# ATTACHMENT-12

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**United States Patent** *5,952,034*
**Buchanan , et al.** **September 14, 1999**

# Increasing the digestibility of food proteins by thioredoxin reduction

**Abstract**

Thioredoxin, a small dithiol protein, is a specific reductant for major food proteins, allergenic proteins and particularly allergenic proteins present in widely used foods from animal and plant sources. All targeted proteins contain disulfide (S--S) bonds that are reduced to the sulfhydryl (SH) level by thioredoxin. The proteins are allergenically active and less digestible in the oxidized (S--S) state. When reduced (SH state), they lose their allergenicity and/or become more digestible. Thioredoxin achieved this reduction when activated (reduced) either by NADPH via NADP-thioredoxin reductase (physiological conditions) or by dithiothreitol, a chemical reductant. Skin tests and feeding experiments carried out with sensitized dogs showed that treatment of the food with reduced thioredoxin prior to ingestion eliminated or decreased the allergenicity of the food. Studies showed increased digestion of food and food proteins by pepsin and trypsin following reduction by thioredoxin.

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Appl. No.: **953703**

Filed: **October 17, 1997**

**Current U.S. Class:** **426/656**; 424/94.4; 426/541; 426/549; 426/574; 426/581

**Intern'l Class:** A23L 001/03

**Field of Search:** 426/656,541,549,574,581 424/94.4

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## *Goverment Interests*

This invention was made with government support under Grant Contract Nos. DCB 8825980 and DMB 88-15980 awarded by the National Science Foundation. The United States Government has certain rights in this invention.

## *Parent Case Text*

CROSS-REFERENCE

This application is a continuation-in-part of application Ser. No. 08/326,976 filed Oct. 21, 1994, now U.S. Pat. No. 5,792,506 which is a continuation-in-part of application Ser. No. 08/211,673, filed Apr. 12, 1994, which is a continuation-in-part of application Ser. No. 07/935,002, filed Aug. 25, 1992 (now abandoned), which is a continuation-in-part application of Ser. No. 07/776,109, filed Oct. 12, 1991 (now abandoned).

## *Claims*

What is claimed is:

1. A method of increasing the digestibility of a food comprising:

"ENZYME"

(a) treating a food with an amount of thioredoxin, nicotinamide adenine dinucleotide phosphate-thioredoxin reductase (NTR) and NADPH effective for increasing the digestibility of the food; and

(b) administering the treated food in step (a) to an animal thereby increasing the digestibility of the food as measured by the symptoms exhibited by said animal as compared to a control.

2. The method of claim 1 wherein the amount of thioredoxin is about 0.01 mg to 4.0 mg, NTR is about 0.01 mg to 4.0 mg and NADPH is about 1 micromole to 250 micromoles per gram of protein in said food.

3. The method of claim 1 wherein the amount of thioredoxin is about 0.05 mg to 2.0 mg, NTR is about 0.10 mg to 2.0 mg and NADPH is about 10 micromole to 100 micromoles per gram of protein in said food.

4. The method of claim 1 wherein the amount of thioredoxin is at least about 0.01 mg, NTR is at least about 0.01 mg and NADPH is at least about 1 micromole per gram of protein in said food.

5. The method of claim 1 wherein said food contains milk or wheat.

6. The method of claim 1 wherein said food is milk.

## *Description*

### FIELD OF THE INVENTION

The present invention relates to the use of thiol redox proteins to reduce seed protein such as cereal proteins, enzyme inhibitor proteins, venom toxin proteins and the intramolecular disulfide bonds of certain other proteins. More particularly, the invention involves use of thioredoxin and glutaredoxin to reduce gliadins, glutenins, albumins and globulins to improve the characteristics of dough and baked goods and create new doughs and to reduce cystine containing proteins such as amylase and trypsin inhibitors so as to improve the quality of feed and cereal products.

Additionally, the invention involves the isolation of a novel protein that inhibits pullulanase and the reduction of that novel protein by thiol redox proteins. The invention further involves the reduction by thioredoxin of 2S albumin proteins characteristic of oil-storing seeds. Also, the invention involves inactivating snake neurotoxins and certain insect and scorpion venom toxins in vitro and treating the corresponding toxicities in individuals. The invention also involves using thioredoxin to decrease the allergenicity of food allergens and to increase the proteolysis of food proteins and the digestibility of food.

