RESEARCH ARTICLE

Digestibility and allergenicity assessment of enzymatically crosslinked β-casein

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Crosslinking enzymes are frequently used in bioprocessing of dairy products. The aim of this study was to examine the effects of enzymatic crosslinking on IgE binding, allergenicity and digestion stability of β-casein (CN). β-CN was crosslinked by transglutaminase, tyrosinase, mushroom tyrosinase/caffeic acid and laccase/caffeic acid. The IgE binding to β-CN was compared *in vitro* by CAP inhibition assay, ELISA inhibition as well as *ex vivo* by basophil activation assay. Crosslinked CNs were digested by simulated gastric fluid for 15 and 60 min and obtained digests analyzed for their ability to inhibit IgE binding by CAP inhibition assay and SDS-PAGE. The ability of crosslinked CNs to activate basophils was significantly reduced in seven patients in the case of CN crosslinked by laccase and moderately reduced in the case of tyrosinase/caffeic acid crosslinked CN (in two cow's milk allergy patients tested with different allergen concentrations). The response to various crosslinked CNs differed individually among patients' sera tested by ELISA inhibition assay. The presence of caffeic acid hampered digestion by pepsin, and this effect was most pronounced for the tyrosinase/caffeic acid crosslinked CN. The laccase/caffeic acid and mushroom tyrosinase/caffeic acid had the highest potential in mitigating IgE binding and allergenicity of the β-CN out of all investigated enzymes. The presence of a small phenolic compound also increased digestion stability of β-CN.

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1 Introduction

Food allergy has become a serious health concern, especially in developed countries. Approximately 4–6% of children and 1–3% of adults are affected with food allergy [1]. The financial and social implications related to these conditions are increasing worldwide. Although the mechanism of food allergy development, *e.g.* sensitization process, remains

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Abbreviations: AbTyr, *Agaricus bisporus* tyrosinase; **Aga,** casein cross-linked by *Agaricus bisporus* tyrosinase; **BA,** basophil activation; **CN,** casein; **Lacc,** casein cross-linked by laccase; **MW,** molecular weight; **PAA,** polyacrylamide; **SGF,** simulated gastric fluid; **TG,** casein cross-linked by transglutaminase; **Tgase,** *Streptoverticillium mobaraense* transglutaminase; **ThL,** *Trameteshirsuta* laccase; **TrTyr,** *Trichodermareesei* tyrosinase; **TTBS,** Tris-buffered saline; **Tr,** casein cross-linked by *Trichoderma reesei* tyrosinase

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currently unclear, it is thought that an allergen is able to survive harsh acidic and proteolytic environment of the stomach in order to reach the immune system of the intestine, or to share the epitopes with common aeroallergens [2]. Many of the food allergens are stable proteins that are very resistant to digestion by gastrointestinal enzymes [3, 4] or can be digested into high molecular weight (MW) peptide fragments which retain the IgE binding and T-cell stimulating capacities [5–7]. Due to proteolysis of some food allergens by gastrointestinal enzymes, their bioavailability and immunogenicity have been reported to be altered as a result of interactions between allergens and other food ingredients, such as phospholipids in milk [8] or polysaccharides in fruits [9]. Additionally, food processing has also been reported to affect the allergenicity of food products [10].

Enzymatic crosslinking of proteins is currently exploited in the food processing industry as means to stabilize food structure, in particular meat and dairy products, as reviewed by Buchert *et al.* [11]. Current commercial applications are based on transglutaminase (Tgase) being able to form an isopeptide bond between glutamine and lysine residues of proteins. In addition to Tgase, oxidative enzymes, such as tyrosinases and laccases, are currently being investigated as crosslinking agents [11]. Laccases and tyrosinases act mostly on tyrosyl residues and on other phenolic substrates leading to crosslinking of proteins, polysaccharides and proteins with polysaccharides [12]. Low MW phenolic compounds have been reported to enhance the crosslinking efficiency of not only laccases but also tyrosinases [12].

Crosslinking of proteins has been shown to affect the allergenic properties [10]. However, not a great amount of research has been published on the impact of different crosslinking enzymes on allergenicity. In the case of peroxidase-treated peanut proteins, resulting crosslinked aggregates have shown reduced IgE binding, thus fostering application of these enzymes in the production of hypoallergenic food formula [13]. By contrast, transglutaminase crosslinking of peanut proteins did not have a profound impact on the IgE binding of peanut proteins [14, 15], whereas transglutaminase treatment of ω-gliadin markedly increased its IgE-binding potential in a study involving a high number of clinical sera with allergy to wheat [16]. Crosslinking of peanut proteins by polyphenol oxidase/caffeic acid reduced their IgE binding [17].

Bovine β-casein (CN) represents a serious health risk to patients with cow's milk allergy (CMA) as it binds IgE in more than 50% of the patients with CMA [18, 19]. This disorder is more common in children, and it is severe but rare in adults [20]. CN is a protein with a very flexible three-dimensional structure that accounts for approximately 25% of the total milk proteins [21]. CN can be crosslinked by different enzymes efficiently [22, 23] due to its random coil structure.

Up to now, no study has addressed digestibility and allergenicity of food proteins, two the most important aspects of food safety, following crosslinking by tyrosinase and laccase. The contradictory data obtained from studies of allergenicity of peroxidase- and transglutaminase-treated peanut proteins justify the need for reassessment of the effects of different crosslinking enzymes in a model food allergen on its peptide-releasing capacity during digestion and IgE-binding activity on a panel of well-defined patients' sera.

In this study, CN was crosslinked by four different enzymes (fungal laccase, two tyrosinases of a different fungal origin and a microbial transglutaminase) to highly polymerized products of various intra- and intermolecular bonds, depending on the enzyme used. The digestibility of the crosslinked CNs in simulated conditions of the gastrointestinal tract was studied under simulated gastric and intestinal conditions and the allergenicity was examined *in vitro* and *ex vivo*.

2 Material and methods

2.1 Chemicals

Bovine β-CN (product number: C6905, purity ≥ 85%) was obtained from Sigma-Aldrich (Taufkirchen, Germany). Pepsin (from porcine stomach mucosa, 2650 U/mg solid), pancreatin (from porcine pancreas), 2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), *N*-carbobenzoxy-L-glutaminyl-glycine/hydroxylamine and 3,4-dihydroxy-L-phenylalanine were purchased from Sigma-Aldrich. Anti-human IgE and anti-rabbit IgG labeled with alkaline phosphatase were also from Sigma. Urea and di-sodium hydrogen phosphate (analysis grade) were purchased from Merck (Darmstadt, Germany). Deionized water used in the experiments was purified in a Milli-Q system (Millipore, Molsheim, France).

