



Enhancement of Tryptic Digestibility of Milk β -Lactoglobulin Through Treatment with Recombinant Rice Glutathione/Thioredoxin and NADPH Thioredoxin Reductase/Thioredoxin Systems

Tahere Shahriari-Farfani¹ · Azar Shahpiri¹ ·
Asghar Taheri-Kafrani²

Received: 27 December 2017 / Accepted: 22 May 2018
© Springer Science+Business Media, LLC, part of Springer Nature 2018

Abstract β -Lactoglobulin (BLG), a member of lipocalin family, is one of the major bovine milk allergens. This protein exists as a dimer of two identical subunits and contains two intramolecular disulfide bonds that are responsible for its resistance to trypsin digestion and allergenicity. This study aimed to evaluate the effect of reduction of disulfide bonds of BLG with different rice thioredoxins (Trxs) on its digestibility and allergenicity. Therefore, the active recombinant forms of three rice Trx isoforms (OsTrx1, OsTrx20, and OsTrx23) and one rice NADPH-dependent Trx reductase isoform (OsNTRB) were expressed in *Escherichia coli*. Based on SDS-PAGE, HPLC analysis, and competitive ELISA, the reduction of disulfide bonds of BLG with OsNTRB/OsTrx23, OsNTRB/OsTrx1, GSH/OsTrx1, or GSH/OsTrx20 increased its trypsin digestibility and reduced its immunoreactivity. The finding of this study opens new insights for application of plant Trxs in the improvement of food protein digestibility. Especially, the use of OsTrx20 and OsTrx1 are more cost-effective than *E. coli* and animal Trxs due to their reduction by GSH and no need to NADPH and Trx reductase as mediator enzyme.

Keywords Milk · β -Lactoglobulin · Allergenicity · Digestibility · Thioredoxin · NADPH-dependent thioredoxin reductase · Glutathione

✉ Azar Shahpiri
a.shahpiri@cc.iut.ac.ir

¹ Department of Biotechnology, College of Agriculture, Isfahan University of Technology, Isfahan 84156-83111, Iran

² Department of Biotechnology, Faculty of advanced Sciences and Technologies, University of Isfahan, Isfahan 81746-73441, Iran

Introduction

Food allergy is known as an important worldwide problem that affects the health of children and adults in both developing and developed countries [1]. Although the diversity of human diets is high, only a small number of foods account for the majority of food allergies [2]. The most common allergic foods are cow's milk- and soy-based formulas [1]. The hypersensitivity reactions against cow's milk proteins affect up to 2.4% of infants [3]. It is very well-documented that the allergic response to cow's milk is caused by the proteins α -lactalbumin (ALA), serum albumin, caseins, and β -lactoglobulin (BLG) [4].

The protein BLG is one of the major bovine milk allergens [5]. This protein belongs to the lipocalin family and is considered as retinol-binding proteins [6]. Like other members of this protein family, BLG has a β -barrel fold with a large internal cavity that binds small hydrophobic molecules [7]. Under physiological conditions, BLG predominantly exists as a dimer of two identical subunits of 162 amino acids with molecular weight of 18.3 kDa. Each monomer contains one free cysteine and two disulfide bridges [8, 9].

The previous studies have shown that treatments like heat and high-pressure microfluidization damage the native globular structure and expose residues for proteases, thus enhancing the digestibility of BLG [10, 11]. Intermolecular disulfide bridges are responsible for the stability of BLG structure [12]. Dissociation of disulfide bridges destabilizes the native rigid globular structure of BLG, rendering it more vulnerable to the enzymatic hydrolysis of digestive proteases [13]. This thereby leads to the reduction of BLG allergenicity [14]. The reducing agents such as dithiothreitol (DTT) can reduce disulfide bonds of BLG; however, due to the toxic effect of these agents, the use of them is not applicable. In 1999, de val et al. showed that disulfide bonds of BLG could be reduced in the presence of NADPH, *E. coli* thioredoxin (Trx), and *E. coli* NADPH-dependent thioredoxin reductase (NTR) [15]. The treatment of milk with the NTR/Trx system was a novel procedure to increase BLG digestibility. This method was independent of heat, chemical agents, and proteolytic enzymes that reduce the food nutritional value and taste [15].

Thioredoxins (Trxs) are small ubiquitous oxidoreductase proteins with two redox-active Cys residues in a conserved active site (WCG/PPC) that regulate numerous target proteins via thiol/disulfide exchanges in the cells of prokaryotes and eukaryotes [16]. Members of the Trx superfamily share a similar fold known as the Trx fold (consisting of a four-stranded β -sheet surrounded by three α -helices), which is in common with the three-dimensional structure of glutaredoxins (Grxs) [17]. Animal, microbial, and plant cytoplasmic Trxs (h-type) are reduced themselves with NADPH via a flavoenzyme, NTR [18, 19].

Higher plants have a very complex NTR/Trx system compared with other organisms due to the presence of multiple isoforms of Trxs and NTRs [20]. For instance, in the genome of rice, 30 genes encode putative OsTrxs. Nine of these 30 genes are predicted to encode h-type Trxs including OsTrx1, OsTrx10, OsTrx15, OsTrx18, OsTrx20, OsTrx23, OsTrx24, OsTrx26, and OsTrx30 [21]. In addition, three isoforms of NTR (OsNTRA, OsNTRB, and OsNTRC) have been identified in rice [22]. Previously, we heterologously expressed OsTrx1, OsTrx20, OsTrx23, OsNTRA, and OsNTRB in *Escherichia coli* as His.tag fusion proteins [23, 24]. Both OsNTRA and OsNTRB were able to reduce OsTrx1 and OsTrx23 [24]. However, the isoform OsTrx20 was not reduced by OsNTR isoforms; but instead, it was reduced by glutathione (GSH). Furthermore, we found that OsTrx1 can be reduced with either OsNTR isoforms or GSH [25].

In the present work, the application of rice NTR/Trx systems in the improvement of milk product digestibility was investigated. Therefore, BLG was pretreated before tryptic digestion with different recombinant OsNTR/OsTrx and GSH/OsTrxs systems including OsNTRB/OsTrx23, OsNTRB/OsTrx1, GSH/OsTrx1, and GSH/OsTrx20, and subsequently, its tryptic digestibility degree was evaluated in comparison to control samples.

