Relationship between Thermocoagulation of Proteins and Amino Acid Compositions

Kazuko Shimada* and Setsuro Matsushita

The thermocoagulations of several protein solutions were investigated with respect to their relationship to protein concentration. For egg albumin, hemoglobin, catalase, and urease, the critical pH on coagulation shifted to either a more acidic or alkaline side with increase of protein concentration. On the other hand, coagulation for bovine serum albumin, soybean protein, conalbumin, prothrombin, and other proteins examined showed no dependence on protein concentration. The critical pH on coagulation, $h_{T=0.5}$, remained constant and did not change even with higher protein concentration. Both coagulation type proteins (concentration dependent) and gelation type proteins (concentration independent) were distinguished by the mole percent of hydrophobic groups in each protein. The relationship between the thermocoagulation and molecular weight of protein was also investigated. Coagulation for low-molecular-weight proteins was not dependent on protein concentration even though the proteins contained a large amount of hydrophobic groups.

Recently, fabricated foods have been developed. In this connection, information relating to the gel formation of proteins is important. Circle et al. (1964) reported that gels were formed by heating 8% or higher dispersions of sodium soy proteinate to temperatures of 80–125 °C for 30 min. Catsimpoolas and Meyer (1970) showed that the slurry of soybean globulins (the sol) was activated to a progel state by heating and a reversible gel was obtained by cooling the progel. With excess heating, it produced a metasol which did not gel on cooling. The soybean 11S globulin produced a firmer gel structure than the 7S globulin when either calcium induced or heat induced (Saio et al., 1973, 1974). The microstructures of soybean protein curds and yeast protein curds were examined by an optical microscope and a scanning electron microscope (Lee and Rha, 1978; Tsintsadze et al., 1978). Poor functional properties of proteins could be overcome by blending with dairy proteins. Gelation of a peanut/whey protein blend was studied in order to expand the utilization of peanut protein (Schmidt, 1978; Schmidt et al., 1978).

Network structures of proteins are formed by various conditions such as calcium coagulation, the isoelectric point precipitation, and heat-induced coagulation or gelation. The properties of heat-induced network structures differ from transparent and thermoreversible gel such as gelatin gel to opaque and thermoirreversible coagulum such as heated egg white. The relation between the properties of network structure and the physicochemical features of protein is not well understood. In this study, the relationship of thermocoagulation to the amino acid composition and that of thermocoagulation to the molecular weights of proteins were investigated.

MATERIALS AND METHODS

Materials. Egg albumin, pepsin, and gelatin (fine powder) were purchased from Nakarai Chemical Ltd., Kyoto. Ovalbumin (grade V, crystalline), hemoglobin (from beef blood, type I, crystallized two times), catalase (from bovine liver), urease (from jack bean, type III), α-chymotrypsin (from bovine pancreas, type II, crystallized three times), conalbumin (from chicken egg white, type I), β-lactoglobulin (from bovine milk, crystallized three times), trypsin inhibitor (ovomucoid, from chicken egg white, type II-O), and ribonucrease-A (from bovine pancreas, crystallized five times, type I-A) were obtained from Sigma Chemical Co. γ-Globulin (human, Fr II), prothrombin bovine (Fr III-2), and human serum albumin (Fr V) were purchased from ICN Pharmaceuticals Inc., Cleveland. γ-Globulin (bovine, Fr II) and bovine serum albumin (demineralized) were obtained from Povite Producten N.V. (Amsterdam, Holland). Casein (Hammarsten) was purchased from Merck (Germany). Soybean protein, prepared from an aqueous extract of defatted soybean meal by isoelectric point precipitation, was dialyzed against distilled water and lyophilized. Other chemicals were reagent grade.

Heat Treatment and Turbidimetry. Heat treatment was performed in the same manner as was reported in a previous paper (Shimada and Matsushita, 1980). Protein solutions were heated in a water bath at 80 °C for 15 min. Turbidity was measured using a Coleman Model 6-20 Junior 2 spectrophotometer at 700 nm for hemoglobin and catalase and at 600 nm for other proteins. Protein concentrations were determined by drying the samples to constant weight at 110 °C. Relative turbidity was expressed as shown in a previous paper (Shimada and Matsushita, 1980).

