



Digestibility and allergenicity of β -lactoglobulin following laccase-mediated cross-linking in the presence of sour cherry phenolics

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ABSTRACT

β -Lactoglobulin (BLG) is an important nutrient of dairy products, but it represents a serious health risk in patients allergic to milk. Sour cherry (*Prunus cerasus* L.) extract (SCE) is frequently added as a natural food colour in composite foods, such as fruit yogurt, ice creams, frappés and milkshakes. The aim of this study was to investigate the potential of laccase to cross-link BLG in the presence of an SCE and to characterise the obtained products for their bioactivity.

Laccase cross-linked BLG in the presence of sour cherry phenolics. In a basophil-activation assay, the allergenicity of the cross-linked protein was shown to decrease in all nine cow's milk-allergic patients, while digestibility of the remaining monomeric BLG in simulated conditions of the gastrointestinal tract increased. Tryptic peptides became immediately available in BLG treated by laccase and laccase/SCE. The hydrolysates obtained by trypsin digestion of BLG/laccase/SCE showed an increase of 57% in radical-scavenging activity, compared to the control BLG.

Enzymatic processing and usage of natural phenolic extracts as mediators of enzymatic reaction may improve BLG safety and the availability of peptides following digestion, while conserving its bioactivity.

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1. Introduction

Enzyme usage for improving food functionality and texture is nowadays a good replacement for chemical additives that are to be avoided as much as possible. Cross-linking enzymes, like transglutaminases that make isopeptidyl bonds or oxidases that make dityrosinyl bonds may improve the texture of skimmed-milk products (Buchert et al., 2007). Oxidases like tyrosinase and laccase act on phenolic compounds and can facilitate cross-linking of proteins (Selinheimo, Autio, Kruus, & Buchert, 2007). The cross-linking of tyrosine-containing peptides by laccase has been shown to proceed via tyrosyl radicals that form primarily isodityrosine and also a small number of dityrosine bonds (Koschorreck et al., 2008). The formation of disulphide bonds by oxidation of cysteines into cystine is another way of cross-linking proteins, which laccase is capable of inducing in flour dough using ferulic acid as a mediator (Selinheimo et al., 2007).

Enzymatic cross-linking of food proteins, besides improving food texture, can beneficially affect their safety (i.e., allergenicity) and influence digestibility by digestive enzymes. Reduction of allergenicity was shown for laccase/cafeic acid and tyrosinase/cafeic acid cross-linked β -casein (Stanic et al., 2010), polyphenol oxidase cross-linked peanut proteins (Chung, Kato, & Champagne, 2005) and tyrosinase exposed major cherry allergen in the presence of various polyphenols (Gruber, Vieths, Wangorsch, Nerkamp, & Hofmann, 2004). Thus, the usage of enzymes has been anticipated as a novel approach to making hypoallergenic foods (Mills & Mackie, 2008).

β -Lactoglobulin (BLG) accounts for approximately 10–15% of total milk proteins (Kontopidis, Holt, & Sawyer, 2004). Besides being an important nutrient of dairy products, it also represents a serious health risk in cow's milk-allergic (CMA) patients as it is known as one of the major milk allergens (Shek, Bardina, Castro, Sampson, & Beyer, 2005). Various bioactive peptides are hidden in BLG, and once liberated by proteolysis, bioactive peptides originating from BLG exert antioxidative properties (Liu, Chen, & Mao, 2007), antimicrobial activity (Pihlanto-Leppala et al., 1999), antihypertensive activity and antiproliferative action in various cell lines (Hernandez-Ledesma, Davalos, Bartolome, & Amigo, 2005).

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Previous studies have shown that BLG is not a good substrate for laccase, presumably by not having adequately exposed phenolic groups of tyrosine at the surface (Yasunobu & Dandliker, 1957), thus requiring phenolic mediators for the formation of intermolecular bonds by laccase (Mattinen et al., 2005). Sour cherries (*P. cerasus* L.) contain significant levels of anthocyanins possessing strong antioxidant, chemopreventive and anti-inflammatory activities (Hou, Fujii, Terahara, & Yoshimoto, 2004).

In this study we investigated the cross-linking potential of laccase on purified BLG in the presence of a sour cherry extract (SCE) as a source of phenolic mediators, and the impact of enzymatic action in the presence of sour cherry phenolic mediators on the allergenicity and digestibility of cross-linked BLG, as well as the bioactivity of peptides released from cross-linked BLG by proteolysis.

The results of the study showed the potential of enzymatic processing in making new and safer foods with improved functional properties.

2. Material and methods

2.1. Chemicals

Pepsin (from porcine stomach mucosa, 2650 U/mg solid), Folin–Ciocalteu's phenol reagent, gallic acid and ABTS (2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid), were purchased from Sigma–Aldrich (Taufkirchen, Germany). Anti-rabbit IgG labelled with alkaline phosphatase was also from Sigma. Urea and disodium hydrogen phosphate (analysis grade) were purchased from Merck (Darmstadt, Germany). Deionised water (DW) used in the experiments was purified in a Milli-Q system (Millipore, Molsheim, France).

2.2. Purification of β -lactoglobulin and determination of protein

BLG was purified from raw milk as described previously (Stanic et al., 2009). Protein purity was assessed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Concentrations of BLG were determined by spectrophotometry at 280 nm ($\epsilon = 0.943 \text{ mg}^{-1} \text{ ml cm}^{-1}$).

2.3. Preparation of anti-BLG rabbit antibodies

The antibodies against pure BLG were raised in rabbits according to Harboe and Ingild (1973). Antibodies were partially purified by ammonium sulphate precipitation. High titre partly purified antibodies were used in immunoblot experiments.