Materials and Methods

Chemicals

The chemicals were in analytical grade and obtained from either Sigma–Aldrich or Merck chemical companies and used without further purification. BLG variant A (used as the reference in all experiments) was purified from homozygote cow's milk following the method of Maillart and Ribadeau Dumas (1988). It was >97% pure as judged by high-performance liquid chromatography and poly acrylamide gel electrophoresis.

Production and Purification of Recombinant OsNTRB and OsTrxs

Recombinant OsNTRB and OsTrxs (OsTrx1, OsTrx20, and OsTrx23) were produced and purified as previously described in detail [24, 25]. To this end, *E. coli* cells containing plasmids pET28a-OsNTRB were grown at 37 °C in Luria-Bertani medium supplemented with 50 µg/mL kanamycin and 5 µg/mL chloramphenicol. For the production of OsTx1, OsTrx20, and OsTrx23, the *E. coli* cells containing pET15b-OsTrx1, pET15b-OsTrx20, and pET15b-OsTrx23 were grown at 37 °C in Luria-Bertani medium supplemented with 5 µg/mL chloramphenicol and 100 µg/mL ampicillin. When OD₆₀₀ reached 0.6, the cultures were induced by 100 µM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h under vigorous agitation. Cells were harvested by centrifugation at 9000×g and frozen at −20 °C until time of use. For extraction of cytoplasmic soluble proteins, the frozen pellets were suspended in pre-cooled buffer (10 mM Tris–HCl, pH 8.0) and disrupted by mild sonication at 0 °C. Soluble and insoluble fractions were separated by centrifugation at 13,500×g for 20 min. Recombinant proteins in the supernatant were purified by a His-Trap HP column (GE, Healthcare) and pre-equilibrated with binding buffer (10 mM imidazole, 500 mM NaCl, 30 mM Tris–HCl, pH 7.5). Afterwards, the bound proteins were eluted using a step gradient of 70–280 mM of imidazole. Eluted fractions were analyzed by 12% SDS–PAGE procedure. Purified proteins were desalted in 10 mM Tris–HCl buffer (pH 8.0) at 4 °C using 12 kDa molecular weight cut-off cellulose acetate membranes (Sigma–Aldrich). The concentrations of the desalted proteins were determined according to the Beer–Lambert law by measuring the UV absorbance of proteins at 280 nm (using the calculated molar extinction coefficient of 28,545 M^{−1} cm^{−1} for OsNTRB, 19,542.5 M^{−1} cm^{−1} for OsTrx1, 17,085 M^{−1} cm^{−1} for OsTrx20, and 8542.5 M^{−1} cm^{−1} for OsTrx23) using a spectrophotometer (Beckman DU530).

The Activity Assays of OsNTRB and OsTrxs

The reduction of OsTrx1 and OsTrx23 by OsNTRB was tested by DTNB assay as described previously [24, 25]. The reduction of OsTrx20 and OsTrx1 by GSH was also confirmed by the method described previously [25].

Reduction and Digestion of Pure BLG

(A) Digestibility of pure BLG without reduction (Control): A 150- μ l reaction solution containing 100 mM potassium phosphate buffer (pH 7.5) and 0.2 mg/ml BLG was incubated with trypsin in the ratio of 100:1 BLG/trypsin at 37 °C for 2 h.

(B) Digestibility of BLG after reduction with DTT: A 150 μ l reaction solution containing 100 mM potassium phosphate buffer (pH 7.5), 0.2 mg/ml BLG and 5 mM DTT was incubated at room temperature for 1 h. Then, in order to digestion, trypsin was added to the reaction medium in the ratio of 100:1 BLG/trypsin and reaction was incubated at 37 °C for 2 h.

(C) Digestibility of pure BLG after reduction with either OsNTRB/OsTrx23 or OsNTRB/OsTrx1: A 150- μ l reaction media containing 100 mM potassium phosphate buffer (pH 7.5), 0.2 mg/ml BLG, 0.1 mg/ml BSA, 0.1 mM NADPH, 160 nM OsNTRB, 7 μ M OsTrx1 or OsTrx23 was incubated at 4 °C overnight or at 25 and 37 °C for 1 h. Then, trypsin was added in the ratio of 100:1 BLG/trypsin and incubated at 37 °C for 2 h.

(D) Digestibility of BLG after reduction with GSH/Trx: A 150- μ l reaction solution containing 100 mM potassium phosphate buffer (pH 7.5), 0.2 mg/ml BLG, 5 μ M OsTrx1 or OsTrx20, and 20 mM GSH was incubated at 4 °C overnight or at 25 and 37 °C for 1 h. The trypsin was then added to the reaction medium in the ratio of 100:1 BLG/trypsin and incubated at 37 °C for 2 h.

Sulfhydryl Quantification

The sulfhydryl content was determined based on the method initially described by Ellman using DTNB (Ellmant's reagent, Thermo Scientific) [26]. The protein BLG was treated with DTT, OsNTRB/OsTrx1, OsNTRB/OsTrx23, GSH/OsTrx1, or GSH/OsTrx20 described in the above section. After reduction for determination of sulfhydryl content, DTNB was added to the samples (200 μ M as final concentration). In addition, DTNB was added to a sample containing untreated BLG as control sample. The reactions were incubated at room temperature for 30 min. The absorbance was monitored spectrophotometrically at 412 nm and the sulfhydryl content was calculated on the basis of the molar extinction coefficient of TNB (14,150 M⁻¹ cm⁻¹). Each data point represents the mean $\pm$ SD obtained from three independent reactions. The means were compared with least significant difference test (LSD, P0.05) test.

SDS-PAGE Analysis

The digested products (20 μ l) were run on 12% Tris–glycine SDS-PAGE. Gels were stained with Coomassie blue. The intensity of BLG bands were quantified using ImageJ software (W.S. Rasband; 1997–2007; National Institutes of Health; <http://rsb.info.nih.gov/ij>). The means were compared with least significant difference test (LSD, P0.05) test.