Opalescence Measurement at A_{340nm}. The protein solution was heated in a vial with a capacity of about 5 mL by using a dry block heater at 80 °C for 15 min. Opalescence was determined at a wavelength of 340 nm according to the procedure of Hegg et al. (1978). A microcell with a capacity of 1 mL was used.

Reduction and Carboxyamidomethylation of Egg Albumin (RCAM-Egg Albumin). Egg albumin (2 g) was dissolved in 50 mL of 0.2 M Tris-HCl buffer containing 8 M urea and 0.2% EDTA (pH 8.5), and 0.5 mL of 2-mercaptoethanol was added. After standing for 4 h at room temperature, 2 g of iodoacetamide and a small amount of crystal tris(hydroxymethyl)aminomethane were added, and the system was allowed to stand overnight in the dark. RCAM-egg albumin was dialyzed against distilled water and lyophilized.

Cyanogen Bromide Treatment of Egg Albumin (CNBr-Egg Albumin). CNBr treatment of egg albumin was performed according to the procedure of Cahnmann et al. (1966). The CNBr-treated egg albumin was submitted to NaDodSO$_4$-polyacrylamide gel electrophoresis and it was found that polypeptide fractions with molecular weights of 35 000, 20 000, and 14 000 existed in a concentration ratio of 1:0.7:0.4, respectively. From the amino acid analysis, CNBr-treated egg albumin showed a two-thirds decrease of methionine compared to native egg albumin but the amounts of other amino acids had not changed.

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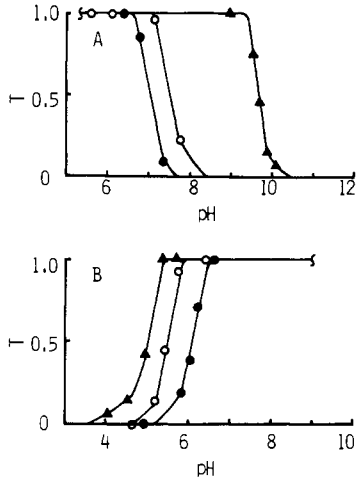


Figure 1. Coagulations of hemoglobin and catalase. T indicates relative turbidity. Changes in turbidity describe only the acidic side for hemoglobin and only the alkaline side for catalase. (A) Catalase: (●) 0.13% protein concentration, (○) 0.42%, and (▲) 2.74%. (B) Hemoglobin: (●) 0.05%, (○) 0.47%, and (▲) 2.91%.

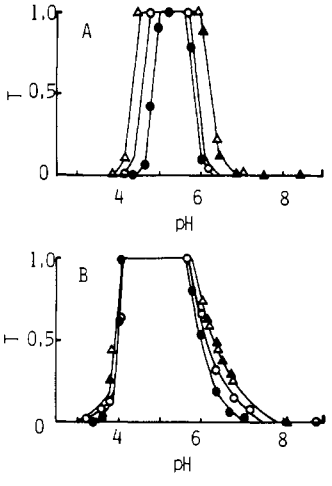


Figure 2. Coagulations of BSA and soybean protein. T indicates relative turbidity. (A) BSA: (●) 0.10% protein concentration, (○) 1.13%, (▲) 4.67%, and (△) 8.36%. (B) Soybean protein: (●) 0.11%, (○) 1.06%, (▲) 4.73%, and (△) 8.57%.

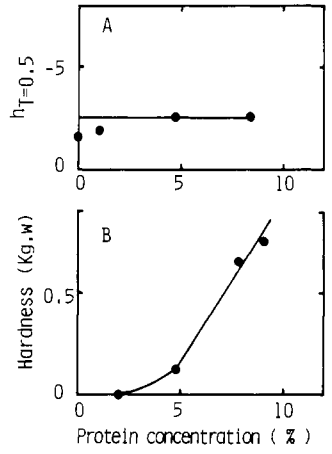


Figure 3. Relationship between (A) $h_{T=0.5}$ and protein concentration; (B) hardness of coagulum and protein concentration for BSA. The $h_{T=0.5}$ refers to only alkaline side. Hardness indicates the maximal value that was measured at each protein concentration.