2.2 Enzymes

Microbial transglutaminase from *Streptoverticillium mobaraense* (Tgase) was obtained from Ajinomoto and further purified according to Lantto *et al.* [24]. Laccase from the fungus *Trametes hirsuta* (ThL) and tyrosinase from *Trichoderma reesei* (TrTyr) were purified and characterized at VTT [25, 26]. Mushroom tyrosinase from *Agaricus bisporus* (AbTyr) was purchased from Fluka Biochemica (Sigma-Aldrich). The activity of the Tgase preparation was assayed according to Folk [27] using 0.03 M *N*-carbobenzoxy-L-glutaminyl-glycine as substrate. Laccase activity was measured using 2,2-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) [28]. Tyrosinase activity was assayed using 3,4-dihydroxy-L-phenylalanine as substrate [28].

2.3 Rabbit polyclonal sera production

Bovine milk was defatted initially by centrifugation (10 min at 13 400 rpm) and followed by chloroform extraction.

Defatted milk was extensively dialyzed against PBS and diluted with PBS to 1 mg/mL (assayed by Bradford protein determination method) [29]. Antibodies against whole bovine milk proteins were raised in rabbits according to Harboe and Ingild [30] at the Institute of Immunology and Virology "Torlak," Belgrade, Serbia. Two rabbits were immunized with milk proteins dissolved in PBS. Presence of IgG antibodies was determined by ELISA using a 1:6000 dilution of anti-rabbit IgG alkaline phosphatase-labeled antibodies and whole milk proteins coupled to the microtiter plates (5 μg/mL). High titer rabbit serum was diluted 1:50 000 in a Tris-buffered saline (pH 7.4) (TTBS) with 0.1% Tween-20 and 0.1% BSA. Antibodies were partially purified by ammonium-sulfate precipitation. Partially purified high titer antibodies were used in the immunoblot and ELISA inhibition experiments.

2.4 Human sera

IgE levels from sera of eight patients with a positive skin prick test to milk assessed to milk proteins with ImmunoCAP® 100 system using ImmunoCAP® code f2 (Pharmacia Diagnostics, Uppsala, Sweden) above 0.35 kAU/L, and documented clinical history of CMA were pooled for ImmunoCAP inhibition experiments.

2.5 Crosslinking of β-CN

β-CN was dissolved in 50 mM sodium phosphate buffer, pH 8.0 (1.7 mg/mL), and incubated with Tgase (100 and 1000 nkat/g of CN), TrTyr (1000 nkat/g), AbTyr (1000 nkat/g) and ThL (1000 nkat/g of CN each). In the cases of AbTyr and ThL, caffeic acid acting as mediator (1 mM) was added. Enzymatic reactions were carried out at 40°C for 24 h. Reaction mixtures were incubated for 24 h. Reaction mixture vessels remained open and constant stirring was ensured. All reactions were stopped by freezing (−20°C). Controls without enzyme addition were run in parallel. All reactions were run in duplicates.

2.6 Electrophoresis

Protein components were resolved on 10% polyacrylamide (PAA) Tris-glycine SDS-PAGE according to Laemmli [31] and 14% PAA Tris-Tricine SDS-PAGE according to Coligan *et al.* [32]. An electroblotter system (Serva, Heidelberg, Germany) was used for protein transfer to nitrocellulose membrane (Millipore, USA, 0.45 μm pore size). Composite agarose–PAA gels were prepared according to a described protocol [33]. Chicken myofibrilar proteins were prepared, as previously described [34], and used as high MW marker proteins for composite slab gel electrophoresis in 0.5% agarose/2.4% PAA and 0.5% agarose/2% PAA SDS electrophoresis.

2.7 Biphasic digestion of crosslinked CN

Gastric and intestinal fluids were prepared according to EU Pharmacopeia. Simulated gastric and intestinal digestions were performed as described previously [35, 36]. Digestion mixtures contained 150 μL of β-CN control or β-CN treated with crosslinking enzymes (1.7 mg/mL) solutions and 50 μL 0.4 M HCl with 8 g/L of NaCl and 6.8 g/L of pepsin (3.64 U/mg of protein, protein:pepsin ratio: 1:1.33). Digestion proceeded at 37°C for 60 min and continuous shaking. Digestion was stopped by addition of 60 μL of 0.3 M Na_2CO_3 to give final pH of 8.0. Aliquot of 140 μL was withdrawn for CAP inhibition assay and SDS-PAGE analysis whereas to the rest of 120 μL of digestion mixture, 40 μL of 200 mM sodium phosphate buffer with 1.8% NaCl, pH 7.4, with 40 g/L of pancreatin was added. The second phase of digestion proceeded at 37°C for 60 min and continuous shaking. Digestion was stopped by addition of 40 μL of five–times-concentrated sample buffer (60 mM Tris, pH 6.8, 25% glycerol, 2% SDS, 14.4 mM beta-mercaptoethanol and 1% bromophenol blue) for SDS-PAGE and incubation at 95°C for 10 min. Pepsin and pancreatin controls were set up in the same manner in absence of allergens. Control samples of native and crosslinked CN were treated the same way without pepsin/pancreatin addition.

For CAP inhibition assay, 25 μL aliquot of digested sample was added to 225 μL of pool sera from patients allergic to cow's milk. For SDS-PAGE analysis, 20 μL of samples were mixed with 5 μL of five-times-concentrated sample buffer and 20 μL of each sample was applied *per* lane (16 μg of protein *per* well).

2.8 Partial gastric digestion of crosslinked CN

Digestion mixtures containing 150 μL of β-CN control or β-CN were treated with crosslinking enzyme (1.7 mg/mL) solutions and 50 μL 0.4 M HCl with 8 g/L of NaCl and 6.8 g/L of pepsin, as described above. Digestion was performed at 37°C for 15 min with continuous shaking. The digestion was stopped with 60 μL of 0.3 M Na_2CO_3 (final pH). Pepsin control was set up in the same manner in the absence of allergens. Control samples of native and crosslinked CN were treated the same way without pepsin/pancreatin addition.

For CAP inhibition assay, 25 μL of digested sample was added to 225 μL of pool sera from patients allergic to cow's milk. For SDS-PAGE analysis, 20 μL of samples were mixed with 5 μL of five-times-concentrated sample buffer and 20 μL of each sample was applied *per* lane (16 μg of control or enzyme-treated CN *per* well).

