spectively. It is clear that X was contained in the dialyzable fraction. Therefore X has a small molecular size and is not chondroitin sulfate itself.

Comparison of the Potency of Acceleration. Table IV shows the comparison of nitrosation-accelerating potency of X with that of thiocyanate or iodide. The reaction mixture containing the similar weight of sodium thiocyanate or potassium iodide formed only 5~6% of NDMA compared with the reaction mixture containing X. Phenols such as gallic acid sometimes showed an accelerating effect and sometimes showed an inhibitory effect depending on their concentrations or pHs of the media (Walker et al., 1975; Yamada et al., 1978). X, however, strongly accelerated the nitrosation reaction under all conditions in our experiments and was different in many ways from known nitrosation-accelerating substances. Nitrosamines and nitrosatable compounds are contained in some foods, and nitrosamines may also be formed in vivo. X or unknown substances having a similar function as X may play an important role in nitrosamine formation in vitro and in vivo.

The knowledge concerning the molecular structure of X is very limited so far, and separation and identification of the nitrosation-accelerating principle are in progress.

Registry No. Sodium chondroitin sulfate, 9082-07-9; dimethylamine, 124-40-3; dibutylamine, 111-92-2; pyrrolidine, 123-75-1; piperidine, 110-89-4; morpholine, 110-91-8; cupric ion, 15158-11-9; silver ion, 14701-21-4.

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Heat Inactivation of Ovoinhibitor in the Alkaline pH Region

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Heat inactivation of ovoinhibitor was studied at the pH region between 8.0 and 9.5. When ovoinhibitor was heated at higher pH and higher ionic strength, the protease inhibitory activity greatly decreased. To study the heat inactivation of the ovoinhibitor molecule, partially heat inactivated ovoinhibitor was applied to either an insolubilized-trypsin column or an insolubilized-chymotrypsin column and both the trypsin and chymotrypsin inhibitory activities were measured in the absorbed and unabsorbed fractions. Trypsin inhibitory activity of the insolubilized trypsin absorbed fraction or chymotrypsin inhibitory activity of the insolubilized chymotrypsin absorbed fraction was the same as that of native ovoinhibitor, and both trypsin and chymotrypsin inhibitory activities remained in the unabsorbed fraction. Circular dichroism and difference scanning calorimetry measurements suggest that heat treatment produces many kinds of denatured ovoinhibitor molecules of which domain structure is destroyed in a different degree.

Many kinds of protease inhibitors have been found in natural food materials. Egg white contains three kinds of protease inhibitors (Osuga and Feeney, 1977), namely, ovomucoid, ovoinhibitor, and cystatin [formerly called ficin–papain inhibitor (Barrett, 1981)]. It is interesting that these three protease inhibitors are relatively heat stable; ovomucoid is resistant to heating at 100 °C (Deutsch and Morton, 1961), cystatin survives even after exposure to 80–100 °C (Sen and Whitaker, 1973), and ovoinhibitor is stable under heating at 100 °C for 30 min when the solution pH is rather low (Matsushima, 1958). The heat stability of ovoinhibitor, however, is pH dependent and decreases rapidly at alkaline pH (Matsushima, 1958). Since the pH of native egg white is between 8.0 and 9.5, this ovoinhibitor property is very interesting. Furthermore, it has been shown that each molecule has independent binding sites for trypsin, chymotrypsin, and elastase (Davis et al., 1969). It is interesting to know whether the effect of heating in alkaline pH region is the same for both activities. In the present study, we extensively investigated the heat inactivation of ovoinhibitor in the pH region between 8.0 and 9.5.

MATERIALS AND METHODS

Materials. Ovoinhibitor was prepared from fresh egg white by the method described by Davis et al. (1969) with a slight modification. Trypsin (Type XI, DPCC treated) was obtained from Sigma Chemical Corp. α-Chymotrypsin (bovine, 3 times crystallized) was obtained from ICN Pharmaceuticals Inc. (Cleveland, OH). Both insolubilized trypsin and insolubilized chymotrypsin were prepared by the method described by Porath et al. (1973).

