

Enzymatic Treatment of Whey Proteins in Cow's Milk Results in Differential Inhibition of IgE-Mediated Mast Cell Activation Compared to T-Cell Activation

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Key Words

Cow's milk · Enzymatic hydrolysis · Mast cell degranulation · T-cell activation · Whey

Abstract

Background: Cow's milk (CM) hydrolysates are frequently used as milk substitutes for children with CM allergy. In hydrolysates, allergenic epitopes within CM proteins are diminished by enzymatic treatment. The aim of this study was to examine the allergenic and immunogenic properties of whey proteins during hydrolysis. **Methods:** During hydrolysis, samples were obtained at 0, 10, 15, 30, 45, 60, 75 and 90 min. Degradation was checked by HPLC and SDS-PAGE. Allergenic potential was analyzed by IgE crosslinking capacity of human Fcε receptor type 1-transduced rat basophilic leukemia cells sensitized with serum of CM-allergic patients. Whey-sensitized C3H/HeOuJ mice were ear challenged intracutaneously with the hydrolysates. Immunogenicity was tested using whey-specific human T-cell clones and T-cell lines at the level of proliferation and release of IL-4, IL-10, IL-13 and IFN-γ. **Results:** After 15 min of hydrolysis, the majority of

the proteins were degraded. Hydrolysis for 15 min resulted in 92% inhibition of mast cell degranulation and in 82% reduction of ear swelling in the mouse model. In contrast, T-cell-stimulatory capacity was less affected by hydrolysis: reduction of human T-cell proliferation was only 9%. This was further reduced to 57 and 74% after 30 and 45 min of hydrolysis, respectively. Cytokine production followed the pattern of T-cell proliferation. **Conclusion:** Via differential analysis of allergenic versus immunogenic properties of the time kinetics of hydrolysis of whey proteins, we have demonstrated specific hydrolysis conditions with reduced IgE-crosslinking responses but retained T-cell activating properties. This approach might be useful in better defining CM hydrolysates.

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Introduction

Cow's milk (CM) allergy (CMA) is the most common allergy in early childhood, with an estimated prevalence of 3% in the pediatric population [1]. Food allergy is defined as an adverse immunological (hypersensitivity) re-

sponse to food and can be divided into IgE-mediated and non-IgE-mediated reactions [2]. Disorders with acute onset of symptoms after ingestion are usually IgE mediated and affect one or more target organs: the skin (urticaria/angio-edema), respiratory tract (rhinitis/asthma), gastrointestinal tract (pain/vomiting/diarrhea) and the cardiovascular system (anaphylactic shock) [3]. In IgE-mediated reactions, an allergen will crosslink IgE on the plasma membrane of basophils and/or mast cells and trigger the release of inflammatory mediators. The optimal conditions for this release depend on the concentration of allergen-specific IgE antibody on the membrane, the concentration of allergen and the affinity of the IgE for the allergen [4, 5].

The only curative treatment for IgE-mediated allergy is specific immunotherapy (IT). The immunological mechanisms by which specific IT is effective include the modulation of T- and B-cell responses and is accompanied by a significant decrease in allergen-specific IgE and an increase in allergen-specific IgG antibodies, mainly IgG4 [6]. However, specific IT is not available for CMA. Several strategies to reduce the allergenicity of therapeutic preparations, while maintaining their therapeutic benefit, are being developed. Peptide IT is one such approach. Short synthetic peptides, comprising T-cell epitopes of major allergens, show a major potential advantage by abolishing the capacity to crosslink allergen-specific IgE and activate mast cells and basophils [7, 8]. Peptide IT studies in cat-allergic and insect-venom-allergic subjects show promising results that support the potential of this approach [7, 9].

