Polymerization of Acetylated Lysozyme and Impairment of Their Amino Acid Residues Due to α -Dicarbonyl and α -Hydroxycarbonyl Compounds

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We studied the mechanism of polymerization of proteins induced by storage with reducing sugars. Glucose alone did not polymerize acetylated lysozyme as a solid (75% relative humidity), but it did when there was free Lys. This indicated that a certain substance generated through a reaction between glucose and amino groups of proteins polymerized the proteins. The substance impaired Arg residues. The impaired Arg residues appeared in three peaks eluting just behind the Phe peak on amino acid analysis.

α -Dicarbonyl and α -hydroxycarbonyl compounds such as 2,3-butanedione, glyoxal, methylglyoxal, dihydroxyacetone, glyceraldehyde, and glycolaldehyde polymerized both lysozyme and acetylated lysozyme, and impaired their Arg residues. However, the elution patterns of the impaired amino acid residues on amino acid analysis were different from that observed with glucose.

Glyoxal, methylglyoxal, and 2,3-butanedione polymerized proteins to a notable extent even under the conditions used conventionally for chemical modification of Arg residues.

The Maillard reaction between proteins and reducing sugars alters the proteins in various ways such as impairment of certain amino acid residues, reduction of digestibility, loss of solubility, browning, and polymerization.^{1~6)} Although much attention has been devoted to these alterations, the mechanism of the accompanying polymerization of proteins is still not completely understood.

Thus, we have been conducting a series of studies to clarify the mechanism of protein polymerization that occurs when lysozyme is stored with glucose in the solid state (75% relative humidity) at 50°C.^{7,8)} Such storage caused impairment of its Arg, Lys, and Trp residues as well as the polymerization of lysozyme. The acetylation of lysozyme prevented these changes, so Lys residues may be involved at the initial stage of the reaction.⁷⁾ To discover the role of Arg residues in the reaction, lysozyme with Arg residues modified with 1,2-cyclohexanedione (CHD) was stored

with and without glucose. The modified lysozyme was polymerized with accompanying impairment of Lys and Trp residues even in the absence of glucose.⁷⁾ These results lead us to speculate that the agents responsible for cross-linking of proteins and impairment of the amino acid residues are dicarbonyl compounds analogous to CHD produced by the reaction of amino groups with glucose.

We did this work to prove our speculation. The work included experiments to investigate how lysozyme was modified when it was stored with various carbonyl compounds known to be generated in amino-carbonyl reactions involving glucose,^{9,10)} with special attention to a similarity between such modification and the glucose-induced modification.

MATERIALS AND METHODS

Materials. Lysozyme from egg white (6 $\times$ crystallized), was obtained from Seikagaku Kogyo, Tokyo, Japan.

Dihydroxyacetone (DHA), glyceraldehyde (GA), and methylglyoxal (MG) were from Nakarai Chemical Co., Kyoto, Japan. Glyoxal (G), 5-hydroxymethylfurfural (HMF), thiobarbituric acid, and 2,3-butanedione (BD) were from Tokyo Kasei Kogyo, Tokyo, Japan. Glycolaldehyde (GCA) and *N*⁶-acetyl-Arg were from the Sigma Co., U.S.A. All other chemicals used were analytical reagent grade.

Preparations of stored proteins. Five milliliters of solutions containing 0.03 mmol of lysozyme or acetylated lysozyme and 0.3 mmol of various carbonyl compounds or 0.6 mmol of glucose (Glc) and/or 0.2 or 1.0 mmol of Lys were lyophilized, and then stored at 50°C and 75% relative humidity (RH) for 5 days. The unreacted carbonyl compounds and free Lys after storage were removed from the proteins by washing with an ether-acetone mixture (3:1 by volume), followed by dialysis against distilled water.

Preparation of acetylated lysozyme. Acetylated lysozyme was prepared by the procedure of Riordan and Vallee.¹¹⁾ The modification ratio of amino groups was more than 99%, when measured by the method of Fields.¹²⁾ We did not examine whether tyrosine residues escaped acetylation.