2.9 Probing of antigenic peptides generated by partial digestion

Nitrocellulose membranes were first blocked with 1% BSA in TTBS. Membranes were probed with sera of rabbits immunized to whole milk proteins, diluted 20 000 times in TTBS containing 0.1% BSA. Bound rabbit IgG was detected using anti-rabbit IgG antibodies labeled with alkaline phosphatase, diluted 2000 times in TTBS–0.1% BSA. The binding patterns were visualized using a substrate solution consisting of 1.5 mg BCIP (5-bromo-4-chloro-3-indolyl phosphate) and 3 mg NBT (nitro-blue-tetrazolium) in 10 mL of 100 mM Tris buffer, containing 150 mM NaCl and 5 mM $MgCl_2$, pH 9.6.

2.10 CAP inhibition of specific IgE binding by crosslinked CNs or its peptides

Sera from eight patients allergic to cow's milk with 0.5–57 kU/L of IgE were pooled for CAP inhibition experiments. CAP inhibition assay was done with undigested and pepsin-digested CN (following 15 and 60 min of digestion) in control and crosslinked CN samples. β-CN sample of 25 μL was mixed with 225 μL of human sera pool to give a final protein concentration of 100 μg/mL (or peptides originating from 100 μg/mL CN) and an IgE level of 11.5 kAU/L. After an overnight incubation at 4°C, CAP results were assessed with ImmunoCAP® 100 system using ImmunoCAP® Allergen nBos d 8 casein code f78 (Pharmacia Diagnostics).

2.11 ELISA inhibition of specific IgE binding by CN and crosslinked CNs

Individual sera from two patients of high IgE level to milk proteins (Table 2) with proven CMA were used. Specific IgE binding to CN crosslinks was determined using polystyrene 96-well microtiter plates (MaxiSorb, Nunc, Denmark) coated with 100 μL of CN at 5 μg/mL in a coupling buffer (50 mM carbonate buffer, pH 9.6) for 15 h at 25°C. Coated wells were blocked with 1% BSA in the TTBS buffer. Serial tenfold dilutions of crosslinked CNs were preincubated with the same volume of sera of each milk-allergic patient (six times diluted). Dilutions were made in TTBS–0.1% BSA. After 1 h at room temperature, 100 μL of each sample was added to microtiter plate and incubated for 2 h at 37°C. After washing with TTBS, wells were incubated with alkaline phosphatase-labeled anti-human IgE (Sigma, Germany) for 2 h at 37°C. Plates were washed again and reaction developed with 100 μL of substrate (1 mg/mL *p*-nitrophenyl phosphate in 10 mM diethanolamine with 0.5 mM MgCl2). Following incubation for 15 h at 4°C, the OD at 405 nm was measured. Positive control (OD 0%) was diluted human serum preincubated with the same volume of TTBS–0.1% BSA, while negative control (OD 100%) was sera of non-allergic patient preincubated with the same volume of TTBS–0.1% BSA. Results were evaluated by means of multiple regression, with percentage inhibition as the dependent variable and concentration (log transformed) and the CN form as predictor variables in the model. Tests were performed in triplicate. Inhibition values were determined as follows:

$$\text{Inhibition}\,(\%) = 100 \times (\text{OD}_{\text{sample}} - [\text{OD0\%} - \text{OD100\%})/$$

$$(\text{OD0\%} - \text{OD100\%})]$$

2.12 Basophil activation (BA) assay in patients with CMA

Heparinized blood samples were taken from seven cow's milk-allergic patients and two non-atopic individuals. Clinical data are given in Table 1. Blood aliquots (100 μL) were incubated with dilutions of β-CN and crosslinked β-CN antigens (50 μg/mL), anti-IgE antibody as a positive control (1 μg/mL), or PBS as a negative control for 15 min (37°C). In three CMA patients, serial dilutions of β-CN and crosslinked β-CN antigens (50, 5, 0.5, and 0.05 μg/mL) were also probed for BA. After incubation, cells were washed with PBS and then incubated with 10 μL of phycoerythrin-labeled CD203c monoclonal antibody 97A6 (Immunotech) and FITC-labeled CD63 monoclonal antibody (Immunotech) for 15 min at room temperature. Thereafter, samples were subjected to erythrocyte lysis with 2 mL of FACS™ lysing solution (BD Biosciences, USA). Cells were then washed, resuspended in PBS and analyzed by flow cytometry using a FACSCalibur (BD Biosciences). The basophil marker CD203c was used to set a basophil gate including only $CD203c^+$ cells. Collected cells were further analyzed for granule release using an anti-CD63 antibody. The magnitude of BA was calculated as the percentage of $CD63^+$ events among the gated basophils ($CD63^+/CD203c^+$ cells). Results were expressed as follows: (magnitude of BA of the sample/magnitude of BA of the positive control (anti-IgE)) × 100.

Table 1. Clinical data on seven patients diagnosed with cow's milk allergy and analyzed for *ex vivo* allergenicity (by basophil activation assay) to casein and cross-linked caseins

Patient no.	IgE to milk kAU/L	Age (years)	Symptoms
1	0.68	2	Anaphylaxis, urticaria
2	>100	3	Anaphylaxis
3	1.72	13	Anaphylaxis, urticaria
4	6.14	3	Urticaria
5	5.1	3	Anaphylaxis
6	0.62	3	Anaphylaxis
7	0.36	2	Urticaria

Differences between BA reactivity in seven CMA patients to β-CN and crosslinked β-CN antigens were compared statistically using the Wilcoxon matched pairs test and the Statistica 9.0 program.

3 Results

3.1 Crosslinking of β-CN by tyrosinases, laccase and transglutaminase

β-CN was crosslinked using four different enzyme systems, *i.e. T. reesei* tyrosinase (TrTyr), *Agaricus bisporus* tyrosinase (AbTyr) together with caffeic acid, ThL together with caffeic acid and Tgase. To achieve extensive crosslinking, high enzyme dosages were used: Tgase 100 nkat/g, TrTyr 1000 nkat/g, AbTyr 1000 nkat/g, ThL 1000 nkat/g. SDS-PAGE was used to monitor the effects of crosslinking of β-CN with Tgase, TrTyr (Fig. 1A, lanes 2, 3 and 4), AbTyr (Fig. 1A, lane 5) and ThL in the presence of caffeic acid as a mediator (Fig. 1A, lane 6). When β-CN was treated with all crosslinking enzymes, high MW reaction products were obtained and crosslinked products remained in the wells of the gel (Fig. 1A). In the case of AbTyr and ThL crosslinking reactions, some of the monomeric unreacted β-CN was still present. In the of ThL/caffeic acid treatment, most of the CN remained monomeric (Fig. 1A, lane 6), although polymers of mass above 200 kDa could be observed at the top of the gel. The formation of high MW crosslinked CN was obvious for all the crosslinking reaction conditions, although to a lesser extent in the case of ThL/caffeic acid.

