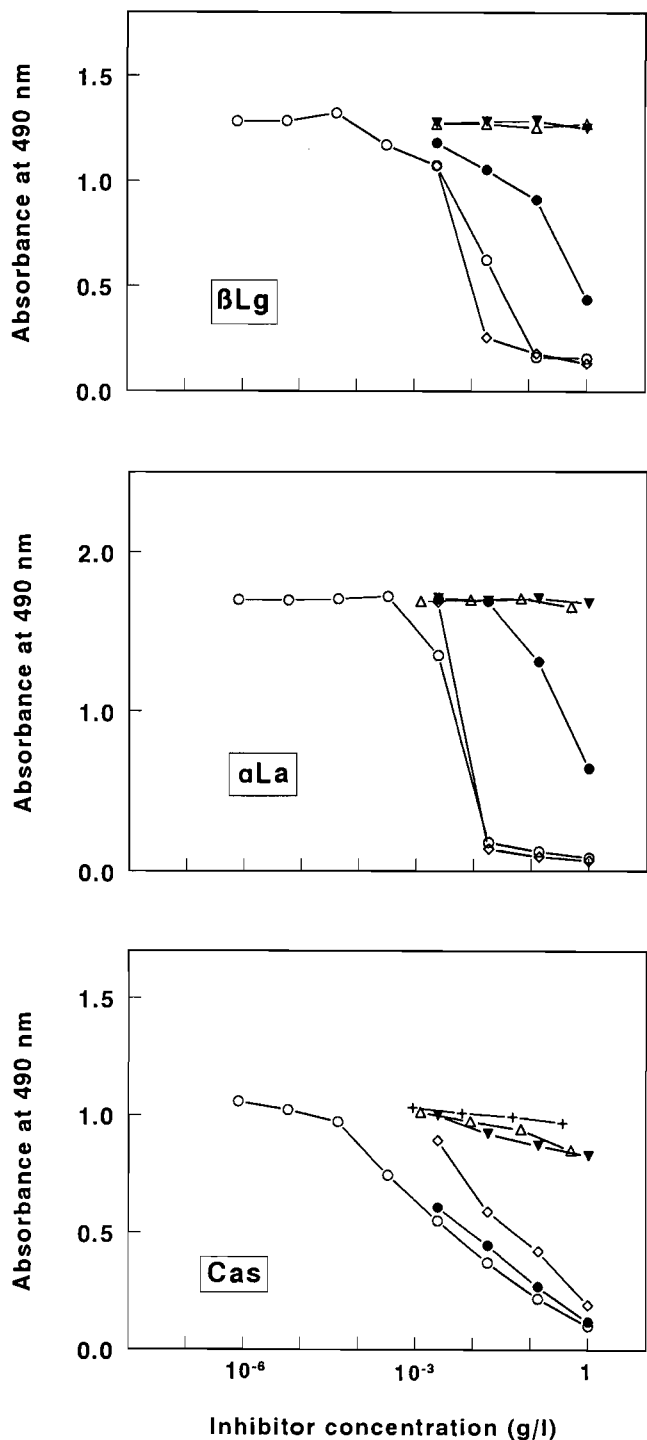


FIGURE 4. Inhibition of the binding to human IgE antibodies against β Lg, α La, or total casein (cas) of retentate and permeate fractions obtained after ultrafiltration of a mixture of cas, α La, and β Lg or of sodium caseinate hydrolyzed with CEP (90 units per mg protein). ($\circ$) intact milk protein: upper panel β Lg, middle panel α La, lower panel cas. ($\diamond$) 5-kDa retentate of mixture cas, α La and β Lg; ($\bullet$) 5-kDa retentate of sodium caseinate; ($\triangle$) 5-kDa permeate of mixture cas, α La, and β Lg; ($\blacktriangledown$) 5-kDa permeate of sodium caseinate; (+) 3-kDa permeate of mixture cas, α La, and β Lg.

not show any IgE binding inhibition with the permeate fractions up to an inhibitor concentration of 1 g/liter, including BSA and IgG (data not shown). In contrast, the retentate fractions and the respective intact milk protein showed 100% inhibition at that protein concentration.

DISCUSSION

We have shown that the lactococcal CEP specifically degrades total casein in the presence of one or more of the whey proteins α La, β Lg, BSA, and IgG. By using the discriminating action of CEP and subsequent ultrafiltration with appropriate membranes, it was possible to produce a casein hydrolysate with different immunological characteristics. Removal of cross-reactive ABBOS antibody-binding sites was possible by using membranes with cutoff values up to 30 kDa. A real "hypoallergenic" casein hydrolysate could be obtained by using membranes ≤ 5 kDa, as it was shown that even the casein itself was degraded in such a way by CEP that the final product was free of peptides with cross-reactive casein-specific IgE antibody-binding sites.

