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Inactivation of Castor Bean Allergen CB-1A by Heating and Chemical Treatment

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Abstract The biological effects of heating and chemical treatment on castor meal were investigated in order to develop a procedure to inactivate its antigenic activity in a way that is suitable for industrial applications. A 1% solution of purified castor bean allergen (CB-1A) was heat-treated with or without exposure to NaOH and NaOCl (250 ppm each). CB-1A exhibited extreme stability when heat-treated alone. In the presence of NaOH and NaOCl, CB-1A showed a drastic decrease in antigenic activity as the temperature surpassed the critical level of 70°C. The gradual disappearance of disc gel electrophoresis bands presumably responsible for the allergenicity of CB-1A, along with the significant losses of the amino acids phenylalanine, methionine, arginine, histidine, and cysteine correlated with the loss of CB-1A activity. CB-1A showed a single symmetrical band in SDS acrylamide gel electrophoresis with an estimated molecular weight of 6,000 daltons. The chemical and heat treatments reduced the disulfide bond content of CB-IA by 9.1% with a coincident increase in sulfhydryl bonds.

Key words: castor meal, allergen, CB-1A, antigen, deallergenation

Introduction

The castor plant *Ricinus communis* L. originated in Asia or Africa, and now grows in Europe and the Americas. The castor bean has a variety of potential uses because it has high content of unique oil. The economics of most oilseed processing plants are based on two major products, oil and meal. However, contrary to the high value of castor oil, castor meal is typically used only as a fertilizer, neglecting its potential value as a feed protein and source of carbohydrate due to the presence of several harmful substances (1-3). A poisonous heat-labile protein called 'ricin', a toxic alkaloid component called 'ricinine', and a powerful and very stable allergen known as CB-1A (4-6) are of major concern in the castor bean industry. In contrast to heat labile ricin and ricinine, the principal castor bean allergen CB-1A is among one of the most heat stable proteins found. In most normal heating conditions, it retains its native antigenic structure, and immune precipitating and allergenic properties (7, 8).

CB-1A was first isolated by Spies and Coulson (9) using the basic lead acetate method. It was found that this allergenic component is heat-stable, soluble in 25% alcohol and lead acetate, precipitated by 75% alcohol, and nontoxic to unsensitized animals. CB-1A is a group of low molecular weight microheterogenous proteins having similar chemical properties (10). It consists of low molecular weight albumin storage proteins from the castor endosperm (11, 12). The CB-1A component compares physico-chemically with the 2S storage protein of seeds. The amino acid composition of CB-1A consists of a relatively high arginine, cystine, and glutamic acid content and the absence of tryptophan (13, 14).

Risks associated with having potent allergen in castor beans and castor pomace are a deep concern to castor bean growers, producers of fertilizers, oil processors, traders, public health officials, and even to residents near such installments (15-21). As a result, deallergenation of castor meal has been proposed by many researchers (22-26). However, practical and effective methods of reducing castor bean allergenic activity have as yet not been reported. This aim of this study is to evaluate changes in the biochemical and immunological properties of the major castor bean allergen, CB-1A, due to heating and chemical treatment.

Materials and Methods

Raw materials Fifty kg of industrially prepressed, hexane defatted castor meal packaged in a craft-paper bag was obtained from Deutsch Rizinus Oelfabrik Baley GMBH & Company (Uerdingen, Germany). The proximate composition of castor meal was: moisture 8.2%, protein 37.3%, oil 1.8%, crude fiber 30.2%, and ash 6.9% as provided by the supplier. All chemicals used in the experiments were reagent grade.

Separation and purification of CB-1A CB-1A antigen was separated from the defatted castor meal and purified according to the procedure outlined by Spies and Coulson (10) with slight modifications as described by Kim (27). The isolation of CB-1A was carried out essentially to obtain the castor meal fraction that was: water soluble, stable in boiling water, soluble in 25% but insoluble in 75% ethanol, and not precipitated by basic lead acetate.

Preparation of CB-1A antiserum (antibody) Purified CB-1A dissolved in physiological saline solution with 'Complete Adjuvant' (28). Fifteen mg of CB-1A was injected subcutaneously into each of seven rabbits (body weight 4-5 kg) once per week. After 3 to 6 weeks, blood was collected and the serum was tested for antibody formation

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using an immunodiffusion technique (28) against the purified CB-1A.