Chemical modification of Arg residues of lysozyme. Using the procedure of Nakaya *et al.*,¹³⁾ 0.29 mmol of glyoxal, methylglyoxal, and 2,3-butanedione were mixed with 1.35 μ mol of lysozyme in 5 ml of 0.3 M bicarbonate-NaOH buffer (pH 8.6). The mixture were left standing at 15°C for 3 hr.

Thiobarbituric acid reaction of stored lysozyme. The thiobarbituric acid (TBA) reaction was done by the method of Keeney and Bassette¹⁴⁾; 1 ml of 0.2% protein solution was mixed with 0.5 ml of 0.3 N oxalic acid and heated for 1 hr in a boiling water bath. After cooling to room temperature, 0.5 ml of 40% trichloroacetic acid was added and resulting precipitates were removed by filtration. After addition of 0.5 ml of 0.05 M TBA into 1 ml of the filtrate, the mixture was incubated at 40°C for 30 min and then the absorbance at 443 nm was measured with a Hitachi model 100-60 spectrophotometer using a cuvette of 1 cm width.

Sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis. Electrophoresis was done as described by Weber and Osborn¹⁵⁾ using 10% slab gels. The rate of polymerization of a protein was found by scanning the bands stained with Coomassie brilliant blue R-250 using a Toyo DMU-33C densitometer. Each oligomer of lysozyme was identified using bovine serum albumin, ovalbumin, α -chymotrypsinogen, and cytochrome *c* as standard proteins.

Amino acid analysis. The stored protein were hydrolyzed

with 6 N HCl in evacuated tubes at 110°C for 24 hr and analyzed with a Hitachi model 835 Amino Acid Analyzer. Trp residues were analyzed by the method of Matsubara and Sasaki.¹⁶⁾

Measurement of discoloration of stored lysozyme. Following the method of Massie and Norris,¹⁷⁾ 0.1 g of stored protein powder was placed into an integrating sphere optical system and its reflectance spectra measured in the visible range with a Nikon model P-250 spectrophotometer.

RESULTS

I. Changes of acetylated lysozyme induced by storage with glucose and free lysine

1) Polymerization of lysozyme and impairment of Arg and Trp residues. As shown in our previous paper⁷⁾ and again in Table I, the acetylation of lysozyme prevented almost completely the browning and polymerization of the protein and the impairment of Arg and Trp residues induced by storage with glucose. On the other hand, the addition of free Lys into the storage system composed of acetylated lysozyme and glucose was found to restore all these changes. Thus, all these changes are caused by a certain compound generated from the reaction of amino groups with glucose.

Moreover, it is noteworthy that addition of a large amount of Lys (1.0 mmol) to the acetylated lysozyme/glucose system was less effective to induce the changes of proteins than that of 0.2 mmol of Lys (Table I).

The acetylated lysozyme which had thus changed was positive for the TBA reaction described in MATERIALS AND METHODS: The absorbance at 443 nm of the samples incubated with and without glucose were 0.02 and 0.03, respectively, while the samples incubated with glucose plus 0.2 mmol of Lys and glucose plus 1.0 mmol of Lys were 0.54 and 0.48, respectively. This result indicated that the acetylated lysozyme gained some carbonyl compounds during storage with glucose and free Lys.

2) Generation of unknown amino acid residues ascribable to impaired Arg residues. The amino acid analysis found that storage of lysozyme with glucose caused a generation of three unknown peaks which eluted just behind

TABLE I. POLYMERIZATION, AMINO ACID COMPOSITION AND DISCOLORATION OF LYSOZYME OR ACETYLATED LYSOZYMES AFTER STORAGE WITH CARBONYL COMPOUNDS AT 50°C AND 75% RH FOR 5 DAYS