Reversed-Phase High-Performance Liquid Chromatography

Reversed-phase high-performance liquid chromatography (RP-HPLC) experiments were carried out using a 1200 series Agilent HPLC and an Agilent automatic injector (Agilent Technologies, Palo Alto, CA), with a diode array detector set at 220 nm. Samples were filtered through a 0.45- μ m syringe filter. Fifty microliters was separated on C18 column (C18, 4.6 $\times$ 150 mm, particle size of 5 μ m) with linear gradients of

solvent A (10% v/v acetonitrile, 0.1% v/v trifluoroacetic acid) and solvent B (90% v/v acetonitrile, 0.1% v/v trifluoroacetic acid) at flow rate of 0.3 mL/min at 20 °C. The gradient was as follows: 3 min 100% A, 45 min 100% A to 50% B, 5 min 50% B to 70% B, 5 min 70% B, 5 min 70% B to 100% A. EZ Chrom Elite software (version 3.3.1) was used for data acquisition.

Competitive Enzyme-Linked Immunosorbent Assay

The pretreatment of BLG with GSH/OsTrx1 and digestion with trypsin was performed as explained above (part 2.4 D). The competitive enzyme-linked immunosorbent assay (ELISA) was used to evaluate the binding strength of the untreated and treated BLG to the immunoglobulin E (IgE) in pool sera of the cow's milk allergy (CMA) patients. Ninety-six-well microtitration plates were pre-coated overnight with native BLG diluted to 5 µg/mL in PBS buffer pH 7.0 at 4 °C. By substituting BLG with the same volume (100 µl) of PBS, the negative control (blank) was prepared. The plates were washed three times with PBS containing 0.1% (v/v) Tween-20 (PBS/T) and then saturated for 2 h with 250 µL of PBS/T containing 1% (w/v) polyvinyl alcohol (PBS/T/PVA). A 14 sera sample pool obtained from BLG allergic patients was used. After a 1:20 ratio dilution, the pool sera were combined with equivalent volumes of untreated or treated BLG in PBS/T/PVA (0 to 250 µg/ml) serial dilutions. A 100-µl portion of the mixture was added to every one of the wells and incubated overnight at 4 °C. After washing three times with PBS/T, the plate was incubated with the secondary antibody (alkaline phosphatase- conjugated polyclonal antihuman IgE, Sigma) diluted 1:1000 on PBS/T/PVA, for 2 h at 37 °C. After washing with PBS/T, binding of the secondary antibody was quantified by adding 4-methylumbelliferyl phosphate (4-MUP) substrate mixed in the ratio of 1:5 with Tris-HCl pH 9.8. After 90 min of incubation at 37 °C, the fluorescence emission was measured with the FLX800 plate reader (Biotech Instruments, Inc., Winooski, VT) fitted with a 360-nm excitation filter and a 440-nm emission filter. The experiment was performed in triplicate, and the fluorescence data were corrected through deduction of the fluorescence of the control with no antigen. Protein (inhibitor) concentration desired for 50% inhibition of IgE binding (IC_{50}) was calculated from the inhibition curves by relating fluorescence intensity to inhibitor concentration.

Digestibility of Milk Lactosum Proteins After Reduction by OsNTR/OsTrxs and GSH/OsTrx

For preparation of milk lactosum (whey), 200 mg skimmed milk powder was solved in 0.1 M sodium acetate buffer (pH 4.8) and incubated at 65 °C for 15 min. Then, the solution was centrifuged three times at 16,000 rpm for 10 min at room temperature. The upper phase containing lactosum proteins was transferred to new tubes. Then, the sodium acetate buffer was removed by dialysis. The concentration of total protein in lactosum fraction was determined by Bradford assay using BSA as standard. Pretreatment of lactosum proteins at four temperatures (4, 25, 37, and 55 °C) with GSH/OsTrxs and digestion with trypsin was performed in reactions containing 2 mg/ml lactosum proteins using the condition described in the “[Reduction and Digestion of Pure BLG](#)” section, D.

Results

Heterologous Expression of OsNTRB and OsTrxs

The recombinant forms of His₆-OsNTRB, His₆-OsTrx1, His₆-OsTrx20, and His₆-OsTrx23 were produced in *E. coli*. They were found in the soluble fraction of the transformed *E. coli* culture after induction by IPTG. The theoretical molecular weight of His₆-OsTrx1, His₆-OsTrx20, His₆-OsTrx23, and His₆-OsNTRB were 16.8, 17.5, 15.3, and 38.4 kDa, respectively. The SDS-PAGE analysis showed sharp protein bands of expected molecular masses (Fig. 1). The recombinant proteins were purified from the soluble fraction using nickel affinity chromatography and desalted using dialysis. The quality of purification was assessed by SDS-PAGE analysis (Fig. 1e). The protein yields for His₆-OsTrx1, His₆-OsTrx20, His₆-OsTrx23, and His₆-OsNTRB were 23, 13, 25, and 30 mg, respectively of purified fusion protein per liter of culture.

DTNB and Insulin Assays

The kinetic parameters of OsNTRB toward either OsTrx1 or OsTrx23 were calculated using DTNB assay. The K_m and k_{cat} values of OsNTRB toward OsTrx1 were 1 μ M and 0.5 S⁻¹,

