# Heat-Induced Formation of Intermolecular Disulfide Linkages between Thaumatin Molecules That Do Not Contain Cysteine Residues

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Thaumatin, a sweet protein that contains no cysteine residues and eight intramolecular disulfide bonds, aggregates upon heating at pH 7.0 above 70 °C, and its sweetness thereby disappears. The aggregate can be solubilized by heating in the presence of both thiol reducing reagent and SDS. This molecular aggregation depended on the protein concentration during heating and was suppressed by the addition of *N*-ethylmaleimide or iodoacetamide, indicating a thiol-catalyzed disulfide interchange reaction between heat-denatured molecules. An amino acid analysis of the aggregates suggested that the cysteine and lysine residues were reduced, and the formation of a cysteine residue and a lysinoalanine residue was confirmed. The reduction and formation of these residues stoichiometrically satisfied the $\beta$-elimination of a cystine residue. The disulfide interchange reaction was catalyzed by cysteine; that is, a free sulfhydryl residue was formed via $\beta$-elimination of a disulfide bond. Intermolecular disulfide bonds were probably formed between thaumatin molecules upon heating at pH 7.0, which led to the aggregation of thaumatin molecules.

**Keywords:** *Thaumatin; heating; β-elimination; aggregation; thiol/disulfide exchange*

## INTRODUCTION

Thaumatin is a sweet protein with a threshold of sweetness of ~48 nM, which is $10^5$-fold lower than that of sucrose on a molar basis (Van der Wel and Loeve, 1972). Thaumatin is obtained from the berry of the West African plant *Thaumatococcus daniellii* Benth (Van der Wel and Loeve, 1972). Although there have been many reports on its properties, structure, and modification of its amino acid residues (Van der Wel, 1994), the mechanism of its sweetness as well as its thermostability has not yet been clarified.

Thaumatin is commercially available as a sweetener and has been readily accepted as a food material because it is a natural protein (Etheridge, 1994); it is believed to be heat-resistant (Staff report, 1996) on the basis of its structural characteristics, that is, eight intramolecular disulfide bonds and no cysteine residue (Van der Wel et al., 1984). These disulfide bonds might be responsible for the formation of a higher order structure that provides stability against denaturation by physical treatment and proteolytic attack (Shamli et al., 1990). Unfolding of the protein molecule at extremely high pH and temperature was investigated in detail using differential scanning calorimetry and spectrophotometry (Lozano et al., 1997; Poklar et al., 1997; Staniforth et al., 1998; Chalikian et al., 1997), whereas the aggregation of partially unfolded proteins has not been investigated extensively (Babu and Bhakuni, 1997; Bohidar, 1998; Shirai et al., 1997).

It is generally accepted that thiol/disulfide exchange reactions, which lead to the formation of intermolecular disulfide bonds, play an important role in the thermal aggregation of protein molecules, and free cysteine residues are needed for such reactions (Shimada and Cheftel, 1989). However, it has been reported that proteins without cysteine residues aggregate upon heating at high temperature with or without the participation of thiol/disulfide inter- and/or intramolecular exchange (Bohidar, 1998; Korver et al., 1973). For example, lysozyme, which has four cystine residues and no cysteine residue, forms an aggregate at high temperature without the involvement of an intermolecular disulfide bond (Babu and Bhakuni, 1997).

Thaumatin contains no cysteine residues but many intramolecular disulfide bonds (Van der Wel and Loeve, 1972; Van der Wel et al., 1984). The sweetness of thaumatin disappears and an aggregate forms upon heating at a high temperature and/or for a long duration (Korver et al., 1973). The mechanism of this thermal inactivation of thaumatin has not been clarified, including the contribution of thiol/disulfide exchange to aggregate formation. In this study, the relationship between thermal denaturation/coagulation and loss of sweetness of thaumatin was investigated, with a particular focus on the formation of disulfide linkages among thaumatin molecules.