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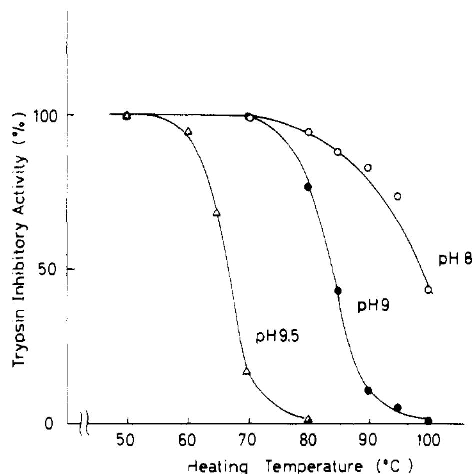

Figure 1. Effect of pH on the heat inactivation of ovoinhibitor. The pH of the solution was adjusted with addition of diluted NaOH. The protein concentration was 1 mg/mL. The heating time was 30 min.

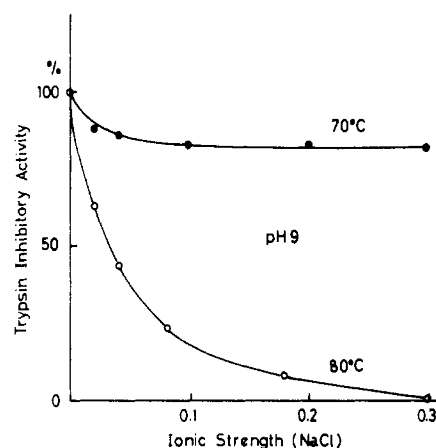

Figure 2. Effect of ionic strength on the heat inactivation of ovoinhibitor. The pH of the solution was adjusted with addition of diluted NaOH, and the ionic strength of the solution was adjusted with addition of a definite amount of NaCl. The protein concentration was 1 mg/mL. The heating time was 30 min.

Inhibitor Activity Assays. Assays for inhibitory activity were performed by measuring the decrease in enzymatic activity caused by preincubation with either native or heated ovoinhibitor. Trypsin activity was assayed with α-*N*-benzoyl-L-arginine *p*-nitroanilide as the substrate by the method described by Waheed and Salahuddin (1975). Chymotrypsin activity was assayed with *N*-acethyl-L-tryosine ethyl ester as the substrate by the method described by Schwert and Takenaka (1955).

Preparation of Partially Heat Inactivated Ovoinhibitor. Ovoinhibitor was dissolved in distilled water to give a protein concentration of 1 mg/mL. The pH of the solution was adjusted to 9.0 by adding 0.5 M NaOH. Immediately after pH adjustment, ovoinhibitor solution (1 mL) was added to a test tube (1.2 × 14 cm) and kept at 90 °C for 10 min. Heat ovoinhibitor solution was cooled immediately by placing the tube in ice water.

Fractionation of Partially Heat Inactivated Ovoinhibitor Using Insolubilized Trypsin or Chymotrypsin. Fractionation of partially heat inactivated ovoinhibitor was carried out using both insolubilized trypsin and chymotrypsin under the similar conditions described by Beeley and McCairns (1972). Briefly, heated ovoinhibitor was applied to the column (1.5 cm × 5 cm) of either insolubilized trypsin or chymotrypsin, washed with 0.1 M Tris-HCl buffer, pH 7.0, and then eluted with 0.1 M glycine–HCl buffer, pH 1.5. The absorbed fraction was obtained in the fraction eluted with 0.1 M glycine buffer, pH 1.5. Both the absorbed and unabsorbed fractions were gathered, dialyzed, and lyophilized.

Circular Dichroism. Circular dichroism (CD) was measured on a Jasco J-40A spectrophotometer at 20 °C. The data were expressed in terms of molar ellipticity, [Θ]. A protein concentration of about 0.07 mg/mL of 0.05 M Tris-HCl buffer (pH 8.0) was used for measurements with a 1.0-mm path length.