To further estimate the size of the obtained crosslinked proteins, a series of composite agarose/PAA slab gel electrophoreses were performed. Figure 1B shows results of crosslinked proteins separated by 0.5% agarose/2.4% PAA electrophoresis. Among all of the analyzed samples, only the ThL+caffeic acid and Tgase crosslinked β-CN entered the gel. The mass range of Tgase crosslinked β-CN (Fig. 1B, lane 3) was observed to be higher than in the case of ThL crosslinked β-CN (Fig. 1B, lane 6). The mass range of the obtained crosslinks was estimated to be above 220 kDa, according to the position of the marker protein at 220 kDa (myosin heavy chain). Due to the lower amount of the high MW crosslinked CN obtained by crosslinking by ThL/caffeic acid (Fig. 1), these polymers were not visible in Fig. 1B. Figure 1C shows results of crosslinked proteins separated by 0.5% agarose/2.0% PAA electrophoresis. Under these conditions, well-separated protein bands were obtained for TrTyr and AbTyr crosslinked β-CN. Their size was found to be comparable to each other (Fig. 1C, lanes 4 and 5), with the highest protein bands observed above 3000 kDa.

3.2 IgE binding potential of crosslinked CNs in CAP and ELISA inhibition assay

Although the size of the obtained crosslinked β-CN varied depending on the crosslinking enzyme used, all the enzymes

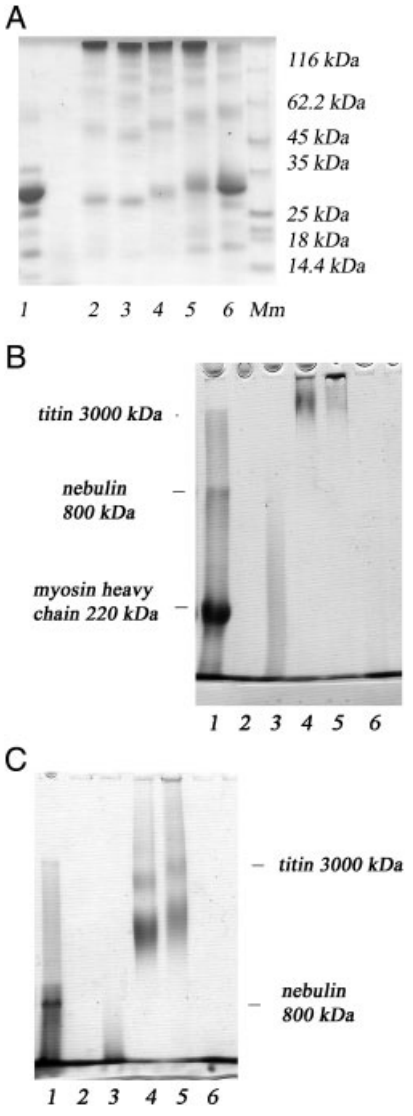

Figure 1. (A) SDS-PAGE of crosslinked β-caseins. Lane 1: untreated β-casein. Lane 2: β-casein treated by Tgase 100 nkat/g. Lane 3: β-casein treated by Tgase 1000 nkat/g. Lane 4: β-casein treated by TrTyr 1000 nkat/g. Lane 5: β-casein treated by AbTyr 1000 nkat/g and caffeic acid. Lane 6: β-casein treated by ThL 1000 nkat/g and caffeic acid. Lane Mm: molecular mass markers. (B) SDS electrophoresis on composite 0.5% agarose and 2.4% polyacrylamide gel: Lane 1: chicken myofibrilar proteins; lane 2: CN; lane 3: TG; lane 4: Tyr; lane 5: Aga; lane 6: Lacc. (C) SDS electrophoresis on composite 0.5% agarose and 2.0% polyacrylamide gel. Lane 1: chicken myofibrilar proteins; lane 2: CN; lane 3: TG; lane 4: Tyr; lane 5: Aga; lane 6: Lacc.

crosslinked proteins to some extent, giving high MW polymers, and only trace amounts of the monomeric CN remained (except for ThL crosslinked CN, whereby majority of the CN remained monomeric) in the mixture that was subsequently investigated for *in vitro* IgE binding potency and allergenicity.

Crosslinked CNs bind a high portion of the IgE specific to casein in the CAP inhibition assay of the pooled CMA

patients' sera. With the high concentration of crosslinks used in the assay, natural protein achieved an almost maximal inhibition of 80% to the CN-coupled discs (Fig. 2A).

