Biosci. Biotechnol. Biochem., **75** (10), 1872–1879, 2011

Yeast Thioredoxin-Enriched Extracts for Mitigating the Allergenicity of Foods

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Received October 12, 2010; Accepted June 26, 2011; Online Publication, October 7, 2011
[doi:10.1271/bbb.100734]

Thioredoxin (TRX) catalyzes the reduction of disulfide bonds in proteins *via* the NADPH-dependent thioredoxin reductase system. Reducing the disulfide bonds of allergenic proteins in food by TRX lowers the allergenicity. We established in this study a method to prepare TRX-enriched extracts from the edible yeast, *Saccharomyces cerevisiae*, on a large and practical scale, with the objective of developing TRX-containing functional foods to mitigate food allergy. Treating with the yeast TRX-enriched extracts together with NADPH and yeast thioredoxin reductase enhanced the pepsin cleavage of β-lactoglobulin and ovomucoid (OM). We also examined whether yeast TRX can mitigate the allergenicity of OM by conducting immediate allergy tests on guinea pigs. The treatment with TRX reduced the anaphylactic symptoms induced by OM in these tests. These results indicate that yeast TRX was beneficial against food allergy, raising the possibility that yeast TRX-enriched extracts can be applied to food materials for mitigating food allergy.

Key words: thioredoxin; yeast extract; food allergen; disulfide bond

Food allergies are immunoreactions induced by the intake of such foods as egg white, cow's milk and wheat.[1)] Food allergies are caused by a limited number of protein families with certain characteristics,[2)] including the presence of intramolecular disulfide bonds that give resistance to heat, acid and proteases; for example, members of the prolamin superfamily in plants (2S albumin, and inhibitors of trypsin and α-amylase) that have more than four disulfide bonds are notable food allergens.[2,3)] Ovomucoid (OM), an egg white allergen, has three domains that are crosslinked by three intradomain disulfide bonds,[4)] and is stable against heat, extreme pH and proteolysis.[4)] IgE-binding epitopes are located in regions with intramolecular disulfide bonds in such cysteine-rich proteins as chitinases in fruits[2,5)] and Ara h 2 protein in peanuts.[6)] Reducing these disulfide bonds may therefore be beneficial for reducing the allergenicity of these proteins.

Thioredoxin (TRX) is a small ubiquitous thiol protein (12 kDa) that was first described as a hydrogen donor for ribonucleotide reductase, an enzyme involved in DNA synthesis.[7)] TRX has subsequently been reported to exert multiple regulatory functions *via* its catalytically active disulfide site (Cys-Gly-Pro-Cys) which is conserved in animals, plants, and bacteria.[8–10)] These cysteines in the active site show a reversible redox change between an oxidized disulfide and a reduced dithiol form. TRX is oxidized when it cleaves disulfide bonds in the target proteins and is reduced back to the dithiol form by the NADPH and NADPH-dependent thioredoxin reductase (NTR)-dependent system (hereinafter referred to as the TRX system).

Buchanan *et al.* have demonstrated mitigating the allergenicity of food allergens by using the TRX system, wheat extracts treated with *E. coli* TRX, *E. coli* NTR and NADPH evoking lower allergic responses in atopic canine models.[11)] Treating components with the TRX system reduced the intramolecular disulfide bonds of the major bovine milk allergen, β-lactoglobulin, and the reduced protein showed increased susceptibility to pepsin digestion and lower allergenicity *in vivo*.[12)] The results of these studies suggest that TRX may be useful for reducing food allergenicity. Since the preparation of *E. coli* TRX may contain such immunoreactive constituents as lipopolysaccharides, it may be desirable if TRX for food applications were itself to be derived from foods. We therefore prepared TRX in this study from edible yeasts which are commonly used in such fermented foods as alcoholic drinks and bread.

Saccharomyces cerevisiae contains three isoforms of TRX: two cytoplasmic types (TRX1 and TRX2) and a mitochondrial type (TRX3). TRX1 and TRX2 regulate cellular redox homeostasis,[13)] and TRX3 protects against the oxidative stress generated during respiratory metabolism.[14)] Although these three TRX isoforms exhibit similar redox activity, the proportion of each isoform in

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Abbreviations: TRX, thioredoxin; OM, ovomucoid; NTR, NADPH-dependent thioredoxin reductase; ASA, active systemic anaphylaxis; PCA, passive cutaneous anaphylaxis; OA, ovalbumin; mBBr, monobromobimane; PMSF, phenylmethyl-sulfonyl fluoride; FCA, Freund's complete adjuvant; GSH, glutathione; TRR, thioredoxin reductase

Saccharomyces cerevisiae is unknown due to the lack of specific monoclonal antibodies.

We established a non-chemical method to prepare yeast TRX-enriched extracts on a large and practical scale, and to check the quality of our extracts, we determined the content of each TRX isoform by using antibodies developed in the study. We examined the effect of recombinant TRX on the digestibility of β-lactoglobulin and OM, and investigated whether the yeast extracts would have a similar effect. We also examined whether yeast TRX could mitigate the allergenicity of OM by measuring the immediate hypersensitivity response in active systemic anaphylaxis (ASA) and passive cutaneous anaphylaxis (PCA) tests on guinea pigs. Based on the results of these tests, we propose the application of yeast TRX-enriched extracts as food materials for mitigating the allergenicity of foods.

Materials and Methods

Materials. Purified recombinant human TRX1, yeast TRX2, yeast NTR and NADPH were obtained from Oriental Yeast Co. (Tokyo, Japan). β-Lactoglobulin, OM, monobromobimane (mBBr), pepsin-agarose, trypsin-agarose and chymotrypsin-agarose were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other biochemicals, reagents, and enzymes were of analytical grade.