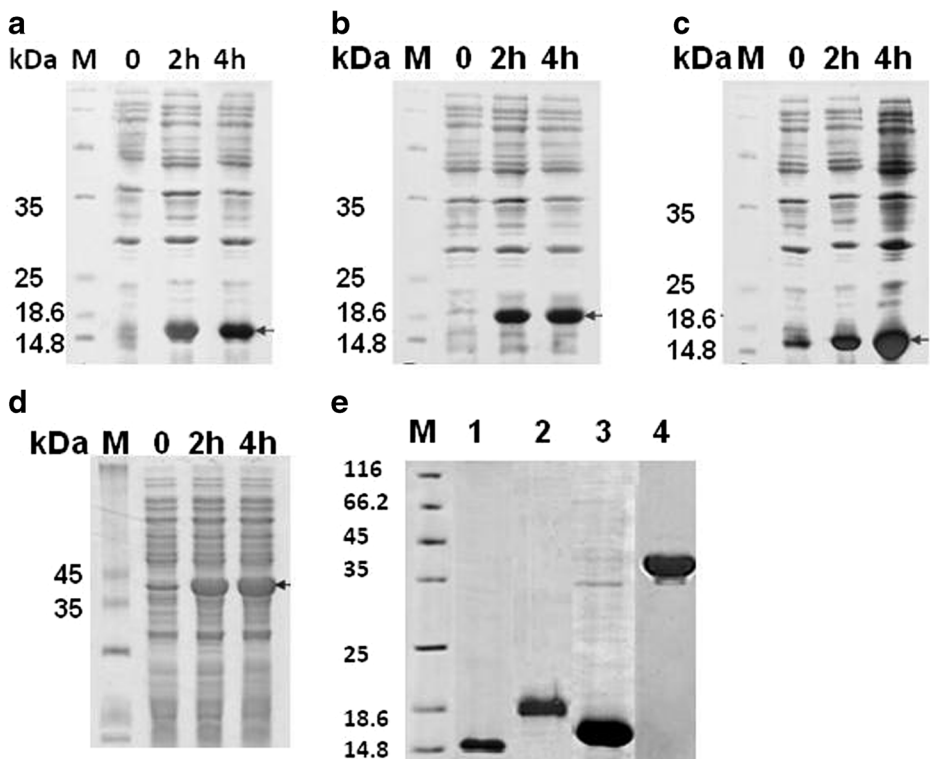


Fig. 1 SDS-PAGE analysis for verification of expression and purification of recombinant proteins. Total soluble protein extracted from *E. coli* harboring **a** pET15b-OsTrx1, **b** pET15b-OsTrx20, **c** pET15b-OsTrx23, and **d** pET28a-OsNTRB before addition of IPTG and 2 or 4 h after addition of IPTG. **e** Purified His-OsTrx1, His-OsTrx20, His-OsTrx23, and His-OsNTRB, respectively

respectively. These parameters for OsNTRB toward OsTrx23 were $4.9 \mu\text{M}$ and 0.48 S^{-1} , respectively. As previously reported, the isoform OsTrx20 was not reduced by OsNTRB. The reduction of OsTrxs was also tested in the presence of GSH. As Fig. 2 shows, the isoforms OsTrx20 and OsTrx1 were reduced by GSH. However, the isoform OsTrx23 was not reduced in the presence of GSH.

Sulfhydryl Quantification

The sulfhydryl content of BLG pretreated with DTT increased almost ninefold as compared to untreated BLG (36.5 vs $4.6 \mu\text{mol/g}$, Fig. 3). In comparison, the sulfhydryl contents of samples containing BLG pretreated with OsNTRB/OsTrx23, OsNTRB/OsTrx1, GSH/OsTrx1, or GSH/OsTrx20 were significantly higher than that for the sample containing untreated BLG (26.5 , 25.25 , 26.4 , and 24.5 , respectively vs $4.6 \mu\text{mol/g}$). These results show the tremendous effect of NTR/Trxs and GSH/Trxs in the reduction of BLG. Nevertheless, the effect of these systems is significantly lower than DTT as shown in Fig. 3.

Trypsin-Digestibility of BLG after Treatment with OsNTRB/OsTrxs and GSH/OsTrxs Systems

(A) SDS-PAGE analysis Figure 4a shows the percentage of intact BLG before and after treatment with trypsin. Almost 50% of BLG was undigested after treatment of BLG with trypsin. However, the amount of undigested BLG was significantly decreased to less than 25%, when, before digestion with trypsin, BLG was pretreated at 25°C with each reducing system including OsNTRB/OsTrx1, OsNTRB/OsTrx23, GSH/OsTrx1, or GSH/OsTrx20. It should be noted that there was no significant difference in BLG digestibility when BLG was pretreated with different systems. In comparison, the pretreatment of BLG with DTT caused the highest increase in BLG digestibility, with only 8% undigested BLG left. These results show that the systems OsNTRB/OsTrxs and GSH/OsTrxs are able to reduce disulfide bonds which thereby can increase BLG digestibility. Furthermore, pretreatment of BLG with different

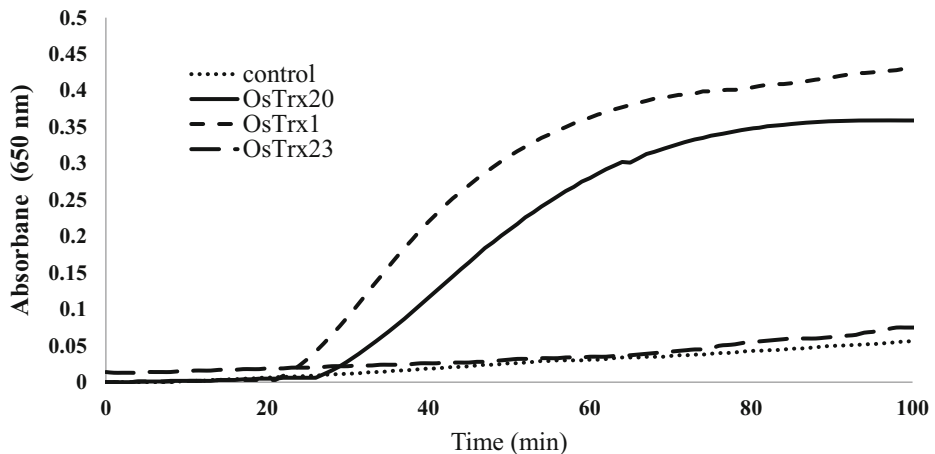


Fig. 2 Insulin reduction by OsTrxs in the presence of GSH. The experiments were performed using $20 \mu\text{M}$ each recombinant OsTrxs in the presence of 10 mM GSH

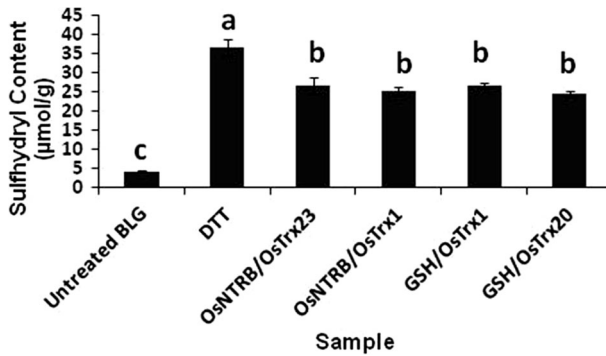


Fig. 3 The sulfhydryl content in untreated and treated BLG

systems was also tested at different temperatures. As Fig. 4b shows, there was no significant variation between BLG digestibilities when BLG is pretreated with different systems at different temperatures.