Lysozyme	Control ^a	Lysozyme									Acetylated Lysozyme										
Carbonyl ^b		None	Glc	BD	MG	G	DHA	HMF	GA	GCA	None	Glc	Glc + Lys ^c	Gly + Lys ^d	BD	MG	G	DHA	HMF	GA	GCA
Polymerization ^e																					
Monomer		99.2	76.2	39.6	17.4	32.3	12.1	66.1	36.3	45.4	98.5	97.1	86.4	89.3	34.6	65.2	57.9	61.9	92.5	61.9	60.7
Dimer		0.8	12.6	24.1	20.3	17.4	14.2	19.9	13.8	17.6	1.5	2.9	11.6	9.9	21.3	24.3	21.6	25.3	6.8	18.8	21.5
Trimer			6.7	16.1	16.5	8.4	14.5	10.3	8.3	10.5			1.9	0.8	13.0	7.4	10.8	8.6	0.6	8.7	9.5
Tetramer			4.5	8.6	12.5	12.2	10.9	4.6	6.8	8.1					8.9	2.1	5.9	2.9		5.3	4.6
Others ^f				11.6	33.2	29.6	48.2		34.6	18.4					22.2	1.0	3.8	1.1		5.5	3.6
Amino acid composition ^{g,h}																					
Asp	(21) 22.0	21.8	20.6	21.6	21.2	19.2	21.3	19.9	20.2	20.9	19.5	19.9	20.6	20.5	22.0	21.7	20.8	21.9	21.6	20.8	21.0
Thr	(7) 7.1	7.0	6.7	6.5	6.4	5.8	7.0	6.4	6.0	6.5	6.3	6.3	6.6	6.7	6.9	6.9	6.9	6.8	7.0	7.0	7.0
Ser	(10) 11.1	10.4	10.5	11.2	11.2	10.2	10.7	10.0	10.5	10.6	10.4	10.3	10.6	10.3	11.4	11.2	11.2	11.2	11.1	10.1	10.5
Glu	(5) 4.9	4.9	4.5	4.7	4.8	4.1	5.0	4.4	4.4	4.6	4.4	4.3	4.6	4.7	4.9	4.9	5.0	4.9	4.9	4.9	4.9
Gly	(12) 14.2	13.6	12.2	14.1	14.1	13.4	13.5	12.4	13.4	13.3	11.9	12.2	11.7	11.7	13.8	13.7	14.1	13.6	13.3	12.5	12.8
Ala	(12) 13.8	13.2	13.5	13.8	13.7	13.6	12.9	12.9	13.6	13.5	13.0	13.5	13.0	12.9	13.8	13.4	13.2	13.3	13.3	12.8	13.0
Cys	(4) 2.4	2.2	2.3	2.4	2.4	2.1	2.7	2.5	2.3	2.4	2.0	2.2	2.5	2.4	2.5	2.6	2.3	2.6	2.5	2.8	3.0
Val	(6) 3.1	3.4	3.3	2.8	3.0	2.5	3.8	3.2	2.7	3.1	3.4	3.3	3.6	3.6	3.0	3.3	3.1	3.1	3.3	3.9	3.7
Met	(2) 2.6	2.5	2.3	2.9	3.2	2.8	2.8	2.4	2.8	2.6	2.4	2.4	2.3	2.2	2.8	2.9	3.0	2.8	2.7	2.2	2.3
Ile	(6) 3.5	3.7	3.5	3.2	3.4	3.2	4.0	3.5	3.2	3.4	3.5	3.6	3.6	3.7	3.3	3.5	3.3	3.4	3.6	4.0	3.9
Leu	(8) 8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
Tyr	(3) 3.5	3.3	3.4	3.4	3.7	3.7	3.4	3.3	3.5	3.4	3.4	3.3	3.2	3.1	3.2	3.5	3.3	3.3	3.3	3.2	3.2
Phe	(3) 3.0	3.1	2.9	2.9	3.6	4.6	4.0	3.2	3.4	3.4	3.0	3.2	3.1	2.8	2.6	3.0	3.1	2.8	2.8	2.8	2.8
Lys	(6) 5.6	5.6	4.0	5.2	5.2	3.2	3.1	5.5	4.7	3.0	5.7	5.7	5.7	5.8	5.2	5.6	5.7	5.6	5.3	5.6	5.5
His	(1) 1.3	1.1	1.5	1.2	1.6	4.0	1.6	1.8	1.7	1.7	1.2	1.2	1.0	1.0	1.1	1.5	4.2	1.3	1.6	1.3	1.4
Trp	(6) 5.2	5.4	4.1	5.5	5.3	2.7	4.3	4.8	4.6	4.3	5.4	5.4	4.4	4.9	5.1	5.4	5.8	4.6	4.3	4.6	4.7
Arg	(11) 10.7	10.6	8.7	7.1	8.2	4.0	9.3	10.3	8.4	9.8	10.7	10.7	3.3	3.8	6.9	4.9	2.7	6.8	10.5	8.0	10.1
Pro	(2) 2.2	1.8	2.3	1.7	1.6	1.6	1.9	1.9	1.6	1.8	2.1	2.1	2.2	2.5	1.9	1.8	1.8	1.8	1.9	2.4	2.4
Discoloration ⁱ																					
		White	Brown	Reddish brown	Yellow	Brown	Brown	Pinkish brown	Brown	Brown	White	White	Brown	Brown	Reddish brown	Yellow	Yellow	Brown	Pale brown	Pale yellow	Yellow