Texturometer Measurements. Textural properties of coagulum were determined by a Texturometer (General Food Co. GXT-2) using a visco-type plunger and a cup of 24-mm diameter. Hardness was measured from the profile of the first chew.

Electrometric Titration. Titration was carried out in a water-jacketed vessel at 25 °C on a Radiometer PHA 943 pH meter using a G2222C glass electrode and a K4112 calomel electrode. The procedure was the same as described in a previous paper (Shimada and Matsushita, 1980).

Isoionic point measurement was performed according to the procedure of Nozaki and Tanford (1967). The h value is defined as the net charge in 100 amino acid residues.

Amino Acid Analysis. Proteins were hydrolyzed in 6 N HCl at 110 °C for 24 h. Analysis was carried out with a Hitachi KLA-5 amino acid analyzer using the method of Spackman et al. (1958). Half-cystine content was determined by the method of Moore (1963).

RESULTS

Effect of Protein Concentration on Coagulation. In a previous paper (Shimada and Matsushita, 1980), it was reported that thermocoagulation of egg albumin was dependent on protein concentration. In this study, other proteins were investigated with respect to the effect of protein concentration on coagulation. In the cases of hemoglobin and catalase, the critical pH on coagulation shifted to either a more acidic or alkaline side as protein concentration increased (Figure 1). Coagulation of bovine serum albumin (BSA) and soybean protein showed that the critical pH of both proteins shifted little even with higher protein concentrations (Figure 2). The critical pH on coagulation of BSA at each concentration level was converted to the net charge obtained from the titration curve, and the $h_{T=0.5}$ values were plotted against protein concentration (Figure 3A). Figure 3B shows the relationship between the hardness of coagulum and protein concentration. Even though protein concentration was increased and coagulum formed, $h_{T=0.5}$ did not increase but remained constant.

Opalescence Measurement at A_{340nm}. Coagulation has been investigated by observing the changes in turbidity at wavelengths of 600 or 700 nm, but this method has the disadvantage of requiring a large quantity of protein. Measurement at a wavelength of 340 nm is highly sensitive and requires only a small quantity of protein, but the coagulated sample cannot be measured by this method. Consequently, the effect of protein concentration on coagulation was investigated by utilizing absorbance at 340 nm. The pH at which opalescence began to be observed by heating differed in each protein concentration of 0.1% (noncoagulable concentration), 2.5% (coagulable concentration), and 5.0% (coagulable concentration) for egg albumin and in each protein concentration of 0.1, 1.0, and 3.9% for urease (Figure 4). In the cases of BSA, human serum albumin, γ-globulin bovine, γ-globulin human, prothrombin, and α-chymotrypsin, the pH at which opalescence began to be observed differed in protein concentrations of 0.1% (noncoagulable concentration) and 2.5% (or 5.0%) (coagulable concentration) but did not separate in protein concentrations of 2.5 and 5.0% (Figure 5). Figure 6 shows that the absorbances at 340 nm increased from the same pH under each protein concentration of 0.1% (noncoagulable concentration), 2.5% (coagulable concentration), and 5.0% (coagulable concentration) for both soybean protein and conalbumin.

Relationship between Thermocoagulation and the Molecular Weight of Protein. Proteins of low molecular weight cannot coagulate but form aggregate or fragile,

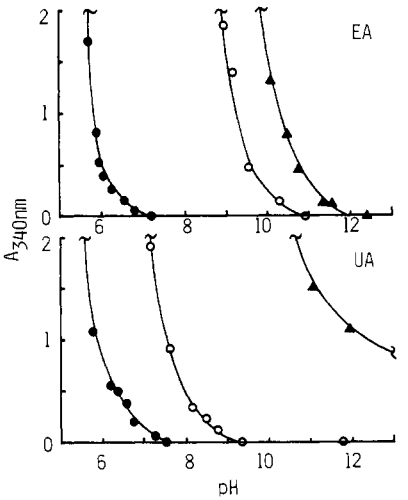


Figure 4. Changes in A_{340nm} by heating for egg albumin and urease: changes in turbidity at alkaline side. Egg albumin (EA): (●) 0.1% protein concentration, (○) 2.5%, and (▲) 5.0%. Urease (UA): (●) 0.1%, (○) 1.0%, and (▲) 3.9%.