2.4. Preparation and characterisation of fruit phenolics

Thirty grams of frozen sour cherries (cultivar Sumadinka) were homogenised using a Polytron homogeniser (PT 10/35, Kinematica, Kriens-Luzern, Switzerland) with 50 ml of 95% ethanol and filtered under vacuum through one layer of Whatman No. 1 filter paper. The ethanol extract was centrifuged for 10 min at 12,000g and supernatant was concentrated ten times by centrifugation under vacuum at 30 °C in an evaporator (Eppendorf, Hamburg, Germany). The extract was further deproteinised by vigorous vortexing with ethanol (10 ml ethanol with 3 ml of extract). Denatured proteins were removed by centrifugation at 12,000g for 20 min. By ultrafiltration of the extract (cut off 3 kDa) ethanol was exchanged with DW and traces of remaining proteins were removed. The obtained deproteinised water extract of sour cherry phenolics (SCE) was concentrated by centrifugation under vacuum and total phenolics were estimated.

Total phenolics of frozen sour cherries were measured by spectrophotometric analysis using Folin–Ciocalteu's reagent. Total phenolics were expressed as mg gallic acid equivalents (GAE)/100 ml of cherry extract.

2.5. Electrophoresis and isoelectrofocusing

SDS–PAGE was carried out using a Hoefer Scientific Instruments apparatus with a discontinuous buffer system. Protein components were resolved on 12% or 6% polyacrylamide gels, which were stained using Coomassie Brilliant Blue R-250 (Sigma–Aldrich). Reducing conditions were achieved with 5% β -mercaptoethanol (Sigma–Aldrich). Chicken myofibril proteins were prepared as previously described (Warren, Krzesinski, & Greaser, 2003) and used as high molecular weight marker proteins for 6% SDS–PAGE.

Isoelectrofocusing was performed in 7% polyacrylamide slab gel in the presence of 2% Amfolines (Pharmacia, Uppsala, Sweden), pH 3.5–10, in a Multiphor electrophoretic system (Pharmacia) at 200 V for 2 h. Each sample was applied in 12.5 μg of proteins per well. The pH gradient was determined by pH measurement by cutting the gel into strips and incubating in 10 mM KCl for 30 min.

Tris–Tricine 14% PAG electrophoresis for analysis of trypsin digests was performed according to Coligan, Dunn, Ploegh, Speicher, and Wingfield (1995).

2.6. Immunoblotting

To examine specific rabbit IgG anti-BLG binding, serum was 30,000 times diluted in TTBS, containing 0.1% of bovine serum albumin (BSA). The nitrocellulose membranes were first blocked with 1% BSA in TTBS, before being probed with primary antibodies. The bound IgG was detected using anti-rabbit IgG antibodies (Sigma–Aldrich), labelled with alkaline phosphatase, diluted in TTBS–0.1% BSA in appropriate dilutions (as recommended by the manufacturer). The binding patterns were visualised with a substrate solution consisting of 1.5 mg BCIP (5-bromo-4-chloro-3-indolyl phosphate; Sigma–Aldrich) and 3 mg NBT (nitroblue tetrazolium; Sigma–Aldrich) in 10 ml of 100 mM Tris buffer, containing 150 mM NaCl, and 5 mM MgCl_2 , pH 9.6.

2.7. Cross-linking of BLG by laccase in the presence of cherry fruit phenolics

Laccase (30.6 U/mg of solid) of fungal origin (*Trametes versicolor*) was supplied by Fluka BioChemika (Sigma–Aldrich). Laccase activity was determined using ABTS as a substrate (Selinheimo et al., 2007). Laccase-treated samples contained 0.8 mg/ml BLG, cherry extract at phenol concentrations of 0.06 mg/ml of GAE up to 0.9 mg/ml and 30.95 U/ml laccase in 40 mM acetate buffer, pH 4.5; in control samples, laccase and the mediator were omitted. Samples were incubated for 2 h at 37 °C with shaking and the reaction was stopped by adding 1 mM EDTA and freezing the sample immediately.

For further analysis, cross-linked BLG was prepared using SCE (0.24 mg/ml GAE).

2.8. Size-exclusion and reverse-phase chromatography

Size-exclusion chromatography (SEC) was performed on an ÄKTA purifier 10 HPLC system using a Superdex 75 PC 3.2/30 (3.2 mm $\times$ 300 mm) column (Amersham Biosciences). The components were eluted with 50 mM Tris buffer, pH 8.2, containing 0.2 M NaCl and 1 mM EDTA, at 0.05 ml/min, and monitored at 215 nm and 280 nm. Reverse-phase HPLC (RP–HPLC) was performed on a Discovery BIO Wide Pore C5 (10 cm $\times$ 4.6 cm) column (Supelco, Sigma–Aldrich). For elution, 0.1% TFA (trifluoroacetic acid) and

0.1% TFA in 90% acetonitrile were used at 0.75 ml/min in a linear gradient, with the second solvent rising from 0 to 100% over 120 min. The eluate was monitored at 280 and 215 nm.

2.9. Mass spectrometry of BLG

For mass spectrometric analysis, BLG fractions from RP-HPLC were evaporated by centrifugation under vacuum at 30 °C in an Eppendorf evaporator and resolved in 0.1% formic acid in 50% acetonitrile at 2.5 mg/ml of analysed protein.

Protein mass measurements were carried out on a mass spectrometry (MS) system consisting of 6210 time-of-flight liquid chromatograph/MS (G1969A, Agilent Technologies, Santa Clara, CA), and samples dissolved in the mobile phase were introduced via a 1200 Series HPLC system (Agilent Technologies). Agilent MassHunter Workstation Software was used for data acquisition, and Agilent MassHunter Workstation Software and Analyst QS were used for data processing. The mobile phase was 50:50 mixture of solvent A (0.2% formic acid in water) and solvent B (acetonitrile) at a flow rate of 0.2 ml/min. The mass spectrometer was run in the positive electron spray ionisation (ESI) mode over a mass range of m/z 100–3200.