### BACKGROUND OF THE INVENTION

Chloroplasts contain a ferredoxin/thioredoxin system comprised of ferredoxin, ferredoxin-thioredoxin reductase and thioredoxins f and m that links light to the regulation of enzymes of photosynthesis (Buchanan, B. B. (1991) "Regulation of CO.sub.2 assimilation in oxygenic photosynthesis: The ferredoxin/thioredoxin system. Perspective on its discovery, present status and future development", Arch. Biochem. Biophys. 288:1-9; Scheibe, R. (1991), "Redox-modulation of chloroplast enzymes. A common principle for individual control", Plant Physiol. 96:1-3). Several studies have shown that plants also contain a system, analogous to the one established for animals and most microorganisms, in which thioredoxin (h-type) is reduced by NADPH and the enzyme, NADP-thioredoxin reductase (NTR) according to the following: ##STR1## (Florencio F. J. et al. (1988), Arch. Biochem. Biophys. 266:496-507; Johnson, T. C. et al. (1987), Plant Physiol. 85:446-451; Suske, G. et al. (1979), Z. Naturforsch. C. 34:214-221). Current evidence suggests that the NADP/thioredoxin system is widely distributed in plant tissues and is housed in the mitochondria, endoplasmic reticulum and cytosol (Bodenstein-Lang, J. et al. (1989), FEBS Lett. 258:22-26; Marcus, F. et al. (1991), Arch. Biochem. Biophys. 287:195-198).

Reactions to Foods--AAAI and NIAD Report, 1984, NIH Pub. No. 84-2442, pp. 2, 3). In some countries, the figures are higher, and, throughout the world, the problem is considered to be increasing, especially in infants (T. Matsuda and R. Nakamura 1993 Molecular structure and immunological properties of Food Allergens, Trends in Food Science & Technology 4, 289-293). The problem extends to a wide range of foods. Food allergies in general have recently achieved an increased profile as a result of the concern about transgenic foods.

Milk represents a significant problem, especially in infants. Wheat and soy allergies are of growing importance as new populations adopt these foods and are of increased concern in pet (especially dog) foods. Beef, rice and egg also cause serious allergies in many individuals and again are of significant concern with respect to pet food.

Many of the major allergenic proteins in the above mentioned foods have intramolecular disulfide (S--S) bonds but so far two treatments have been applied commercially to minimize food allergies: (1) heat, and (2) enzymatic proteolysis. In both cases, success has been only partial. While lowering allergenicity, heat treatment has not eliminated the problem, even in the best of cases, because the responsible proteins are typically heat stable. Moreover, heat lowers product quality by destroying nutritionally important amino acids such as lysine, cysteine and arginine. Enzymatic proteolysis is more successful in reducing allergenicity, but desirable food properties such as flavor are usually lost and treatment is costly. Therefore a physiologically safe system that would bring about a decrease in or loss of allergenicity when applied to allergenic foods without a resulting loss in flavor and nutrition would be extremely valuable.

SUMMARY OF THE INVENTION

It is an object herein to provide a method for reducing a non thionin cystine containing protein.

It is a second object herein to provide methods utilizing a thiol redox protein alone or in combination with a reductant or reduction system to reduce glutenins or gliadins present in flour or seeds.

It is also an object herein to provide methods using a thiol redox protein alone or in combination with a reductant or reduction system to improve dough strength and baked goods characteristics such as better crumb quality, softness of the baked good and higher loaf volume.

It is a further object herein to provide formulations containing a thiol redox protein useful in practicing such methods.

Still a further object herein is to provide a method for producing a dough from rice, corn, soybean, barley, oat, cassava, sorghum or millet flour.

Yet, another object is to provide a method for producing an improved gluten or for producing a gluten-like product from cereal grains other than wheat and rye.

It is further an object herein to provide a method of reducing an enzyme inhibitor protein having disulfide bonds.

Still another object herein is to provide yeast cells genetically engineered to express or overexpress thioredoxin.

Still yet another object herein is to provide yeast cells genetically engineered to express or overexpress NADP-thioredoxin reductase.

Still yet a further object herein is to provide a method for improving the quality of dough or a baked good using such genetically engineered yeast cells.

Yet still another object herein is to provide a method of reducing the intramolecular disulfide bonds of a non-thionin, non chloroplast protein containing more than one intramolecular cystine comprising adding a thiol redox protein to a liquid or substance containing the cystines containing protein, reducing the thiol redox protein and reducing the cystines containing protein by means of the thiol redox protein.

Another object herein is to provide an isolated pullulanase inhibitor protein having disulfide bonds and a molecular weight of between 8 to 15 kDa.

Still another object herein is to provide a method of increasing the activity of pullulanase derived from barley or wheat endosperm comprising adding thioredoxin to a liquid or substance containing the pullulanase and reducing the thioredoxin thereby increasing the pullulanase activity.