Animals. Hartley guinea pigs and Balb/c mice were purchased from Charles River Laboratories Japan (Kanagawa, Japan). These animals were housed at 23 ± 3 °C and 40–60% humidity under a 12-h light cycle, with access to solid laboratory feed (Oriental Yeast Co.) and tap water *ad libitum*. All animal experiments were approved by the Animal Care and Use Committee at Oriental Yeast Co., and the animals were treated according to the guidelines issued by this committee.

Treatment with the TRX system and protease digestion. β-Lactoglobulin or OM in PBS was reduced by treating with human TRX1, yeast TRX2, or a yeast extract with or without yeast NTR and NADPH. The reactions were carried out at 37 °C. The TRX-treated or untreated allergen was subjected to protease digestion. Trypsin and chymotrypsin digestion was conducted by incubating the allergens with the respective proteases coupled to agarose in 20 mM Tris–HCl (pH 8.0) for the indicated time at 30 °C. The reaction was stopped by removing the protease by centrifugation at $800 \times g$ for 10 min. Pepsin digestion of the allergens was carried out by incubating with pepsin-agarose in simulated gastric fluid (70 mM NaCl at pH 1.2) at 37 °C, stopping the reaction by neutralization with 160 mM $NaCO_3$ and removing the protease by centrifugation at $800 \times g$ for 10 min. The digested allergens were separated by SDS–PAGE, using Laemmli's method.[15] After electrophoresis, the gel was stained with a CBB staining kit (Nacalai Tesque, Kyoto, Japan) or a silver staining kit (Cosmo Bio Co., Tokyo, Japan) according to the protocols recommended by the respective manufacturers. β-Lactoglobulin was also detected by western blotting, using the anti-β-lactoglobulin antibody.

Production of yeast TRX-enriched extracts. Industrial baker's yeast, *Saccharomyces cerevisiae*, was cultured, centrifuged to collect the cells, and dried at Oriental Yeast Co. based on company specifications (http://www.oyc.co.jp/en/index.html). The dried yeast powder (50 kg) was suspended in 500 L of water and homogenized with a Dyno-Mill (Shinmaru Enterprises Corp., Osaka, Japan). The powder residue was removed by centrifugation with a disc bowl device (Saito Separator, Tokyo, Japan), and the supernatant was ultrafiltered through a 1000-kDa cutoff membrane. The resulting filtrate was concentrated with a 5-kDa cutoff membrane and then sterilized with a 0.2-μm-pore filter. The extract was freeze-dried for preservation and then diluted in water for each experiment. The TRX content of the product at each stage of the process was measured by an assay of the TRX reducing activity as described later.

Expression and purification of recombinant yeast TRX1 and yeast TRX3. DNA encoding for yeast TRX1 or TRX3 was amplified by PCR with yeast genomic DNA as a template. TRX1-His (5′-CAT CAC CAT CAC CAT CAC ATG GTT ACT CAA TTC AAA ACT GCC AG-3′) and TRX1-SalI (5′-AAA GTC GAC TTA AGC ATT AGC AGC AAT GGC TTG C-3′) were used as primers for amplifying yeast TRX1, and TRX3-His (5′-CAT CAC CAT CAC CAT CAC TCC TCA TAC ACC AGT ATT AC-3′) and TRX3-SalI (5′-AAA GTC GAC CCT GTG TTT CTG AAT ATT GC-3′) were used as primers for yeast TRX3 DNA which lacked a mitochondrial insertion signal sequence. The amplified DNAs were cloned into a pGEM-T vector (Promega, Madison, WI, USA). The cloned DNA coding yeast TRX1 or TRX3 with a His tag was inserted into a pET-28 expression vector (Novagen, Merck, Darmstadt, Germany). *E. coli* BL21 (DE3) was transformed with each vector, and the two transformants were grown at 30 °C and then treated for 4 h with 0.5 mM isopropyl β-D-thiogalactoside. The cells were lysed in buffer A (50 mM NaH_2PO_4 at pH 8.0 and 300 mM NaCl) containing 10 mM imidazole and clarified by centrifugation. The resulting supernatant containing yeast TRX1-His or yeast TRX3-His was loaded into an Ni-NTA agarose column (Qiagen, Hilden, Germany). Each recombinant protein was then prepared in the same way by washing the column with buffer A containing 10 mM imidazole and eluting the fusion protein with a 10–500 mM imidazole gradient in buffer A. The eluted protein was then dialyzed against buffer B (20 mM NaH_2PO_4 at pH 7.5) and loaded into a DEAE-sepharose CL-6B column (GE Healthcare UK, Amersham, UK). The column was washed with buffer B, and the protein was eluted with a 0–450 mM NaCl gradient in buffer B.

Preparation of the monoclonal antibodies against yeast TRX isoforms. Mouse monoclonal antibodies that recognized each yeast TRX isoform were prepared by immunizing Balb/c mice with each purified recombinant. Hybridoma cell lines that secreted antibodies against each yeast TRX were prepared by fusing lymphocytes from the immunized mice with the P3-X63-Ag8-U1 mouse myeloma cell line. Conditioning media for the cells were screened by ELISA, using the three recombinant TRX isoforms as a coating. Hybridomas highly reactive with the isoform used as the antigen without cross-reactivity to the other isoforms were selected and cloned by limiting dilution. Anti-yeast TRX monoclonal antibodies against yeast TRX1 (T1-1D7), TRX2 (T2-2A6) and TRX3 (T3-3D9) were finally prepared and used in the study.