Measurement of CB-1A antigenic activity The antigenicity of CB-1A was determined based upon the double immunodiffusion technique of Ouchterlony as described by Spies and Barren (29) with modifications. The formation of immunodiffusion precipitin lines between antigen and antibody on the gel plate (Fig. 1) after incubation for 24 hr at room temperature was interpreted as the presence of antigenic CB-1A. For quantitative determination, the original CB-1A solution (1.0%) was diluted to 1/2, 1/4, 1/10, 1/50, and 1/100 of the original concentration. The peripheral wells of the agar plate were then filled with these diluted solutions along with the treated samples. After development of the precipitin lines between antibody and antigen, the dilution which most closely matched the precipitin line intensity of the unknown sample was used to estimate the relative amount of antigen present in the unknown sample.

Inactivation of CB-1A antigenic activity A 1% CB-1A solution was prepared in phosphate buffer (pH 6.7) using purified CB-1A powder. To study the effects of chemical and heat treatment on the biochemical and immunological properties of purified CB-1A, 2 mL each of 10% sodium hydroxide (NaOH) and 10% sodium hypochlorite (NaOCl) solution was added to 9.85 mL phosphate buffer to obtain a solution containing 250 ppm of each chemical. The CB-1A solution containing each chemical was then heat-treated in a water bath at 50, 60, 70, 80, 90, and 100°C for up to 10 min. The heated samples were immediately cooled in ice water and analyzed for residual CB-1A antigenic activity by the immunodiffusion method including the dilution technique described above. To analyze changes in CB-1A properties by disc gel electrophoresis, a 1% CB-1A solution was first prepared by dissolving 0.1 g of purified CB-1A in 9.9 mL of Tris buffer (pH 6.7). For the SDS acrylamide gel electrophoresis, 0.01 g of purified CB-IA was dissolved in 10 mL of phosphate buffer (pH 7.1). Aliquots of this solution (1 mL) were then transferred to a 10 mL test tube and heat-treated. Heated samples were then immediately cooled using an ice-water bath and stored at 4°C until electrophoresis.

Disc gel electrophoresis The procedure reported by Pomeranz and Meloan (30) was used for disc gel electrophoresis. Briefly, each protein sample (0.04 mL of 1% solution in Tris buffer, pH 8.6) was loaded on top of the sample gel. Electrophoresis was carried out using a running gel (7.5% acrylamide) at a current of 3 mA/gel for about 2 hr, until the tracking dye was 10 mm from the gel bottom.

SDS-PAGE The procedure reported by Weber and Osborn (31) was used to carry out SDS-PAGE (32). Briefly, each protein sample (0.04 mL of 0.1% solution in phosphate buffer, pH 7.1) was loaded on the top of the running gel (12% acrylamide). Electrophoresis was carried out at a current of 8 mA/gel. Normally, 8 hr was required for the tracking dye to reach ca.10 mm from the gel bottom. The molecular weight of the sample protein (CB-1A) was determined by comparing its electrophoretic mobility (33) with the mobilities of marker proteins with known molecular weights ranging from 2,510 to 16,950 daltons (MW-SDS-17 kit; Sigma Chemical Company, St. Louis, MO, USA). The relative mobility (R_f) was then calculated by dividing the distance of sample migration by the distance of tracking dye migration. The R_f values (abscissa) were then plotted against the known molecular weights (ordinate).

Amino acid analysis Amino acid analysis of purified CB-1A was performed according to the method of Kwon and Yoon (34). Each sample (50 mg) was placed in a glass to which 6 N HCl (6 mL) was added. The samples were sealed with nitrogen and hydrolyzed at 110°C for 24 hr to allow for complete hydrolysis and then filtered through a Millipore filter (0.45 μm, pore size) and brought up to 200 mL with deionized water. Aliquots of 0.5 mL were mixed with 195 mL of HPLC water and then analyzed with a Waters® HPLC (Milford, MA, USA). Tryptophan content was determined by the barium hydroxide analysis method (35) and cysteine was analyzed after oxidation by performic acid.