Differential Scanning Calorimetry. Differential scanning calorimetry (DSC) thermograms were recorded on a Daini Seikosha Model SSC/560 thermal analyzer with a DSC cell programmed at the rate of 1.0 °C/min temperature increase Samples (50 μL) of protein solutions solubilized in 10 mM phosphate buffer (pH 7.0) were pressure sealed in silver pans weighing approximately 1.47 g. A sealed pan that contained a volume of the buffer equal to that of the sample was used as a reference. Amounts of ovoinhibitor used for DSC are given under Results and Discussion.

RESULTS AND DISCUSSION

As shown in Figure 1, heat inactivation of ovoinhibitor was dependent on pH even in the alkaline pH region; the inhibitory activity remained after heating at 100 °C for 30 min when the pH of the solution was 8.0, but it completely disappeared with heating at 80 °C for 30 min when the pH of the solution was 9.5. When the ionic strength of the solution was increased, the inhibitory activity of ovoinhibitor was greatly decreased (Figure 2). The effect of ionic strength was rather large at the higher heating temperature. Since the ionic strength of egg white was estimated to be about 0.1 when calculated from the salt concentration according to Sato et al. (1960), these results show that the inhibitory activity of ovoinhibitor is destroyed easily during the usual cooking of an egg.

Ovoinhibitor possesses both trypsin and chymotrypsin inhibitory activities, so it is interesting to know whether the effect of heating is the same for both activities. Since both the effects of pH and ionic strength on the trypsin inhibitory activity were similar to the chymotrypsin inhibitory activity, an exact comparison can be made. To investigate this problem, partially heat inactivated ovoinhibitor was applied to an insolubilized-trypsin or an insolubilized-chymotrypsin column, and the inhibitory activities of the absorbed and unabsorbed fractions were measured (Figures 3 and 4).

In this experiment, about 50% of heated ovoinhibitor was absorbed on an insolubilized-trypsin column or an insolubilized-chymotrypsin column. This value is a little smaller than the expected value from the results of trypsin or chymotrypsin inhibitory activity measurement (Table I, column 2). Table I shows that trypsin inhibitory activity remained in the unabsorbed fraction on the insolubilized trypsin and chymotrypsin inhibitory activity remained in the unabsorbed fraction on the insolubilized chymotrypsin. Trypsin- or chymotrypsin-binding sites of the ovoinhibitor molecule might be destroyed in a different degree by heating, and a part of the heated ovoinhibitor molecule having trypsin or chymotrypsin inhibitory activity might not be absorbed on the insolubilized trypsin or chymotrypsin.

It seems to be strange that the chymotrypsin inhibitory activity of the absorbed fraction on the insolubilized trypsin was about half of native one and that of unabsorbed fraction was not so large. A similar phenomenon was also noted for the result of the trypsin inhibitory activity measurement of both absorbed and unabsorbed

Table I. Trypsin and Chymotrypsin Inhibitory Activities[c] of Native and Heat-Treated Ovoinhibitors[a]

	native ovo-inhibitor	heat-treated ovo-inhibitor[b]	fractionation with insolubilized-trypsin column: adsorbed fraction	fractionation with insolubilized-trypsin column: unadsorbed fraction	fractionation with insolubilized-chymotrypsin column: adsorbed fraction	fractionation with insolubilized-chymotrypsin column: unadsorbed fraction
trypsin inhibitory activity	1.15	0.72	1.17	0.16	0.58	0.21
	(100)[d]	(63)	(100)	(14)	(50)	(18)
chymotrypsin inhibitory activity	0.95	0.55	0.52	0.19	0.94	0.14
	(100)	(58)	(55)	(20)	(100)	(15)

[a] Calculated from the values shown in Figures 3 and 4. [b] Inhibitory activities were measured immediately after heat treatment. [c] Proteinase inhibitory activity was presented as an activity (unit) of each protease that was inhibited by the microgram of native or heated ovoinhibitor. [d] Values in parentheses are in percent.