Detection of the yeast TRX isoforms by western blotting. Yeast cell lysates were prepared by sonication. Cells (150 mg wet weight) were incubated in a lysis buffer (50 mM NaH_2PO_4 at pH 7.4, 1 mM EDTA, 5% (v/v) glycerol, and 1 mM PMSF) and cleared by centrifugation at $20{,}000 \times g$ for 10 min at 4 °C. These lysates were measured for their total TRX content by an assay of the TRX reducing activity. The lysates, three recombinant yeast TRXs, and the yeast TRX-enriched extracts were separated by SDS–PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% skim milk in TBS containing 0.05% Tween 20 (TBS-T), the membrane was incubated for 1 h in a conditioning medium of the hybridoma or with purified anti-yeast TRX monoclonal antibodies (1:5,000), before being incubated with the HRP-conjugated anti-mouse IgG antibody (1:4,000). Chemiluminescence signals were detected with an ECL Plus western blot detection kit (GE Healthcare UK). The amount of each TRX isoform in the yeast TRX-enriched extracts was semi-quantitatively estimated from an analysis of the density of each band by using a computerized densitometer.

Assay of the TRX reducing activity. The total TRX content in each yeast TRX-enriched extract was measured by an assay of the TRX reducing activity. A reaction buffer (100 mM potassium phosphate at pH 7.0, 2 mM EDTA, 1.125 mg/mL of insulin, and 200 μM NADPH) was mixed with a sample (or a dilution series of recombinant yeast TRX2 as standards) to a final volume of 980 μL. Twenty μL of 1 mg/mL of yeast NTR was added to the mixture, and the change in absorbance at 340 nm due to the oxidation of NADPH at 25 °C was monitored.

mBBr labeling of the proteins. Sulfhydryl groups were visualized as their fluorescent mBBr derivatives.[16] mBBr (800 nmol) was added to

200 μg of yeast TRX-treated OM or untreated OM. After incubating for 30 min at room temperature, the reaction was stopped by adding an SDS–PAGE sample buffer (62.5 mM Tris–HCl at pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.01% bromophenol blue) and then incubating for 15 min more at 37 °C. The proteins were separated by SDS–PAGE. After electrophoresis, the gels were placed in 12% trichloroacetic acid for 1 h for fixation and then soaked overnight in 40% (v/v) methanol and 10% (v/v) acetic acid for washing. The gels were placed under UV light to visualize the fluorescent bands. Each gel was stained with a CBB staining kit to compare the amount of protein applied to each lane.

Immunization and induction of active systemic anaphylaxis (ASA). The antigenicity of TRX system-treated OM was measured by using an ASA test. Yeast TRX system-treated OM was prepared by dissolving OM in a reaction buffer (PBS, 0.8 mg/mL of yeast TRX2, 0.7 mg/mL of yeast NTR, and 42 mM NADPH) at a final concentration of 10 mg/mL, and then incubating the solution for 1 h at 37 °C. Untreated OM was prepared by dissolving OM in PBS at a final concentration of 10 mg/mL and incubating for 1 h at 37 °C. Male Hartley guinea pigs (200–250 g body weight) were used after a 2-week quarantine and acclimatization period. Two groups of 10 animals each were injected with yeast TRX system-treated OM as a sensitizing or challenge antigen. Five animals sensitized and challenged with untreated OM were used as a control group. The guinea pigs were sensitized for 3 weeks by a weekly subcutaneous injection of 2 mg/kg body weight of yeast TRX system-treated OM or untreated OM with Freund's complete adjuvant (FCA; Difco Laboratories, Detroit, MI, USA). An ASA challenge was performed 14 d later by an intravenous injection of 2 mg/kg body weight of yeast TRX system-treated OM or untreated OM, and the appearance times for anaphylactic shock and mortality were measured for 24 h.

Induction of passive cutaneous anaphylaxis (PCA). Blood from the guinea pigs sensitized as just described was withdrawn from the heart on the day before the ASA challenge. The sensitized serum from the guinea pigs was separated by centrifugation at 1,500 × *g* for 15 min at 4 °C. Serum from the test with TRX system-treated OM was diluted 2000, 4000, 6000, and 8000 times with 0.9% NaCl. Each 50 μL of the diluted serum samples was intradermally injected into a site on the dorsal skin of untreated guinea pigs. At 4 h after the serum injection, the guinea pigs were intravenously injected with 10 mg/kg body weight of Evans blue and then immediately challenged with 2 mg/kg body weight of TRX system-treated OM or untreated OM. The skin was exfoliated after 30 min, and the blue dye leakage was examined. The PCA titer is expressed as the maximum dilution causing a skin lesion >5 mm in diameter [(major axis + minor axis)/2].

Toxicity tests on the yeast TRX-enriched extracts. The toxicity of the yeast TRX-enriched extracts was examined by using a bacterial reverse mutation test, single oral dose toxicity test, ASA test and PCA test. See supplemental information for details of our experimental procedures (*Biosci. Biotechnol. Biochem.* Web site).

Statistical analysis. A statistical analysis was performed by using Dunnett's test, a p value <0.05 being considered significant.

Results

Effects of human TRX on the digestibility of β-lactoglobulin

del Val *et al.* have reported that reduction by TRX, NTR and NADPH improved the digestibility of β-lactoglobulin, a milk allergen.[12] We examined the digestibility of β-lactoglobulin reduced by human TRX, yeast NTR and NADPH (referred to as the human TRX system) to confirm this finding. We had ascertained by an assay of the TRX reducing activity that human TRX could cross-react with yeast NTR (data not shown). Treatment with the human TRX system caused a decrease in the band for β-lactoglobulin, consistent with the result of the previous study (Fig. 1, lanes 1–3). Glutathione (GSH), a peptide thiol, was much less effective, if any, than the human TRX system for increasing the digestibility of β-lactoglobulin (Fig. 1, lanes 4–7).