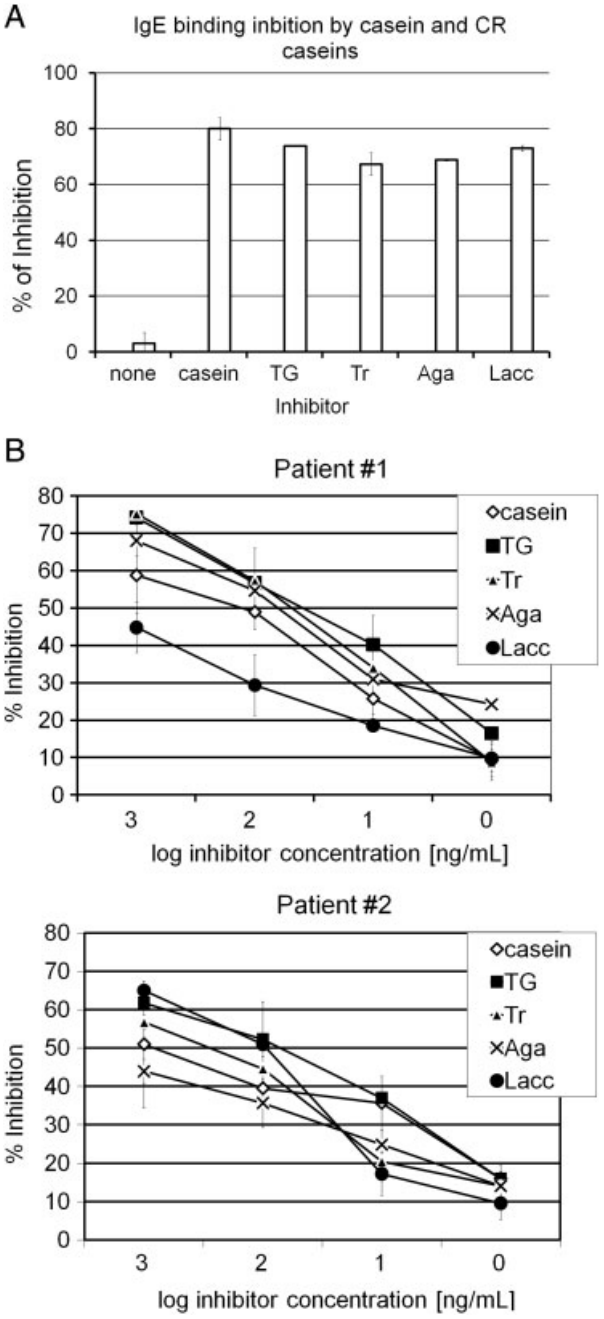

Figure 2. (A) CAP inhibition of casein-specific IgE by control casein, transglutaminase crosslinked casein (TG), tyrosinase crosslinked casein (Tr), tyrosinase/caffeic acid crosslinked casein (Aga), and laccase/caffeic acid crosslinked casein (Lacc). (B) Inhibition of IgE binding to casein-coupled microtiter plates by crosslinked caseins in two CMA patients.

In some crosslinked CNs (*i.e.* Aga and Tr), IgE binding from the pool of CMA patients was reduced in the *in vitro* assay for 14 and 16%, when compared with the control CN.

Crosslinked CNs were analyzed individually in ELISA inhibition assay for their ability to inhibit IgE binding to the CN-coupled microtiter plate. The response of two patients' sera was quite different showing a fine difference in epitope specificity for CN and crosslinked CNs. Results of ELISA inhibition of the IgE binding are shown in Fig. 2B and the IC_{50} values calculated from the inhibition slopes are presented in Table 2. When comparing the *in vitro* ability to inhibit IgE binding to the CN-coupled plate, the laccase-treated CN has reduced IC_{50} value (patient no. 1), while the Aga-treated CN showed reduced ability to inhibit IgE binding (patient no. 2). In both tested patients' sera, all highly crosslinked CNs (TG and Tr) showed a stronger inhibition potential in ELISA inhibition assay than the control CN.

3.3 Partial digestion of crosslinked CNs in simulated gastric fluid (SGF) by pepsin

Short incubation time digestion of the crosslinked CNs was analyzed by Tris-Tricine PAGE (Fig. 3A). A high portion of the control CN was already digested in 15 min, in agreement with the described lability of CN to gastric fluid digestion [3, 4], while crosslinked CNs of high MW were still visible at the top of the gel. Remaining monomeric CN in samples treated with laccase/caffeic acid was digested most efficiently.

3.4 Detection of peptic peptides by immunoblot

CN fragments generated by pepsin digestion were further visualized by polyclonal rabbit antibodies (Fig. 3B). The major fragment in the control CN had a MW below 25 kD (Fig. 3B, lane 2) and fragment of a similar MW could be seen with Tgase-treated CN (Fig. 3B, lane 4). The AbTyr, ThL and TrTyr-treated CNs gave more discrete fragmentation patterns with pepsin (Fig. 3B, lanes 6, 8 and 10), as detected by polyclonal rabbit antibodies. Digested laccase crosslinked protein generated a high portion of lower MW products in the range of monomeric CN, as detected by antibodies and visible also by CBB-stained Tris-Tricine

Table 2. IC_{50} values (ng/mL) obtained for casein and cross-linked caseins in ELISA inhibition of IgE binding to casein-coupled microtiter plates in two CMA patients' sera

	Casein	TG	Tr	Aga	Lacc
Patient 1, IgE to milk: >100 kAU/L	215.3	46.0	60.0	71.9	3981.1
Patient 2, IgE to milk: 3.48 kAU/L	676.1	109.7	359.7	3357.2	166.0

PAGE gel (Fig. 3A). Presence of a phenolic mediator of the crosslinking reaction in the case of ThL and TrTyr crosslinking was noticeable and stabilized the protein in SGF for the first 15 min of exposure.

3.5 IgE binding potential of peptic digests in CAP inhibition

Analysis of the IgE-binding properties of the partially digested CN and crosslinked CN by CAP inhibition is shown in Fig. 4A. Crosslinked CNs, in accordance with the slower digestion pattern (Fig. 3), possess high capacity for the IgE binding. Fragments obtained after 15 min pepsin digestion of control CN were still able to inhibit a portion of IgE, while crosslinked CNs changed their IgE binding properties just for up to 15%. The highest IgE-binding ability remained

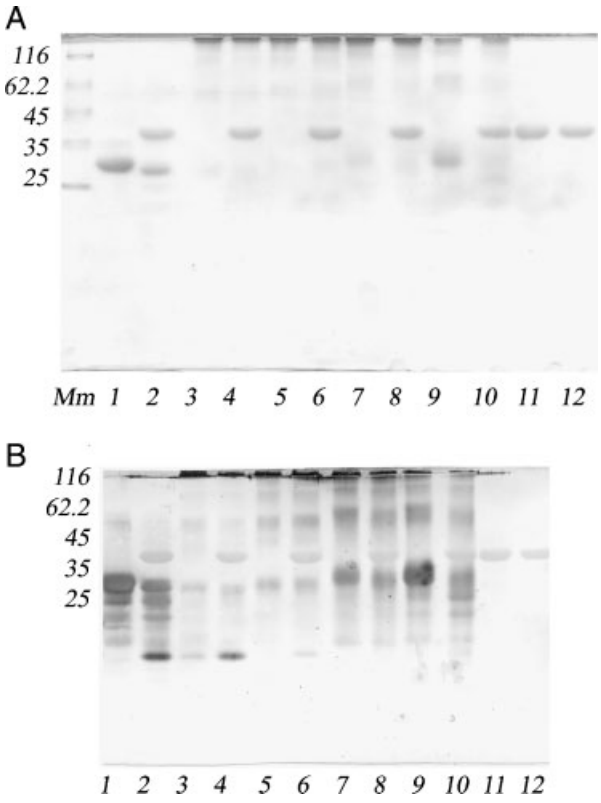

Figure 3. (A) Tris-Tricine SDS-PAGE of 15 min SGF digested crosslinked caseins. Lane 1: CN. Lane 2: digested CN. Lane 3: TG. Lane 4: digested TG. Lane 5: Tr. Lane 6: digested Tr. Lane 7: Aga. Lane 8: digested Aga. Lane 9: Lacc. Lane 10: digested Lacc. Lane 11: pepsin. Lane 12: pepsin exposed to SGF for 15 min. Mm – molecular weight markers. (B) Immunoblot detection of pepsin fragments generated by 15-min digestion by rabbit polyclonal sera to whole milk proteins. Lane 1: CN. Lane 2: digested CN. Lane 3: TG. Lane 4: digested TG. Lane 5: Tr. Lane 6: digested Tr. Lane 7: Aga. Lane 8: digested Aga. Lane 9: Lacc. Lane 10: digested Lacc. Lane 11: pepsin. Lane 12: pepsin exposed to SGF for 15 min.