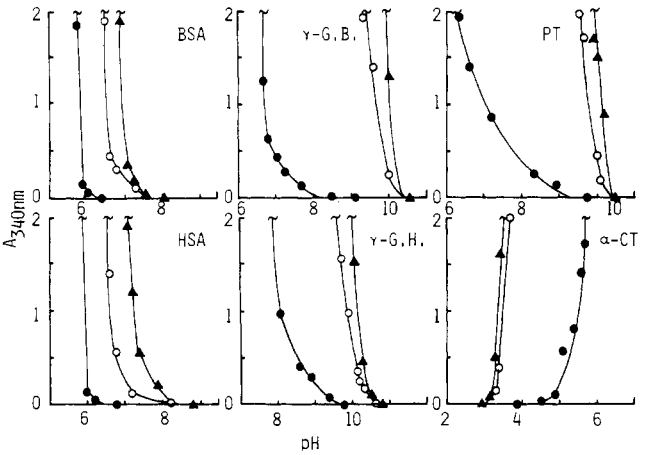


Figure 5. Changes in A_{340nm} by heating for BSA, human serum albumin (HSA), γ-globulin bovine (γ-G.B.), γ-globulin human (γ-G.H.), prothrombin (PT), and α-chymotrypsin (α-CT): changes in turbidity at alkaline side except acidic side for α-chymotrypsin; (●) 0.1% protein concentration, (○) 2.5%, and (▲) 5.0% for each protein.

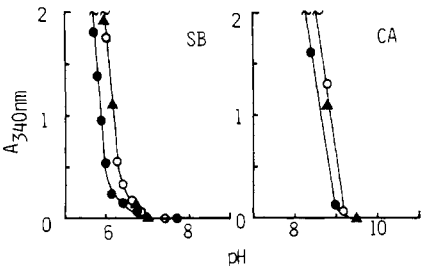


Figure 6. Changes in A_{340nm} by heating for soybean protein (SB) and conalbumin (CA): changes in turbidity at alkaline side; (●) 0.1% protein concentration, (○) 2.5%, and (▲) 5.0% for both proteins.

heterogeneous coagulum. So far thermocoagulation had been investigated by the use of proteins with relatively high molecular weights. α-Chymotrypsin has the lowest molecular weight [25 000, Matthews et al. (1967)] of all proteins investigated. But α-chymotrypsin is known to associate to dimer in solution (Schwert, 1949). Therefore, the protein with the lowest molecular weight is ovalbumin [45 000, Fothergill and Fothergill (1970)] in egg albumin, and this egg albumin was found to consist of 80% oval-

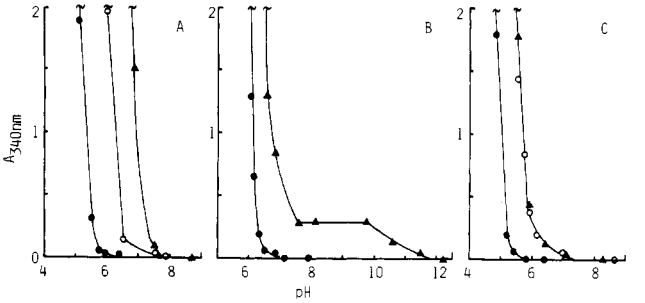


Figure 7. Changes in A_{340nm} by heating for (A) ovalbumin, (B) RCAM-egg albumin, and (C) CNBr-egg albumin: changes in turbidity at alkaline side; (●) 0.1% protein concentration, (○) 2.5%, and (▲) 5.0% for each protein.