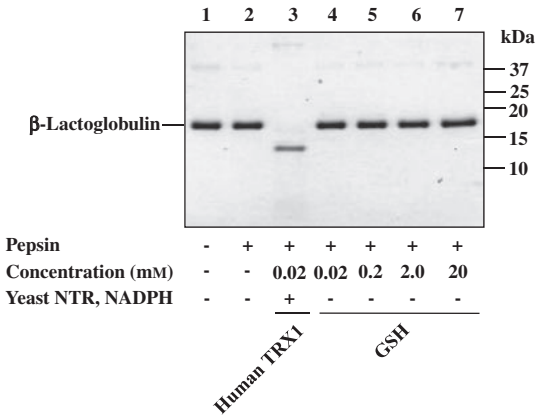

Fig. 1. Effects of Treating with the Human TRX System on the Digestibility of β-Lactoglobulin.

β-Lactoglobulin (0.75 mg/mL) was incubated for 0.5 h at 37 °C with 0.02 mM human TRX, 42 mM NADPH and 0.2 mg/mL of yeast NTR, or with GSH, and then digested for 1 h at 37 °C with pepsin-agarose. After stopping the reaction by removing pepsin by centrifugation, the digested samples were subjected to SDS–PAGE. The gel was stained with a CBB staining kit.

Table 1. Preparation of the Yeast TRX-Enriched Extracts for Foods

	Volume (L)	Protein (g)	TRX (g)	Purity[d] (%)
Extract[a]	315	1,031	6.9	0.7
1000 K[b]	296	278	5.7	2.1
5 K[c]	9	183	3.0	1.6
Freeze-dried powder	602 g	167	2.9	1.7

[a]The supernatant separated from the homogenate of 50 kg of yeast powder in 500 L of water.
[b]The filtrate of "a" after ultrafiltration with a 1000-kDa cutoff membrane.
[c]The concentrate of "b" filtered through a 5-kDa cutoff membrane.
[d]The percentage yeast TRX content in the protein.

Preparation of yeast TRX-enriched extracts from yeast

Yeast TRX-enriched extracts were produced as described in the Materials and Methods section and obtained as 602 g of freeze-dried powder. This powder contained 2.9 g of yeast TRX, while an extract of 50 kg of yeast powder contained 6.9 g of yeast TRX (Table 1). The percentage yeast TRX content was augmented about twofold (0.7–1.7%) from the extract of yeast powder to the freeze-dried TRX-enriched powder.

Yeast contains three isoforms of TRX. We established monoclonal antibodies against each isoform to discriminate among the isoforms. Figure 2 shows that each hybridoma recognized only the respective recombinant protein used in each immunization and the respective native TRX isoform in the yeast cell lysates by western blotting. Native TRX3 in the yeast cell lysates was hardly detected, suggesting that the yeast contained a smaller amount of TRX3 than of the other TRX isoforms. We had ascertained that the anti-yeast TRX3 monoclonal antibody recognized both recombinant yeast TRX3 and native TRX3 in the yeast cells (data not shown).

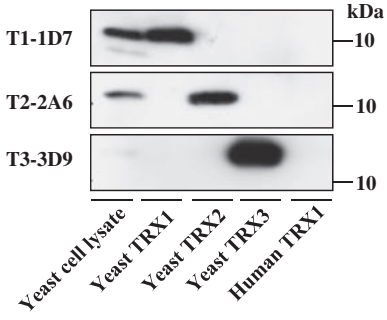

Fig. 2. Characterization of Monoclonal Antibodies against the Three Yeast TRX Isoforms.

The total TRX content in 100 ng was determined from the thioredoxin reduction activity in yeast lysates. The yeast cell lysates, 100 ng of the three recombinant yeast TRX isoforms and 100 ng of human TRX1 were separated by SDS–PAGE and detected by western blotting, using a conditioning medium from each hybridoma.

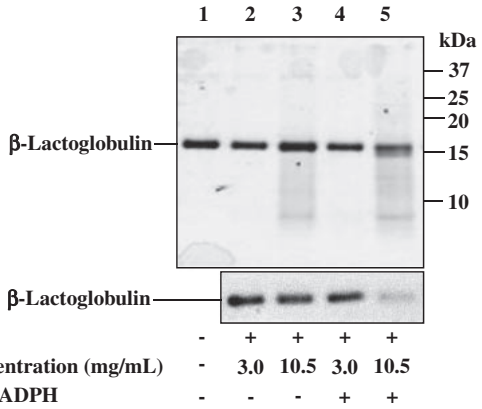

Fig. 3. Effects of the Yeast TRX-Enriched Extracts on the Digestibility of β-Lactoglobulin.

β-Lactoglobulin (0.4 mg/mL) was incubated for 0.5 h at 37 °C with the yeast TRX-enriched extracts (0.02 mM total TRX) with or without 42 mM NADPH and 0.2 mg/mL of yeast NTR, and then digested for 2 h at 37 °C with pepsin-agarose. The digested samples were subjected to SDS–PAGE after stopping the reaction by centrifugation to remove pepsin. β-Lactoglobulin was detected by CBB staining (upper figure), or western blotting (lower figure).

We calculated the isoform contents in the yeast TRX-enriched extracts by the semi-quantitative method for measuring each band density in western blots when using the monoclonal antibodies. The contents in yeast powder were 62.3% for TRX1 and 37.7% for TRX2, with TRX3 being undetectable.