2.10. Basophil-activation assay in patients with CMA

Basophil-activation assay was performed as described previously (Stanic et al., 2010). Briefly, heparinised blood samples were taken from nine cow's milk-allergic patients. Patients were selected on the basis of positive skin prick testing to cow's milk, a documented clinical history of cow's milk allergy and positive *in vitro* testing to cow's milk: immunoglobulin E (IgE) levels were assessed in milk proteins with ImmunoCAP® 100 system using the ImmunoCAP® code f2 (Pharmacia Diagnostics, Uppsala, Sweden) above 0.35 kAU/L.

Blood aliquots (100 µl) were incubated with dilutions of BLG and cross-linked BLG antigens (50 µg/ml), with anti-IgE antibody as a positive control (1 µg/ml), or PBS as a negative control for 15 min (37 °C). After incubation, cells were washed in PBS and then incubated with 10 µl of phycoerythrin-labelled CD203c monoclonal antibody 97A6 (Immunotech, Marseille, France) and FITC-labelled CD63 monoclonal antibody (Immunotech) for 15 min at room temperature. Thereafter samples were subjected to erythrocyte lysis with 2 ml of FACS TM lysing solution (BD Biosciences). Cells were then washed, resuspended in PBS, and analysed by flow cytometry, using a FACSCalibur (BD Biosciences, Franklin Lakes, NJ). The basophil marker CD203c was used to set a basophil gate including only CD203c+ cells. The collected cells were further analysed for granule release using an anti-CD63 antibody. The magnitude of basophil activation (BA) was calculated as the percentage of CD63+ events among the gated basophils (CD63+/CD203c+ cells). The results were expressed as:

(magnitude of BA of the sample/magnitude of BA of the positive control(anti-IgE)) × 100.

In addition, upregulation of CD203c was analysed in each sample, using the mean fluorescence intensity.

Statistical comparisons were done using the Wilcoxon matched pairs test and the Statistica 9.0 program.

2.11. Simulation of gastric fluid digestion

In vitro gastric fluid digestion of BLG was performed as described previously (Polovic et al., 2009). Briefly, 50 µl of BLG control and laccase-treated BLG (0.8 mg/ml) with or without SCE solutions (0.24 mg/ml GAE) were diluted with 10 µl of DW,

warmed to 37 °C, and 20 µl of prewarmed 0.4 M HCl containing 8 g/l of NaCl and 12.8 g/l of pepsin A (Sigma–Aldrich) were added. The mass ratio of protein: pepsin was 1:6.5. Digestion proceeded at 37 °C, pH 1.2, with continuous shaking; an aliquot (16 µl) of the digest was periodically withdrawn (at 15 and 30 min and 1, 2 and 3 h). The control samples (0 min) were prepared in the same way, without addition of the pepsin. The digestion was stopped with 5 µl of 0.2 M Na₂CO₃, and samples were mixed with 5 µl of five times concentrated sample buffer for SDS–PAGE analysis. Thirteen microlitres of each sample were applied per lane (5 µg of BLG, laccase-treated BLG or laccase/SCE-treated BLG per well). A pepsin control was set up in the same manner but with no addition of BLG. BLG, laccase-treated BLG and laccase/SCE-treated BLG controls were prepared with addition of deionised water instead of the pepsin solution.

2.12. Trypsin digestion of BLG and cross-linked BLG

BLG, BLG incubated with SCE and BLG/SCE exposed to laccase were dissolved in 20 mM NH₄HCO₃ buffer, pH 8.0, up to a concentration of 5 mg/ml. By ultrafiltration through a 10 kDa Millipore membrane (Millipore, Carrigtwohill, Co Cork, Ireland), the remaining phenols were removed. Trypsin (sequencing grade, Merck) was added in 1:100 mass ratio to proteins in the reaction mixture. The reaction took place at 37 °C with continuous shaking. Aliquots were withdrawn from the reaction mixture at time points of 1 min up to 20 h; the reaction was immediately quenched by heating to 95 °C for 5 min and freezing the samples immediately thereafter. The samples for Tris–Tricine PAGE were prepared in a reducing electrophoresis buffer by boiling for 5 min at 95 °C; 10 µg of protein hydrolysates were applied per lane.

Trypsin-digested samples were analysed for immunoreactivity with anti-BLG antibodies, as described above. Control samples and 60 min trypsin-digested samples were resolved by 12% SDS–PAGE and transferred to the membrane. An electroblotter system (Serva) was used for transfer to nitrocellulose membrane and checked by staining the proteins with 0.5% Ponceau S (Sigma–Aldrich) for 15 min. The membrane was probed as described in Section 2.6.

2.13. ABTS+ radical-scavenging activity determination of peptides obtained by trypsin digestion

The antioxidative properties of trypsin-generated BLG and BLG/SCE peptides were assayed by the ABTS method. After 20 h hydrolysis by trypsin, under conditions described above, Chelex 100 beads (Sigma–Aldrich) were added to the hydrolysis mixtures. Traces of metal ions were removed according to the manufacturer's recommendation.