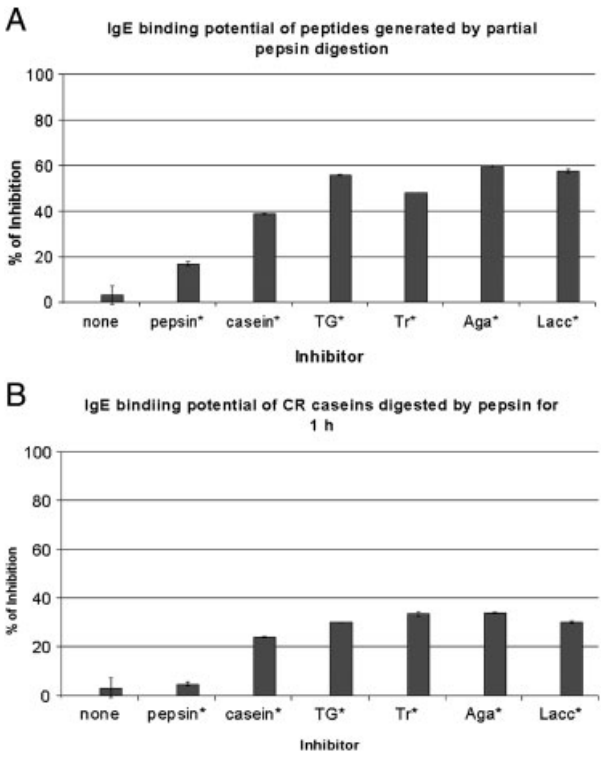

Figure 4. (A) CAP inhibition of casein-specific IgE by 15 min pepsin digested control casein (casein∗), pepsin control (pepsin∗) 15 min pepsin digested transglutaminase crosslinked casein (TG∗), 15 min pepsin digested tyrosinase crosslinked casein (Tr∗), 15 min pepsin digested tyrosinase/caffeic acid crosslinked casein (Aga∗) and 15 min pepsin digested laccase/caffeic acid crosslinked casein (Lacc∗). (B) CAP inhibition of casein-specific IgE by 60 min pepsin digested control casein (casein∗), pepsin control (pepsin∗), 60 min pepsin digested transglutaminase crosslinked casein (TG∗), 60 min pepsin digested tyrosinase crosslinked casein (Tr∗), 60 min pepsin digested tyrosinase/caffeic acid crosslinked casein (Aga∗) and 60 min pepsin digested laccase/caffeic acid crosslinked casein (Lacc∗).

with the partially digested AbTyr-treated CN that also exhibited slower digestion in SGF.

3.6 Complete biphasic digestion of crosslinked CNs

The complete 1 h digestion of CN and crosslinked CNs by pepsin was analyzed by Tris-Tricine PAGE and the results are shown in Fig. 5. All the crosslinked proteins were readily digested after 1 h proteolysis, and no discrete fragments were visualized by CBB staining or by immunological detection. In the case of AbTyr-treated CN, after 1 h proteolysis, a smear of peptide fragments was noticed in the MW range of 3–10 kDa (Fig. 5A, lane 12). None of these smeared protein fragments were detectable by immunoblotting (Fig. 5B). The intestinal digestion completed proteolysis in all analyzed samples.

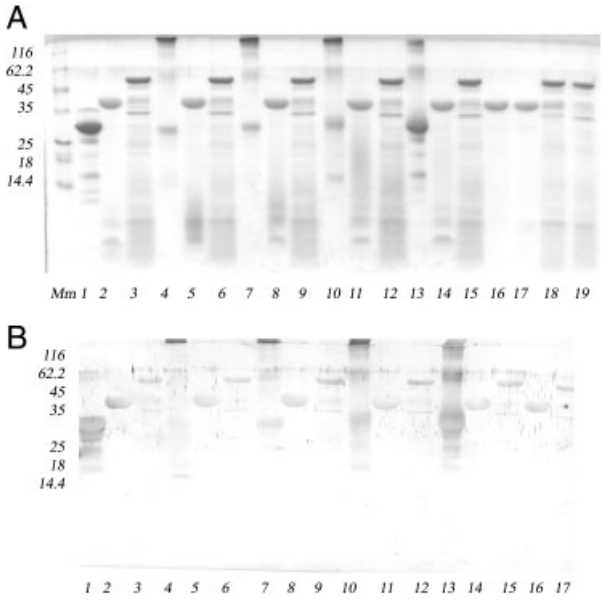

Figure 5. (A) SDS-PAGE of gastric and intestinal digestions of casein and crosslinked caseins. Lane 1: CN. Lane 2: SGF digested CN. Lane 3: SGF/SIF digested CN. Lane 4: TG. Lane 5: SGF digested TG. Lane 6: SGF/SIF digested TG. Lane 7: Tr. Lane 8: SGF digested Tr. Lane 9: SGF/SIF digested Tr. Lane 10: Aga. Lane 11: SGF digested Aga. Lane 12: SGF/SIF digested Aga. Lane 13: Lacc. Lane 14: SGF digested Lacc. Lane 15: SGF/SIF digested Lacc. Lane 16: pepsin. Lane 17: pepsin control. Lane 18: pankreatin. Lane 19: pancreatin control. Mm – molecular weight markers. (B) Immunoblot detection of pepsin fragments generated by 60 min digestion by rabbit polyclonal sera to whole milk proteins. Lane 1: CN. Lane 2: SGF digested CN. Lane 3: SGF/SIF digested CN. Lane 4: TG. Lane 5: SGF digested TG. Lane 6: SGF/SIF digested TG. Lane 7: Tr. Lane 8: SGF digested Tr. Lane 9: SGF/SIF digested Tr. Lane 10: Aga. Lane 11: SGF digested Aga. Lane 12: SGF/SIF digested Aga. Lane 13: Lacc. Lane 14: SGF digested Lacc. Lane 15: SGF/SIF digested Lacc. Lane 16: pepsin control. Lane 17: pancreatin control.

