

Influence of Enzymatic Cow Milk Hydrolysates on IgA and IgG Response of Balb/c Mice Organism

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Abstract

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The manuscript presents the application of an animal model, Balb/c mice, in studies aimed at identifying among enzymatic hydrolysates of sodium caseinate and whey proteins the product with the most beneficial impact on the animal organism. One- and two-step hydrolyses were carried out using the following enzymes: Alcalase (Novo Nordisk), papain (Sigma), and Lactozym (Novo Nordisk). Estimations were also made with a peptide fraction, the so-called II fraction, of WPC (Whey Protein Concentrate) and Alcalase hydrolysate with molecular masses lower than 12.4 kDa. The levels of specific IgG and IgA were determined in blood serum and intestinal extracts of mice. The hydrolysis of sodium caseinate with the use of Alcalase was found to affect the reduction in the material allergenicity. Of all whey hydrolysates, the most promising results were obtained upon the application of the II fraction of WPC and Alcalase hydrolysate (M.W. <12.4 kDa).

Keywords: milk protein hydrolysates; Alcalase; papain; Lactozym; Balb/c mice; IgA; IgG

Food allergy is triggered by an aberrant immune response elicited by oral administration of dietary antigens, e.g. milk proteins. It is generally accepted that food allergies are generated only when immunological barriers such as oral tolerance are breached. Thus, oral tolerance is thought to be an immune mechanism which serves to suppress food allergy. The interaction of orally-administered antigens with the gut-associated lymphoid tissue (GALT) induces characteristic immunological responses such as the production of immunoglobulin A (IgA) and the induction of oral tolerance. The role of IgA in food allergy is not known definitely. The protective effect of IgA antibodies against infections, antigens, and

parasites in the gut is well documented. Mucosal IgA prevents the adhesion of bacteria and reduces the absorption of food antigens in the intestine (OTANI *et al.* 2000).

The sensitising capacity as well as the residual tolerogenicity of a formula can, however, be evaluated only with *in vivo* animal models (FRITSCHÉ 2003). Mice and men respond similarly to an allergic kind of food. GAUDRY *et al.* (2004) estimated a significant IgE and IgG response to shrimp, peanut, walnut, and cashew (allergenic foods) and minimal IgE antibody – and a lower IgG antibody-response to cod, rice, chicken, and beef (non-allergenic foods). The immunological reaction of mice was similar with 4 out of 5 allergens

and 3 out of 3 non-allergens. There is a significant obstacle to the development of oral murine models of food allergy, namely the strong innate tendency to develop oral tolerance to ingested antigens. Oral tolerance is the basic mechanism commonly accepted to be the normal immune reaction of the host to food. Its breakdown leads to food allergy (FRITSCHÉ 2003).

Mice, especially Balb/c, have been widely used in the feeding experiments dealing with the immune response to dietary compounds such as cow milk allergens (ADEL-PATIENT *et al.* 2000; BERNARD *et al.* 2000). VON DER WEID *et al.* (2001) immunised orally mice against β -lactoglobulin using cholera toxin as an adjuvant. Before oral sensitisation, mice were administered milk whey proteins. The authors concluded that bystander suppression was observed at the lowest doses of proteins. Mucosally-induced IgE responses were suppressed by a mechanism that was distinct from that operational in the periphery (VON DER WEID *et al.* 2001). MIZUMACHI and KURISAKI (2002) fed mice continuously for 58 days with β -lactoglobulin and milk instead of drinking water. The mice were immunised with β -lactoglobulin emulsified with complete Freund's adjuvant to examine their antibody response. The authors concluded that this schedule of feeding induces the suppression of Th1 and Th2-dependent responses, hence the mice can achieve the state of oral tolerance.

The mechanisms of the acute and late allergic reactions were shown in Adel-Patient's article (ADEL-PATIENT *et al.* 2003). She used female Balb/c mice sensitised intraperitoneally with β -lactoglobulin to study the active phase of allergy in the respiratory tract after a single airway challenge or denatured β -lactoglobulin which contained only linear epitopes. The production of peptide-leukotriens or PGD2 may result from distinct activation intensities of mast cells, leading to a distinct late reaction 24/72 h after the challenge. There are also studies developed in C3H/HeN mice and Sprague-Dawley rats (PENG *et al.* 2004). The animals were fed such hydrolysate formulas as Nan, Neoangelac FL, and Nan-HA. The authors concluded that the suppression of IgE responses to whey proteins was readily induced in native or Nan-HA for one week, and hardly induced in sensitised mice even after prolonged feeding with Nan for 12 weeks. However, some authors proved that food-induced hypersensitivity reactions are strain-dependent (MORAFO

et al. 2003). They found that C3H/HeJ mice were susceptible and Balb/c mice were resistant to cow milk or peanut administered intragastrically with cholera toxin as the adjuvant.

From time to time, new articles appear whose authors try to sum up the knowledge on the immunoreactivity of food components in experimental animals (MILLER *et al.* 1998), oral tolerance to protein antigens (MAYER *et al.* 2001), animal models in food allergy (FRITSCHÉ 2003), or information about hypoallergenic formulas (HØST & HALKEN 2004).

The aim of this study was focused on the ability of cow milk protein hydrolysates, prepared from whey protein concentrate (WPC) and sodium caseinate, to induce immune specific reaction as oral tolerance to the native protein, β -lactoglobulin.

MATERIALS AND METHODS

Hydrolysates of whey protein concentrate and sodium caseinate isolate. Commercial whey protein concentrate (WPC) and sodium caseinate isolate (SCI) were obtained from Laktopol Company, (Suwałki, Poland). Protease *Subtilisina carlsberg* – Alcalase 2.4 FG and Lactozym 3000 L HP-G were purchased from Novo Nordisk, and papain EC 3.4.22.2 – from Sigma.

The conditions of the enzymatic process were as follows: temperature 50°C, pH 8.0, enzyme addition 15 mAU/g of protein (MEJBAUM-KATZENELLENBOGEN & MOCHNACKA 1969). During hydrolysis, the pH was maintained by the addition of 1M NaOH from a burette. Hydrolysis was continued for 120 min. The sample was heated at 90°C/5 min to inactivate the enzyme, then cooled immediately and lyophilised. During "two-step" hydrolysis, the second enzyme (15 mAU/g) was added after 100 min. The conditions of hydrolysis were as described above. To determine free α -amino groups, TNBS method (PANASIUK *et al.* 1998) was used.