The procedure followed the method of Re et al. (1999) with some modifications. ABTS+ solution (7.4 mM) and 2.6 mM potassium persulfate solution were mixed in equal quantities and allowed to react for 12 h at room temperature in the dark. The dilution of the ABTS+ solution was set up with DW to obtain an absorbance of 0.7 units at 734 nm for the uninhibited sample, under the test conditions. A fresh ABTS+ solution was prepared for each assay. Trypsin hydrolysates (100 µl) were allowed to react with 100 µl of the ABTS+ solution for 5 min in the dark, after which absorbance was measured at 734 nm (A_{sample}). The assay was performed in triplicate. The absorbance of the buffer reacting with ABTS+ solution for 5 min (A_{blank}) was set up as 0% inhibition. The percentage inhibition of the sample of absorbance at 734 nm was calculated as: $100 - ((A_{\text{sample}}/A_{\text{blank}}) \times 100)$. Ascorbic acid was used as the standard antioxidant in the assay. Ascorbic acid equivalents per mg of sample protein tested were taken into account for comparison of the radical-scavenging activity of tryptic peptides.

3. Results

3.1. Cross-linking of β -LG by laccase in the presence of sour cherry phenolics and characterisation of the obtained products

BLG in the presence of sour cherry phenolics is efficiently cross-linked by laccase (Fig. 1), and cross-linking increases with the increase of phenolics concentration up to 0.3 mg/ml GAE. For higher concentrations of mediators, cross-linking of BLG was inhibited, as mediators present in the reaction mixture preferably formed poly-phenol compounds.

The obtained protein cross-links were observed in both reducing and non-reducing conditions (Fig. 2A). The adducts obtained were covalently cross-linked, predominantly by non-disulphide linkages; higher molecular weight proteins were observed in reducing SDS–PAGE analysis, as well. Detection of BLG in the cross-links was also observed by immunodetection by anti-BLG antibodies. Smears of cross-linked BLG, having masses starting from 50 kDa (corresponding to BLG trimer) were observed in immunoblotting (Fig. 2B). Highly cross-linked BLG exhibited an apparent molecular mass of above 400 kDa in 6% SDS–PAGE (Fig. 2D). No significant shift was observed in the pI value of laccase/SCE-treated BLG, while a portion of the cross-linked proteins remained at the application spot (Fig. 2C).

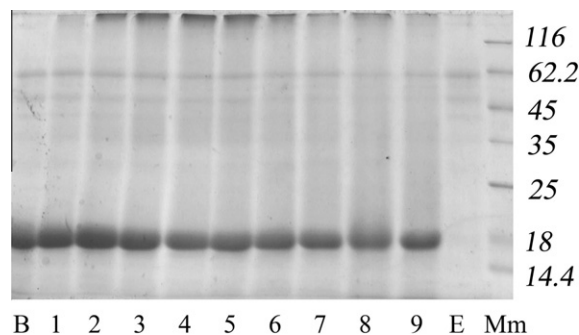


Fig. 1. Cross-linking of BLG by laccase in the presence of different concentrations of SCE analysed by 12% SDS PAGE. B – BLG and laccase only. Lanes 1–9: BLG/laccase and various concentrations of SCE; 1: 0.06 mg ml⁻¹ SCE, 2: 0.12 mg ml⁻¹ SCE, 3: 0.18 mg ml⁻¹ SCE, 4: 0.24 mg ml⁻¹ SCE, 5: 0.3 mg ml⁻¹ SCE, 6: 0.45 mg ml⁻¹ SCE, 7: 0.6 mg ml⁻¹ SCE, 8: 0.75 mg ml⁻¹ SCE, 9: 0.9 mg ml⁻¹ SCE. E – laccase. M – molecular weight marker proteins.

3.2. Characterisation of the remaining monomeric BLG

Considering that a significant fraction of BLG remained in monomeric form, we wanted to examine if it was modified by phenolics. Thus, monomeric BLG was isolated from all preparations