## MATERIALS AND METHODS

**Materials.** Crude thaumatin powder was obtained from San-Ei Gen F.F.I., Inc. (Osaka, Japan). Bicinchoninic Acid (BCA) protein assay reagent was purchased from Pierce (Rockford, IL). $^{14}$C-Labeled iodoacetamide and EN$^3$HANCE (autoradiography enhancer) were from DuPont/NEN Life Science Products (Boston, MA), and X-Omat AR film was from Kodak (New York, NY). *S*-(Carboxymethyl)cysteine was obtained from Aldrich Chemical Co. (Milwaukee, WI). Lysinoalanine was purchased from Sigma Chemical Co. (St. Louis, MO). Amino acid standard solution (Type H) was from Wako Pure Chemical Industries (Osaka, Japan). Ammonium sulfate, urea,

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iodoacetamide (IAM), *N*-ethylmaleimide (NEM), 2-mercaptoethanol (2ME) of specially prepared reagent grade, and other chemicals of guaranteed reagent grade were obtained from Nacalai Tesque (Kyoto, Japan).

**Purification of Thaumatin.** Thaumatin I was purified from crude thaumatin by SP-Sephadex C-25 (Pharmacia, Uppsala, Sweden) ion exchange column chromatography (Van der Wel and Loeve, 1972) and Sephadex G-75 superfine (Pharmacia) gel filtration column chromatography. The resulting thaumatin I sample gave a single band on both sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and nondenaturing polyacrylamide gel electrophoresis (native PAGE) and was stored as a precipitate from ammonium sulfate at 4 °C until use. This purified thaumatin I was used throughout the study and is referred to as simply thaumatin hereafter.

**Protein Concentration.** The protein concentration of native thaumatin was measured spectrophotometrically at 278 nm based on $E^{1\%}_{1cm} = 7.69$ (Van der Wel and Loeve, 1972). For heated thaumatin, the protein concentration was determined according to the BCA procedure using native thaumatin as a standard.

**Heating of Thaumatin.** Thaumatin stored as a precipitate was desalted by dialysis against 5 mM sodium phosphate buffer, pH 7.0, at 4 °C. The pH of thaumatin solution after dialysis was adjusted to 7.0 by adding dilute NaOH. The protein concentration of thaumatin solution was adjusted to a given value with 5 mM sodium phosphate buffer, pH 7.0. After the protein concentration was adjusted, 1 mL of the solution was heated at a given temperature for 15 min in a 2.0-mL screw-top polypropylene microcentrifuge tube (Quality Scientific Plastics, San Francisco, CA). To see the change in the amount of specific amino acid residues upon heating, the sample was heated for various durations. After heating, samples were cooled in a water bath at 15−20 °C for 15 min.

**PAGE.** SDS−PAGE of thaumatin was carried out according to the method of Laemmli (1970). The samples were mixed with an equal volume of 2× reducing SDS sample buffer (0.1 M Tris-HCl, pH 6.8, 4% SDS, 0.6 M 2ME) and then boiled at 100 °C for 5 min to denature protein samples. In nonreducing SDS−PAGE, 20 mM IAM was added instead of 2ME. Native PAGE was performed using a system for basic protein (Reisfeld et al., 1962). Five micrograms of protein sample was loaded onto each lane of 15% homogeneous SDS polyacrylamide gel or 7.5% homogeneous native polyacrylamide gel. After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250.

**Autoradiography.** Autoradiography was performed as follows. The samples were subjected to reducing SDS−PAGE. The gel after electrophoresis was treated with EN$^3$HANCE according to the supplier's instructions to intensify the screen and then dried. The dried gels were exposed to Kodak X-Omat AR film at room temperature for 18 days.

**Acid Hydrolysis and Amino Acid Analysis.** A protein sample of 3 nmol was hydrolyzed in the gas phase with 6 N HCl containing 1% (v/v) phenol for 22 h at 110 °C under vacuum. After hydrolysis, HCl was evaporated and the residue was dissolved in sodium citrate buffer, pH 2.2. An amount of sample equivalent to 1.5 nmol of thaumatin was analyzed on an L-8500A high-speed amino acid analyzer (Hitachi, Tokyo, Japan). The amino acids were detected with postcolumn derivatization with ninhydrin and determined using an amino acid standard solution supplemented with *S*-(carboxymethyl)-cysteine as a standard. Cystine residue was determined as *S*-(carboxymethyl)cysteine, after reduction, carboxymethylation, and acid hydrolysis of the sample. Lysinoalanine residue was determined with authentic lysinoalanine as a standard.