^a Lysozyme before storage. ^b Abbreviated in the MATERIALS AND METHODS. ^c 0.2 mmol of lys was added. ^d 1.0 mmol of Lys was added. ^e Percentage ratio. ^f Oligomers larger than tetramer. ^g Expressed as a molar ratio taking the value for leucine as 8.0. ^h Numbers in parenthesis are the values reported by Canfield.¹⁷⁾ ⁱ Visual observation.

FIG. 1. Amino Acid Analysis of Lysozyme Stored with Glucose (I) or Acetylated lysozyme Stored with Glucose and Lys (II) at 50°C and 75% RH for 5 Days.

* Unknown compounds, painted in black.

FIG. 2. Amino Acid Analysis of a HCl-Hydrolysate of the N^{α} -Acetyl-Arg after Storage with lysozyme and Glucose at 50°C and 75% RH for 10 Days.

The elution positions of known amino acids are also indicated in the chart.

the Phe peak (Fig. 1). The acetylated lysozyme stored with both glucose and free Lys also had exactly the same unknown peaks as observed for the lysozyme stored with glucose (Fig. 1). These results indicated that the unknown peaks originated from the impaired Arg and/or Trp residues but not from Lys residues.

Thus, we prepared a lyophilized mixture of lysozyme (0.5 g), glucose (0.2 g) and N^{α} -acetyl-Arg (0.04 g), and then stored the mixture. Then we concentrated the outer solution after dialysis of the stored mixture and ana-

lyzed the amino acids of concentrate after HCl hydrolysis. We found three unknown peaks ascribable to the impaired N^{α} -acetyl-Arg. These peaks were identical in elution position to the peaks that appeared in the reaction between glucose and lysozyme (Fig. 2).

The same experiment was done using N^{α} -acetyl-Trp in place of N^{α} -acetyl-Arg but none of the unknown peaks appeared on amino acid analysis.

Thus we concluded that the unknown peaks were ascribed to the impaired Arg residues.

II. Polymerization and impairment of amino acid residues of acetylated lysozyme due to carbonyl compounds

To find whether carbonyl compounds polymerize proteins and impair their Arg and Trp residues, we incubated acetylated lysozyme with α -dicarbonyl compounds such as glyoxal, methylglyoxal, and 2,3-butanedione, and α -hydroxycarbonyl compounds such as dihydroxyacetone, glyceraldehyde, glycolaldehyde, and 5-hydroxymethylfurfural. All these substances are generated from amino-carbonyl reactions involving reducing sugars (Table I and Fig. 3). All of them were found to polymerize the acetylated lysozyme. The impairment of a notable amount of Arg residues was also recognized for all these substances except for 5-hydroxymethylfurfural and glycolaldehyde. Trp residues were also impaired slightly by all of the hydroxycarbonyl compounds, but not by any of the α -dicarbonyl compounds.

When the amino acids of acetylated lysozyme which had been stored with these substances were analyzed, some unknown peaks appeared between the Phe and Trp peaks (Fig. 4). However, those peaks were not identical in number or elution position to the unknown peaks observed for the lysozyme stored with glucose.

Since there was no decrease in amino acid residues other than Lys residues, those unknown peaks appeared on storage with α -dicarbonyl compounds was ascribed to the impaired Arg residues. By the same reasoning,

FIG. 3. SDS Polyacrylamide Gel Electrophoresis of Lysozyme or Acetylated Lysozyme after Storage with Various Carbonyl Compounds at 50°C and 75% RH for 5 Days.