Sulfhydryl (-SH) and disulfide (-SS) group analysis The procedure of Beveridge *et al.* (36) was used to determine -SH and -SS group content using Ellman's reagent (5,5'-dithiobis-nitrobenzoic acid in Tris-glycine buffer, 4 mg/mL). Purified CB-1A (200 mg) was dissolved in 10 mL of 1% NaCl in Tris-glycine buffer (10.4 g Tris, 6.9 g glycine, and 1.2 g EDTA per liter, pH 8.0). For the analysis of -SH groups, 2.9 mL of 0.5% SDS in Tris-glycine buffer, 0.1 mL of the diluted CB-1A solution, and 0.02 mL of Ellman's reagent were mixed followed by color development. For the analysis of -SS groups, 0.2 mL of diluted CB-1A solution, 1 mL of 10 M urea in Tris-

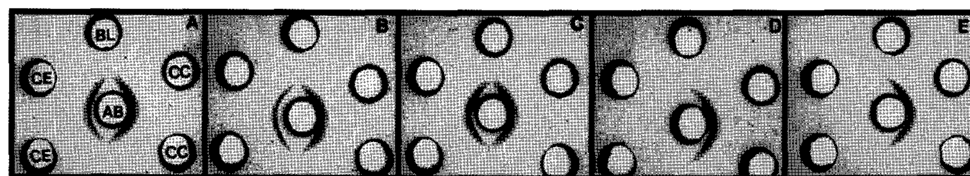

Fig. 1. Immunodiffusion patterns of residual CB-1A as affected by heating at various conditions without (A-B) and with (C-E) chemical treatment. AB, antibody; BL, blank; CC, control, 1% CB-1A solution; CE, chemically-treated (NaOH+NaOCl; 250 ppm, each) and heat treated sample; A, 2-hr heating in boiling water bath; B, 30-min autoclaving; C, control, no heat treatment; D, 20-sec heating in boiling water bath; E, 40-sec heating in 90°C water bath.

glycine buffer, and 0.02 mL of 2-mercaptoethanol were mixed and allowed to stand for 1 hr at 25°C. After an additional 1 hr incubation with 10 mL of 12% TCA solution, the tubes were centrifuged at 5,000×g for 10 min. The precipitate was twice resuspended in 5 mL of 12% TCA solution and centrifuged (5,000×g, 10 min) in order to remove 2-mercaptoethanol. The precipitate was dissolved in 3 mL of 0.5% SDS in Tris-glycine buffer, and 1 mL was then diluted to 10 mL with the same SDS solution. Color was developed by adding 0.05 mL of Ellman's reagent. Absorbance was measured for both –SS and –SH groups at 412 nm on a spectrophotometer.

Results and Discussion

Establishment of a model system Most of the studies reporting on the deallergenation of castor meal include the cooking of castor bean cakes or meals under various conditions of moisture, pH, temperature, steam pressure, and treatment with chemicals like sodium hydroxide, sodium hypochlorite, hydrochloric acid, and so forth.

It is known that commercial castor meal usually contains 0.092-4.2% allergen (6, 37). The routine CB-1A immunodiffusion test procedures previously used by Kim (27) for assaying CB-1A involved dissolving 20 g of castor meal sample in 50 mL of 0.85% saline solution. Thus, in theory, the extract would contain approximately 0.04-1.68% allergen under ideal conditions. In this study, to elucidate a treatment process with a similar CB-1A content, a 1% CB-1A solution was prepared in phosphate buffer (pH 6.7) using purified CB-1A powder.

To verify the effects of chemical treatment on CB-1A activity, 10% NaOH and 10% NaOCl solutions were added to the CB-1A solution to give a final concentration of 250 ppm each, a much milder treatment than those employed in actual deallergenation procedures for castor meal. The reason for using such mild conditions is the possibility that chemicals in model systems might react more freely with CB-1A than actual castor meal treatment, resulting in more effective deallergenation of antigen than in the castor meal.

Effect of heat and/or chemical treatment on the allergenicity of CB-1A Figure 1 shows the immunodiffusion pattern of residual CB-1A following various heat treatments. A pure CB-1A solution lacking any added chemicals showed extreme heat stability. As shown in Fig. 1, thick precipitin lines of immunodiffusion still remained even after 2 hr of heating in boiling water at 100°C (Fig. 1 A) or autoclaving 30 min at 120°C (Fig. 1, B). However, after adding NaOH and NaOCl, the immuno-diffusion precipitin line between antigen and antibody, clearly apparent without heating (Fig. 1, C), was diminished following 20 sec of heating at 100°C (Fig. 1, D) and disappeared completely again following 40 sec of heating at 90°C each (Fig. 1, E). No immunodiffusion precipitation line was further detected with heat-dependent manner when these chemicals were added into the solution. These results indicate that either heat treatment or chemical treatment (with NaOH or NaOCl) alone (Fig. 1, A-C) is not effective, but in combination are sufficient to destroy CB-1A antigenic activity.