Effects of the yeast TRX-enriched extracts on the digestibility of β-lactoglobulin

Treating with the yeast TRX-enriched extracts did not significantly change the digestibility of β-lactoglobulin (Fig. 3). However, treating β-lactoglobulin with 10.5 mg/mL of the extracts in combination with NADPH and yeast NTR increased the digestibility. These results indicate that TRX could improve protein digestibility in the presence of NADPH and NTR.

Effects of the yeast TRX system on the reduction and digestibility of OM

OM has 9 disulfide bonds, while β-lactoglobulin has only 2. We therefore examined whether the digestibility

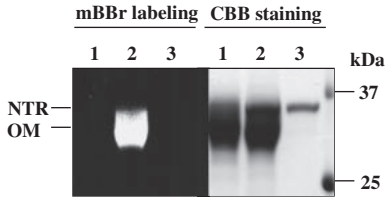

Fig. 4. Reduction of OM by the Yeast TRX System Determined by SDS–PAGE and mBBr Labeling.

OM was labeled with mBBr after incubating under different conditions, and the fluorescent bands were visualized after SDS–PAGE. The gel was stained with a CBB staining kit to determine the amount of protein in each lane. Lane 1, OM alone; lane 2, OM, TRX, NADPH and yeast NTR; lane 3, TRX, NADPH and yeast NTR.

of OM was more dependent on reduction by the yeast TRX system than that of β-lactoglobulin. We first determined whether the yeast TRX system reduced the disulfide bonds in OM by labeling the reduced thiol form with mBBr. Figure 4 shows that OM treated with the yeast TRX system appeared as a fluorescent band, whereas untreated OM was not detected with fluorescence. Both OM samples were detected by CBB staining. These results show that OM was reduced by the yeast TRX system. We next examined the effect of the yeast TRX system on the digestibility of OM by treating the OM samples with pepsin, trypsin or chymotrypsin. Yeast TRX system-treated OM was digested by pepsin after 0.5 h and by trypsin or chymotrypsin after 1 h (Fig. 5A). In contrast, untreated OM was not completely digested by pepsin after 1 h, or by trypsin or chymotrypsin after 5 h. Samples treated with the yeast TRX-enriched extracts were also digested by pepsin, and OM treated with the extracts combined with NADPH and yeast NTR were more digestible than OM treated with the extracts alone, similar to the results for β-lactoglobulin (Fig. 5B). These results suggest that reduction by the yeast TRX system or yeast TRX-enriched extracts with NADPH and yeast NTR improved the digestibility of OM.

Allergenicity of OM treated with the yeast TRX system

We examined whether treatment with the yeast TRX system would contribute to mitigating the allergenicity by OM *in vivo* by identifying the effect of TRX system-treated OM on guinea pigs with ASA and PCA tests. The animals were injected with yeast TRX system-treated OM as a sensitizing antigen and as a challenge antigen, and the anaphylactic symptoms were examined in the ASA test. In the group injected with yeast TRX system-treated OM as the sensitizing antigen, 8/10 animals died, the mean time for appearance of the symptoms being 52.9 s. In the group injected with yeast TRX system-treated OM as the challenge antigen, 3/10 animals died, the mean time for appearance of the symptoms being 66.7 s. In the group sensitized and challenged with untreated OM, all the guinea pigs died, the mean time for appearance of the symptoms being 12.0 s (Table 2). The PCA test showed respective titers for the group injected with yeast TRX system-treated OM as the sensitizing antigen and challenge antigen of 4000–8000 and 2000–6000. The titers were $\geq$8000 in the group

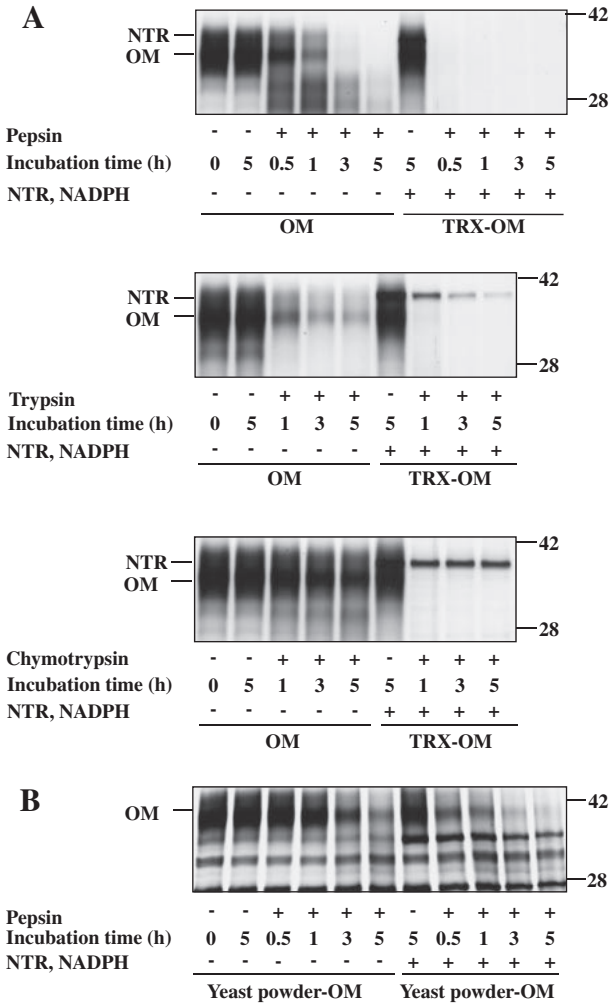

Fig. 5. Effects of the Yeast TRX System and Yeast TRX-Enriched Extracts on the Digestibility of OM.