**Determination of Dehydroalanine.** First, dehydroalanine was converted to pyruvate by acid catalysis, and the resulting pyruvate was measured with lactate dehydrogenase. Using this method, quantities below 0.1 μmol could not be detected (Patchornik and Sokolovsky, 1964; Sokolovsky et al., 1964).

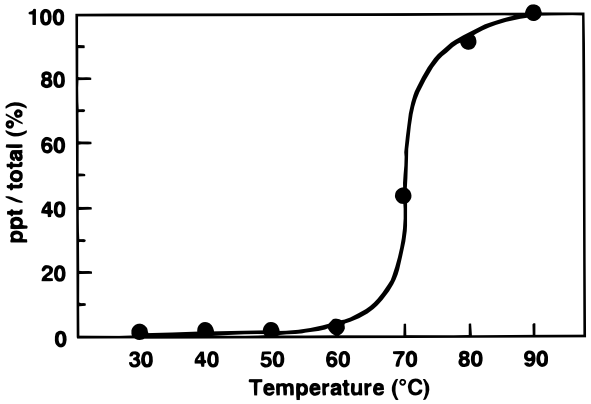


**Figure 1.** Temperature dependence of the precipitation of thaumatin. Thaumatin solution (45 μM, pH 7.0) was incubated at various temperatures. After cooling, the samples were centrifuged to collect the precipitate. The percentage of thaumatin precipitated was plotted against the temperature.

**Sensory Analysis of the Sweetness of Thaumatin.** Five milliliters of test solution was added to paper cups that were coated with polyethylene and kept at room temperature. The protein concentration of the test solution was adjusted with 5 mM sodium phosphate buffer, pH 7.0, and ranged from 5 to 500 nM. A well-trained subject tested the sweetness of the sample solution from the lowest to the highest concentration of thaumatin to determine the threshold of sweetness. The subject has normal response to native thaumatin because he detected sweetness of native thaumatin at 50 nM, which is almost the same value reported previously (Van der Wel and Loeve, 1972). After tasting the sample, the subject thoroughly rinsed his mouth with distilled water and 0.1 M NaCl solution until no aftertaste was detected.

## RESULTS

**Precipitation of Thaumatin by Heating.** Thaumatin solutions (45 μM, pH 7.0) were heated from 30 to 90 °C for 15 min. After cooling to 15−20 °C, the resulting precipitate was collected by centrifugation (5000*g*, 10 min, 4 °C). The amounts of protein recovered in both the supernatant and precipitate were determined. Figure 1 shows the percentage of protein precipitated at each temperature plotted against the heating temperature. No protein was detected in the precipitate fraction with incubation below 60 °C. The amount of protein in the precipitate fraction markedly increased above 70 °C. The precipitate fraction was not sweet, whereas the supernatant fraction was determined to be sweet at 500 nM, which is 10 times higher than the concentration of native thaumatin.

The effects of protein concentration on thermal precipitation are shown in Figure 2. Thaumatin solutions from 0.5 to 45 μM were heated at pH 7.0 and 80 °C for 15 min. The amount of protein in the precipitate fraction increased as the protein concentration of the original solution increased, and most of the proteins aggregated and precipitated above 40 μM. Precipitation appeared to be caused by aggregation of thaumatin molecules after thermal denaturation.