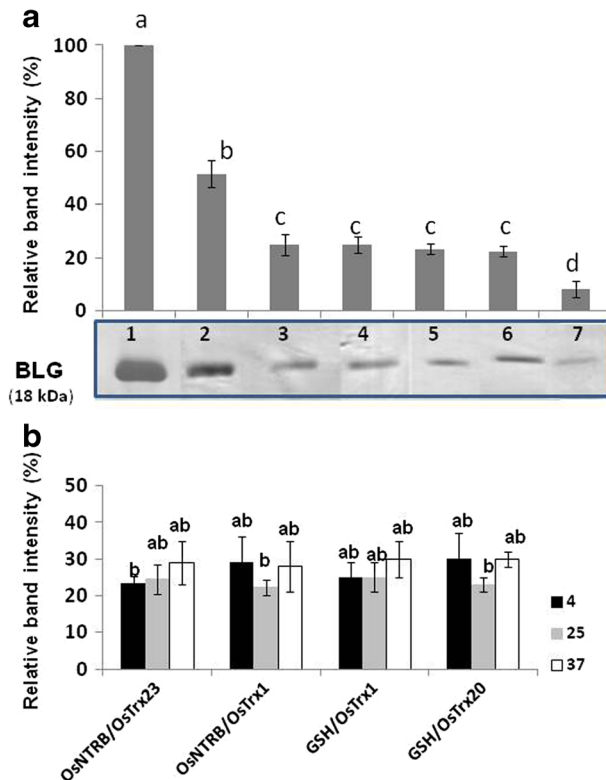


Fig. 4 Effect of Trx reduction systems on digestibility of BLG determined by SDS (12% SDS–PAGE gels). The percent of intact BLG was determined by relative band intensity and shown on vertical axis. **a** Line 1, BLG (untreated and undigested); line 2, untreated BLG was incubated with trypsin; lines 3–6, the pretreated BLG with either OsNTRB/OsTrx1, OsNTRB/OsTrx23, GSH/OsTrx1, or GSH/OsTrx20 at 25 °C was incubated with trypsin; line 7, pretreated BLG with DTT was incubated with trypsin. **b** Comparison of trypsin-digestibility of BLG after pretreatment with different systems at different temperature

(B) HPLC analysis Figure 4 shows the RP-HPLC profiles of BLG before and after digestion with trypsin. Undigested BLG showed a single peak with a retention time of about 48 min (Fig. 5a). The tryptic digestion of BLG leads to 50% decrease in BLG peak area and appearance of new small peaks (Fig. 5b). The pretreatment of BLG with GSH/OsTrx1 and DTT at 25 °C before digestion with trypsin resulted in almost 80 and 90% decrease in BLG peak area, respectively (Fig. 5c, d). This decrease in peak area was also observed for BLG pretreated with OsNTRB/OsTrx23, OsNTRB/OsTrx1, and GSH/OsTrx20 (data not shown). These results demonstrate that the systems OsNTRB/OsTrxs and GSH/OsTrxs effectively reduce BLG which consequently lead to considerable increase in the digestibility of BLG. Furthermore, based on the results, there was no significant difference between the effects of different systems.

Response of Patient Sera to Treated BLG with GSH/OsTrx1

The consequence of protein treatment of BLG on its binding affinity to IgE was investigated through a competitive ELISA experiment. Figure 6 shows the binding ability of the IgE of CMA patients' pool sera to tryptic digestions of untreated and treated BLG with GSH/OsTrx1. It can be noted that treated BLG's recognition and binding to the serum IgE was significantly weaker in comparison with the untreated BLG. Inhibitor concentration, IC_{50} , denotes the BLG concentration binding 50% of the IgE in the sera of the CMA patients. According to Fig. 6, the IC_{50} value of intact BLG was calculated to approximately 1.43 $\mu\text{g/ml}$ whereas the treated protein had an IC_{50} value of 17.34 $\mu\text{g/ml}$ high. These results demonstrate that IgE binding to the tryptic digests of treated BLG is approximately 12 times inferior as compared to tryptic

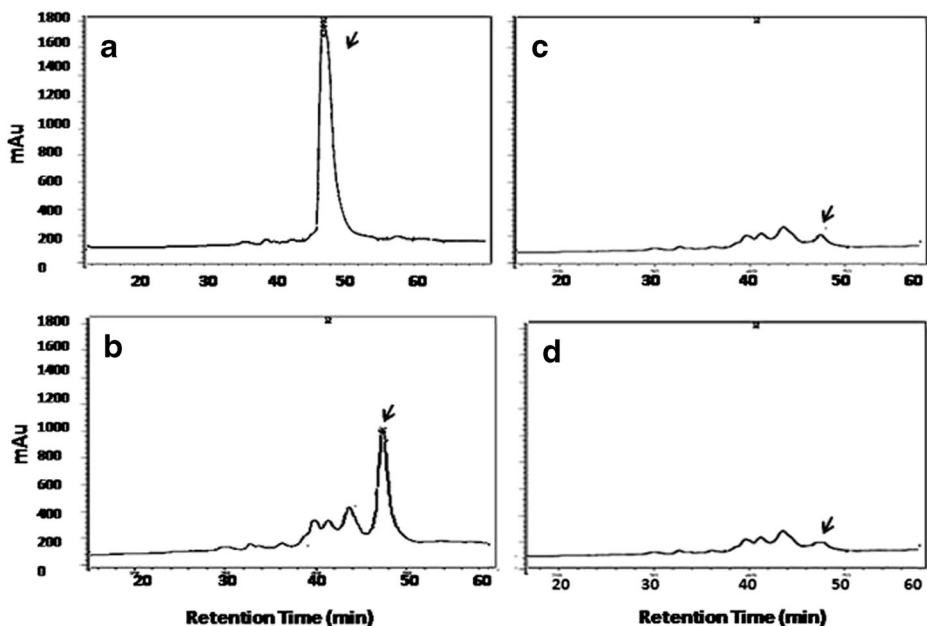


Fig. 5 Effect of GSH/OsTrx1 on digestibility of BLG determined by HPLC analysis. The arrows show the peak of intact BLG. **a** BLG (untreated and undigested). **b** Untreated BLG was incubated with trypsin. **c** The pretreated BLG with GSH/OsTrx1 at 25 °C was incubated with trypsin