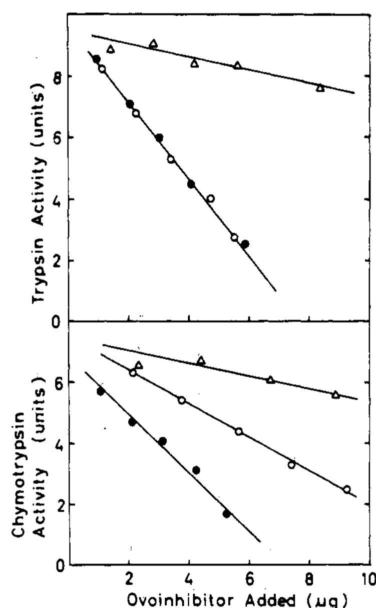

Figure 3. Trypsin and chymotrypsin inhibitory activities of native and heat-treated ovoinhibitors (insolubilized-trypsin column): (○) native ovoinhibitor; (●) insolubilized trypsin adsorbed fraction; (△) insolubilized trypsin unadsorbed fraction.

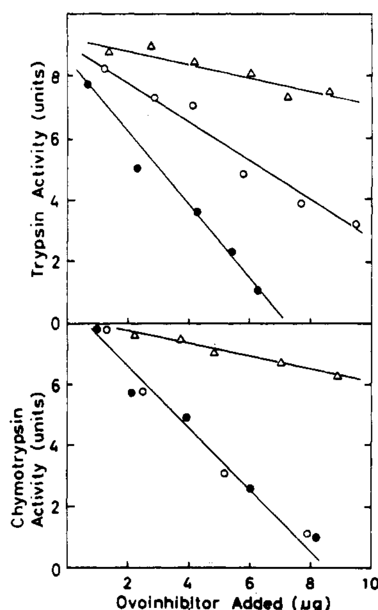

Figure 4. Trypsin and chymotrypsin inhibitory activities of native and heat-treated ovoinhibitors (insolubilized-chymotrypsin column): (○) native ovoinhibitor; (●) insolubilized chymotrypsin adsorbed fraction; (△) insolubilized chymotrypsin unadsorbed fraction.

fractions on the insolubilized chymotrypsin. Laskowski and Kato (1980) showed that ovoinhibitor has a domain structure and each domain has a different kind of inhibitory activity. The reason why the above phenomenon occurs may be due to a cooperative effect of denaturation in the domain structure of ovoinhibitor. That is, denaturation of one domain of the ovoinhibitor molecule predisposes the remaining domains of that molecule to denaturation so that there is some predominance of either totally undenatured molecules or totally denatured molecules and a lower proportion of molecules that have all trypsin inhibitor sites native and all chymotrypsin sites denatured (or vice versa). This interpretation is supported by the present results showing that 55% of the chymotrypsin inhibitory activity is retained by the molecules that bind to immobilized trypsin columns and a nearly equal amount, 50%, of trypsin inhibitory activity is retained by molecules that bind to the immobilized chymotrypsin column (Table I). Furthermore, the fact that unabsorbed fractions on the immobilized trypsin column still retain some trypsin inhibitory activity (Table I) may show that molecules having only one trypsin inhibitory domain undenatured would not absorb to the immobilized trypsin column and would produce some trypsin inhibitory activity.

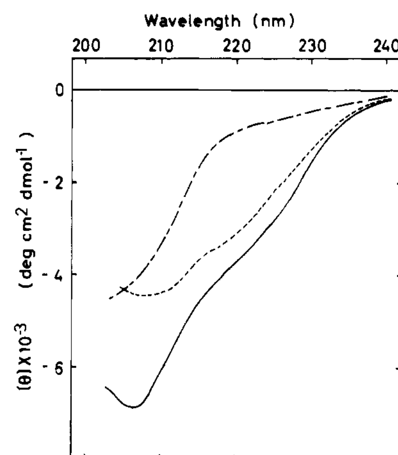

Figure 5. CD spectra of native and heat-treated ovoinhibitors: (—) native ovoinhibitor; (- - -) insolubilized trypsin adsorbed fraction; (- - -) insolubilized trypsin unadsorbed fraction.