Whey proteins represent nearly 20% of the total bovine milk proteins. The known major allergenic proteins of whey are β -lactoglobulin (β -Lg) and α -lactalbumin (α -La). Several peptides with considerable biological roles have been identified in enzymatic hydrolysates of whey, of which some seem to have immunomodulatory potential and might therefore be beneficial for humans [10–13]. Hydrolysates, also called hypoallergenic formulas, are intended for use by infants at risk for CMA (partially hydrolyzed formulas) or with diagnosed CMA (extensively hydrolyzed formulas). Hypoallergenic formulas are processed by enzymatic hydrolysis of protein sources, such as bovine whey and/or casein, followed by further processing, such as heat treatment and/or ultrafiltration. The products have been classified according to the degree of protein hydrolysis as ‘extensively’ or ‘partially’ hydrolyzed products. Product properties may be characterized by biochemical techniques, and reduction of allergenicity may be assessed in vitro with various im-

munological methods and in vivo with skin prick, patch or challenge tests [14]. It is known that processing of milk leads to changes in allergenicity. For example, heat treatment reduces the allergenicity of β -Lg by inducing conformational changes and by increasing its susceptibility to enzymatic digestion, both of which disrupted B-cell epitopes [15]. The optimal hydrolysis process for the current study was selected from previous studies in which several different enzymes, enzyme concentrations, enzyme mixes, hydrolysis time, temperature and pH have been tested. The peptide profiles were analyzed by high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry, gel permeation chromatography and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the antigenicity was tested by β -Lg ELISA and Western blotting plus immune incubation with β -Lg and α -La (data not shown).

In the current study, we have examined the allergenic and immunological properties of whey proteins during hydrolysis for different periods of time. By careful differentiation between IgE-mediated responses and T-cell activation, we aimed at selecting hydrolysates with retained immunogenicity but reduced allergenic properties.

Materials and Methods

Hydrolysis of Whey

The bovine whey used in this study was a mixture of Deminal 90 (Friesland Foods Domo, Zwolle, The Netherlands) and whey protein concentrate (WPC 60; Milei, Leutkirch, Germany); 1,277 g Deminal 90 and 551 g WPC 60 were dissolved in 8.12 liters of deionized water and heat-treated for 2 min at 72°C. Whey was enzymatically treated with an established mixture of microbial endo- and exopeptidases (enzyme composition confidential by Danone) at 58°C and adjusted to pH 7.75 with lye [12.3 g $\text{Ca}(\text{OH})_2$, 9.6 g $\text{Mg}(\text{OH})_2$ and 9.3 g KOH in 360 ml H_2O]. During hydrolysis, protein samples were obtained at 0, 10, 15, 30, 45, 60, 75 and 90 min. At each time point (also $t = 0$), hydrolysis was stopped by inactivation of the enzymes through heating to 100°C.

HPLC Analysis of the Hydrolysates

Analytical HPLC (Akta Explorer; Pharmacia, Uppsala, Sweden) was performed to analyze the chemical fingerprint of the whey hydrolysates (time points 0, 10, 15 and 30 min) according to the ‘WPI-macrospher’ method. A Macrospher RP300 (C4) 5 μm 250 $\times$ 4.6 mm column (Alltech, Lexington, Ky., USA) was used with two solvent systems: 0.15% (v/v) trifluoroacetic acid (Sigma-Aldrich, St. Louis, Mo., USA) in demiwater (A) and 0.13% (v/v) trifluoroacetic acid in acetonitril (B). The elution gradient profile was set as follows: 0–1 min 17.5% B, 1–3 min 30% B, 3–10 min linear to 65% B and 10–12 min 17.5% B, while flow rate was set at 1.0 ml/min and the detector wavelength was set to 220 and 280

nm. α -La and β -Lg were identified according to their retention time and comparison to pure α -La (Sigma-Aldrich) and β -Lg (Sigma-Aldrich).

SDS-PAGE and Colloidal Gold Staining

The pattern of the residual proteins of the whey hydrolysates (time points 0, 10, 15 and 30 min) was analyzed with SDS-PAGE. A Bio-Rad (Hercules, Calif., USA) Mini-PROTEAN II system was used with a 15% Tris-HCl gel. Molecular weight markers were used as a reference. The proteins were diluted at a 1:3 ratio with reducing sample buffer (6.05 g Tris, 8.0 g SDS, 3.2 g dithiothreitol, 20 mg bromophenol blue in 60 ml H₂O and 40 ml glycerol 87%, pH 6.8), and 10 μ g of protein were loaded on the gel. The proteins were blotted to a PVDF membrane (Roche Diagnostics, Basel, Switzerland), and the total protein pattern was visualized with colloidal gold staining (AuroDye™ Forte kit; GE Healthcare, Uppsala, Sweden).