**Native PAGE and SDS−PAGE Analysis of Heated Thaumatin.** Thermally denatured thaumatin molecules seem to interact with each other by hydrophobic interaction, hydrogen bonding, electrostatic interaction, and/or some other covalent bonding. To clarify the contribution of each effect, the supernatant and precipitate fractions were analyzed by native PAGE, SDS−PAGE under nonreducing conditions, and SDS−PAGE

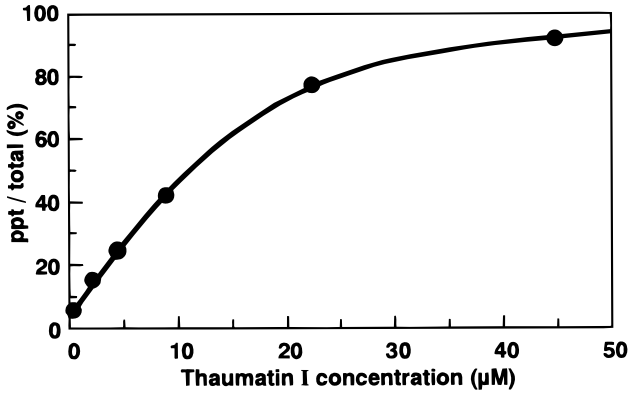


**Figure 2.** Thermal precipitation of thaumatin as a function of concentration. Thaumatin solutions (0.5−45 $\mu$M, pH 7.0) were heated at 80 °C for 15 min.

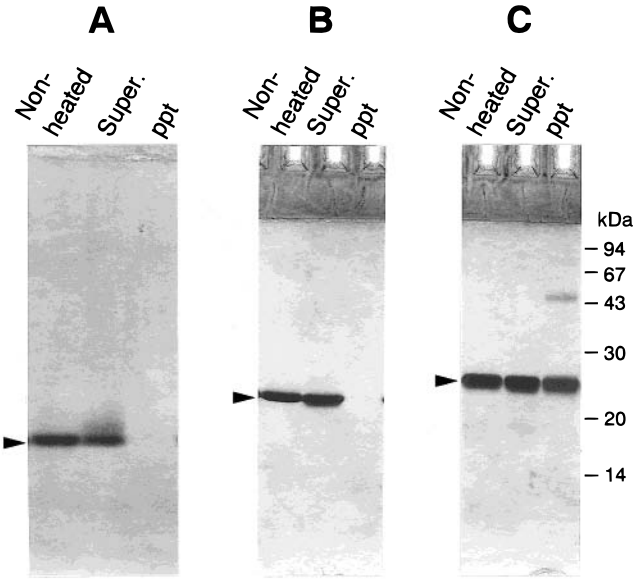


**Figure 3.** Native PAGE and SDS−PAGE analysis of heated thaumatin: (A) native PAGE, 7.5% acrylamide; (B) SDS−PAGE under nonreducing conditions, 15% acrylamide; (C) SDS−PAGE under reducing conditions, 15% acrylamide. Five micrograms of protein was applied to each lane in PAGE. A band of unheated thaumatin is indicated by an arrow.

under reducing conditions. The supernatant fraction gave a single band corresponding to unheated native thaumatin in all three PAGEs (Figure 3). The precipitate fraction gave no band in native PAGE and SDS−PAGE under nonreducing conditions (Figure 3A,B). Upon treatment with 2ME, SDS−PAGE of the precipitate fraction gave several bands. Although most of the protein was in a monomeric state, a faint band corresponding to dimeric thaumatin was also observed (Figure 3C). Native PAGE revealed no aggregate in the supernatant fraction. On the other hand, the precipitate fraction in SDS solution did not give any band by nonreducing SDS−PAGE. Furthermore, precipitate fractions that were incubated at room temperature in either 8 M urea or 6 M guanidine hydrochloride for 1 week also did not give any band in nonreducing SDS−PAGE (data not shown). These results suggest that the intermolecular linkage of thaumatin molecules was due to disulfide bonds induced by heating and gave aggregates of molecules.