Van Beresteijn et al. (37) have shown that many commercially available hypoallergenic infant formulas based on hydrolyzed casein and/or whey proteins still contain variable numbers of immunoreactive epitopes against the individual milk proteins. With the modern processing techniques (nonspecific enzymatic hydrolysis with subsequent ultrafiltration) it appeared, in particular, difficult to remove cross-reactive IgE antibody-binding sites of α La and β Lg. Apparently, the food-grade enzymes used so far do not have the specificity to eliminate their epitopes and these are not completely removed in the subsequent ultrafiltration step. The milk-protein-specific residual IgE antigenicity of these products together with the individual sensitization pattern of the patient allergic to cow's milk might explain reported cases of hypersensitivity (4, 5, 9, 28, 30). Furthermore, it was recently found that these products may also contain protein fragments with cross-reactive ABBOS antibody-binding sites (36). Their use in ongoing and forthcoming intervention trials to elucidate the role of BSA as a possible environmental triggering agent of IDDM (2) is, therefore, at least questionable.

Lactococci are used in the production of many types of fermentation products. For optimal growth in milk they are dependent on a cell-envelope-located serine proteinase for the supply of small peptides. These peptides are the primary substrates for translocation by the peptide-transport systems of *Lactococcus lactis*, and consequently the main source of essential amino acids and nitrogen for this organism. CEP of *L. lactis* subsp. *cremoris* WG2 has been shown to hydrolyze β -casein into more than a hundred different oligopeptides (20). CEPs of various *L. lactis* strains degrade casein in different ways. Visser et al. (39) distinguished two types of CEP on the basis of their ability to degrade α _{s1}-, β - and κ -casein. Type CEP_I, including the CEP of *L. lactis* subsp. *cremoris* WG2, preferentially degrades β -casein and type CEP_{III} also degrades β -casein, but at the same time splits α _{s1}- and κ -casein more extensively than does CEP_I. Exterkate et al. (14) distinguished seven different groups of CEPs on the basis of their specificity towards the α _{s1}-casein fragment 1-23, in which the CEP of *L. lactis* subsp. *cremoris* WG2 had an intermediate specificity. This strain was chosen because of the positive release and stability characteristics of the CEP obtained from this strain (12) and also for its food-grade nature. On the other hand, the slower degradation of α _{s1} and κ -casein by WG2-CEP as compared to

β -casein might be a disadvantage. The use of other types of CEP, with other preferences (14, 39), or the application of other hydrolysis conditions (13) may offer possibilities to optimize the degradation of the different types of casein.

The preference of the lactococcal CEP for degrading casein rather than the whey proteins might be a characteristic unique to this enzyme. Whether other proteolytic enzymes possess the same characteristic is to our knowledge not known. Schmidt and Poll (32) showed that chymosin (main constituent of rennet) in solution, in contrast with CEP, exhibited at least some activity towards α La. Moreover, conversion of α_{s1} -casein by chymosin appeared to be directly inhibited by the whey proteins α La and β Lg (24).

During the different treatments of the lactococcal cells to obtain the released CEP, some lysis occurred as measured by the activity of an intracellular exopeptidase, PepN. The presence of exopeptidase activity may lead to the formation of free amino acids in the final hydrolysate. This might be a disadvantage for the practical application of such a hydrolysate. Free amino acids are not as well absorbed as small peptides (25), and high amounts of free amino acids should also be avoided, because they will further increase the osmolarity of the final product. Application of a more pure CEP fraction, such as the MonoQ-fraction, will avoid the formation of free amino acids.

The present method for the preparation of the lactococcal CEP could have some other practical limitations. Specifically the release of CEP from the lactococcal cells will need primary attention if scaling up of the method is required. Controlled overproduction of the enzyme by *L. lactis* in a truncated form which facilitates secretion of the enzyme in the medium will allow the preparation of larger amounts of the enzyme. A recently developed controlled gene expression system for *L. lactis*, in which the food-grade inducer nisin is used, is a feasible possibility (8, 22).

In conclusion, the discrimination for the different milk proteins by the lactococcal WG2-CEP led to the exclusive degradation of casein in the presence of the major whey proteins. Depending on the application, removal of one or more of the intact whey proteins is then made possible by ultrafiltration. The method can be applied for the preparation of a casein hydrolysate formula free of protein fragments with cross-reactive milk-protein-specific IgE or ABBOS antibody-binding sites. The product could find application in infant formulas for therapeutic and preventive treatment of children with cow's milk allergy; whereas the preventive use of such formulas in children genetically susceptible to the development of IDDM should be considered if a relationship between the consumption of BSA and IDDM were to become more apparent. The method is also applicable for preparing casein-free whey protein preparations.

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