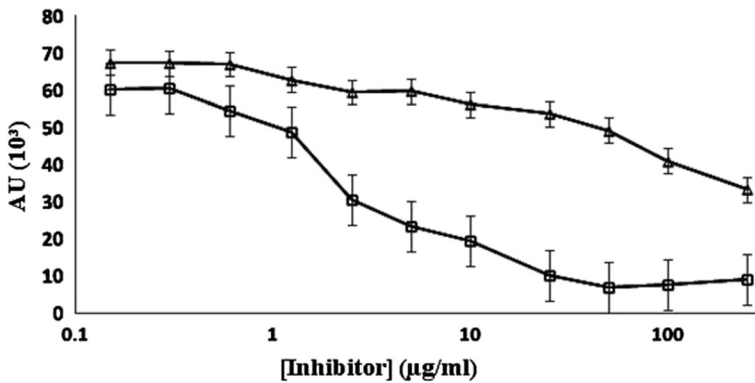


Fig. 6 Inhibition of anti-BLG IgE in serum pool. The signal (AU) results from the binding of IgE on native BLG coated plates. Comparison between trypsin-digested product of untreated BLG (square) and trypsin-digested product of treated BLG with GSH/OsTrx1 (triangle)

digests of untreated protein. The results illustrated that the reduction of BLG by GSH/OsTrx1 before trypsin digestion could reduce its allergenicity. This decrease in allergenicity is related to the effect of this pretreatment on the BLG digestibility. Indeed, the lower amount of intact BLG in the treated samples resulted in a decrease in allergenicity.

Effect of GSH/OsTrxs on Milk Lactosernum Proteins

The lactosernum fraction was isolated from milk as described previously. The SDS-PAGE analysis of lactosernum proteins showed two sharp proteins with the size of 18 and 14 kDa corresponding to the molecular weight of two major lactosernum proteins, BLG and ALA, respectively. As shown in Fig. 7, the pretreatment of lactosernum with GSH/OsTrx1 and GSH/OsTrx20 before digestion with trypsin lead to a considerable decrease in the intensity of BLG and ALA bands, indicating the effect of these reducing systems improves the digestibility of lactosernum proteins. Pretreatment with GSH/OsTrx1 and GSH/OsTrx20 was also tested at different temperature. Whereas the digestibility of BLG was not affected by pretreatment temperature, the digestibility of ALA was at the highest level when pretreatment was performed at 50 °C.

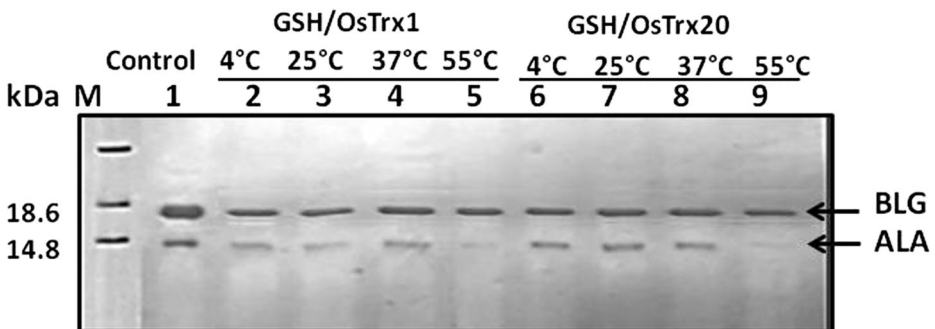


Fig. 7 Effect of GSH/OsTrx1 and reduction systems on the digestibility of BLG and ALA in lactosernum determined by SDS (12% SDS-PAGE gels). The arrows show the bands correspond to intact BLG and ALA in untreated lactosernum incubated with trypsin (control, line 1), treated lactosernum with either GSH/OsTrx1 (lines 2–5) or GSH/OsTrx20 at different temperatures (lines 6–9), and then incubated with trypsin

Discussion

Trxs are ubiquitous proteins that participate in thiol disulfide reactions via two active site cysteine residues (WCXXC), allowing Trx to reduce disulfide bonds in target proteins [16]. In higher plants, Trx isoforms are divided into six subfamilies based on their primary sequences and subcellular localization [27]. The Trxh isoforms make the largest subfamily in plants which further subdivided into three groups [16]. Groups I and II of Trxsh are reduced by NADPH in the presence of NTR [16, 28]. In contrast, group III contains Trxh isoforms with the unusual active site (WCXXS) and is reduced by GSH or glutaredoxins [16]. Recent progress in proteome analysis has resulted in identification of numerous Trxh target proteins in different species. For instance, Trxh isoforms play a critical role in the reduction of disulfides in seed storage proteins upon germination, making them more soluble and accessible for proteolytic digestion [29]. Furthermore, during seed germination, Trxh activate hydrolytic enzymes and inactivate hydrolytic enzyme inhibitors through the reduction of disulfide bonds, resulting in the mobilization of carbohydrate and protein reserves [29].