**Heat-Induced Disulfide Interchange in Thaumatin.** It has been reported that thaumatin does not

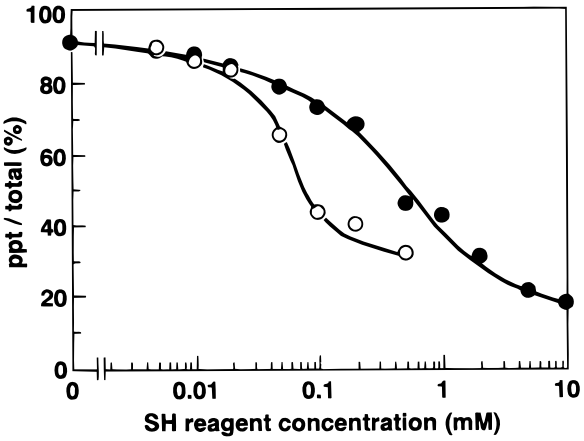


**Figure 4.** Effects of IAM and NEM on the thermal precipitation of thaumatin. After thaumatin solution (45 $\mu$M, pH 7.0) had been heated in the presence of IAM (●) and NEM (○) at 80 °C for 15 min, the amount of precipitate collected by centrifugation was measured.

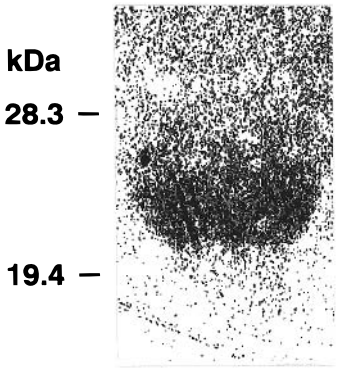


**Figure 5.** Autoradiogram of thaumatin heated with $^{14}$C-labeled IAM. After thaumatin solution (45 $\mu$M, pH 7.0) had been heated with IAM (1.0 mM) at 80 °C for 15 min, the supernatant (100 $\mu$g of protein) was subjected to SDS−PAGE, followed by autoradiography.

contain a cysteine residue (Van der Wel and Loeve, 1972; Van der Wel et al., 1984), which we also confirmed by measuring the number of thiol residues in thaumatin using the 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) titration method in the presence of 8 M urea or 6 M guanidine hydrochloride. However, our electrophoresis experiments with heated thaumatin indicate that disulfide bonds are involved in intermolecular aggregation. To clarify whether a thiol residue appears during heating and is involved in the formation of aggregates, thaumatin solution (45 $\mu$M) was heated at 80 °C for 15 min with IAM (5 $\mu$M to 10 mM) or NEM (5 $\mu$M to 0.5 mM) at pH 7.0. Figure 4 shows that the addition of IAM or NEM suppressed the formation of precipitate in a concentration-dependent manner, suggesting that the thermal precipitation of thaumatin arose from thiol-catalyzed disulfide interchange among thaumatin molecules (Ryle and Sanger, 1955). We then confirmed that IAM was indeed bound to thaumatin as follows. Thaumatin solution was heated in the presence of 1.0 mM $^{14}$C-labeled IAM and then cooled and centrifuged. The supernatant was subjected to reducing SDS−PAGE and autoradiography. As shown in Figure 5, only one band with the same molecular weight as that of thaumatin was detected, indicating that IAM was bound to thaumatin. The most potent amino acid residue that can be modified by IAM is cysteine, which changes to *S*-

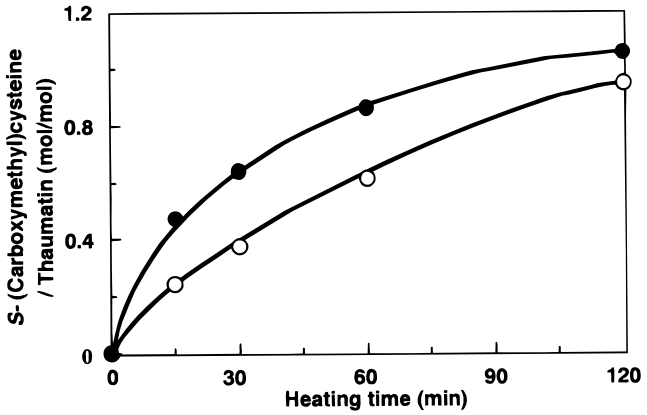


**Figure 6.** Increase in *S*-(carboxymethyl)cysteine residues in thaumatin recovered in the soluble fraction (○) and in the insoluble fraction (●) after heating. Thaumatin solution (45 $\mu$M, pH 7.0) was heated in the presence of IAM (10 mM) at 80 °C, and *S*-(carboxymethyl)cysteine residue was then assayed by an amino acid analysis.