Degranulation of Human Fc ϵ Receptor Type 1 RBL Cells (RBL-hEla-2B12 Cells)

The RBL-hEla-2B12 cell line, which has a mucosal mast cell phenotype [16], was transfected with the α -chain of human Fc ϵ receptor type 1 (huFc ϵ R1) complex (a kind gift from Dr. Teshima, Japan) [17]. Degranulation of RBL-hEla-2B12 cells with pooled serum from CMA patients was performed as described previously [18]. In short, confluent growing RBL-hEla-2B12 cells (1×10^5 /well) in 96-well flat-bottom culture plates were sensitized with 5 μ g/ml human purified IgE (Chemicon/Millipore, Billerica, Mass., USA) or with 1:50 diluted serum of a pool of 10 CMA patients (β -Lg-specific IgE 74.6 kUA/l measured by ImmunoCAP, Phadia, Uppsala, Sweden) for 16 h at 37°C. CMA was confirmed in these patients by double-blind, placebo-controlled food challenge [19]. The cells sensitized with purified IgE were stimulated with 5 μ g/ml anti-human IgE antibody (BD Pharmingen, Franklin Lakes, N.J., USA) in Tyrode's buffer with 0.1% HSA (Sigma-Aldrich), pH 7.4, for 1 h. The release induced by α -IgE on purified IgE-sensitized cells served as maximum amount of release. The sensitized cells with CMA pooled serum were stimulated with anti-human IgE (5 μ g/ml), whey or the whey hydrolysates sampled at different time points; 0, 10, 15, 30, 45, 60, 75 and 90 min (1 μ g/ml Tyrode's buffer) for 1 h. β -Hexosaminidase activity was determined by a fluorescence assay using 4-methylumbelliferyl-N-acetyl- α -D-glucosamine (Fluka/Sigma-Aldrich) as a substrate. The β -hexosaminidase activity released into the medium was expressed as a percentage of the maximum amount of release with anti-IgE.

Oral Sensitization and Challenge of Mice with Whey

Three- to five-week-old specific pathogen-free female C3H/HeOuJ mice ($n = 4$ in the non-sensitized group and $n = 6$ in the sensitized group) were purchased from Charles River Laboratories (Wilmington, Mass., USA), maintained on CM protein-free mouse chow (Special Diets Services, Witham, UK) and housed in the animal facility at the University of Utrecht. Animal care and use were performed in accordance with the guidelines of the Dutch Committee of Animal Experiments. As previously described [20], mice were sensitized intragastrically on days 0, 7, 14, 21 and 28 with 0.5 ml homogenized whey (40 mg/ml PBS; DMV International, Veghel, The Netherlands) with cholera toxin (20 μ g/ml PBS; Quadragech Diagnostics, Epsom, UK) as an adjuvant,

using a blunt needle. Control mice received cholera toxin only. Mice were boosted weekly for a period of 5 weeks.

Allergen-Specific Skin Responses

The acute allergic skin response (ear swelling at 1 h) is a measurement of the immediate IgE-mediated allergic response to evaluate the allergenicity of whey hydrolysates [21]. This was determined in anesthetized mice after intradermal injection of 10 μ g/20 μ l PBS (0.5 mg/ml) whey hydrolysates (time points 0, 10 and 15 min of hydrolysis) and compared to the ear swelling after whey skin challenge (10 μ g/20 μ l PBS). As a negative control, sham-sensitized mice were skin challenged with whey. The allergen-specific net ear swelling was calculated by correcting the allergen-induced increase in ear thickness with the non-specific ear swelling due to local injection in the non-sensitized mice (Δ ear swelling is expressed in μ m).

Human T-Cell Cultures

Epstein-Barr-virus-transformed human B lymphocytes (EBV-B cells) were cultured in RPMI-1640 (Gibco/Life Technologies) supplemented with 10% FBS (Gibco). Established T-cell clones (TCCs) and T-cell lines (TCLs) [22] were maintained in Iscove's modified Dulbecco's medium (Gibco) supplemented with 2% pooled human AB serum, 5% Yssel's medium [23] and 50 U/ml interleukin-2 (IL-2) and IL-4 (a kind gift from Novartis Research Institute, Vienna, Austria). TCCs and TCLs were specific for β -Lg. As a control, a casein-specific TCC was included. For the experiments performed to test antigen specificity and cytokine production, IL-2 and IL-4 were omitted from the medium. All media were supplemented with penicillin (100 IU/ml), streptomycin (100 mg/ml) and glutamine (1 mM; Gibco).