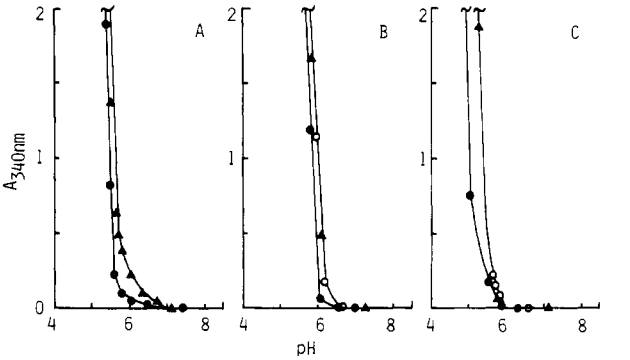
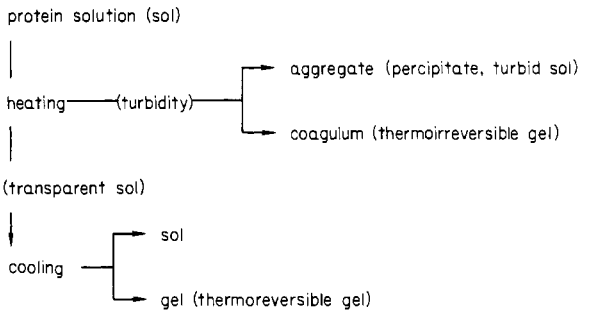


Figure 8. Changes in A_{340nm} by heating for (A) casein, (B) β-lactoglobulin, and (C) pepsin: changes in turbidity at alkaline side. Molecular weight of casein (30 000) was determined by $NaDodSO_4$–polyacrylamide gel electrophoresis. Molecular weights of β-lactoglobulin and pepsin were 18 000 (Braunitzer et al., 1972) and 35 000 (Ryle and Hamilton, 1966), respectively. The mole percent of hydrophobic groups for casein, β-lactoglobulin, and pepsin calculated from the results of amino acid analysis were 38.0%, 34.6%, and 34.0%, respectively; (●) 0.1% protein concentration, (○) 2.5%, and (▲) 5.0% for each protein.

Scheme I



bumin and 20% conalbumin (Shimada and Matsushita, 1980). The coagulation of ovalbumin was not concentration dependent (Figure 7). The absorbance at 340 nm by heat treatment of RCAM-egg albumin began to increase slightly from pH 12 but most of the turbidity increase was observed from about pH 7.5 (Figure 7). The slight absorbance from pH 12 may be due to a small amount of unmodified SH groups. The results from heated CNBr-treated egg albumin are shown in Figure 7. The turbidity of CNBr-treated egg albumin did not shift to a more acidic side even with higher protein concentration. Figure 8 shows that proteins with low molecular weights, namely casein, β-lactoglobulin, and pepsin, are not dependent on protein concentration.

DISCUSSION

The changes occurring in protein solution upon heating are shown in Scheme I. When protein solution (sol) is

heated, the sol becomes opaque or transparent. When it becomes opaque, the protein solution under the conditions of low molecular weight and low protein concentration produces an aggregate, while under the conditions of high molecular weight and high protein concentration, it forms a coagulum (thermoirreversible gel). On the other hand, the transparent protein solution remains in sol state under the conditions of low molecular weight and low protein concentration and forms a gel (thermoreversible gel) when it is cooled and favored by a high molecular weight and high protein concentration. Jensen et al. (1950) reported that, depending on the pH of the medium, heat-denatured solutions of serum albumin formed an opaque coagulum, clear gel, or an intermediate clot between these two types. Therefore, the changes undergone by proteins upon heating are roughly divided into two states depending on varying conditions such as pH, ionic strength, presence or absence of denaturant, and others. Protein, however, tends to prefer either of the two states by its inherent characteristics. Whether a coagulum or a gel is apt to be formed is determined by whether it is concentration dependent or not.

Egg albumin, hemoglobin, catalase, and urease which indicated dependence on protein concentration are classified as coagulation type proteins, while other proteins independent of protein concentration are regarded as gelation type proteins (Figures 1, 2, 4, 5, and 6). Furthermore, gelation type proteins can be divided into BSA types and soybean protein types (Figures 5 and 6). Gelatin and ovomucoid (trypsin inhibitor) remained in transparent sol and did not form a precipitate even when the proteins were heated at the isoelectric point. These proteins are considered to be of another type (gelatin-type proteins).