(carboxamidomethyl)cysteine. To confirm the presence of *S*-(carboxamidomethyl)cysteine in thaumatin after heating with IAM for 2 h, heated sample was hydrolyzed and subjected to an amino acid analysis. *S*-(Carboxamidomethyl)cysteine is deamidated during acid hydrolysis and was detected as *S*-(carboxymethyl)cysteine by amino acid analysis. The production of *S*-(carboxymethyl)cysteine residue in both the supernatant and precipitate was then plotted versus the duration of heating (Figure 6). The amount of *S*-(carboxymethyl)-cysteine increased with the duration of heating, strongly indicating the presence of cysteine residues in thaumatin during heating at pH 7.0 and 80 °C. The amount of *S*-(carboxymethyl)cysteine in the precipitate fraction was greater than that in the supernatant fraction. Thus, the precipitate was formed through free cysteine residues that appeared upon heating and catalyzed the intermolecular disulfide interchange reaction, which led to the thermal aggregation of thaumatin molecules.

**Formation of Lysinoalanine Residue from Lysine Residue and Cystine Residue by $\beta$-Elimination.** On heating at pH 7.0, cysteine residue(s) were produced in thaumatin. It has been shown that cystine residues in protein are degraded under alkaline conditions by $\beta$-elimination to form cysteine residues (Florence, 1980). Based on the mechanism of $\beta$-elimination, alkaline conditions and high temperature are favorable for this reaction, although it has been reported that this reaction even occurs at neutral pH at high temperature (Ahern and Klibanov, 1985; Zale and Klibanov, 1986; Tomazic and Klibanov, 1988; Volkin and Klibanov, 1987). Hence, it has been postulated that the $\beta$-elimination of a disulfide bond of cystine induces one persulfide and one dehydroalanine residue, and a lysinoalanine residue is stoichiometrically formed from this dehydroalanine residue and one lysine residue (Bohak, 1964; Masri and Friedman, 1982), whereas one cysteine residue is formed from one persulfide residue (Figure 7) (Florence, 1980; Masri and Friedman, 1982). The amino acid composition of thaumatin after heating was analyzed. The amino acid residues, except for cystine residues and lysine residues, were not changed. The number of cystine and lysine residues in thaumatin decreased with the duration of heating. To count the number of cystine residues in thaumatin, samples were reduced, carboxymethylated, and subjected to acid hydrolysis followed by amino

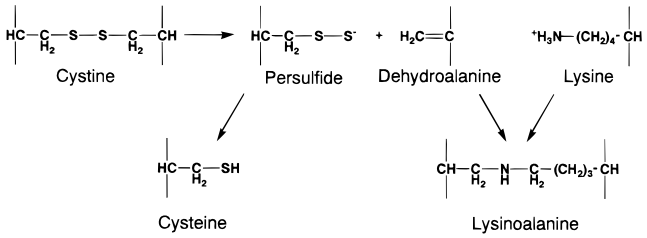


**Figure 7.** Formation of cysteine and lysinoalanine residues from cystine and lysine via $\beta$-elimination of cystine.

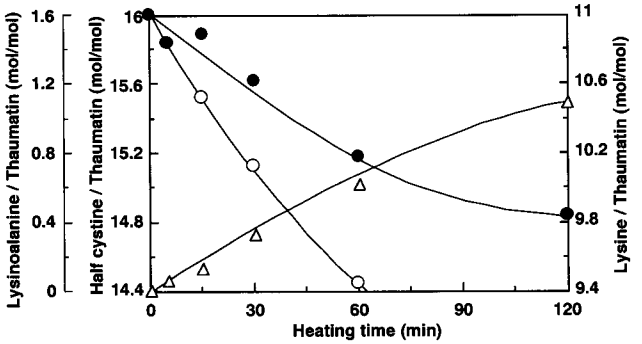


**Figure 8.** Changes in the number of half-cystine residues (○), lysine residues (●), and lysinoalanine residues (△) per mole of thaumatin on heating.

acid analysis. The number of cystine residues was counted as the number of half-cystine residues. Figure 8 shows the decrease in the number of half-cystine residues with heating. After 60 min of heating, ~1.6 mol of half-cystine residues and ~0.8 mol of lysine residues per mole of thaumatin were reduced. Thus, 0.8 mol of cystine residues and lysine residues was degraded in 60 min of heating. About 0.8 mol of lysinoalanine residues appeared after 60 min of heating. According to Figure 7, 1 mol of lysinoalanine residues is formed from 1 mol of lysine residues and 1 mol of dehydroalanine residues. These results are consistent with the stoichiometry of the expected reaction.