3.7 IgE-binding potential of digested crosslinked CNs after 1 h pepsin digestion in CAP inhibition

Pepsin-digested samples of CN and crosslinked β-CNs could still bind a small portion of CN-specific IgE in the CAP inhibition experiment (Fig. 4B). The IgE-binding potential of fragments generated by proteolysis of β-CN or enzymatically crosslinked β-CNs remained the same compared with control β-CN digested for 1 h. The apparent higher IgE-binding potential of the partially digested samples seen after 15 min of digestion reduced to the level of control CN after 1 h digestion by pepsin.

3.8 BA assay in seven CMA patients with CN and crosslinked β-CNs

To measure the ability of allergens to activate degranulation of the effector cells (basophils), the number of $CD63^+$ cells among $CD203^+$ cells (basophil specific marker) was taken into consideration. The magnitude of BA to anti-IgE antibody was taken as 100% activation, and obtained results were compared statistically. In all analyzed patients, only ThL-treated CN showed statistically significant reduction in the ability to activate basophils of allergic patients ($p<0.05$) (Fig. 6). AbTyr-treated CN showed a trend in reduction (median value for Aga 41.25 compared to 63.4 obtained for CN), although this difference was not statistically significant ($p=0.176$). When analyzing patients' response to serially diluted concentrations of allergen and crosslinked allergens, the response varied individually from patient to patient (Fig. 7). Patients differed in their fine specificity toward various allergens and in a highly reactive patient (patient 2), all analyzed allergens were able to activate basophils to a high extent even with the lowest concentration of allergen used for activation (0.05 μg/mL). A small reduction in the functional properties of laccase crosslinked β-CN were noticed in two out of three patients at all tested concentrations of allergens. In the same two patients, the basophil response to AbTyr-treated CN was comparable to the BA seen in the negative control (autoactivation of basophils), thus not inducing activation in those patients (Fig. 7). The non-atopic subjects did not react to any of the crosslinked CNs, control CN or anti-IgE.

4 Discussion

Crosslinking enzymes have become a very useful bioprocessing tool in the food industry. TrTyr is an enzyme favoring crosslinking of proteins rather than of low MW peptides [37]. Polymerization occurs through the formation of covalent bonds between two tyrosine side chains or between a tyrosine side chain and a free amino group [38, 39]. The molecular mass of β-CN, after crosslinking by TrTyr, is around 1500 kDa [40], thus corresponding to

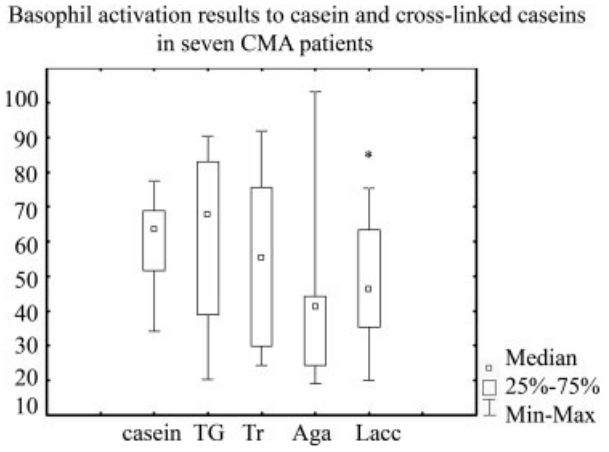

Figure 6. Basophil activation to casein and crosslinked caseins in seven patients with CMA. $p<0.05$ was statistically significant difference in a Wilcoxon matched pairs test.

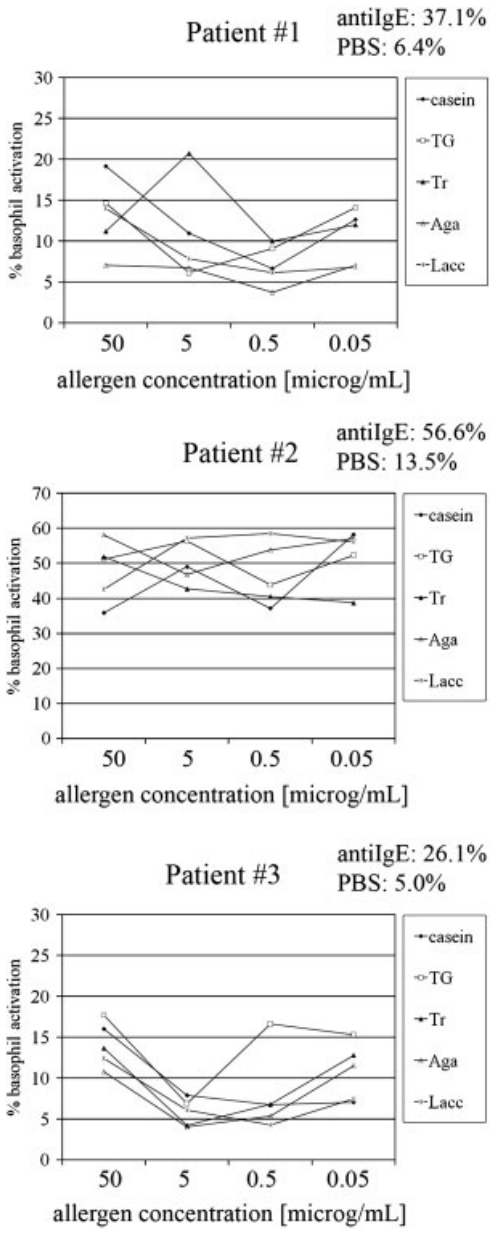

Figure 7. Basophil activation to different concentrations of casein and crosslinked caseins in three patients with CMA.

molecular mass observed by SDS-PAGE and to the major band noticed after agarose/PAA electrophoresis (Fig. 1B).

Crosslinking of peptides and proteins by AbTyr has been extensively studied during recent years [38, 41]. AbTyr has been found to be less efficient at crosslinking of proteins than TrTyr [37, 42]. Mattinen *et al.* (2008) have reported the inability of AbTyr to effectively crosslink β-CN. However, with the use of caffeic acid as a mediator, the resulting product is a polymer of high MW, with the mass range being almost equal to that of TrTyr crosslinked β-CN after agarose/PAA electrophoresis (Fig. 1C).