D + L; 0.6 mmol of glucose and 0.2 mmol of Lys were added. D + LL; 0.6 mmol of glucose and 1.0 mmol of Lys were added. Other abbreviations are shown in MATERIALS AND METHODS.

FIG. 4. Amino Acid Analysis of Lysozyme or Acetylated Lysozyme after Storage with Various Carbonyl Compounds at 50°C and 75% RH for 5 Days.

Abbreviations of carbonyl compounds are shown in MATERIALS AND METHODS. The generated unknown peaks are painted in black.

a marked rise of His content observed on storage with glyoxal was attributable to the unknown peaks which had been generated from the impaired Arg residues and eluted at the position of His.

On the other hand, the origins of the unknown peaks on storage with α -hydroxycarbonyl compounds and 5-hydroxymethylfurfural were to be ascribed to impaired Arg and Trp residues, or either of them.

III. Changes in lysozymes induced by carbonyl compounds

1) *Polymerization and impairment of Lys, Arg, and Trp residues.* We examined the reactivities of the abovementioned carbonyl compounds towards lysozyme (Table I and Fig. 3). The polymerizing effects were higher than those observed for the acetylated lysozyme except for 2,3-butanedione which had the same effects on both lysozyme and acetylated lysozyme. These higher effects indicate that Lys residues can also contribute to the polymerization of proteins caused by these agents. The participation of Lys residues in the polymerization reaction seemed to be a cause for the reduction of Lys content detected on amino acid analysis in the case of glyoxal, dihydroxyacetone, glyceraldehyde, and glycolaldehyde. However, in the case of methyl-

FIG. 5. Second Derivatives of Reflectance Spectra of Lysozyme after Storage with Various Carbonyl Compounds at 50°C and 75% RH for Days.

Abbreviation of carbonyl compounds are shown in MATERIALS AND METHODS.

glyoxal and 5-hydroxymethylfurfural the reduction of Lys content was small or almost nothing. This suggests that bondings between Lys residues and these agents are labile to HCl hydrolysis.

The response of Trp residues towards all the carbonyl compounds except glyoxal was almost the same as in the case of the acetylated lysozyme. Glyoxal caused a notable reduction of Trp content. Its effect seemed to be brought about by a concerted action of Lys residues and glyoxal.

The response of Arg residues towards all the carbonyl compounds except methylglyoxal, glyoxal, and dihydroxyacetone was almost the same as in the case of the acetylated lysozyme. Methylglyoxal, glyoxal, and dihydroxyacetone became less effective in impairing Arg residues. A reaction of these three compounds with Lys residues might reduce the amount available to impair Arg residues.

The generation of unknown peaks on amino acid analysis was also recognized for all the carbonyl compounds (Fig. 4). These unknown peaks were the same as generated from the acetylated lysozyme in elution position and number. This means that none of the unknown peaks ascribable to the Lys residues impaired by glyoxal, dihydroxyacetone, glyceraldehyde, and glycolaldehyde appeared on the conventional amino acid analysis. The peaks ascrib-

FIG. 6. SDS Polyacrylamide Gel Electrophoresis of Lysozyme after Storage with α -Dicarbonyl Compounds at 15°C in Bicarbonate Buffer (pH 8.6) for 3 Hr.

Abbreviation of carbonyl compounds are shown in MATERIALS AND METHODS.

able to the Trp residues impaired by glyoxal did not appear either.

2) *Discoloration of stored protein mixture.* The mixtures of lysozyme and various carbonyl compounds were various colors after storage (Table I). The stored mixtures could be dissolved neither by water nor by solutions such as guanidine-HCl, SDS/urea,

or dimethylsulfoxide. Thus, we measured the color of the whole mixture in the solid state by a reflectance method. The obtained data were expressed as the second derivatives of reflectance spectra (Fig. 5). The curves from all the samples were different. It was noteworthy that none of the curves was identical to the curve from lysozyme stored with glucose.