Due to the effective role of Trxs in the reduction of disulfide bonds, the application of recombinant forms of *E. coli* NTR/Trx system for improvement of food digestibility has been already evaluated [15, 30]. One of the major food allergens is BLG in the bovine milk. The solved structure of this protein shows the binding of two IgE/Fab molecules to the dimeric BLG [31]. The BLG epitope consists of six different short fragments of the polypeptide chain, which are located especially in the β strands, covering a flat area on the allergen surface. Because of BLG stability under acidic pH conditions, BLG is resistant to acid digestion and remain intact after passing through the stomach [32, 33]. When proteins are denatured by heat, the original tertiary structure is often lost, leading to the disappearance of the native protein epitopes recognized by antibodies [34]. Therefore, thermal treatment alters the digestibility of milk, which makes it more susceptible for degradation by hydrolyzing enzymes [35]. However, the aggregation of BLG which improves its stability is considered as an important side effect of high temperature processing of milk [36]. Based on previous studies, the presence of two disulfide bonds in the BLG structure, one clearly accessible on the surface (Cys66-Cys160) and the other close to the core (Cys106-Cys119), is an important factor for the BLG stability [15]. Reduction of disulfide bridges destabilizes the native rigid globular structure of BLG, making it more accessible for enzymatic hydrolysis [13]. In agreement, a computer modeling revealed that the disruption of the exposed disulfide bond of BLG (Cys66-Cys160) changes the conformation and accessibility of the major human IgE epitope [15]. Therefore, finding efficient and safe ways for reduction of BLG disulfide bonds can be extremely beneficial. The treatment of milk BLG with *E. coli* NTR/Trx system effectively reduced BLG disulfide bonds which led to enhancing tryptic digestibility and decreasing the allergenicity of BLG [15]. The treatment of wheat proteins and soy flour with this system effectively reduced disulfide bonds, increased their susceptibility to proteolysis, and decreased their allergenicity [30, 37]. Despite the high potential of plant NTR/Trx system in reduction of disulfide bonds, the treatment of food proteins with plant NTR/Trx has not yet been examined. Therefore, in the present work, we studied the in vitro effects of OsTrx23, OsTrx1, and OsTrx20 as three rice Trxh isoforms on the digestibility of BLG by trypsin. The isoform OsTrx23 belongs to the subgroup I of h-type Trxs and is effectively reduced by OsNTRB in the presence of NADPH [25]. The isoform OsTrx20 and OsTrx1 are members of subgroup III. Whereas OsTrx1 is reduced by both OsNTRB and GSH, the isoform OsTrx20 is reduced only by GSH [25]. The production and purification of recombinant forms of these three Trx

isoforms and OsNTRB as His.tag fusion proteins enabled us to study the effects of systems OsNTRB/OsTrx23, OsNTRB/OsTrx1, GSH/OsTrx1, and GSH/OsTrx20 on the BLG digestibility and allergenicity. The pretreatment of BLG, either pure or in milk lactoserum, by these reduction systems enhanced digestibility of BLG as shown by both SDS-PAGE and HPLC analysis and decreased the allergenicity in comparison to untreated BLG. There was no significant difference between the effects of different systems. However, in order to practically use these reduction systems and large-scale pretreatment for improvement of BLG digestibility, the cost should be considered. It seems that the lower cost of GSH than NADPH and the need to produce only one recombinant protein make the use of GSH/OsTrx1 and GSH/OsTrx2 more economical than the use of OsNTRB/OsTrx23 and OsNTRB/OsTrx1.

In conclusion, in the present work, we found that plant NTR/Trx and GSH/Trx systems are efficient systems for reduction of BLG disulfide bonds. Therefore, the pretreatment of milk BLG with these systems improves significantly the digestibility of BLG and decreased its allergenicity. However, there are still barriers to the practical use of these systems. The most important barrier is the need for a treatment system to do reduction without direct addition of GSH and OsTrxs. Further work is necessary to overcome these problems.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

References

1. Sampson, H. (2004). Update on food allergy. *Journal of Allergy and Clinical Immunology*, 113(5), 805–819.
2. Rytönen, J. (2006). Effect of heat denaturation of bovine milk betalactoglobulin on its epithelial transport and allergenicity. *Acta Universitatis Ouluensis Series D*, 883, 17–18.
3. Saarinen, K. M., Juntunen-Backman, K., Järvenpää, A. L., Kuitunen, P., Lope, L., Renlund, M., Siivola, M., & Savilahti, E. (1999). Supplementary feeding in maternity hospitals and the risk of cow's milk allergy: a prospective study of 6209 infants. *Journal of Allergy and Clinical Immunology*, 104(2), 457–461.
4. Docena, G. H., Fernandez, R., Chirido, F. G., & Fossati, C. A. (1996). Identification of casein as the major allergenic and antigenic protein of cow's milk. *Allergy*, 51(6), 412–416.
5. Savilahti, E., & Kuitunen, M. (1992). Allergenicity of cow milk proteins. *Journal of Pediatrics*, 121(5), S12–S20.
6. Perez, M. D., & Calvo, M. (1995). Interaction of B-lactoglobulin with retinol and fatty acids and its role as a possible biological function for this protein. *Journal of Dairy Science*, 78(5), 978–988.
7. Flower, D. R. (1996). The lipocalin protein family: structure and function. *Biochemical Journal*, 318(1), 1–14.
8. Fogolari, F., Ragona, L., Zetta, L., Romagnoli, S., De Kruijff, K. G., & Molinari, H. (1998). Monomeric bovine beta-lactoglobulin adopts a beta barrel fold at pH 2. *FEBS Letters*, 436(2), 149–154.
9. Hambling, S. G., McAlpine, A. S., Sawyer, L., & Fox, P. (1992). β -Lactoglobulin. *Adv. Dairy Chemistry*, 1 Protein 2, 141–190.
10. Rahaman, T., Vasiljevic, T., & Ramchandran, L. (2016). Digestibility and antigenicity of β -lactoglobulin as affected by heat, pH and applied shear. *Food Chemistry*, 217, 517–523.
11. Zhong, J., Luo, S. H., Liu, C. H., & Liu, W. (2014). Steady-state kinetics of tryptic hydrolysis of β -lactoglobulin after dynamic high-pressure microfluidization treatment in relation to antigenicity. *Journal European Food Research and Technology*, 239(3), 525–531.
12. Lowe, E. K., Anema, S. G., Bienvenue, A., Boland, M. J., Creamer, L. K., & Jiménez-Flores, R. (2004). Heat-induced redistribution of disulfide bonds in milk proteins. 2. Disulfide bonding patterns between bovine β -Lactoglobulin and κ -casein. *Journal of Agriculture and Food Chemistry*, 52(25), 7669–7680.
13. Zhao, D., T Le, T., Nielsen, S. D., & Larsen, L. B. (2017). Effect of storage on lactase-treated B-casein and B-lactoglobulin with respect to bitter peptide formation and subsequent *in vitro* digestibility. *Journal of Agricultural and Food Chemistry*, 65(38), 8409–8417.