Still another object herein is to provide a method of reducing an animal venom toxic protein having one or more intramolecular cystines comprising contacting the cystine containing protein with an amount of a thiol redox (SH) agent effective for reducing the protein, and maintaining the contact for a time sufficient to reduce one or more disulfide bridges of the one or more intramolecular cystines thereby reducing the neurotoxin protein. The thiol redox (SH) agent may be a reduced thioredoxin, reduced lipoic acid in the presence of a thioredoxin, DTT or DTT in the presence of a thioredoxin and the snake neurotoxin protein may be a presynaptic or postsynaptic neurotoxin.

Still a further object of the invention is to provide a composition comprising a snake neurotoxin protein and a thiol redox (SH) agent.

Still yet another object of the invention is to provide a method of reducing an animal venom toxic protein having one or more intramolecular cystines comprising contacting the protein with amounts of NADP-thioredoxin reductase, NADPH or an NADPH generator system and a thioredoxin effective for reducing the protein, and maintaining the contact for a time sufficient to reduce one or more disulfide bridges of the one or more intramolecular cystines thereby reducing the protein.

Yet another object herein is to provide a method of inactivating, in vitro, a snake neurotoxin having one or more intramolecular cystines comprising adding a thiol redox (SH) agent to a liquid containing the toxin wherein the amount of the agent is effective for reducing the toxin.

Yet a further object herein is to provide a method of treating venom toxicity in an individual comprising administering, to an individual suffering from venom toxicity, amounts of a thiol redox (SH) agent effective for reducing or alleviating the venom toxicity.

In accordance with the objects of the invention, methods are provided for improving dough characteristics comprising the steps of mixing a thiol redox protein with dough ingredients to form a dough and baking said dough.

Also, in accordance with the objects of the invention, a method is provided for inactivating an enzyme inhibitor protein in a grain food product comprising the steps of mixing a thiol redox protein with the seed product, reducing the thiol redox protein by a reductant or reduction system and reducing the enzyme inhibitor by the reduced thiol redox protein, the reduction of the enzyme inhibitor inactivating the enzyme inhibitor.

BACTERIA DO PRODUCE THIOREDOX.

The thiol redox proteins in use herein can include thioredoxin and glutaredoxin. The thioredoxin includes but is not exclusive of E. coli thioredoxin, thioredoxin h, f and m and animal thioredoxins. A reductant of thioredoxin used herein can include lipoic acid or a reduction system such as NADPH in combination with NADP thioredoxin reductase (NTR). The reductant of glutaredoxin can include reduced glutathione in conjunction with the reduction system NADPH and glutathione reductase. NADPH can be replaced with an NADPH generator or generator composition such as one consisting of glucose 6-phosphate, NADP and glucose 6-phosphate dehydrogenase from a source such as yeast. The NADPH generator is added together with thioredoxin and NADP-thioredoxin reductase at the start of the dough making process.

It should be noted that the invention can also be practiced with cysteine containing proteins. The cysteines can first be oxidized and then reduced via thiol redox protein.

Further in accordance with the objects of the invention, a method is provided for decreasing the allergenicity of an allergenic food protein comprising the steps of contacting the protein with an amount of thioredoxin, NTR and NADPH or an amount of DTT in the presence of thioredoxin effective for decreasing the allergenicity of the protein and administering the contacted protein in step (a) to an animal, thereby decreasing the allergenic symptoms in said animal that would otherwise occur if the animal received the untreated protein.

Another object of the invention is to provide a hypo-allergenic ingestible food. The food was made hypo-allergenic by prior treatment with thioredoxin in the presence of NTR and NADPH. The food can be beef, milk, soy, egg, rice or wheat.

A further object of the invention is to provide a method for improving the proteolysis and therefore the digestibility of food proteins and consequently to also provide more digestible foods. The foods are made more susceptible to proteolysis and more digestible by prior treatment with thioredoxin in the presence of reductants of thioredoxin such as those described above. Appropriate foods include soy, nuts, milk, whey, beef, egg, bread, other wheat products, and other grain products.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a bar graph showing the mitigation of a skin test response to soy allergen by treating soy allergenic extract with reduced thioredoxin.

FIG. 2 is a bar graph showing the mitigation of a skin test response to milk allergen by treating milk allergenic extract with reduced thioredoxin.

FIG. 3 is a bar graph showing the mitigation of a skin test response to wheat allergen by treating wheat allergenic extract with reduced thioredoxin.

FIG. 4 is a bar graph showing the mitigation of a skin test response to beef allergen by treating beef allergenic extract with reduced thioredoxin at room temperature.

FIG. 5 is a bar graph showing the mitigation of a skin test response to beef allergen by treating beef allergenic extract with reduced thioredoxin at 37.degree. C.

FIG. 6 is a bar graph showing the mitigation of a gastrointestinal allergenic response to soy and wheat by treating diets with reduced thioredoxin.

FIG. 7 is a photograph of an SDS polyacrylamide electrophoretic gel showing the extent of thioredoxin-