Separation of proteins of whey protein hydrolysates by gel chromatography. Gel chromatography was run on a 600-ml chromatographic column from Pharmacia LKB (Uppsala, Sweden). The column (85 cm in length, 3 cm in diameter) was filled with Sephadex G-75 gel. As the material obtained from gel chromatography was to be used in an animal-model experiment, distilled water was used as eluent. The column was calibrated with the following standards: dextrane (2 000 000 Da),

hen egg ovalbumin (43 000 Da), α -lactoalbumin (12 400 Da), and vitamin B₁₂ (1355 Da). The absorbance of the eluate was measured at a wavelength of 214 nm using a Spectrophotometer Dn 7500 apparatus (Beckman). The eluates of WPC and Alcalase hydrolysate, containing peptide fractions with molecular masses lower than 14.2 kDa, were subjected to gel chromatography in an FPLC system on SuperdexTM 74 HR 10/30 column. The chromatographic column was calibrated with the following standards: cytochrome C (12 500 Da), aprotinin (6 500 Da), gastrin I (2 126 Da), P substance (1 348 Da), glycine 6 (360 Da), glycine 3 (189 Da), glycine (75 Da), which enabled the determination of approximate molecular masses of peptides formed during hydrolysis.

Experiment of mice. Three-week-old female Balb/c mice, obtained from the Animal House–Warsaw (Poland), were used in the experiment. All mice were acclimatised before immunisation, then bred and raised on a cow milk-free diet. Water was offered *ad libitum* in their drinking bottles. Mice were divided into 10 groups (7 mice per one group). Each group was fed orally with different kinds of milk proteins (100 μ l of solution contained 50 μ g of protein): C – control group, WPC, A – hydrolysate of WPC produced with Alcalase 2.4 FG, 2A – hydrolysate of WPC produced with a double dose of Alcalase, AP – two-step hydrolysate of WPC produced with Alcalase and papain, AL – two-step hydrolysate of WPC produced with Alcalase and Lactozym 3000 L HP-G, II – second fraction (M.W. < 12.4 kDa) of WPC hydrolysate produced with Alcalase, KS – sodium caseinate, KA – sodium caseinate produced with Alcalase, I – group of immunised mice with β -lg without oral stimulation. All groups of mice were weighed on days 0, 7, 14 and 28. The animals (all except the control group) were immunised intraperitoneally on day 0 with 5 μ g of β -lg adsorbed onto alum (Alhydrogel 3%). A booster immunisation was performed 7, 14 and 28 days later. Seven days after the final injection, blood samples and guts were collected.

Enzyme immunometric assay for IgA and IgG levels. The following procedure was used: the microtitre plate was coated with 100 μ l/well of antigen (β -lactoglobulin or α -casein) diluted in 50mM carbonate buffer, pH 9.8, and incubated for 12–18 h at 4°C. The plate was then washed 2 times with 10mM phosphate buffered saline, pH 7.4, containing 0.1% Tween-20 (PBS-T), and 2 times with PBS without Tween. This washing system

was used after each analytical step. The residual free binding sites were blocked with 200 μ l/well of 1.5% gelatine in coating buffer for 30 min at 25°C. The plate was washed, coated with 100 μ l/well of diluted mice serum or gut extract and incubated for 1 h at 37°C. After washing, the plate was incubated for 1 h at 37°C with 100 μ l/well of goat anti-mouse IgA biotin conjugate (Sigma) or goat anti-mouse IgG biotin conjugate (Sigma). After washing, 100 μ l/well of ExtrAvidin peroxidase conjugate was added. Incubation was continued for 1 h/37°C. Then 3,3',5,5'-tetramethylbenzidine (TMB) in a 9mM citrate buffer, pH 5.0, was used as a substrate. After incubating the plates for 30 min, 50 μ l of 2M sulphuric acid was added to stop the reaction. The absorbance was read at 450 nm on an automated plate reader (Sunrise, Tecan).

Statistical analysis. Specific IgA and IgG antibody titres were expressed as means of three determinations. Comparative analyses of all groups for specific IgA and IgG response antibodies estimated in serum and gut of mice were carried out with one-factor analysis of variance ANOVA and Duncan's multiple range test using Statistica 6.0 software by Statsoft.

RESULTS AND DISCUSSION

Evaluation of the hydrolysis degree of whey proteins WPC-65

Protein hydrolysis is a process that results in the formation of peptides with different molecular masses and immunoreactive properties. Final traits of a product depend on the type of enzymes or enzymatic preparations used, the time extent of hydrolysis, and the conditions provided during its course (temperature, pH, application of mixing etc.). The degree of hydrolysis (DH) of milk proteins was calculated using the method elaborated by Adler-Nissen (ADLER-NIESSEN 1986).

One-step hydrolysis with Alcalase was carried out under conditions optimal for the enzyme. After 180 minutes, the hydrolysis degree of WPC accounted for ca. 14.5% (Figure 1). The results obtained indicate that 140 min of hydrolysis were sufficient to obtain a relatively stable degree of hydrolysis, maintaining a negligibly ascending tendency. The rate of hydrolysis reached its maximum within the first 80 min. In that period, DH reached the value of 13%. In the final phase of hydrolysis, from the 110th min, the degree of hy-