14. Del Val, G., Buchanan, B. B., Yee, B. C., Lozano, R. M., Ermel, R. W., & Frick, O. L. (1997). Production of hypoallergenic, hyperdigestible milk by a new biotechnology. In: XVI International Congress of Allergology and Clinical Immunology, (p. 26).
15. Del Val, G., Yee, B. C., Lozano, R. M., Buchanan, B. B., Ermel, R. W., Lee, Y. M., & Frick, O. L. (1999). Thioredoxin treatment increases digestibility and lowers allergenicity of milk. *Journal of Allergy and Clinical Immunology*, 103(4), 690–697.
16. Gelhaye, E., Rouhier, N., & Jacquot, J. P. (2004). The thioredoxin h system of higher plants. *Plant Physiology and Biochemistry*, 42(4), 265–271.
17. Meyer, Y., Siala, W., Bashandy, T., Riondet, C., Vignols, F., & Reichheld, J. P. (2008). Glutaredoxins and thioredoxins in plants. *Biochimica et Biophysica Acta*, 1783(4), 589–600.
18. Reichheld, J. P., Meyer, E., Khafif, M., Bonnard, G., & Meyer, Y. (2005). AtNTRB is the major mitochondrial thioredoxin reductase in *Arabidopsis thaliana*. *FEBS Letters*, 579(2), 337–342.
19. Bréhélin, C., Laloi, C., Setterdahl, A. T., Knaff, D. B., & Meyer, Y. (2004). Cytosolic, mitochondrial thioredoxins and thioredoxin reductases in *Arabidopsis thaliana*. *Photosynthesis Research*, 79(3), 295–304.
20. Meyer, Y., Vignols, F., & Reichheld, J. P. (2002). Classification of plant thioredoxins by sequence similarity and intron position. *Methods in Enzymology*, 347, 394–402.
21. Nuruzzaman, M., Gupta, M., Zhang, C., Wang, L., Xie, W., Xiong, L., Zhang, Q., & Lian, X. (2008). Sequence and expression analysis of the thioredoxin protein gene family in rice. *Molecular Genetics and Genomics*, 280(2), 139–151.
22. Serrato, A. J., Pérez-Ruiz, J. M., Spínola, M. C., & Cejudo, F. J. (2004). A novel NADPH thioredoxin reductase, localized in the chloroplast, which deficiency causes hypersensitivity to abiotic stress in *Arabidopsis thaliana*. *Journal of Biological Chemistry*, 279(42), 43821–43827.
23. Papzan, Z., & Shahpiri, A. (2012). Cloning, heterologous expression and characterization of three thioredoxin h isoforms (OsTrx1, OsTrx20 and OsTrx23) from rice. *Plant Omics*, 5, 238–243.
24. Eslampanah, H., & Shahpiri, A. (2012). Molecular cloning and characterization of two isoforms of cytoplasmic/mitochondrial type NADPH-dependent thioredoxin reductase from rice ('*Oryza sativa*' L. ssp. 'indica'). *Australian Journal of Crop Science*, 6, 1045–1050.
25. Shaykholeslam Esfahani, E., & Shahpiri, A. (2015). Thioredoxin h isoforms from rice are differentially reduced by NADPH/thioredoxin or GSH/glutaredoxin systems. *International Journal of Biological Macromolecules*, 74, 243–248.
26. Ellman, G. (1959). Tissue sulphydryl groups. *Archives of Biochemistry and Biophysics*, 82(1), 70–77.
27. Chibani, K., Wingsle, G., Jacquot, J.-P., Gelhaye, E., & Rouhier, N. (2011). Biochemical properties of poplar thioredoxin z. *FEBS Letters*, 34569, 1–6.
28. Gelhaye, E., Rouhier, N., Gérard, J., Jolivet, Y., Gualberto, J., Navrot, N., Ohlsson, P. I., Wingsle, G., Hirasawa, M., & Knaff, D. B. (2004). A specific form of thioredoxin h occurs in plant mitochondria and regulates the alternative oxidase. *PNAS*, 101(40), 14545–14550.
29. Buchanan, B. B., & Balmer, Y. (2005). Redox regulation: a broadening horizon. *Annual Review of Plant Biology*, 56(1), 187–220.
30. Faris, R. J., Wang, H., & Wang, T. (2008). Improving digestibility of soy flour by reducing disulfide bonds with thioredoxin. *Journal of Agricultural and Food Chemistry*, 56(16), 7146–7150.
31. Niemi, M., Jylhä, S., Laukkanen, M. L., Söderlund, H., Kiljunen, S. M., Kallio, J. M., Hakulinen, N., Haahtela, T., Takkinen, K., & Rouvinen, J. (2007). Molecular interaction between a recombinant IgE antibody and the β -lactoglobulin allergen. *Structure*, 15(11), 1413–1421.
32. Fuquay, J. W., Fox, P. F., & McSweeney, P. L. (2011). *Encyclopedia of Dairy Sciences* (2nd ed.). Cambridge: Academic.
33. Stanley, J., & Bannon, G. (1999). Biochemistry of food allergens. *Clinical Reviews in Allergy & Immunology*, 17, 279–291.
34. Song, C. Y., Chen, W. L., Yang, M. C., Huang, J. P., & Mao, S. J. (2005). Epitope mapping of a monoclonal antibody specific to bovine dry milk: involvement of residues 66–76 of strand D in thermal denatured β -lactoglobulin. *Journal of Biology and Chemistry*, 280(5), 3574–3582.
35. Takagi, K., Teshima, R., Okunuki, H., & Sawada, J. (2003). Comparative study of in vitro digestibility of food proteins and effect of preheating on the digestion. *Biological and Pharmaceutical Bulletin*, 26(7), 969–973.
36. Peram, M. R., Loveday, S. M., & Ye, A. (2013). In vitro gastric digestion of heat-induced aggregates of β -lactoglobulin. *Journal of Dairy Science*, 96(1), 63–74.
37. Buchanan, B. B., Adamidi, C., Lozano, R. M., Yee, B. C., Momma, M., Kobrehel, K., Ermel, R., & Frick, O. L. (1997). Thioredoxin-linked mitigation of allergic responses to wheat. *Production National Academy of Science*, 94(10), 5372–7377.