3) *Polymerization by a conventional method employed for chemical modification of Arg residues.* Some α -dicarbonyl compounds are being used¹⁹⁻²²⁾ for chemical modification of Arg residues of proteins in the field of protein chemistry. Using the method of Nakaya *et al.*¹³⁾ for the modification of Arg residues, we incubated lysozyme with some α -dicarbonyl compounds for 3 hr at 15°C in bicarbonate buffer (pH 8.6), and found that the extent of the modification of Arg residues was 75% by glyoxal, 93% by methylglyoxal, and 28% by 2,3-butanedione. The values for glyoxal and 2,3-butanedione were in good agreement with those reported by Nakaya *et al.*¹³⁾ Impairment of Lys residues was also recognized for glyoxal and 2,3-butanedione, the extent being 28% and 5%, respectively, whereas there was no impairment of Trp residues. It was noteworthy that fairly large amounts of polymerized lysozymes were observed in all the cases (Fig. 6). Their amounts were 23% of the whole lysozyme for 2,3-butanedione, 25% for methylglyoxal, and 27% for glyoxal.

DISCUSSION

This study gave us some valuable information to clarify the mechanism of polymerization of proteins induced by storage with glucose. The results in Table I showed that a certain compound generated from a reaction of amino groups with glucose is responsible for glucose-induced modifications of proteins, *i.e.* impairment of Arg and Trp residues and browning and polymerization of proteins. Furthermore, various carbonyl compounds known to be generated from amino-carbonyl reactions were shown to polymerize acetylated lysozyme accompanying the impairment of

Arg and Trp residues.

On basis of those results including the data from the TBA reaction, it was strongly suggested that a reaction of free amino groups of proteins with glucose occurring at the initial stage of the storage produces a certain carbonyl compound, which subsequently combined with Arg and Trp residues and polymerized the protein. However, the suggested carbonyl compounds was not found among the various carbonyl compounds tested in this study. Thus, further studies are needed to find the cross-linker responsible for glucose-induced polymerization of proteins.

Nevertheless, we obtained the elution patterns of the unknown peaks on amino acid analysis of the protein modified by various carbonyl compounds are useful to find what kind of carbonyl compounds, if any, are involved, when proteins deteriorate during storage or processing of foods.

α -Dicarbonyl compounds are assumed to cross-link protein molecules through a combination of their two carbonyl groups with two amino acid residues such as Arg, Lys, or Trp residues. α -Hydroxycarbonyl compounds seem to react with Lys residues as a ketose or aldose and then to be converted to α -dicarbonyl compounds. However, this speculation is not applicable to explain why they reacted with the acetylated lysozyme. Thus, they seem also able to be converted to α -dicarbonyl compounds through a reaction not requiring Lys residues. 5-Hydroxymethylfurfural does not seem to be converted to an α -dicarbonyl compound. This may be why it is less reactive with acetylated lysozyme, while fairly reactive to lysozyme as a monoaldehyde.

Our study showed that polymerization of proteins occurred to a notable extent even under the conditions used conventionally for the chemical modification of Arg residues. This result should be noted when such a method is employed to investigate the role of Arg residues in the biological action of proteins.

Furthermore, the fact that such a method caused polymerization of proteins without the

impairment of Trp residues suggested that Arg residues were the most reactive binding sites for α -dicarbonyl compounds to cross-link proteins. Thus, we are doing further studies to reveal the structure of the unknown compounds which eluted just behind the Phe peak on amino acid analysis and was ascribed to the impaired Arg residues of the lysozyme stored with glucose.

As to the role of Lys residues, the results of the reaction between various carbonyl compounds and lysozyme revealed that Lys residues were also able to be the sites for those compounds to bind on cross-linking proteins. This seemed to explain why an excessive addition of free Lys into the acetylated lysozyme/glucose system lessened its own effect to induce modification of the protein (Table I): A certain carbonyl compound generated through a reaction of Lys with glucose reacted again with the remaining Lys, so that its availability to react to Arg and Trp residues was reduced. There is the possibility that on the glucose-induced polymerization of proteins Lys residues also become binding sites of the cross-linker which is generated by themselves through a reaction with glucose. Thus, further study on the impaired Lys is also needed to understand the mechanism of the glucose-induced polymerization of proteins.

Moreover, a study is also needed to examine whether or not the impaired Trp residues are involved in cross-linking of proteins.

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