Figure 2 demonstrates the qualitative disappearance of the antigenic properties of CB-1A as measured by the immunodiffusion method with dilution. This effect was dependent on the heating temperature and heating time in a stepwise fashion when 1% NaOH and NaOCl solution was added. At 50 and 60°C, no loss of CB-1A activity was observed up to 10 min of heating (Fig. 2). But at 70°C, the antigenic activity of CB-1A started to decrease at 3 min heating, and became almost undetectable at 3.5 min. At 80, 90, and 100°C, the destruction of CB-1A antigenic activity was so drastic that only 90, 60, and 20 sec, respectively, were necessary to destroy all antigenic activity. This indicates that the antigenic activity of CB-1A decreased almost exponentially with incremental temperature increases of 10°C. These results also show that heat treatment, to be effective, must be at temperatures above a critical level (70°C, in this system), rather than for longer times at temperatures below this level.

Disc gel electrophoresis analysis Figure 3 shows the disc gel electrophoresis patterns of CB-1A as affected by heating time and temperature. In the control (sample A), three major bands that presumably contain allergenicity were easily discernible, but traces of one or more additional bands were also present in the 3.0-4.0 cm region. According to Layton *et al.* (2), five bands visible in the electrophoretic fractionation of soluble antigenic proteins from the seeds of castor bean are thought to be allergenic in humans.

The major bands remained largely unaffected after the sample had been placed in boiling water for 2 hr (sample B). Autoclaving the samples at 120°C for 10 (sample C) to 30 min (sample D) caused a slight diffusion of these bands and resulted in a new distinct band (band in the 3.0-4.0 cm region of sample D), but the general pattern was not greatly altered. Overall, the immunodiffusion lines were detectable for heat-treated samples without added chemicals (samples A-D). In contrast, samples heated with NaOH and NaOCl showed new bands that migrated to the regions of 3.5-5.5 cm in the electrophoresis gel (samples F-H). Significantly, the integrity of existing bands (region 0-3.0 cm, samples A-D) decreased, with the greatest

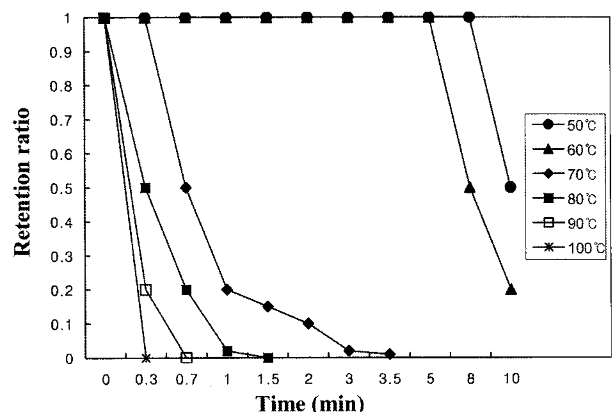

Fig. 2. Effects of heating time and temperature on the destruction of CB-1A antigenic activity in the presence of added chemicals (NaOH+NaOCl; 250 ppm, each).

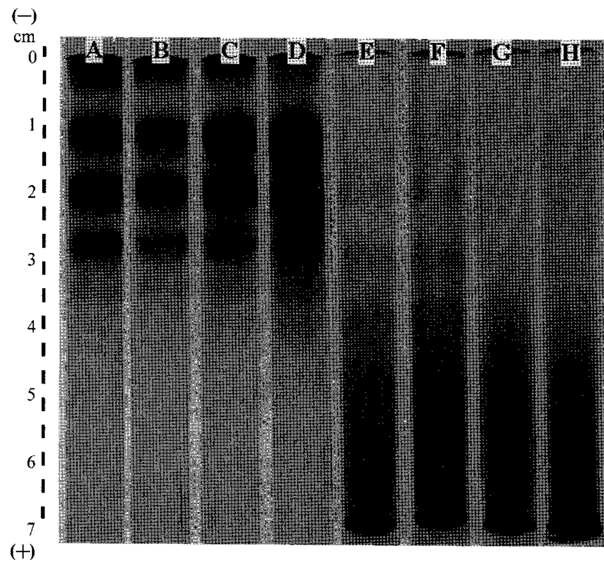

Fig. 3. Disc gel electrophoresis patterns of CB-1A as affected by heating time and temperature with (E-H) and without (A-D) addition of chemicals (NaOH+NaOCl; 250 ppm, each). A, control, no heat treatment; B, 2-hr heating in boiling water bath; C, autoclaving for 10 min; D, autoclaving for 30 min; E, control, unheated; F, 20-sec heating in boiling water bath; G, 40-sec heating in boiling water bath; H, 60-sec heating in boiling water bath.