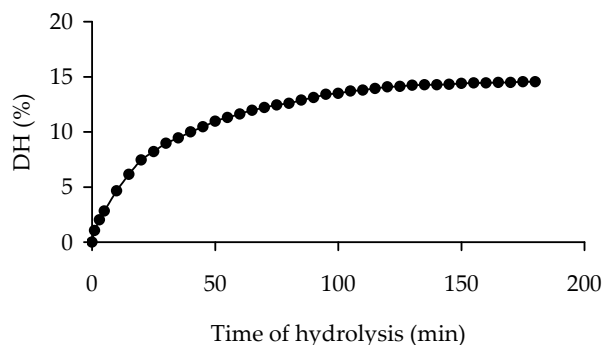


Figure 1. Evaluation of the degree of hydrolysis (DH) throughout incubation time of whey proteins with Alcalase 2.4L (pH 8, temperature 50°C, 15 mAU Alcalase 2.4 l/g protein)

drolysis increased by as little as 1%. The obtained plot of the course of the enzymatic reaction was typical for the course of hydrolysis described by the Michaelis-Menten constant. SPELLMAN *et al.* (2003) recorded a similar hydrolysis degree of WPC (14%) in an experiment carried out in a pH-stat with the use of Alcalase 2.4L. In that research, the yield of hydrolysis was the highest within the first 160 min and reached DH of 13%. Over the next 200 min, the DH value negligibly increased up to 15%. Incubation of WPC-65 with Alcalase used in a dose of 30 mAU/g of protein evoked an increase in the hydrolysis degree to 18% (an increase by 4% compared to hydrolysis run with the amount of enzyme recommended by the producer). The research by CASTRO *et al.* (1996) indicates, however, that even a twofold increase in the hydrolysis degree is possible upon applying a double dose of *Bacillus subtilis* cell extract in WPC solution. Hydrolysis of sodium caseinate run for 160 min with Alcalase enabled to obtain a DH of ca. 15%. A steady increasing tendency of DH was observed until ca. 130th minute. Thereafter, the DH gain was observed to be slower. The incubation of WPC hydrolysate with Alcalase with the addition of an amylolytic enzyme, Lactozym, raised the level of glucose by over 80% which may be significant for the production of nutrients for patients with lactose intolerance.

Separation by gel chromatography of proteins of WPC hydrolysate obtained with Alcalase

Chromatographic separation of proteins of WPC preparation obtained with Alcalase on a Sephadex G-75 column enabled the identification of two

main fractions: the first one including proteins with high molecular masses up to 12.4 kDa, and the second one containing proteins and peptides with molecular masses of 6 kDa and less (Figure 2). This indicates great proteolytic abilities of the enzyme used. Literature data reveal that the whey proteins with the molecular mass lower than 10 kDa are characterised by reduced allergenicity (KANANEN *et al.* 2000). It is thus highly desirable to obtain fractions with molecular masses below 5 kDa in the hydrolysis process. An experiment carried out by SVENNING *et al.* (2000) proves that the most efficient reduction of the allergenicity of the protein fractions of β -lactoglobulin hydrolysates was accomplished in the case of fractions with molecular masses ranging from 1 to 5 kDa (SVENNING 2000). SMYTH and FITZGERALD (1998) hydrolysed a WPC preparation using the addition of proteolytic enzymes including Alcalase 0.6 l. After 30 min of hydrolysis with Alcalase 6.0 l, they obtained 38% of fractions with molecular masses over 10 kDa, 11.5% of fractions with molecular masses ranging from 10 to 5 kDa, and 61.64% of fractions with masses lower than 5 kDa. Successive hydrolysis (run for 8 hours) considerably increased the concentration of peptides with molecular masses below 5 kDa (to 81.42%), and decreased the contents of the other fractions to 12.84% and 5.73%, respectively.

The eluate collected after chromatographic separation on a Sephadex G-75 column (the so-called II fraction of WPC hydrolysate obtained with Alcalase) was lyophilised and used for mice feeding. It was also subjected to chromatographic analysis

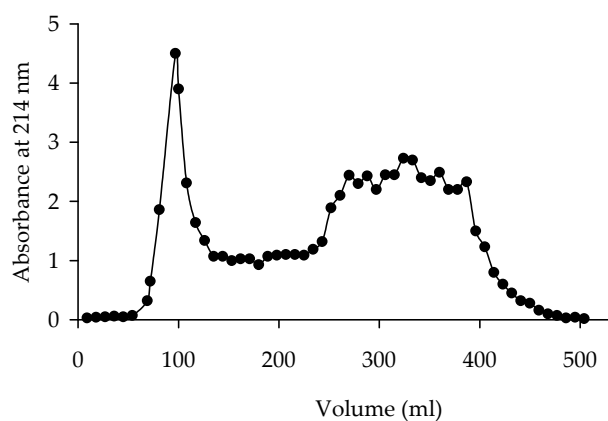


Figure 2. Gel chromatography of whey proteins (WPC) hydrolysate with Alcalase on Sephadex G-75 (flow rate 9 ml, λ = 214 nm)

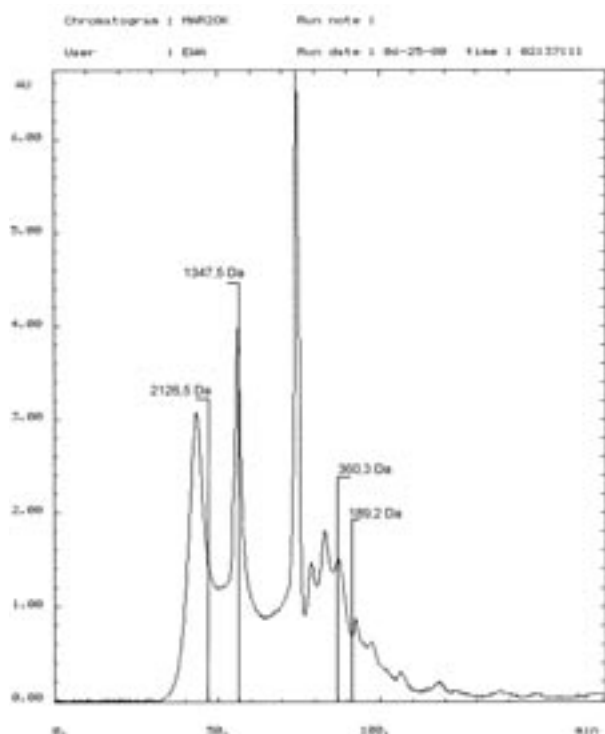


Figure 3. FPLC profile of low mass fraction (< 12.4 kDa) of hydrolysates of WPC with Alcalase, buffer: 0.5M NaCl in 0.02 phosphate buffer, pH 7.2, flow rate 0.25 ml/min, UV 214 nm

in a FPLC system with the use of a SuperdexTM74 HR 10/30 column (Figure 3). Three distinct peaks in the range of 360–2200 kDa and a peptide fraction with molecules smaller than 189.2 kDa were obtained. CALVO and GOMEZ (2002) explored the allergenicity of fractionated hypoallergenic preparations of whey proteins, caseins, and their

mixtures. They showed that peptides with molecular masses of 5000–500 Da, and in the case of casein – even below 500 Da, also display immunoreactive properties. Literature data indicate that they should not trigger allergies but might unfavourably affect the organoleptic properties of hydrolysates, by giving them a bitter after-taste (KORHONEN *et al.* 1998).