T-Cell Stimulation and Cytokine Production

T-cell stimulation was performed in triplicate in 96-well U-bottom plates (Greiner Bio-one, Monroe, N.C., USA). Each well contained 4×10^4 T cells and 4×10^4 irradiated (55 Gy) autologous EBV-B cells, as antigen-presenting cells, pre-incubated overnight with 50 μ g/ml total CM proteins, casein, β -Lg or whey hydrolysates. EBV-B cells incubated without antigen were used as negative controls. After 24 h, proliferation was measured by [³H]-thymidine (TdR) incorporation (1 μ Ci/well; Amersham/GE Healthcare, Little Chalfont, UK), which was added for additional 18 h. Proliferative responses are measured as the mean [³H]-TdR incorporation (counts/minute) of triplicate wells $\pm$ SEM. Prior to addition of [³H]-TdR, supernatant was collected for the measurement of cytokine production from the triplicate wells and stored at -20°C. Cytokine production was measured by ELISA according to the manufacturer's recommendations (Sanquin, Amsterdam, The Netherlands). The detection limit was 0.6 pg/ml for IL-4, 1.2 pg/ml for IL-10, 2 pg/ml for IFN- γ and 0.5 pg/ml for IL-13.

Statistical Analysis

The results in non-sensitized ($n = 4$) and sensitized mice ($n = 6$) and T-cell stimulation were analyzed using one-way ANOVA and the post hoc Dunnett's test. Data are presented as means $\pm$ SEM. Differences in in vitro mast cell degranulation were assessed by Student's *t* tests. Statistical analyses were conducted using GraphPad Prism software (version 4.03). Values of $p < 0.05$ were considered significant.