## DISCUSSION

In this study, the disulfide bonds in thaumatin were degraded upon heating at pH 7.0. Three different mechanisms have been proposed for the degradation of disulfide bonds: hydrolysis, α-elimination, and $\beta$-elimination (Florence, 1980). In this case, $\beta$-elimination is the most probable mechanism, because the $\beta$-hydrogen of the cystine residue in a peptide linkage is very labile and easily eliminated. On the other hand, it has been reported that hydrolysis and α-elimination of cystine residue in a peptide linkage rarely occur by heating at neutral pH. The identification of lysinoalanine by an amino acid analysis of heated thaumatin clearly indicates the participation of $\beta$-elimination; that is, lysinoalanine is formed upon the $\beta$-elimination of a disulfide bond. According to Florence (1980), the elimination of a $\beta$-hydrogen from a cystine residue is the first step in the degradation of a disulfide bond under weak alkaline conditions at ambient temperature. Cystine residues undergo degradation by $\beta$-elimination even at neutral pH when heated at high temperature, that is, 100 °C. The degradation of cystine at neutral pH and higher temperature is observed in unrelated proteins, including hen egg white lysozyme (Ahern and Klibanov, 1985), insulin (Volkin and Klibanov, 1987), ovalbumin (Volkin

and Klibanov, 1987), ribonuclease (Zale and Klibanov, 1986), and transferrin (Volkin and Klibanov, 1987). The degradation of cystine residues in protein upon heating at neutral pH and high temperature via $\beta$-elimination is quite likely, and similar reactions may occur in the case of thaumatin, as shown in this study.

The base-catalyzed subtraction of a $\beta$-proton from the disulfide bond results in cleavage of the cystine residue and formation of dehydroalanine and persulfide residues (Figure 7). Dehydroalanine, another product of $\beta$-elimination and an intermediate in lysinoalanine formation, could not be measured in this study, suggesting that the dehydroalanine was immediately converted to lysinoalanine. This is confirmed by the fact that the amount of lysinoalanine is almost equal to that of the cystine reduced. Dehydroalanine can be produced from serine (Patchornik and Sokolovsky, 1964; Sokolovsky et al., 1964). However, no change was observed in the amount of serine in this study (data not shown). Therefore, the only source of the dehydroalanine residues observed in this study should be cystine.

These results show that cysteine residues, the sulfhydryl groups of which catalyze the heat-induced disulfide interchange of thaumatin at pH 7.0, are formed via the $\beta$-elimination of disulfide bonds. This creates intermolecular disulfide linkages and induces aggregation, which results in a precipitate. A disulfide bond does not always contribute to the stabilization of a protein.

## CONCLUSIONS

(1) The thermal aggregation of thaumatin at pH 7.0 occurs above 70 °C.

(2) The thermal aggregation of thaumatin depends on the concentration of thaumatin and is mainly induced by the formation of intermolecular disulfide bonds.

(3) Thiol-catalyzed disulfide interchange reactions contribute to the aggregation process.

(4) The disulfide interchange reaction is catalyzed by cysteine; that is, a free sulfhydryl residue is formed via the $\beta$-elimination of a disulfide bond. Intermolecular disulfide bonds between thaumatin molecules that do not contain free sulfhydryl groups are also formed by heating at pH 7.0, which leads to the aggregation of thaumatin molecules.

## ABBREVIATIONS USED

IAM, iodoacetamide; NEM, *N*-ethylmaleimide; 2ME, 2-mercaptoethanol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; native PAGE, nondenaturing PAGE; BCA, bicinchoninic acid.

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