OM (10 mg/mL) was incubated for 1 h at 37 °C with 0.8 mg/mL of yeast TRX, 42 mM NADPH and 0.7 mg/mL of yeast NTR, and then digested for the indicated time with each protease coupled to agarose (A). OM (10 mg/mL) was incubated for 1 h at 37 °C with 0.8 mg/mL of total TRX in the yeast TRX-enriched extracts with or without 42 mM NADPH and 0.7 mg/mL of yeast NTR, and then digested by pepsin-agarose for the indicated time (B). The digested samples were subjected to SDS–PAGE after stopping the reaction by removing the proteases by centrifugation. The gel was stained with a silver staining kit.

sensitized and challenged with untreated OM (Table 3, Fig. 6). These PCA titers are consistent with the results of the ASA test and suggest slight mitigation of the anaphylactic symptoms in guinea pigs injected with yeast TRX system-treated OM as the sensitizing antigen or challenge antigen.

Toxicity tests on the yeast TRX-enriched extracts

We examined the toxicity of the extracts to investigate the use of the yeast TRX-enriched extracts in foods. A bacterial mutation assay (Ames test) showed a less than twofold increase in reverse mutated colonies at each dose level in the presence and absence of S9 activation when compared with the untreated groups, while a marked increase in the number of colonies was observed in the positive control groups (Supplemental Table 1). A single-dose toxicity test on both male and female rats orally administered with 2000 or 5000 mg/kg of the yeast TRX-enriched extract showed no significant change in body weight compared with the untreated rats, all the rats showing normal general signs (Supplemental Table 2).

The allergenicity of the orally administered yeast extracts was examined by ASA and PCA tests on guinea pigs. The animals sensitized by a subcutaneous injection of ovalbumin (OA) and challenged by an intravenous injection of OA as a positive control all died in the ASA test and showed blue dye leakage in the PCA test. In contrast, none of the guinea pigs died (Supplemental Table 3) or showed anaphylactic signs (data not shown) after oral sensitization with the yeast TRX-enriched extracts and challenge by the same extracts. Moreover, none of these guinea pigs showed any blue dye leakage in the PCA test, indicating that the yeast TRX-enriched extracts given orally did not pose serious allergenicity.

Discussion

Intramolecular disulfide bonds stabilize proteins against heat, acid and proteases, and this may cause the allergenicity of dietary proteins rich in disulfide bonds. TRX has been used in previous studies to reduce these disulfide bonds and mitigate allergenicity,[11,12] although its practical use in foods has not been examined. We tried in this study a non-chemical approach to the use of yeast TRX in food materials to mitigate food allergy; we had some success and identified several problems.

Recent studies on reducing the allergenicity of foods can be assigned to four categories: (i) eliminating the addition of allergenic components in food, (ii) eliminating the allergenicity from allergenic components, (iii) suppressing the allergy by immune tolerance, and (iv) preventing the allergy by controlling the intestinal immune system with such additives as probiotics.[1,17–19] Hypoallergenic foods have mainly been developed by the category (ii) method (*e.g.*, enzymatic hydrolysis, thermal denaturation and fermentation of the allergenic components to denature epitopes). Denaturation of such a food as cow's milk has been widely examined,[20,21] and products are available.[17] Reducing the disulfide bonds in allergenic proteins is an effective tool to denature the epitopes, but is still not available for industrial use. We focused the present study on TRX, because it has high reductive activity[8] and can be conveniently obtained from such edible microbes as yeasts.[22]

TRX is a ubiquitous sulfhydryl compound that is conserved from lower to higher organisms.[8–10] Glutathione (GSH) is another major sulfhydryl compound that is related to redox regulation[23] and is present in cells in millimolar concentrations, higher than that of TRX. However, the rate of reactions catalyzed by TRX is faster than that for dithiothreitol or GSH.[8] TRX is more effective in reducing exposed disulfides in proteins, while the GSH system is more effective in reducing disulfides in small molecules.[23] We observed that 20 mM GSH did not increase the digestibility of β-lactoglobulin (Fig. 1), and del Val *et al.* have found that reducing β-lactoglobulin by the GSH system (GSH, NADPH and glutathione reductase) was much less effective than with the TRX system.[12] TRX is thus more efficient than GSH for reducing allergens according to