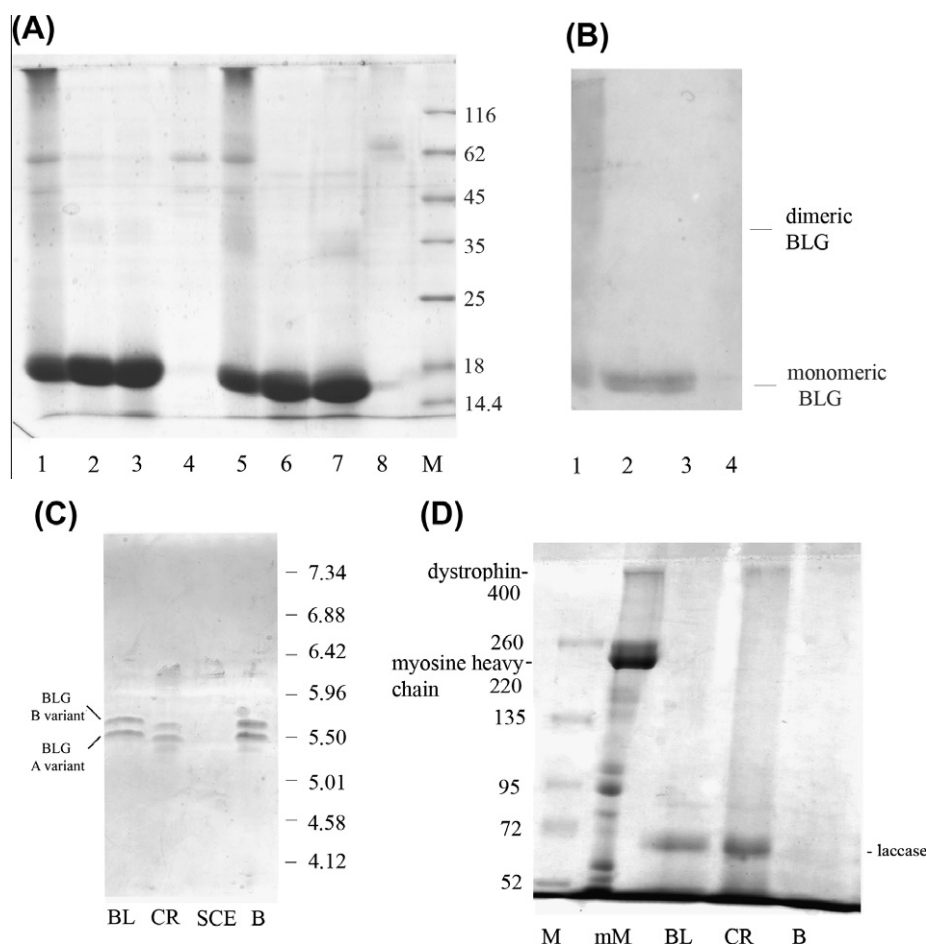


Fig. 2. (A) Laccase mediated cross-linking of BLG in both reducing and non-reducing conditions, analysed by 12% SDS PAGE. Samples: 1 – BLG/SCE laccase, 2 – BLG/laccase, 3 – BLG/SCE, 4 – laccase, in reducing conditions, 5 – BLG/SCE laccase, 6 – BLG/laccase, 7 – BLG/SCE, 8 – laccase, in non-reducing conditions. Proteins (12 μ g) were applied per lane. (B) Detection of BLG and cross-linked BLG by anti-BLG rabbit antibodies in immunoblotting. Samples: 1 – BLG/SCE/laccase, 2 – BLG/laccase, 3 – BLG/SCE, 4 – laccase, in reducing conditions. (C) Isoelectrofocusing of untreated and laccase-treated BLG; BL-laccase-treated BLG, CR-laccase-treated BLG with sour cherry extract, B-untreated BLG, SCE-sour cherry extract. Proteins (12.5 μ g) were applied per lane. (D) Laccase mediated cross-linking of BLG in analysed by 6% SDS PAGE under reducing conditions. BL-laccase-treated BLG, CR-laccase-treated BLG with SCE, B-untreated BLG; 32 μ g of proteins were applied per lane. M – molecular weight marker proteins. mM – chicken myofibril proteins.

Table 1
ESI-MS analysis of the BLG treated with laccase with or without the presence of SCE.

BLG/SCE	BLG/laccase	BLG/laccase/SCE
18363.69 ^a	17809.11 ^{a,*}	17808.36 ^{a,*}
18277.59 ^b	17722.88 ^{b,*}	17722.34 ^{b,*}
		18074.71 [#]
		17988.74 [#]

^a BLG variant A.

^b BLG variant B.

* Truncated form of BLG (Stanic et al., 2009).

[#] Minor component.

(treated with laccase with or without phenolics and incubated with SCE only, as a control) by a combination of SEC and RP-HPLC. The retention times of the remaining monomeric BLG in both SEC and RP-HPLC did not differ significantly from the control BLG (results not shown). A negligible shift was observed in the spectral characteristics of purified BLG, suggesting that a modification took place in the presence of laccase and phenolics only in a minor fraction of BLG. Both control and laccase/SCE-treated BLG were analysed by MS.

BLG treated with laccase (with or without phenolics) was truncated for a pentapeptide (MW of 554) from the N-terminus of both variants, because of the action of contaminating protease (Stanic et al., 2009). However, in the sample treated with laccase in the presence of phenolics a small portion of BLG appeared conjugated to a compound of sour cherries, exhibiting an MW increase of 266.4 for both variants (Table 1). The same MW molecular species were not observed in any of the control BLG preparations (treated by laccase or phenolics in separate experiments), implying that a minor fraction of BLG was intermolecularly modified by sour cherry phenolics.

3.3. *In vitro* digestibility of BLG treated with laccase/SCE

BLG is known as a protein very resistant to *in vitro* conditions of gastrointestinal digestion. However, it is a source of a variety of potent bioactive peptides. Therefore, we wanted to explore the effect of laccase and cross-linking in the presence of SCE on the digestibility pattern of BLG in simulated conditions of the gut (Fig. 3).

Pepsin digestion of remaining monomeric BLG in a preparation of BLG treated by laccase in the presence of sour cherry phenolics proceeds more rapidly than proteolysis of control BLG or BLG exposed to laccase. Untreated BLG is a quite a stable protein, and remains resistant to pepsin digestion for the time period of 3 h, while in the presence of pepsin, laccase-exposed BLG becomes more susceptible to digestion. In contrast to untreated and laccase-exposed BLG, most laccase/SCE-treated BLG was digested after 15 min and almost completely digested after 60 min (Fig. 3A). As proposed in our previous work, the presence of contaminating protease can also facilitate digestion of BLG in acidic conditions (Stanic et al., 2009).

3.4. Trypsin digestion of BLG and BLG/SCE

Further, we analysed the effect of laccase cross-linking in the presence of SCE on BLG digestibility by trypsin, the main intestinal protease. The reaction mixture was digested by trypsin for 1 min up to 20 h. The results obtained are shown in Fig. 3B. A notable difference in digestion of laccase-treated BLG can be noted. Within 1 min, BLG exposed to laccase, with or without SCE, started being degraded by trypsin. Control BLG (exposed to SCE, without addition of the laccase) remained stable to digestion for up to 20 h. The mixture of highly cross-linked BLG and monomeric BLG started degrading from the first minute of exposure to trypsin, being

mostly degraded within 30 min. A small fraction of cross-linked BLG remained undigested up to 20 h, and these trace amounts of proteins were not recognised by anti-BLG antibodies (Fig. 3C).