A previous paper (Shimada and Matsushita, 1980) indicated that the hydrophobic groups of protein participated in thermocoagulable matrix formation. Therefore, by use of the results of amino acid analysis, the mole percent of hydrophobic groups (Val, Pro, Leu, Ile, Phe, Trp) are calculated for each protein. These six hydrophobic amino acids were chosen from those with higher hydrophobicity among nonpolar groups (Tanford, 1962; Bigelow, 1967). The relationship between the mole percent and coagulation type or gelation type proteins is shown in Figure 9. Both types can be divided near 31.5 mol %, and in gelation type proteins, the mole percent also distinguishes among BSA, soybean protein, and gelatin types.

Gelatin and ovomucoid with lower contents of hydrophobic groups did not show turbidity and did not form precipitate or aggregate upon heating even under the conditions of high protein concentration and the isoelectric point. The mole percent of hydrophobic groups of ribonucrease was 16.9% (Dayhoff, 1972), but the protein was thermocoagulable under the conditions of 5% protein concentration and the isoelectric point (pH 9.3). This result cannot be interpreted only from the content of hydrophobic groups. Ribonucrease contains much glutamine and asparagine (Dayhoff, 1972). The amide form of the carboxyl group is known to have a low polarity (Tanford, 1962). Therefore, it seems likely that the actual hydrophobicity of ribonucrease is higher than the calculated value. But, generally a protein with a low content of hydrophobic groups such as gelatin is considered not to thermocoagulate even under the condition of the isoelectric point.

The net charge of egg albumin in which coagulum could be maintained increased with protein concentration (Shimada and Matsushita, 1980). That is, a protein solution of higher concentration can form a coagulum even

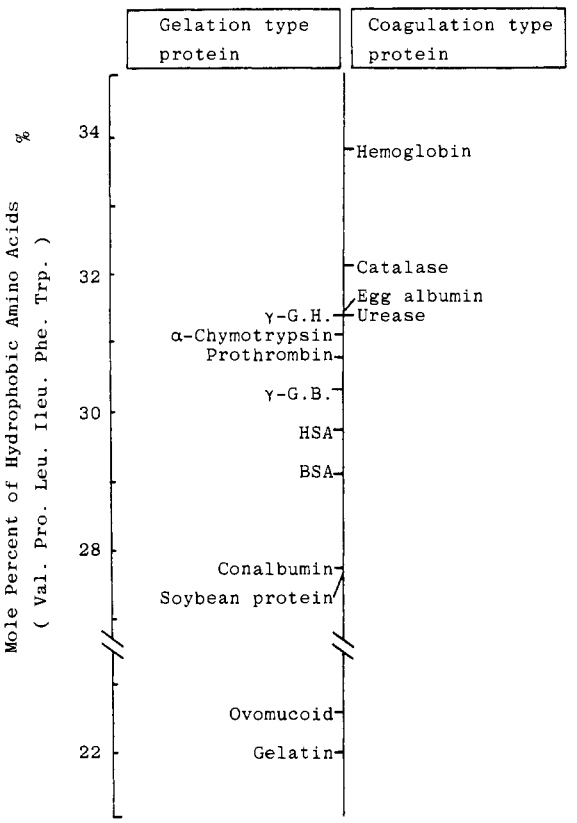


Figure 9. Relationship between network structure type and the mole percent of hydrophobic amino acids. The mole percent of hydrophobic amino acids was calculated from the results of amino acid analysis. HSA, human serum albumin; BSA, bovine serum albumin; γ-G.H., γ-globulin human; γ-G.B., γ-globulin bovine.

under a high net charge. The forces overcoming the electrostatic repulsion may be stronger in coagulum under high protein concentration. Proteins showing concentration-dependent coagulation have a larger amount of hydrophobic groups than those without concentration dependence (Figure 9). The hydrophobic interactions form a denser network structure and individually separate the charged amino acid residues; consequently the interactions may suppress electrostatic repulsion.