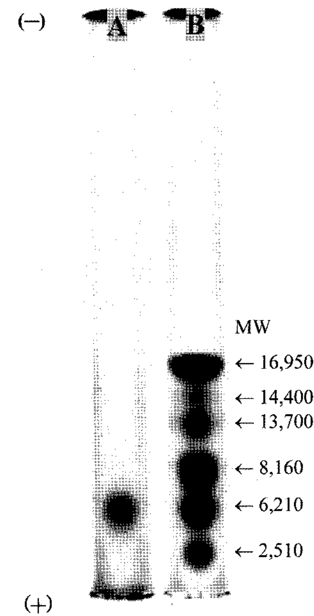

Fig. 4. Molecular weight estimation of CB-1A (A) by SDS polyacrylamide gel electrophoresis using standard polypeptide mixtures (B).

reduction occurring in the bands at upper regions. At the same time, two or possibly more bands appeared in the anodal section (3.5-4.5 cm, sample E). Furthermore, the appearance of a band at R_f 1.0 (bromophenol blue marker front, samples E-H) became very obvious. These changes might have been due to a conformational change caused by chemical breakdown and/or increased exposure of surface-charged groups on the protein (38) as a result of chemical treatment. When the chemically treated samples were heated for longer times (100°C for 20 sec, sample F), the intensity of the original bands decreased sharply and was accompanied by the appearance of additional bands in the regions of 3.5-6.0 cm as shown in sample F. After heating for 40 sec (sample G), the bands in the 1.0-3.0 cm region became difficult to discern, and at the same time, a broadly diffused band was visible throughout the gels. These changes in banding pattern coincided with the degradation of antigenicity as shown in Fig. 1 (D and E). The disappearance of bands in the cathode section, being accompanied by the simultaneous loss of antigenic properties, indicates that the bands in the 0-3.5 cm region are primarily responsible for the antigenic properties of CB-1A.

SDS-PAGE analysis Sodium dodecyl sulfate is usually used to resolve and characterize the number and size of protein chains or subunits in a protein preparation (39). Purified CB-1A protein was analyzed for its homogeneity and molecular weight estimation using the SDS electrophoresis technique. The SDS gel electrophoresis pattern of CB-1A, regardless of chemical and/or heat treatment, showed only a single symmetrical band whose width is constant with each treatment as shown in sample A, Fig. 4. It was recognized that CB-1A and the other 1A fractions from different sources contained the principal allergenic components from their respective source materials and are a complex mixture of low molecular weight proteins and glycosylated proteins (10-12, 29). The single band indicates that the isolated CB-1A from defatted castor meal is in a highly purified form of protein subunits with similar molecular and electrochemical properties. It is also possible that CB-1A is composed of a single polypeptide chain or a group of identical subunits, the latter of which is not a common characteristic of proteins.

It is known that during SDS-PAGE, the electrophoresis velocity is dependent on the retardation coefficient (*Kr*) of the protein, which is related to size and shape (39). The fact that the position of the CB-1A protein band is unchanged following heating with sodium hydroxide and sodium hypochlorite indicates that no dissociation or cleavage of the protein occurred as a result. The bracketing of CB-1A in the linear plot by its molecular mobilities showed that the estimated molecular weight of the single peptide chain of CB-1A is approximately 6,000 daltons (Fig. 5). This estimate is supported by Lehrer *et al.* (40) who reported that the heat-labile allergen in castor bean was of a large molecular weight while the heat-stable allergen was of a smaller molecular weight.