3.5. Antioxidative determination of tryptic peptides of BLG and BLG/SCE

As protein modification by phenolics may increase its free radical-scavenging potential we determined the antioxidative activity of peptides liberated by trypsin digestion. Antioxidative activity determination of peptides liberated by 20-h trypsin digestion showed that BLG/SCE laccase-treated sample digestion resulted in peptides having a higher radical-scavenging activity in the ABTS assay compared to control BLG exposed to laccase only and BLG incubated with SCE only (Fig. 4). Differences reached up to 57%, compared to laccase-treated BLG (Fig. 4), when calculating percent inhibition for 150 µg/ml concentration of initial protein digested.

3.6. Basophil activation capacity of laccase/SCE-treated BLG

To measure the ability of allergens to activate degranulation of effector cells (basophils) *ex vivo*, the magnitude of CD63+ cells among CD203+ cells (constitutively expressed basophil specific marker) in blood of CMA patients was taken into consideration. The magnitude of basophil activation to anti-IgE antibody was taken as a positive control, while spontaneous degranulation in the presence of medium only, was considered as a negative control. The magnitude of 10% activation was considered to be a positive reaction to the allergen/cross-linked allergen. In all nine tested patients, the reactivity to BLG/SCE was significantly reduced compared to intact BLG ($p < 0.05$) (Fig. 5A). In all tested patients, BLG was able to induce activation and degranulation of basophils ($p < 0.05$), while BLG/SCE degranulated and activated basophils only induced activation in one out of 9 tested patients. The marker of basophil activation (CD203) showed upregulation to the presence of BLG ($p < 0.05$), but also to BLG/SCE (Fig. 5B) ($p < 0.05$). Upregulation of the CD203c marker was significantly reduced to BLG/SCE, compared to the intact BLG in the nine patients tested ($p < 0.05$; Fig. 5B).

4. Discussion

In this work, we have shown that phenolic compounds present in SCE can mediate cross-linking of BLG by laccase. Besides being rich in phenolic compounds of high antioxidative and radical-scavenging activity, SCE as a possible mediator of the cross-linking reaction was also chosen as it is a rich source of anthocyanins, natural food colours of growing importance for food industry (Delgado-Vargas, Jimenez, & Paredes-Lopez, 2000; Giusti & Wrolstad, 2003).

When BLG was treated with laccase in the presence of SCE, the protein bands showed a smear towards higher masses. Phenolic compounds are natural substrates of laccase. Tyrosines were therefore expected to be the prime targets in proteins, resulting in the formation of tyrosyl radicals that can lead to inter- or intra-molecular protein cross-links through dityrosine bonds. The results of this study showed that the majority of cross-linked BLG was formed by non-disulphide covalent linkages.

Although efficient in creating polymerised products of BLG, the cross-linking reaction was inhibited in the presence of a higher amount of SCE. The products of laccase action on BLG in the presence of SCE were aggregated, cross-linked proteins and a monomeric BLG, which did not change its pI value and retention time in SEC and RP-HPLC compared to the control BLG (results not shown). The obtained mass spectra of the BLG isolated from the