Coagulation of egg albumin was protein concentration dependent but that of ovalbumin only was not concentration dependent (Figures 4 and 7). The free sulfhydryl groups in egg albumin decreased with heating, and the proteins were polymerized by intermolecular sulfhydryl–disulfide exchange (Shimada and Matsushita, 1980). Ovalbumin contains 6 mol of half-cystine (Fothergill and Fothergill, 1970) and conalbumin contains 22 mol of half-cystine per molecule (Osuga and Feeney, 1974). Therefore, it seems likely that many sulfhydryl groups or disulfide bonds in conalbumin react with sulfhydryl groups (or disulfide bonds) of ovalbumin during heating, and these proteins are cross-linked. Coagulation of ovalbumin is protein concentration independent because of its low molecular weight, but when a small amount of conalbumin is contained, the proteins are cross-linked into polymers by reactive SH groups of conalbumin and then the coagulum may become protein concentration dependent. Since conalbumin has a relatively high molecular weight [76000, Osuga and Feeney (1974)], egg albumin containing conalbumin has a higher average molecular weight. As a result, coagulation of egg albumin may be protein concentration dependent. When reduced and carboxyamidomethylated egg albumin was investigated for the effect of protein concentration, the modified protein in-

dicated no dependence on protein concentration (Figure 7). Turbidity of conalbumin occurred at about pH 9 by heating (Figure 6), but the result of RCAM-egg albumin showed no increase of absorbance at about pH 9. It seems probable that soluble ovalbumin under around pH 9 dissolves conalbumin by functioning like a salt. Coagulation of CNBr-egg albumin was completely independent of protein concentration (Figure 7). As mentioned above, concentration-dependent coagulation of egg albumin may occur due to the intermolecular interactions between ovalbumin and conalbumin. Coagulation of proteins with low molecular weights indicated no dependence on protein concentration even when having a high content of hydrophobic groups (above 34%) (Figure 8). Consequently, concentration dependence seems to require two aspects of coagulable conditions. The protein should have a high molecular weight above 60 000 and a high content of hydrophobic groups.

From the results obtained here, it is possible to predict that wheat protein, one of the typical food proteins, has an excellent thermocoagulable property with concentration dependence because the mole percent of hydrophobic groups calculated from the amino acid composition (Tkachuk and Irvine, 1969) is 32.5%. Actually, wheat protein is largely utilized as a food ingredient due to its excellent thermocoagulable property.

If the amino acid composition of an unknown protein is determined, the thermocoagulable properties of the protein can then be predicted.

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Effects of Storage Temperature and Duration on Total Vitamin C Content of Canned Single-Strength Grapefruit Juice

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Commercially canned single-strength grapefruit juices were stored at 10, 20, 30, 40, and 50 °C. Their "total active" vitamin C (TAVC) contents [L-ascorbic acid (AA) plus dehydroascorbic acid (DHA)] and diketogulonic acid (DKA) contents were evaluated at 3-week intervals. At the end of 12 weeks the loss of TAVC ranged from less than 3% at 10 °C to greater than 68% at 50 °C. AA was continuously lost during the storage time, and the rate of loss increased as storage temperature increased. Polynomial expressions were calculated for the degradation rate of AA at each storage temperature. Large levels of DHA and DKA were not observed in stored canned juices because breakdown of AA apparently proceeded by an anaerobic pathway. The levels of DHA and DKA remained essentially unchanged during the 12-week storage period.

Grapefruit juice is a refreshing beverage with nutritional benefits as well-known as those of orange juice. One of the major nutritional values of grapefruit juice is its vitamin C content. Vitamin C potency (antiscorbutic activity) of natural products is based on the combined levels of L-ascorbic acid (reduced form of vitamin C, or AA) and dehydro-L-ascorbic acid (oxidized form of vitamin C, or DHA). Therefore, the combined contents of AA and DHA in a food product can be considered as "total active vitamin C" (TAVC). Under aerobic conditions, DHA is the first breakdown product of AA (Bauernfeind and Pinkert, 1970). DHA potency is considered between 75% and 100% of AA potency (Mills et al., 1949).

The official AOAC procedure (AOAC, 1970) for vitamin C analysis involves an indophenol dye titration procedure, which measures only the reduced form of vitamin C. For TAVC to be measured a procedure using 2,4-dinitrophenylhydrazine (2,4-DNPH) must be used in addition to indophenol titration. 2,3-Diketo-L-gulonic acid (DKA), a

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