To study the conformational properties of heated ovoinhibitors, both CD and DSC studies were made. The CD spectra of the insolubilized trypsin absorbed and unabsorbed fractions were different from that of native ovoinhibitor (Figure 5). These spectra show both the insolubilized trypsin absorbed and unabsorbed fractions had less adsorption of circularly polarized light in the range of 200–235 nm than native ovoinhibitor. This change may have been caused by some loss of either α-helix or β-structure or by some other conformational change in the molecule. DSC diagrams of all ovoinhibitor samples gave a broad peak, showing the presence of various components

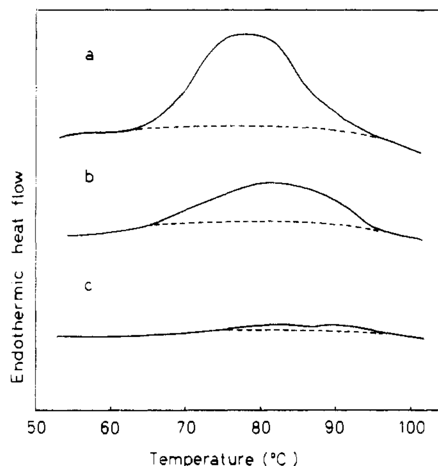

Figure 6. DSC patterns of native and heat-treated ovoinhibitors: (a) native ovoinhibitor; (b) insolubilized trypsin adsorbed fraction; (c) insolubilized trypsin unadsorbed fraction. Amounts of ovoinhibitor used for experiment were 3.43, 6.17, and 4.46 mg for native, insolubilized trypsin adsorbed fraction, and insolubilized trypsin unadsorbed fraction, respectively.

(Figure 6). The relative peak areas per protein weight of the absorbed and unabsorbed fractions on the insolubilized trypsin were 27% and 4%, respectively, of that of the native ovoinhibitor. Both the results of CD and DSC studies showed that the insolubilized trypsin absorbed fraction of heated ovoinhibitor was conformationally different from the native ovoinhibitor.

Similar results were obtained about partially heat-inactivated ovoinhibitor by using an insolubilised chymotrypsin column (data not shown). This seems to show that parts of the ovoinhibitor molecule having a chymotrypsin inhibitory activity behave the same way as those having a trypsin inhibitory activity. All these results suggest that heat treatment produces many kinds of denatured ovoinhibitor molecules of which domain structure is destroyed to a different degree.

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Registry No. Trypsin inhibitor, 9035-81-8; chymotrypsin, 9004-07-3; ovoinhibitor, 62449-23-4.

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Improvement of Water Absorption of Soybean Protein by Treatment with Bromelain

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When 5 mL of 5% acid-precipitated soybean protein and 11S globulin were heated at 100 °C for 10 min and then treated with 0.5 mL of 5 mg/mL bromelain, the viscosity of the protein solutions gradually increased until the solutions coagulated after reaction times of 110 and 15 min, respectively. The coagula were soft gel and floated when shaken. The degradation product of 11S globulin treated with bromelain has a molecular weight of about 15000. These fragments associated mainly through hydrophobic interaction and disulfide bonds. It was also observed through an electron microscope that the coagulation of 11S globulin formed a network structure accompanied by aggregation. The formation mechanism of the coagulum is believed to have begun when the protein strands formed by heat treatment were broken down and then varied strands were exposed to the surface and associated readily, until finally the network structure was formed. This enzymatic treatment improved water absorption of acid-precipitated protein and 11S globulin about 2–2.5 times that of the native ones.

Soybean protein isolates have high nutritional value and various functional properties. As a result, their consumption has increased steadily. In particular they have been used as an additive to meat and marine products because of their functional properties such as emulsifying, foaming, hydration, and other properties (Kato and Nakai, 1980; Nakai et al., 1980; Kato et al., 1981; Furukawa and Ohta, 1981; Voutsinas et al., 1983). Many researchers (Aoki et al., 1977; Hermansson, 1977; Yanagi et al., 1978; Kinsella, 1979; Furukawa and Ohta, 1981; Voutsinas et al., 1983) have tried to improve these functional properties by means of heat denaturation. But enzymatic modification

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