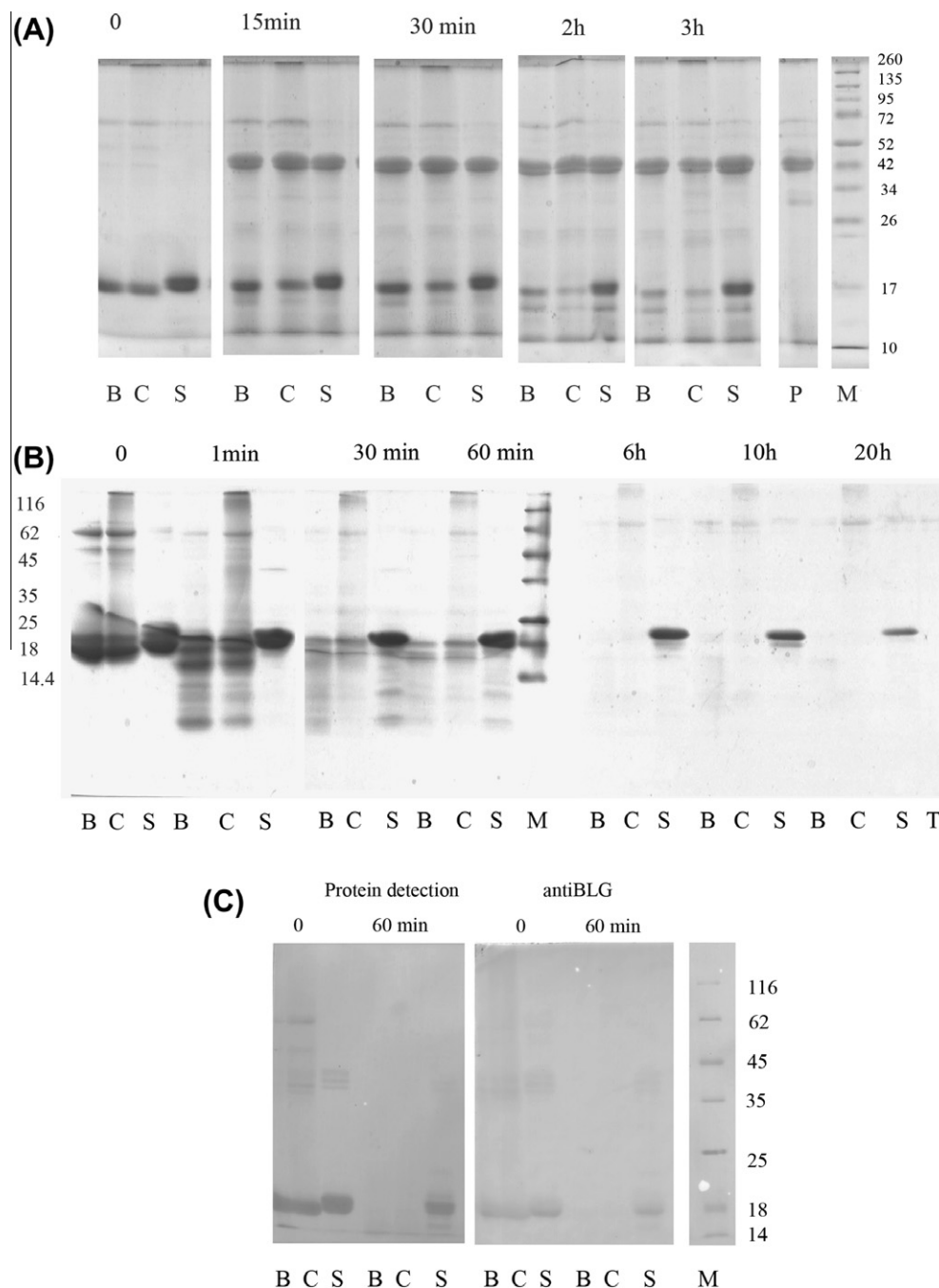


Fig. 3. (A) Digestion of BLG, BLG exposed to laccase and BLG exposed to laccase in the presence of SCE in simulated gastric fluid analysed by reducing SDS–PAGE. Digestion lasted from 15 min to 3 h. 0 – Control samples prepared without addition of pepsin. P-pepsin control incubated in the reaction mixture; 5 μ g of protein were applied per lane. (B) Trypsin digestion of BLG and BLG cross-linked in the presence of SCE, analysed by 14% Tris–Tricine PAGE. B – BLG treated with laccase, C – BLG treated with laccase in the presence of SCE. S – BLG incubated with SCE. T – trypsin incubated in the reaction mixture; 10 μ g of protein were applied per lane. (C) Immunodetection of trypsin digested BLG and BLG cross-linked in the presence of SCE. Proteins were analysed by 12% SDS PAGE. B – BLG treated with laccase, C – BLG treated with laccase in the presence of SCE. S – BLG incubated with SCE. M – Molecular weight marker proteins. Proteins were detected with Ponceau S staining (left), BLG was probed with anti-BLG rabbit antibodies (right).

reaction mixture with laccase/SCE did not suggest a high extent of covalent linkage of any phenolics to the monomeric protein. As previously described, truncation of BLG by a trace contaminant in commercial laccase preparation was also observed (Stanic et al., 2009) and occurs regardless of the presence of SCE in the reaction mixture.

Very complex linkages generated in the presence of phenolic mediators and by enzyme action may have an influence on structural properties, digestibility and biological activity of BLG and peptides liberated from BLG.

The digestibility by pepsin in simulated gastric fluid was also influenced, as only trace amounts of intact BLG remained in the digestion mixture after the exposure of laccase-treated BLG/SCE to pepsin for up to 2 h. Pepsin digestion of a BLG/SCE laccase-treated sample proceeded even more rapidly than laccase-only treated BLG. However, traces of high molecular weight cross-linked proteins also remained undigested after pepsin digestion, although monomeric BLG was almost completely digested under the conditions applied in this study. A dual mode of BLG decay was observed. Monomeric BLG digests more rapidly, while a small

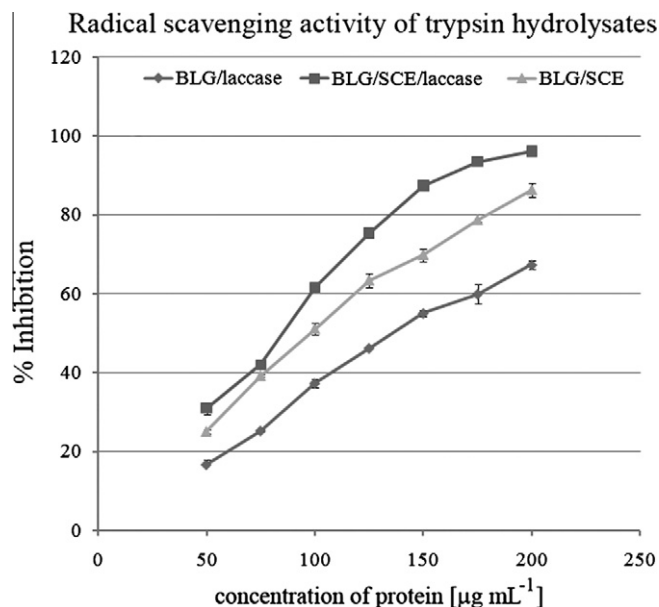


Fig. 4. Radical-scavenging activity of trypsin digests of BLG, BLG preincubated with SCE and BLG cross-linked by laccase, mediated by SCE.

portion of cross-linked proteins remained undigested, even after prolonged exposure to pepsin.

BLG is less prone to digestion by pepsin, due to its very compact structure, but it is labile to digestion by pancreatic enzymes (Fu, Abbott, & Hatzos, 2002). Once hydrolysed, it liberates sets of peptides with various bioactive roles, such as antibactericidal, antioxidative and antiproliferative. Proteases of different specificity have been explored for generation of different sets of bioactive peptides (Hernandez-Ledesma et al., 2005).

Peptides generated by trypsin digestion of laccase-treated BLG/SCE possess increased antioxidative properties, compared to control BLG, which could be due to the incorporation of SCE polyphenols into cross-linked BLG. In addition, treatment of BLG with laccase/SCE influenced its digestion by trypsin, leading to facilitated liberation of tryptic peptides.