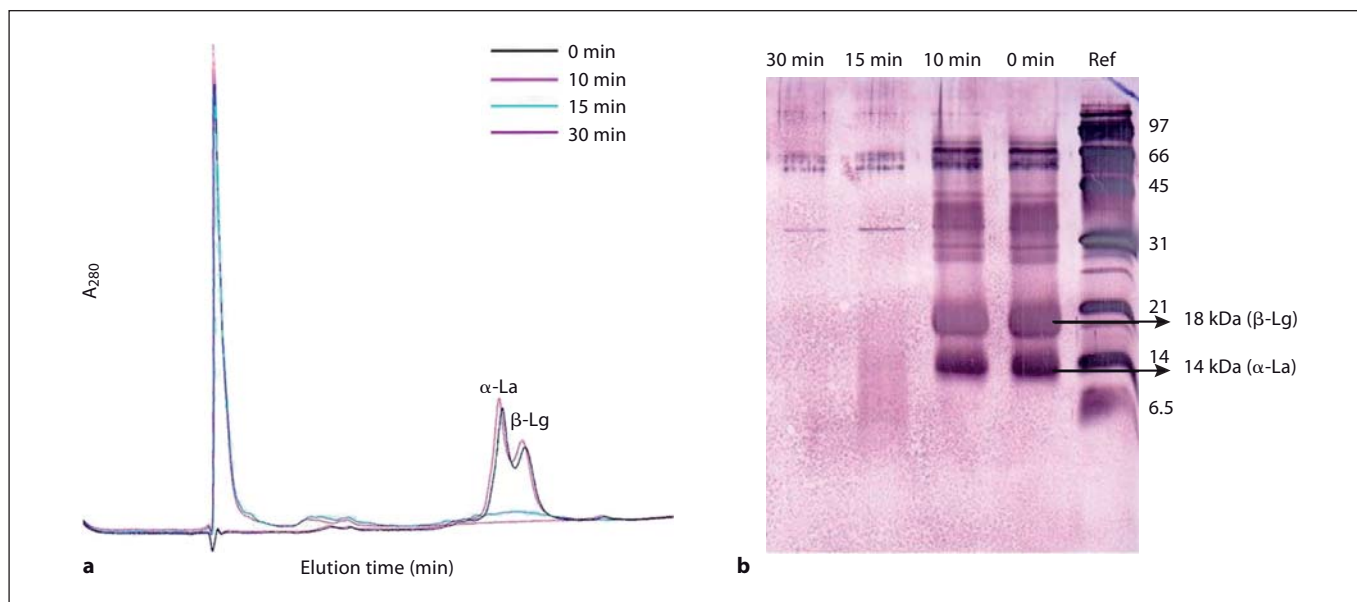


Fig. 1. HPLC chromatograms (**a**) and SDS-PAGE protein pattern (**b**) of whey hydrolysis. Samples were withdrawn at 0, 10, 15 and 30 min of hydrolysis. The HPLC peaks corresponding to α -La and β -Lg were determined by comparison to pure α -La and β -Lg. The SDS-PAGE reference (Ref) is a standard broad-range marker: phosphorylase b (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21 kDa), lysozyme (14 kDa) and aprotinin (6.5 kDa).

Results

Proteolytic Pattern of the Hydrolysates Analyzed by HPLC and SDS-PAGE

Hydrolysis of bovine whey was carried out for 0, 5, 10, 15, 30, 45, 60, 75 and 90 min. Figure 1 shows the proteolytic pattern analyzed by HPLC (fig. 1a) and SDS-PAGE (fig. 1b) corresponding to the whey hydrolysates obtained after 0, 10, 15 and 30 min of hydrolysis. After hydrolysis for 10 min, the protein patterns of α -La (14 kDa) and β -Lg (18 kDa) were still comparable to the unprocessed whey. After hydrolysis for 15 min, the majority of α -La and β -Lg was already partially degraded and after hydrolysis for 30 min, both proteins were largely degraded.

IgE Crosslinking and Mast Cell Activation Potential of the Hydrolysates

RBL cells transfected with huFc ϵ RI- α were incubated with pooled serum from CMA individuals and subsequently incubated with whey hydrolysates. Mast cell degranulation was reduced by 12% when whey was added that was hydrolyzed for 10 min, whereas 15 min of hydrolysis resulted in 98% reduction (fig. 2). No further reduction was observed at later time points.

Acute Allergen-Specific Skin Reactions

In order to study allergic skin responses, whey-sensitized mice were challenged intradermally with complete whey or whey hydrolysates in the ear pinnae 1 week after the last oral sensitization. Acute mouse ear edema was markedly reduced when whey was applied that was hydrolyzed for 10 min (by 62%), and even further reduced (by 82%) when whey was used that had been hydrolyzed for 15 min (fig. 3).

T-Cell Stimulation and Cytokine Production

The different hydrolysates were tested for their ability to activate β -Lg-specific TCCs and TCLs. Whey that had been hydrolyzed for 10 and 15 min demonstrated only a slight reduction in T-cell activation compared to unprocessed whey (7 and 9%, respectively). After 30 min of hydrolysis, a pronounced reduction in T-cell activation by 63% was seen, followed by a reduction by 82 and 88% for the 45- and 60-min hydrolysates, respectively. No further inhibition was observed at later time points (fig. 4). No T-cell stimulation was found when casein-specific TCCs were stimulated with the hydrolyzed whey material (data not shown).