Changes of mean body weights in mice

Particular groups of mice were weighed at days 0, 7, 14 and 28 of the experiment (Figure 4). Initially, the mean body weights in the animal groups ranged from 98 to 112 g. The control group was made of mice with relatively lower body weights taking into account the potential negative, and even destructive, effect of the hydrolysis products analysed. This group was fed only plant proteins and had *ad libitum* access to water. A rapid body gain in the first 14 days and a constant body weight level afterwards were observed. With the experimental period proceeding, diversified body gain tendencies were reported in the other groups. Body weights of the immunised mice (I) were observed to increase until day 21, and then they dropped distinctly from 115 to 110 g. The groups fed whey proteins, sodium caseinate and their hydrolysates (WPC, A, AP, AL, II, KS, and KA) were gaining weight until day 21 after which their body weights declined negligibly. Only the group fed WPC hydrolysate produced with a double dose of Alcalase, despite a good initial condition of mice, were losing weights throughout the experi-

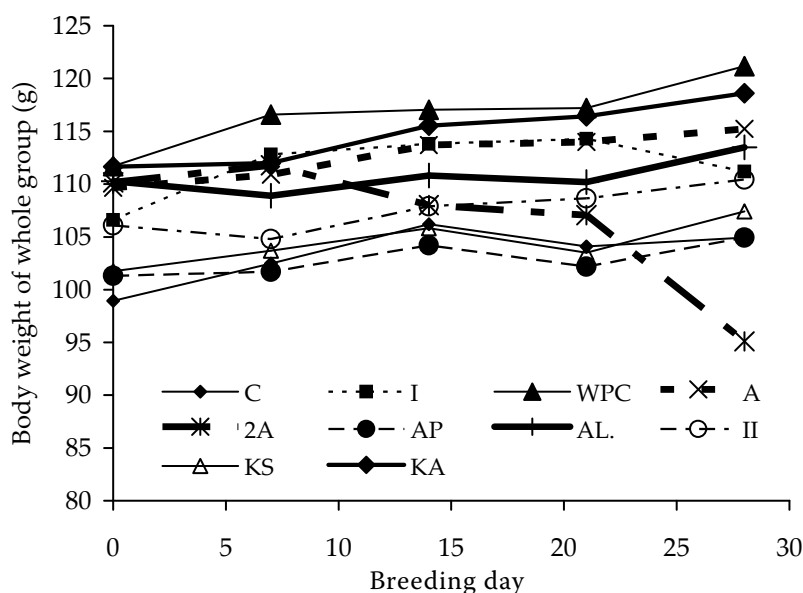


Figure 4. Changes of mean body weights in mice during experimental period

mental period, from 112 g to 95 g on day 28. Most probably, this type of hydrolysate did not exert a favourable effect on the gain of muscle-fat mass. The amount of food administered intragastrically filled up the stomach, evoking the feeling of satiety, and did not mobilise the animals to intake food by themselves. It is hard to identify any other cause-and-effect interaction evoking such a body weight loss. This signals at the same time that, during the production process, hydrolysates should be supplemented with compounds essential for the organism development. OTANI *et al.* (2000) carried out a 60-day experiment separately on females and males. They reported that the mean body weights in both groups were increasing to approximately day 30, and remained at a similar level in the further part of the experiment. So elongated time of the experiment seems unnecessary for the observation of immunological changes

resulting, among others, from the maturation and the growth of the organism.

Effect of sodium caseinate hydrolysates on the immune response of mice

The experiment carried out on Balb/c mice was mainly aimed at providing information on the effect of orally-administered hydrolysates on either gaining tolerance or causing an inverse reaction to enzymatically-modified proteins. In addition, the mice were immunised with one of the strongest milk allergens, β -lg, which was to stimulate additionally the animals towards aggravating the state of allergy. The intake of the hydrolysed proteins was supposed to alleviate the organism's response (to cause tolerance acquisition).

Within the so-called "casein groups", observations were made with respect to the group fed sodium