In a recent study, four different cross-linking enzymes were examined for the effects of enzymatic cross-linking on IgE-binding, allergenicity and digestion stability of β -casein (Stanic et al., 2010). β -Casein was cross-linked by transglutaminase, tyrosinase, mushroom tyrosinase/cafeic acid and laccase/cafeic acid. The presence of caffeic acid (mediator) hampered digestion by pepsin, and this effect was most pronounced for tyrosinase/cafeic acid cross-linked casein. Laccase/cafeic acid and mushroom tyrosinase/cafeic acid had the highest potential in mitigating IgE-binding and allergenicity of β -casein out of all investigated enzymes. The presence of a small phenolic compound as a mediator of the cross-linking reaction also increased the digestion stability of β -casein.

The action of laccase/SCE on BLG influenced its allergenicity in a similar manner. The ability of the cross-linked protein to degranulate basophils (monitored by upregulation of the CD63 marker) was markedly reduced in BA in all tested patients. BLG/SCE failed to degranulate basophils in all tested patients. The upregulation of CD63 and CD203c in response to specific activators and inhibitors appears to follow different kinetics and seems to be directed through alternative signal transduction pathways (Hennersdorf et al., 2005) with CD63 more related to anaphylactic degranulation of basophils. A constitutively expressed marker of basophil activation, CD203c, was upregulated to BLG/SCE, compared to the negative control, although the level of upregulation was significantly lower than for BLG alone. Impairment of CD63 and CD203c upregulation

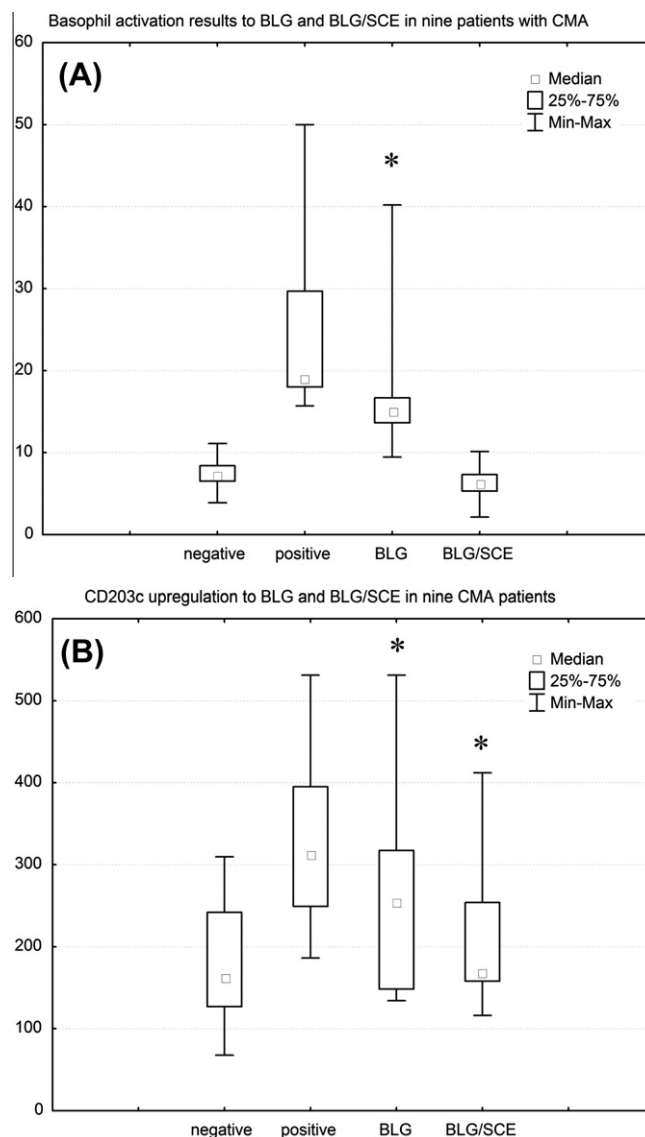


Fig. 5. Upregulation of CD63+ among CD203+ cells (basophil activation) and upregulation of CD203+ cells in blood of allergic patients incubated with BLG and BLG/SCE/laccase. * $p < 0.05$.

ulation was noticed for prostaglandin D₂, which is able to activate basophils, without the involvement of histamine-release and degranulation of basophils, in an anaphylactic response to stimuli (Monneret et al., 2005). Such a response of basophils by a highly cross-linked allergen may be explained by hindering of IgE epitopes on the surface of the cross-linked allergen, preventing it from successful degranulation of basophils. At least in part, these effects could be ascribed to the presence of polyphenols, that could decrease lipid bilayer fluidity, thus stabilising the membrane or directly decreasing histamine release from effector cells by dose-response inhibition of IgE-binding to the high affinity receptor for IgE (Tokura et al., 2005).

One approach to characterise the allergenicity of a food protein refers to the complementary investigation of its digestibility and its immunological properties (Cirkovic Velickovic, Radosavljevic, & Stanic, 2009). BLG treatment with laccase/SCE significantly reduced BLG allergenic capacity in two ways, by increasing the susceptibility of BLG towards peptic digestion, thus reducing its stability, and by reducing BLG ability to degranulate basophils. According to our results, cross-linking of allergenic protein by lac-

case might offer a promising way for reducing its allergenic potential. Besides that, this way of protein processing at the same time boosts the protein antioxidative capacity using quite natural tools. Utilisation of protein hydrolysates or peptides for improvement of antioxidant activity in functional foods presents additional advantages over other natural and synthetic antioxidants, since they also confer an additional nutritional value, as well as, other desired functional properties.

The possibility that proteins can be tailored and their fragments modelled to achieve a particular function has recently given rise to increased interest. Enzymatic processing by cross-linking enzymes and the usage of natural extracts rich in antioxidants as mediators of enzymatic reactions may be used to tailor BLG properties, by improving its safety and facilitating its digestibility, while conserving beneficial health effects, such as the radical-scavenging activity of peptides released during enzymatic digestion.

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