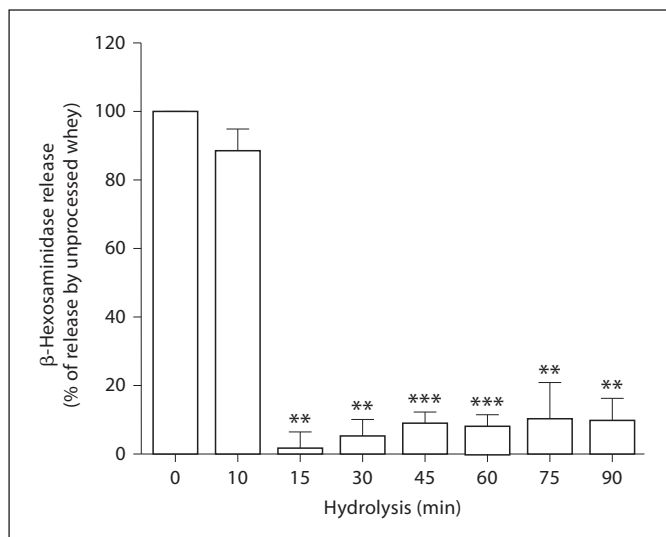


Fig. 2. Effect of hydrolyzed whey proteins on IgE-mediated degranulation of huFcεRI-α-transfected RBL cells. Degranulation was determined by measuring the amount of β-hexosaminidase released in the cell-free supernatant. β-Hexosaminidase release of unprocessed whey (0 min) was set to 100% and samples were expressed as a percentage of unprocessed whey release. Spontaneous β-hexosaminidase release from the cells was $11.6 \pm 9.2\%$ (mean $\pm$ SD), the background β-hexosaminidase release by the serum alone was $24.6 \pm 10.2\%$ (mean $\pm$ SD) and the maximum β-hexosaminidase release by Triton X-100 was $169.1 \pm 11.2\%$ (mean $\pm$ SD) of unprocessed whey. Bars represent the average of 3 different measurements of β-hexosaminidase release $\pm$ SEM. ** $p < 0.01$, *** $p < 0.001$.

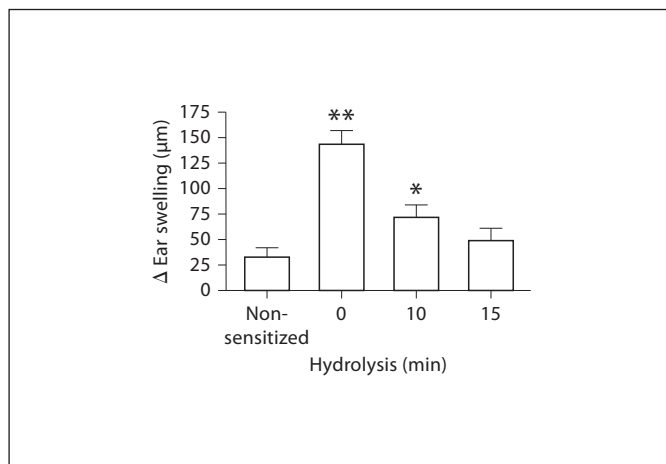


Fig. 3. Allergic skin responses in mice challenged intradermally in the ear pinnae, 1 week after the last oral whey sensitization, with whey hydrolysates sampled at 0, 10 and 15 min of hydrolysis. Bars represent average Δ ear swelling of one experiment ($n = 4$ in the non-sensitized group and $n = 6$ in the sensitized groups) $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$.

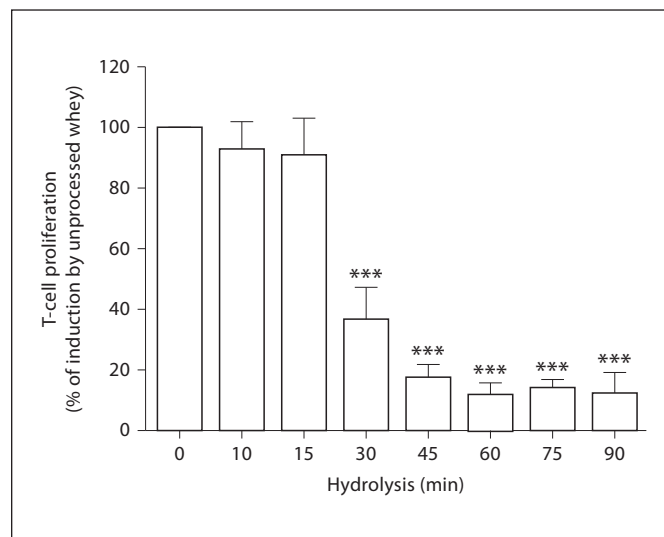


Fig. 4. Percentage of proliferation of β-Lg-specific TCCs and TCLs incubated with whey hydrolysates. Proliferation of unprocessed whey (0 min) was set to 100% and the samples were expressed as a percentage of unprocessed whey-induced proliferation. Spontaneous proliferation was $7,666 \pm 1,395$ counts/min (mean $\pm$ SEM) and unprocessed whey-induced proliferation was $44,438 \pm 7,403$ counts/min (mean $\pm$ SEM). Bars represent average results from 4 different TCCs/TCLs tested in duplicate $\pm$ SEM. *** $p < 0.001$.