Tgase is a well-described enzyme. Tgase is known for its ability to crosslink proteins and CNs in particular [24, 38, 43]. Crosslinking of CN by Tgase occurs through formation of an isopeptide bond between the γ-carboxyamine of a glutamine side chain and a free amino group in the lysine side chain [44]. Polymerized β-CN after treatment with 100 nkat/g of the enzyme and 24 h incubation time is of a molecular mass in the range of 1100 kDa [40]. Under the conditions used in this study, the Tgase effectively polymerized β-CN, but the obtained products were of a broad mass range (Fig. 1B).

Laccase-catalyzed reactions are brought about through the formation of radicals in the hydroxyl group of the phenolic ring of tyrosine [45]. Laccase has low reactivity with proteins [46] and therefore, the presence of a small molecule acting as a mediator is needed. Molecules that are easily oxidized by laccase are usually used as mediators, producing radicals that are then capable of reacting with the substrate. For example, Færgemand *et al.* [47] have reported polymerization of α-lactalbumin and β-lactoglobulin by *Polyporus pinsitus* laccase in the presence of chlorogenic acid. In our study, caffeic acid was used as a mediator enhancing the crosslinking ability of laccase on β-CN, however, not achieving the complete polymerization of the monomeric β-CN.

Processing procedures may also modulate the allergenic properties of food. However, our lack of knowledge of the effects of procedures makes it difficult to predict or minimize the impact of processing on allergenicity of foods [10]. So far, data have been presented on the mitigating effects of peroxidase on the allergenic properties of peanut proteins [13]. The transglutaminase crosslinking has been reported to have no effect on IgE-binding properties of peanut allergens [14].

No study so far has compared the IgE-binding potential of the crosslinked products of different crosslinking enzymes on a well-defined model allergen, such as CN. In addition, data are lacking on the impact of crosslinking on the digestibility of crosslinked food proteins/allergens and the remaining IgE-binding potential of the pepsin-generated fragments.

In this study, we used four different enzyme systems to crosslink the major cow's milk allergen, β-CN. All these crosslinked products were compared for their *in vitro* IgE-binding potency in CAP inhibition assay and ELISA inhibition assay and for their ability to activate basophils in individual patients with well-defined CMA. The slope of the inhibition curves in ELISA inhibition assay differed among tested allergens, suggesting a change in the protein structure in the region of the epitope compared with the untreated CN. Highly polymerized CNs (crosslinked by Tgase and TrTyr) showed a higher potential to inhibit IgE binding in both tested patients than the untreated CN. Results proved the potential of some of the crosslinking enzymes in mitigating the allergenicity of CN, in particular the treatment with laccase and AbTyr together with caffeic

acid as mediator (Figs. 6 and 7), although the patients' response showed a strong individual variations in both *in vitro* and *ex vivo* tests applied to analyze the individual response. The difference is notable between two tyrosinases, TrTyr and AbTyr, both giving efficient polymerization of the tested protein (Fig. 1C), in the IgE-binding properties of the obtained crosslinked allergens. Possible explanation for the obtained results is that the effect of mediators is dual and lies in both the modification of the nucleophilic surface residues in the protein and the hindering of the epitopes in a highly aggregated product by covalent attachment of small phenolic compounds (caffeic acid in our study). On the other hand, the Tgase-treated CN showed no mitigated IgE-binding reactivity compared with the untreated CN. Our results are also in agreement with the mild effects shown for reduction of the allergenicity of the Tgase-treated peanut allergens [14] and more marked effects shown for tyrosinase/caffeic acid mitigated allergenicity of peanut allergens [17]. CN aggregated by disulfide bond formation is also frequently recognized by allergic patients in immunoblotting under non-reducing conditions [19] and the aggregated state of CN, as well as CN micelles, important in the process of allergic sensitization, possibly resembles the amorphous structure of the crosslinked CN. Aggregation of CN monomers reveals new determinants that seem to be absent in the monomeric forms, since some patients' sera show higher IgE reactivity only against these aggregates but not against their individual components [19]. ELISA inhibition results suggest the same phenomenon. The crosslinking treatment with laccase was not very efficient, leaving mostly monomeric CN modified by caffeic acid. Reduced inhibition potential is possibly due to hindering of epitopes by monomer modification. In contrast, high-efficiency crosslinking has, as a consequence, an increased inhibition of IgE binding to the monomeric CN in both tested patients. Another explanation could be that the high local concentration of antigen determinants on a crosslinked molecule provides antibodies with low affinity binding.

The IgE-binding ability is less affected by enzymatic processing than by the ability of the crosslinked β-CNs to activate charged basophils of allergic patients, whereby AbTyr with caffeic acid-treated β-CN appeared less allergenic than the control β-CN (Figs. 6 and 7) and laccase-treated CN showed a significant mitigation of *ex vivo* allergenicity in seven tested patients (Fig. 6). It should also be noted that in a non-atopic subject, the reactivity to control β-CN was equal to the reactivity to the crosslinked CNs, *i.e.* the allergenicity was not enhanced in non-atopic individuals.

Kinetics of pepsin digestion of crosslinked CNs showed a transient phase, immediately following pepsin addition, whereby fragments of a certain size were noticed. Compared with native readily digestible allergen, the digestion of the crosslinked proteins was found to proceed slower, followed by a higher retention of the IgE binding ability. However, in completely digested samples, after 1 h exposure to the simulated gastric conditions, all proteins were fully digested and residual fragments were too small to bind a high portion of the CN-specific IgE in CAP inhibition.

Crosslinking effects on the uptake of proteins in the lower part of the intestine remain to be elucidated. The aggregated form of allergens appears to be more ''allergy'' promoting, as it is taken preferentially by Payer's patches that are able to Th2-drive immune system of the gut [48]. On the contrary, soluble proteins are transcytosed *via* mucosal surfaces and promote Th1 response.

In conclusion, the laccase and AbTyr with caffeic acid as mediator have the highest potential in mitigating the IgE binding and allergenicity of the β-CN of all analyzed enzymes. The effects of crosslinking, especially isopeptide bonds formed by transglutaminase action, are seemingly less able to modify the IgE binding epitopes of the crosslinked CNs than the presence of small phenolic mediators in the crosslinking process. At the same time, the kinetics of AbTyr and ThL crosslinked CNs digestion by pepsin were hampered, making it difficult to predict the effects of this processing on the sensitization potential of the crosslinked food products. The efficacy of processing in reduction of food allergenicity also needs to be demonstrated in allergic individuals and tested in an animal model of food allergy.

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