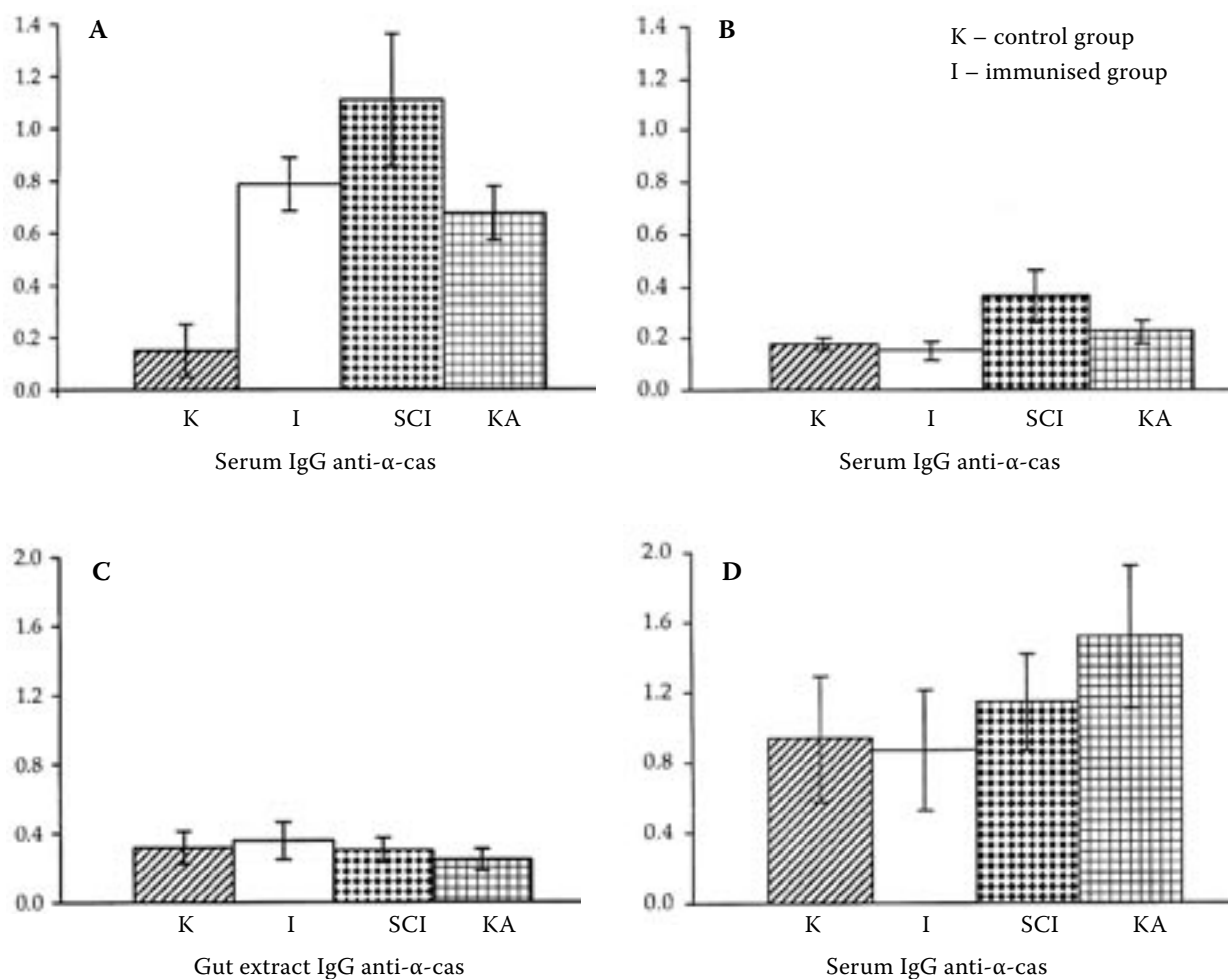


Figure 5. Effect of dietary sodium caseinate isolate (SCI) and its Alcalase hydrolysate (KA) on serum and gut extract IgG and IgA toward α -casein

Table 1. Statistical analysis of the level of immunoglobulin (IgA and IgG) in sera and gut extracts obtained from mice fed with sodium caseinate isolate and its Alcalase hydrolysate

Level of immunoglobulin	Control (K)	Mice only immunised (I)	Mice fed with sodium caseinate isolate (KS)	Mice fed with Alcalase hydrolysate of sodium caseinate isolate (KA)	SEM
α -casein IgG sera	0.1503 ^C	0.7870 ^B	1.1109 ^A	0.6770 ^B	0.0715
α -casein IgA sera	0.1803 ^{Bbc}	0.1534 ^{Bc}	0.3621 ^{Aa}	0.2236 ^{Bb}	0.0187
α -casein IgG gut extracts	0.3196 ^{ab}	0.3587 ^a	0.3033 ^{ab}	0.2476 ^b	0.0172
α -casein IgA gut extracts	0.9326 ^B	0.8650 ^B	1.1410 ^{AB}	1.5167 ^A	0.0792

a, b, c values within rows followed by the same letter are not significantly different at $P = 0.05$

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caseinate and the group fed caseinate hydrolysate (SCI) obtained as a result of a reaction with Alcalase, as compared to the control group and the group immunised with β -lg (Figure 5). Casein and β -lg are able to cross-react, hence their activities toward the organism were expected to be similar (CONTI *et al.* 2000; BERNARD *et al.* 2000).

Considering the level of anti- α -casein IgG in blood serum, a substantial increase of anti- α -casein antibodies was observed both in the group of the mice immunised with β -lg and that fed SCI caseinates and, in addition, periodically immunised with β -lg. The group fed casein hydrolysate obtained with Alcalase demonstrated a lower level of anti- α -casein IgG in blood serum, which might indicate that the hydrolysate applied was not as immunogenic as the initial material (Figure 5A). Immunisation with β -lg caused no IgG increase in the casein group. It may be concluded that the administration of the casein hydrolysate obtained with Alcalase alleviated the effects of immunisation. Statistical results confirm highly significant differences between the individual groups and indicate that the organism's response is more favourably affected by the hydrolysate than the raw material (Table 1).

The level of anti- α -casein IgA in blood serum determined in the group of mice fed sodium caseinate was statistically significant and twice as high as in the other groups (Table 1). An increase of anti- α -casein IgG and the simultaneous low level of anti- α -casein IgA in blood serum of the immunised animals clearly indicate hypersensitivity acquired by the respective animals. In the case of SCI material, the IgG level was higher than in the case of SCI casein hydrolysate obtained with Alcalase which confirms the diminishing impact of

the hydrolysis process on the antigenic properties of the hydrolysate examined.

Alcalase hydrolysate of sodium caseinate (KA) induced the lowest production of IgG against α -casein in intestinal mucosa. Moreover, a decreased level of anti- α -cas IgG observed in the intestinal extract was accompanied by an increase of anti α -cas IgA which was statistically the highest for Alcalase casein hydrolysate. This indicates redirection of the antibody production by Peyer's patches towards the reduction of the food intolerance during the administration of a protein hydrolysate (Figures 5C and 5D). The level of IgA against α -casein produced in gut as estimated in the mice group fed with KS and a higher one for KA group pointed to the stimulation of the local immune response. The more modified was the milk protein product, the higher was the IgA level. Oral administration of antigen and the penetration into the gastric tract induced local immune response.