The whey-induced cytokine production of IL-4, IL-10, IL-13 and IFN- γ by activated T cells was measured in the TCC and TCL supernatants. Cytokine release by T cell upon activation with the whey hydrolysate samples was reduced to the same extent as T-cell proliferation (fig. 5). No differential effect of the hydrolysates for individual cytokines was found.

Discussion

The current study shows that hydrolysis of whey proteins has a time-dependent effect on their capacity to cause IgE-mediated mast and T-cell activation, which coincides with their size decrease. Although this study focused on the manipulation of whey in CM, we anticipate that our approach might also work for other proteins, including CM caseins.

CM hydrolysates that are currently available display strongly reduced immunogenicity and improved digestibility. The extensive, amino-acid-based hypoallergenic

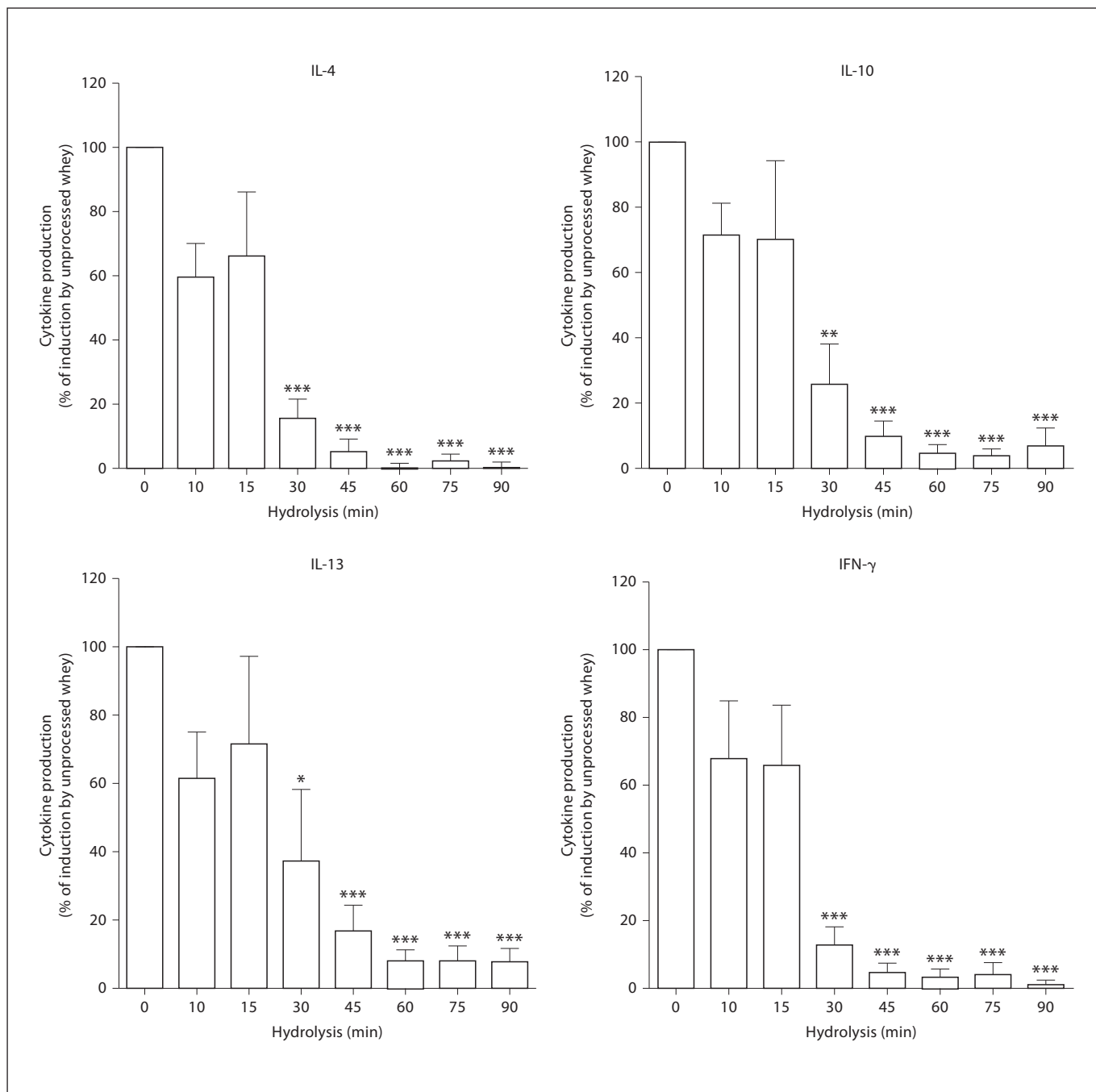


Fig. 5. Effect of different hydrolysates on the release of IL-4, IL-10, IL-13 and IFN- γ in supernatants of TCC/TCL proliferation cultures. Cytokine production of unprocessed whey (0 min) was set to 100% and samples were expressed as a percentage of unprocessed whey cytokine production. Spontaneous release of cytokines was 3.2 ± 1.0 , 8.2 ± 2.5 , 19.4 ± 9.8 and 14.8 ± 9.9 pg/ml

(mean $\pm$ SEM) for IL-4, IL-10, IL-13 and IFN- γ , respectively. For unprocessed whey, cytokine release was 340 ± 166 , 639 ± 135 , 953 ± 279 and 341 ± 166 pg/ml (mean $\pm$ SEM) for IL-4, IL-10, IL-13 and IFN- γ , respectively. Bars represent average results from cytokine production from 4 different TCCs/TCLs $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

formulas have no apparent immunogenic properties and are well tolerated by CMA infants [14]. In the current study, we aimed to elucidate the changes induced by hydrolysis of whey proteins and we demonstrated differential effects by studying time-dependent effects of the process of hydrolysis on different allergenicity and immunogenicity parameters. The most important finding was that the effects of the hydrolysis on the in vitro and in vivo IgE allergen interactions were observed at time points distinct from the effect on T-cell activation. After 15 min of hydrolysis, the IgE binding and crosslinking capacity was diminished without affecting T-cell activation. IgE crosslinking was assessed using an in vitro RBL assay and was confirmed using an in vivo mouse model of CMA. In contrast, cytokine production and proliferation of human TCCs was only reduced after stimulation with whey hydrolysates that were hydrolyzed for at least 30 min.