Table 2. Effects of Yeast TRX System-Treated OM in an ASA Study on Guinea Pigs

Sensitization	Challenge	Symptoms[a]													
Antigen dose (route), frequency	Antigen dose (route)	a	b	c	d	e	f	g	h	i	j	k	Time to death (min)	Judg-ment[b]	Time to appearance (sec)
TRX-OM + FCA 2 mg/kg (sc), 3 times	OM 2 mg/kg (iv)	+	+	−	+	+	−	−	−	+	−	+	3	III	32
		+	−	−	−	+	−	−	+	−	+	−	−	II	73
		+	+	+	+	+	−	+	−	−	+	+	4	III	44
		−	+	−	+	+	−	+	−	+	−	+	3	III	42
		+	+	−	+	−	−	−	−	+	−	+	4	III	56
		+	+	−	+	+	+	−	+	−	+	−	−	II	88
		+	−	+	+	+	+	−	+	+	−	+	4	III	35
		+	−	−	+	−	−	−	+	+	−	+	3	III	52
		+	+	+	+	+	−	−	−	+	−	+	3	III	49
		+	+	+	+	−	+	−	−	+	−	+	5	III	58
													mean ± SD		52.9* ± 17.1
OM + FCA 2 mg/kg (sc), 3 times	TRX-OM 2 mg/kg (iv)	+	+	+	+	−	−	−	+	+	−	+	6	III	46
		+	+	+	+	+	+	+	−	−	+	−	−	III	85
		+	+	−	+	−	−	−	+	+	−	+	3	III	50
		+	+	+	+	+	+	−	+	+	−	+	5	III	43
		+	+	−	+	−	−	−	+	−	+	−	−	II	77
		+	+	−	+	+	+	−	+	−	+	−	−	II	75
		+	+	+	+	+	+	+	−	+	+	−	−	III	62
		+	+	+	+	+	+	+	−	−	+	−	−	III	68
		+	+	−	+	+	−	−	+	−	+	−	−	II	70
		+	+	−	+	+	+	−	+	−	+	−	−	II	91
													mean ± SD		66.7* ± 16.3
OM + FCA 2 mg/kg (sc), 3 times	OM 2 mg/kg (iv)	+	+	−	+	−	−	−	−	+	−	+	3	III	3
		+	+	−	+	−	−	−	−	+	−	+	3	III	12
		+	+	−	+	−	−	−	−	+	−	+	3	III	19
		+	+	−	+	−	−	−	−	+	−	+	3	III	14
		+	−	−	+	−	−	−	−	+	−	+	2	III	12
													mean ± SD		12.0 ± 5.7

[a]a, licking nose, rubbing nose; b, ruffling fur; c, weakness, diminished tone; d, labored breathing; e, sneezing, coughing; f, retching; g, rales; h, evacuation of feces, micturition; I, convulsion; j, prostration; k, death

[b]0, No detectable symptoms; I, 1 to 3 symptoms; II, 4 to 7 symptoms; III, >8 symptoms, or death. −, negative; +, positive

*Significantly different from the positive control at $p < 0.01$.

FCA, Freund's complete adjuvant

Table 3. Effects of Yeast TRX System-Treated OM in a PCA Study on Guinea Pigs Treated with the Serum of Sensitized Guinea Pigs

Sensitization	Challenge	*N*	PCA titer[a] (number of animals)			
Antigen dose (route), frequency	Antigen dose (route)		2000	4000	6000	≥8000
TRX-OM + FCA 2 mg/kg (sc), 3 times	OM 2 mg/kg (iv)	10		2	2	6
OM + FCA 2 mg/kg (sc), 3 times	TRX-OM 2 mg/kg (iv)	10	1	6	3	
OM + FCA 2 mg/kg (sc), 3 times	OM 2 mg/kg (iv)	5				5

[a]The highest serum dilution giving a positive response.

FCA, Freund's complete adjuvant

these findings. Whereas GSH is already marketed as a functional food material, TRX has not been yet, and this study is the first attempt to use TRX in foods.

We did not adopt the chromatographic method, which is equipment-, effort- and time-intensive, to prepare the yeast TRX-enriched extracts. Instead, we concentrated TRX by filtration (a molecular weight cutoff >1000 kDa and <5 kDa), which is simpler and more effective for industrial-scale preparation, and obtained 4.8 mg of yeast TRX/g of powder (Table 1). Calf liver contains 50–100 μg of TRX/g wet weight of tissue, and a similar TRX level has been found in tissue from male Sprague-Dawley rats.[24,25] TRX in plants has been purified with a yield of 0.5–0.9 mg from 1 kg of material, representing a 3–9% recovery rate.[26,27] We measured the amount of yeast TRX and obtained a level of 50–150 μg of TRX/g wet weight (data not shown). These findings suggest that humans ingest TRX daily at an upper level of hundreds of micrograms/g of food weight. The TRX content of our yeast extract powder is higher than that of other foods, although the purity is low. The digestibility of β-lactoglobulin by treating with the yeast TRX-enriched extracts only was therefore low because of interference to the pepsin reaction by impurities in the extracts

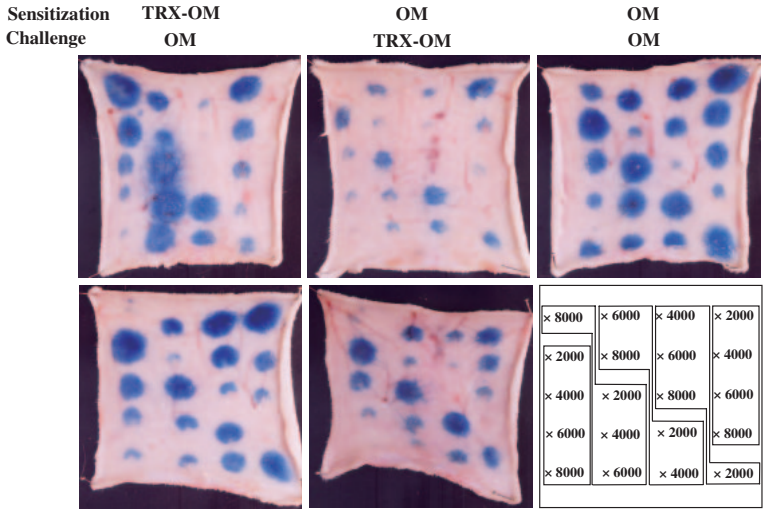

Fig. 6. Effects of Yeast TRX System-Treated OM on Passive Cutaneous Anaphylaxis (PCA) in Guinea Pigs.