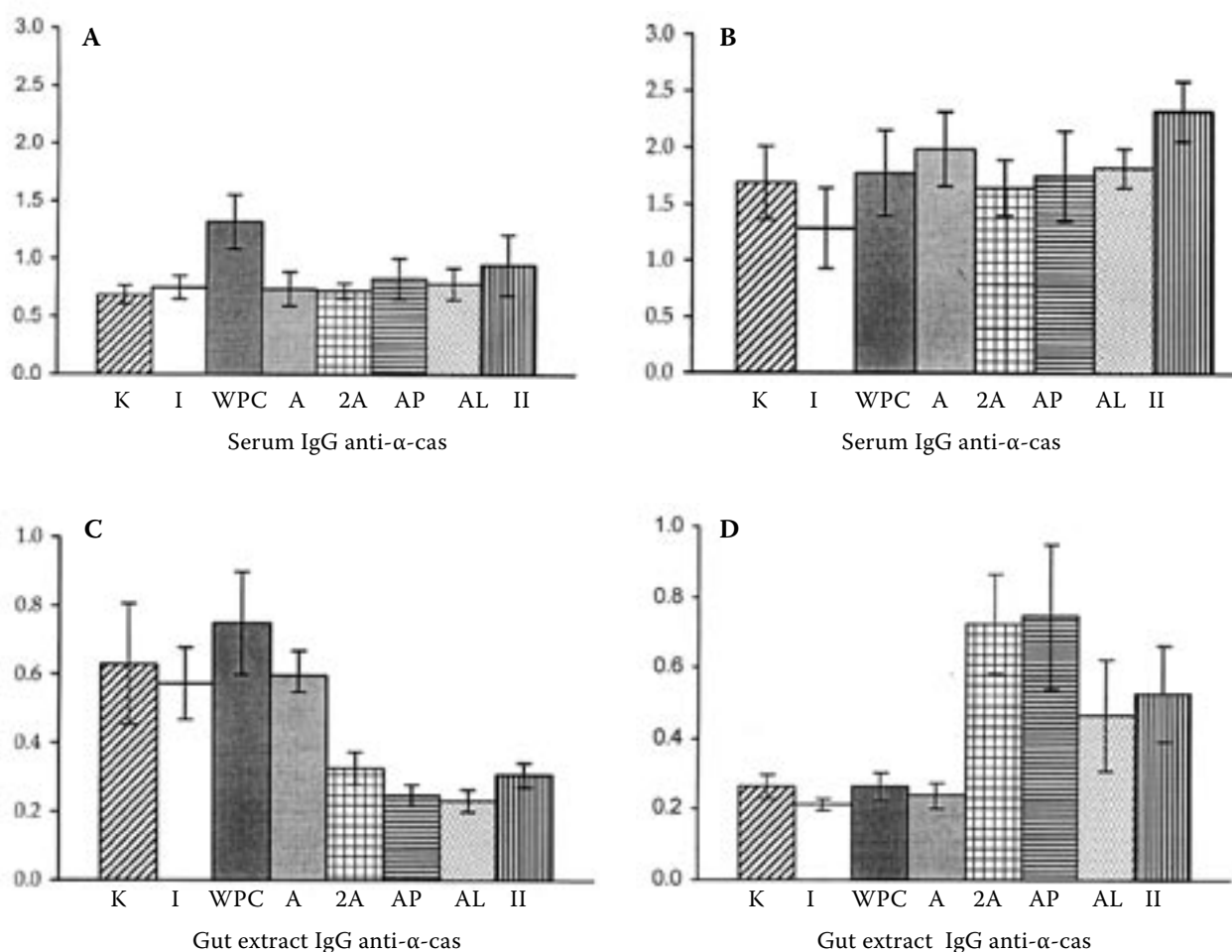
NENTWICH *et al.* (2004) incubated casein and casein hydrolysate in an IgG-ELISA assay with the sera from five patients with cow milk intolerance and a pooled control serum from milk-drinking healthy individuals. The antigenicity of the casein hydrolysate compared to that of the non-hydrolysed casein was reduced to 18.5 and 41.8% as found with patient and control sera, respectively. There were traces of intact protein in the hydrolysate. This research demonstrates that the comparative analysis of the protein material, namely sodium caseinate, and its hydrolysate reveals a distinctively more beneficial response of the organism to the latter which confirms the significance of hydrolysis as a process lowering the allergenicity of milk casein.

Effect of WPC hydrolysates on the immune response of mice

Figure 6 presents the levels of specific anti- β -lg IgG and IgA estimated in sera and intestinal extracts of mice. Mice fed the control diet without milk proteins showed a low proliferative response to β -lg. In blood serum, a very low level of β -lg recognition by IgG class antibodies was observed which was most likely due to the after-birth period where mice were still fed by their mothers. The feeding with a cereal diet over the experimental period was the direct cause of the lack of anti- β -lg IgG production. The immunisation of animals with β -lg together with adjuvant increased the IgG production in mice serum. A tenfold increase of

the anti- β -lg IgG level in serum was observed in comparison to the control group (mean absorbance = 0.81 and 0.076, respectively), which manifests a high immunogenicity of that protein. Statistical analyses confirm a statistically significant difference between those groups (Table 2).

A significant decrease of anti- β -lg IgG was observed in mice serum fed WPC and its hydrolysates as compared to the control group, which denotes the acquisition of the physiological response to orally-administered antigen not necessarily meaning intolerance. The group obtaining orally WPC hydrolysate produced with a double dose of Alcalase (2A) demonstrated the highest level of IgG antibodies against β -lg produced. Simultaneously, a significant decrease was observed in the mean



K – control group, I – immunised group, A – hydrolysate of WPC produced with Alcalase 2.4 FG, 2A – hydrolysate of WPC produced with a double dose of Alcalase, AP – two-step hydrolysate of WPC produced with Alcalase and papain, AL – two-step hydrolysate of WPC produced with Alcalase and Lactozym 3000 L HP-G, II – second fraction (< 12.4 kDa) of WPC hydrolysate produced with Alcalase

Figure 6. Effect of dietary whey protein concentrate (WPC) and its hydrolysates on serum and gut extract IgG and IgA toward β -lactoglobulin

Table 2. Statistical analysis of the level of immunoglobulin (IgA and IgG) in sera and gut extracts obtained from mice fed with whey protein concentrate and its hydrolysates