In allergen IT, several studies have examined the differential effects of reducing allergen size, most particularly by producing peptides. For IgE crosslinking, it has been concluded from several studies that the distance between 2 FcεRI molecules is within 8–24 nm, corresponding to approximately 30–100 amino acids [4]. Peptides below this size are still able to stimulate allergen-specific T cells, but unable to crosslink IgE on mast cells. Peptide IT for cat allergy has been investigated in great detail by Larché et al. [7, 8] who found that pro-inflammatory cytokines were reduced while the immunosuppressive cytokine IL-10 was increased. In addition, skin, lung and nasal sensitivity were decreased. Also, for the bee venom allergen PLA2, IT with 3 different peptides resulted in clinical benefit in 3 of 5 treated patients that now could tolerate a live bee sting challenge without reaction [24]. However, the potential pitfall of the peptide-based IT is that there is wide variation in the types of MHC-II expressed between individuals, leading to the definition of relatively large mixes of peptides to cover all relevant MHC-II pheno-

types of patients. It is likely, although not proven, that by the partial hydrolysis of the whey proteins the large majority of the MHC-II binding structures in the whey proteins are still intact. In contrast to the study by Larché [7], hydrolysates did not exert a differential effect on T-cell cytokines in our study (fig. 5). This could be due to the more fixed cytokine profile in the TCCs and TCLs compared to the primary T cells used in the peptide IT studies.

In addition to the application of hydrolyzed CM protein in the treatment of established CMA, there is also the possibility to use hydrolysates for CMA prevention. To date, different results with different ranges of CM protein hydrolysis have been reported in rodent models [21, 25–27] and clinical studies [28–31]. In prevention strategies, there is not really a need to check for IgE binding, because specific IgE antibodies have not yet been produced. However, better insight into the activation of T cells might be of importance to actively induce tolerance, also in this preventive approach. Consequently, the immunologic activation profiles of these hydrolysates require elucidation. One could hypothesize that for induction of immunological tolerance some immunogenic activity within the hydrolysate is required. Therefore, we aimed to analyze hydrolysis in more detail by in vitro and in vivo methods. Although the current approach seems promising, it should be evaluated further in in vitro studies as well as in CMA patients to confirm that the partial hydrolysate has the preferred tolerogenic activity.

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