The sera of guinea pigs sensitized for 3 weeks by a weekly subcutaneous injection of 2 mg/kg body weight of yeast TRX system-treated OM or untreated OM were diluted 2000, 4000, 6000, or 8000 times, and then intradermally injected into a site on the dorsal skin of untreated guinea pigs. The guinea pigs were intravenously injected after 4 h with 10 mg/kg body weight of Evans blue, and then challenged with 2 mg/kg body weight of TRX system-treated OM or untreated OM. The skin was exfoliated and blue dye leakage was examined after 30 min. One guinea pig was injected with the sera from five animals, and the positions of blue spots derived from individual animals are described on the lower right side of the figure.

(Fig. 3). This problem was solved by adding NTR and NADPH. Oxidized TRX is returned to the reduced form by electrons from NADPH *via* NTR, and reduced TRX can then reduce other proteins.[8,28] TRX can reduce more proteins through its own redox cycle in the presence of NTR and NADPH. Disappointingly, however, the yeast TRX-enriched extracts contained no active yeast NTR (data not shown), because the yeast used to produce the extracts was heated for drying. We found that yeast NTR was inactivated above 60 °C (data not shown), while yeast TRX retained its activity at 60 °C.[29] Improving the methodology to increase the NTR concentration is therefore needed to use the extracts in foods.

The two cytoplasmic isoforms of yeast TRX (TRX1 and TRX2) have 78% amino acid sequence similarity,[30] while the mitochondrial isoform (TRX3) is 50% similar to the homologous region of TRX1 or TRX2.[14] Of the yeast NTR proteins, TRR1 reacts with TRX1 and TRX2, while TRR2 reacts with TRX3 in mitochondria. TRR1 is 84% similar to TRR2,[14] and the yeast NTR used in this study (TRR1) can also react with TRX3 (data not shown). Therefore, an assay of TRX reducing activity cannot discriminate among the three isoforms in the yeast TRX-enriched extracts. Using the monoclonal antibodies obtained in this study showed that the proportions of each isoform in the extracts were approximately 62% for TRX1, 38% for TRX2, and none (undetectable) for TRX3. This suggests that the TRX3 content in yeast cells was much less than that of each other of the two isoforms, or that the method for suspending and homogenizing dried yeast powder in water was not adequate to extract mitochondrial TRX3. Inoue *et al.* have recently demonstrated that TRX containing TRX3 could be efficiently extracted from yeast by incubating with ethanol.[22] TRX may therefore be more efficiently collected by extracting in combination with ethanol, and further improvement in the production of the yeast TRX-enriched extracts is needed. The monoclonal antibodies produced in this study may be useful for quality control of the improved yeast extracts.

Injecting guinea pigs with yeast TRX system-treated OM mitigated the anaphylactic symptoms (Table 2). The injected OM was only treated with yeast TRX, and not with any proteases, suggesting that loosening the protein structure by reduction with TRX resulted in cleavage of the allergenic epitopes. However, this mitigation of anaphylactic symptoms by the TRX treatment cannot be fully explained by a conformational change in OM, because it has been reported that IgG or IgE in sera from patients with allergy to egg mainly recognized the primary structure of OM, rather than the conformation.[31,32] The anti-allergic activity of TRX itself may also play a role, since Hoshino *et al.* have found that injecting recombinant human TRX inhibited airway hyper-responsiveness and airway inflammation in a mouse model of asthma induced by OA.[33] Hoshino *et al.* injected recombinant human TRX at 40 µg per day, whereas yeast TRX system-treated OM used in the ASA and PCA tests in the present study contained 80 µg of yeast TRX per mg of OM. The guinea pigs were therefore injected with 160 µg/kg body weight of yeast TRX simultaneously with OM, and this amount of yeast TRX may have been sufficient to exert the anti-allergic activity. The yeast TRX system-treated OM also contained yeast NTR which might have reacted with TRX of guinea pigs in the blood. The redox system of TRX, NADPH and NTR is ubiquitous from bacteria to humans,[8] and the TRX amino acid sequence in each species has 27–69% homology.[34] TRX of one species can therefore cross-react with NTR of another,[26,35] as demonstrated by the reaction between human TRX and yeast NTR in Fig. 1.

Recent studies have shown TRX itself to be a novel allergenic protein family in baker's asthma.[36,37] The active site of TRX has a conserved sequence (Cys-Gly-Pro-Cys) from *E. coli* to human and is conformationally

stable, because its reducing activity is essential for many organisms. This site may therefore be the origin of the allergenic properties of TRX. We have shown in this study that the yeast TRX-enriched extracts had no allergenicity in toxicity tests, although further examination of this issue is required. On the other hand, TRX injection reduced the sensitivity to OA as already mentioned.[33] Consistent with our observations, Matsumoto *et al.* have demonstrated that TRX-treated salt-soluble wheat allergens mitigated the binding of IgE to wheat in a child with baker's asthma.[38] These findings support our data for yeast TRX being effective in mitigating allergenicity.

The yeast TRX-enriched extracts produced in this study also contained yeast-derived principles other than TRX. Besides TRX having an anti-allergic effect by its own anti-inflammatory activity,[33] our preparation may have possibly contained a minute amount of such other anti-allergenic factors as β-1,3-glucan, a major component of the yeast cell wall involved in innate immunity through binding dectin-1.[39] The possible contribution of such components in coordination with TRX to reducing allergic responses is to be identified in future. The combined results of this study enable us to propose that yeast TRX-enriched extracts could be used for reducing the effects of food allergens, and that the extracts themselves may be useful as functional food for minimizing food allergy.

Acknowledgments

We thank Prof. Mitsufumi Mayumi (University of Fukui, Fukui, Japan) and Prof. Yoshiharu Inoue (Kyoto University, Kyoto, Japan) for helpful discussions and suggestions. This study was supported by a Grant-Aid from the Research and Development Program for New Bio-Industry Initiatives.

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