	A	K	L	I	WPC	2A	AP	II	SEM
α -la IgG sera	0.7371 ^{Bc}	0.6857 ^{Bc}	0.7550 ^{Bbc}	0.7512 ^{Bc}	1.3186 ^{Aa}	0.7235 ^{Bc}	0.8282 ^{Bbc}	0.9481 ^{Bb}	0.0332
α -la IgA sera			1.8226 ^{Bb}	1.2863 ^{Cc}	1.7776 ^{BCb}	1.6497 ^{BCb}	1.7529 ^{BCb}	2.3260 ^{Aa}	0.0544
α -la IgG gut extracts	0.5961 ^{Bb}		0.2329 ^{Cc}	0.5738 ^{Bb}	0.7488 ^{Aa}	0.3260 ^{Cc}	0.2484 ^{Cc}	0.3081 ^{Cc}	0.0280
α -la IgA gut extracts	0.2391 ^C	0.2639 ^C	0.4670 ^B	0.2157 ^C	0.2643 ^C	0.7247 ^A	0.7457 ^A	0.5286 ^B	0.0312
β -lg IgG sera		0.0763 ^{De}			0.4532 ^{Cd}	0.9316 ^{Aa}	0.6126 ^{BCcd}		0.0385
β -lg IgA sera	0.7554 ^{Aa}		0.7397 ^{ABa}					0.3480 ^{Cc}	0.0324
β -lg IgG gut extracts	1.2737 ^{ABb}	0.2267 ^{Cc}	1.0536 ^{Bb}	1.2508 ^{ABb}	0.8553 ^{Bb}	1.7435 ^{Aa}	1.2208 ^{ABb}	1.1370 ^{Bb}	0.0698
β -lg IgA gut extracts	0.8097 ^{Aa}	0.5028 ^{Bb}	0.7021 ^{ABab}	0.5546 ^{ABb}	0.7728 ^{ABa}	0.7846 ^{Aa}	0.6418 ^{ABab}		0.0255

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^{A,B,C} values within rows followed by the same letter are not significantly different at $P = 0.01$

body weights of mice consuming the 2A diet while the body weights of mice from the other groups tended to increase over the experimental period (Figure 6). Such observations may imply that the process of hydrolysis should be carefully planned so that its effects could be foreseen. Increasing the dose of the enzyme may produce unfavourable nutritional consequences including a body weight loss, thus leading to malnutrition.

The lowest level of anti- β -lg IgA in blood serum was reported for the so-called II fraction of WPC hydrolysate obtained with Alcalase (M.W. < 12.4 kDa). The mean absorbance value in this group, relating to the level of anti- β -lg IgA, was 0.35, whereas it appeared to be twice as high in the other hydrolysates and the control group (Figure 6B, Table 2). IgA is an ideal antibody for destroying particles that penetrate occasionally into the blood circulation. It may be supposed that the II fraction of WPC hydrolysate obtained with Alcalase (M.W. < 12.4 kDa) is less allergenic as there is no need to produce an increased amount of IgA in blood serum since mice fed that fraction acquired tolerance. FROSSARD *et al.* (2004a) in

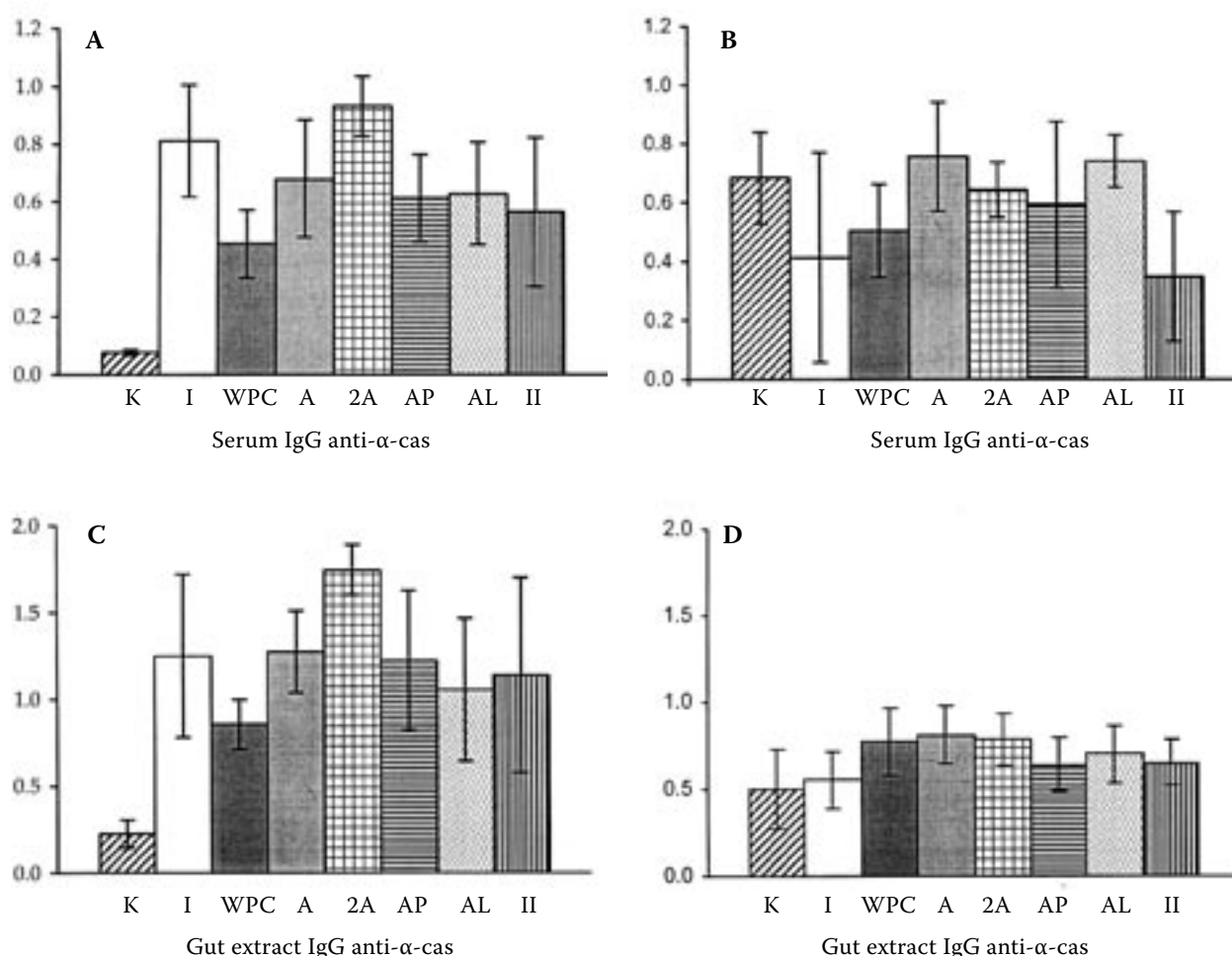
this study proved that β -lg-anaphylactic mice had increased β -lg-specific serum IgA titres and a low level of β -lg-specific secretory IgA in the faeces. The next article of the same group of researchers (FROSSARD *et al.* 2004b) showed that the food-tolerant animals have a specific IgA antibody profile with low β -lg-specific serum IgA titres and high titres of β -lg-specific secretory IgA antibody in their faeces. Our observations of the changes in immunoglobulin levels are similar, which substantiates the fairness of the experimental assumptions and enables to determine the effects of particular hydrolysates on the organism. Most likely, the allergenic protein (β -lg) administered by means of intraperitoneal injection was not recognised as a not-own protein by the mouse organism subjected to a constant oral stimulation with the examined fraction II. Hence, relatively lowest levels of anti- β -lg IgA were reported in the blood serum.