Amino acid analysis The amino acid composition of purified CB-1A was determined before and after the loss of its antigenic activity (Table 1). Results showed that CB-1A contains large amounts of glutamic acid (32.78%) and arginine (12.84%), followed by cysteine (7.25% as cysteic acid) and serine (6.75%). Castor bean protein isolate also contains a high amount of glutamic acid, aspartic acid, and arginine (13). The relatively high proportions of arginine

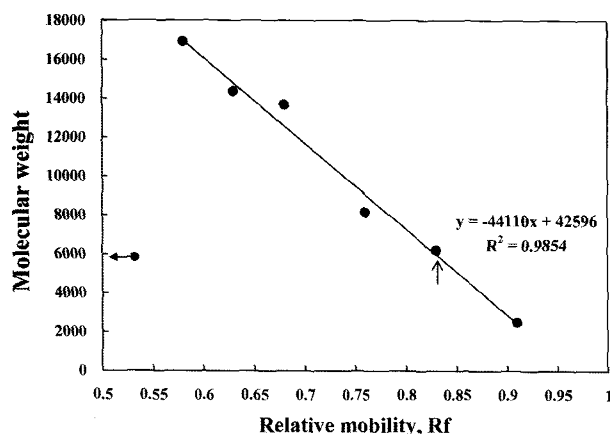

Fig. 5. Calibration curve obtained from SDS polyacrylamide gel electrophoresis with polypeptides from a commercial molecular weight determination kit and purified CB-1A.

and cystine in CB-1A were similar to the results of Coulson *et al.* (8). The amino acid composition of CB-1A changed upon chemical and heat treatments resulting in the loss of antigenicity.

Significant reductions of phenylalanine (1.59 to 0%), methionine (1.59 to 0.03%), arginine (12.84 to 9.84%), histidine (1.05 to 0.55%), and cysteine (7.25 to 1.92%) from the original were noteworthy although the changing mechanism is unknown this time. However, the relationship between these specific amino acids and the allergenicity of CB-1A is not known at this time.

Sulfhydryl (-SH) and disulfide (-SS) group analysis Sulfhydryl (-SH) groups, unless masked as in some proteins, are the most chemically active groups found in the cells. Disulfide (-SS) groups formed from specific cysteine residues are important components of the active sites of many enzymes and -SH groups are capable of reacting with heavy metals or reagents containing heavy metals. Disulfide groups are much less active than sulfhydryl groups and function as stabilizing elements in the structure in proteins. Protein conformation is highly sensitive to minor changes in structure. A minor change in conformation often exerts appreciable effects on the biological function of a protein. For example, trypsin inhibitors became inactive upon the cleavage of -SS bonds, which often control protein structure (41).

The -SH and -SS contents of purified CB-1A were determined before and after chemical and heat treatments under identical conditions to those used in the amino acid profile determination above. The results in Table 2 show that almost no -SH was detected in CB-1A whether or not it was treated. The data also show that the chemical and heat treatments reduced the -SS content of CB-1A by 9.1%, but this value gives insufficient information to explain the possible role of -SS bonds in CB-1A antigenic activity. To confirm the accuracy of the measurement, data for ovalbumin are also included as a reference. For ovalbumin, the value for -SH was about 4 times larger than that of -SS which is in excellent agreement with the published data that ovalbumin contains 4 moles of -SH and 1 mole of -SS per mole of protein (36). In addition, the amount of -SH and -SS groups present in CB-1A at this point cannot be calculated exactly since additional data on molecular absorptivity (or the molecular extinction coefficient) of the purified CB-1A is needed. Further research in this area is necessary.

Table 1. Amino acid profiles of CB-1A before and after chemical and heat treatment

Amino acid	CB-1A	
	Untreated	Treated[1)]
Lysine	2.90	2.93
Histidine	1.05	0.55
(Ammonia)	2.65	4.22
Arginine	12.84	9.84
Aspartic acid	3.70	4.56
Threonine	1.02	1.11
Serine	6.75	6.64
Glutamic acid	32.78	37.19
Proline	2.58	2.07
Glycine	3.91	4.57
Alanine	2.36	2.70
Valine	3.32	3.77
Methionine	1.59	0.03
Isoleucine	3.07	3.16
Leucine	4.68	4.98
Tyrosine	1.72	0.20
Phenylalanine	1.59	-
Cysteic acid	7.25	1.92
Tryptophane	0.55	0.72
Total	88.51	88.52

[1)]Heated at 100°C for 40 sec with added NaOH and NaOCl, 250 ppm each.

Table 2. Relative contents of sulfhydryl (-SH) and disulfide (-SS) bonds in CB-1A before and after chemical and heat treatment (Unit: absorbance)

	-SH(A_{412})	-SS(A_{412})
CB-1A		
Control	0	0.71
Treated[1)]	0.01	0.65
Ovalbumin	0.73	0.20

[1)]Heated at 100°C for 40 sec with added NaOH and NaOCl, 250 ppm each.

Acknowledgments

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