Figure 6C implies that the highest level of anti- β -lg IgG in the intestinal extract was evoked by the hydrolysate produced with a double dose of Alcalase (2A). The level was significantly higher (at a confidence level of $P < 0.01$) than the IgG levels

reported with the other hydrolysates (Table 2). The same observation, referring to a high level of anti- β -lg IgG, applied to the blood serum results. Hence, it may be concluded that the 2A hydrolysate was the least beneficial of all the products tested on animals.

It was observed that the anti- β -lg IgA level in the intestinal extract was characterised by a diminishing tendency along with the application of hydrolysates with a higher degree of hydrolysis and the reduction of proteins with high molecular masses (Figure 6D). Still, these results were at a similar level and differences between them were statistically insignificant (Table 2).

The graphical presentation of the results referring to anti- α -la IgG and IgA is shown in Figure 7. The analysis of the results with the specific anti- α -la IgG in serum indicated that the consumption of WPC material by mice triggers a rapid defence reaction, namely the production of a high level of anti- α -la IgG (abs = 1.32, Table 2), whereas the administration of WPC hydrolysates does not elevate the IgG titre which remains at the level of the control sample. The statistical analysis confirmed insignificant differences between the individual groups (Table 2). Periodical intraperitoneal stimulation of mice with β -lg did not increase the production of specific anti- α -la IgG in blood serum during



K – control group, I- immunised group, A – hydrolysate of WPC produced with Alcalase 2.4 FG, 2A – hydrolysate of WPC produced with a double dose of Alcalase, AP – two-step hydrolysate of WPC produced with Alcalase and papain, AL – two-step hydrolysate of WPC produced with Alcalase and Lactozym 3000 L HP-G, II – second fraction (< 12.4 kDa) of WPC hydrolysate produced with Alcalase

Figure 7. Effect of dietary whey protein concentrate (WPC) and its hydrolysates on serum and gut extract IgG and IgA toward α -lactalbumin

feeding with hydrolysates, which may point to the tolerance acquisition. Proteins of WPC raw material, including technologically intact β -lg, produce a more rapid organism's response than do hydrolysates. The highest level of anti- α -la IgA in blood serum was reported for the fraction II of Alcalase hydrolysate (M.W. < 12.4 Da) (II). Simultaneously, no statistically significant serum level of anti- α -la IgG was observed. Such a condition corresponds to the induction of tolerance to food taken.

A reduced level of anti- α -la IgG in the intestinal extract and simultaneously an increased level of anti- α -la IgA upon the administration of WPC hydrolysates obtained with: Alcalase (A), double Alcalase (2A), Alcalase with Lactozymes (AL), and the so-called II fraction of Alcalase hydrolysate (M.W.<12.4 kDa) (II), speak volumes for the alleviation of the potential intolerance within intestines.

In this experiment, the type and quality of the hydrolysate used was observed to affect the immune system of mice. Of the so-called "whey" hydrolysates analysed, the so-called II fraction of Alcalase hydrolysate (M.W. < 12.4 kDa) (II) proved to be the most favourable.

The determination of tolerogenic fractions was described in a paper by PECQUET *et al.* (2000). The main objectives of that research included hydrolysis of β -lg by trypsin, the identification of tolerogenic peptides with reduced allergenicity, and the measurement of specific IgE in serum and intestinal extracts of mice fed with β -lg hydrolysates and with particular fractions of the hydrolysate. The results obtained indicated that the tolerogenic peptides possess molecular masses lower than 4500 D. The hydrolysed products containing only amino acids are not capable of inducing oral tolerance, although they are in fact non-allergenic. Interesting observations were given in a paper by LEBLANC *et al.* (2002). The authors investigated the immunomodulative effect of peptide fractions obtained from milk fermented with *Lactobacillus helveticus*. They reported that the so-called II fractions had a direct impact on diminishing fibrosarcoma in mice, and that fraction I decreased, although negligibly, the size of the tumor after 35 days of growth. The research papers aimed at identifying selected protein fractions with specified immunomodulative properties seem a very promising direction for future studies. This applies especially to the investigations searching technological solutions for children suffering from cow milk allergy, where hydrolysis is only a

preliminary step during milk protein processing aimed at a complete reduction of antigenicity.

CONCLUSIONS

In vivo animal model experiments were helpful tools in evaluating residual allergenicity of milk proteins modified by enzymatic hydrolysis. Hydrolysates of WPC whey proteins and sodium caseinates were found to exert a diversified effect on the production of immunoglobulins. Casein hydrolysate (KA) evoked a negligible increase of the serum level of IgG, although the increase was lower than that produced by the initial material. An elevated level of anti- α -casein IgA in gut extract points to a tolerogenic effect of the KA hydrolysate administered to mice. In the case of whey protein hydrolysates, the raw material (WPC) was found to stimulate the organism to produce serum anti- β -lg IgG, nevertheless at a lower level than when stimulated by some other hydrolysates (A, 2A, AP and AL). A substantial decrease in serum anti- β -lg IgA may indicate the mice response directed to the acquirement of tolerance. It may then be supposed that intraperitoneally-administered β -lg caused the immunostimulation of the organism against that protein. The most beneficial option of the mice feeding was the whey protein-based preparation, the so-called II fraction of WPC hydrolysate obtained with Alcalase (M.W. < 12.4 kDa).

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