1,5-Anhydro-D-fructose, a New Material for Modification of Amino Groups as Demonstrated Using Amino Acid and Proteins

Kazuhiro Yoshinaga, Mami Fujisue,¹ Jun-ichi Abe,^{2,*}
Yasuhito Takeda² and Susumu Hizukuri³

*The United Graduate School of Agricultural Sciences, Kagoshima University
(1-21-24, Korimoto, Kagoshima 890-0065, Japan)*

¹*Nihondenpun Kogyo Co.*

(3-20, Nanei, Kagoshima 891-0196, Japan)

²*Department of Biochemical Science and Technology, Faculty of Agriculture, Kagoshima University
(1-21-24, Korimoto, Kagoshima 890-0065, Japan)*

³*Faculty of Home Economics, Kobe Women's University
(2-1, Aoyama, Higashisuma, Kobe 654-8585, Japan)*

1,5-Anhydro-D-fructose (1,5-anhydro-D-arabino-hexo-2-ulose, 1,5-AF) reacted well with the amino group and seemed to be useful as a reagent for modification of the amino compounds. The rate of browning of 1,5-AF by reaction with glycine was higher than those of glucose and xylose at 50°C and pH 7.5. Forty-five percent of the amino group of glycine was modified by the incubation of a lyophilized mixture of 1,5-AF and glycine at 30°C for 48 h, whereas the degree of modification by glucose was less than 5%. This modification was applied to lysozyme. A mass spectrum indicated that the sugar moiety was incorporated into the lysozyme. The optimum pH values of the modified and unmodified lysozyme on glycolchitin were pH 4.5 and 5.0, respectively. On the other hand, the optimum pH of the modified enzyme on freeze-dried cells of *Micrococcus lysodeikticus* shifted from pH 7.8 to 6.0. Wheat proteins, gliadin and glutenin, were also modified. The modified gliadin had higher emulsification ability and solubility than the unmodified ones.

1,5-AF is a sugar that was isolated from several fungi¹⁾ and an edible seaweed²⁾ as an intermediate of the biosynthetic pathway for microthecin, and now the sugar is considered to be distributed widely among organisms, including animals.³⁾ The sugar is prepared conveniently from α -1,4-glucan by the action of α -1,4-glucan lyase^{1,4)} and regarded to be highly reactive, since it has an ethylenic linkage in its molecule.¹⁾ Yu *et al.* have reported that 1,5-AF was a highly reactive sugar.⁵⁾ We have reported that 1,5-AF has higher antioxidative activity than ascorbic acid does by weight⁶⁾ and reduces cupric and ferric ions even at 35°C. The latter reactivity allows sensitive and selective determination of 1,5-AF.⁷⁾

It is well known that the carbonyl group reacts with the amino group (Maillard reaction). The preparation of some conjugates of food proteins and sugars for functional improvement has been reported. For example, the thermal stability and emulsification ability of ovalbumin are improved by modification with glucose 6-phosphate,⁸⁾ and the modification of lysozyme with glucose-stearic acid monoester enhances its ability to emulsify.⁹⁾ The problems in this procedure are a rather high temperature and/or long incubation times needed for the reaction. Since the reactivity of 1,5-AF is higher than of other reducing sugars such as glucose, fructose, and xylose, 1,5-AF may overcome these drawbacks and be more suitable for the production of glycosylated food proteins.

In this study we examined the properties of 1,5-AF as a new modifier of amino groups of an

* To whom all correspondence should be addressed
(J.abbe@chem.agri.kagoshima-u.ac.jp).

amino acid and protein. To begin with, we examined suitable conditions for the conjugation, using glycine as a target. We then modified lysozyme, which is often used as a preservative in the food industry, in expectation of an improvement of the enzymatic nature of lysozyme such as substrate specificity, pH profile and heat stability. We also modified the major proteins in wheat flour in order to improve the properties of wheat flour. We may be able to expect an increase of water holding capacities and an improvement of textures in breads and noodles by introducing sugar moieties to those proteins.

MATERIALS AND METHODS

Materials. 1,5-AF was prepared from waxy-corn starch by the action of α -1,4-glucan lyase.⁶⁾ The purity of the 1,5-AF produced was 98.5% on HPLC (Mitsubishi Chemical Co., MCI Gel CK 08 S). Chicken egg-white lysozyme (crystal grade) was purchased from Sigma. Glycolchitin was prepared from chitin.¹⁰⁾ The lyophilized cells of *Micrococcus lysodeikticus* were from Nacalai Tesque Inc. Protein assay reagent was purchased from Bio-Rad Laboratories. Glutenin and gliadin were prepared from wheat flour.¹¹⁾ Other chemicals were of reagent grade.

Browning of sugars. The mixture of a sugar (5%, w/v) and glycine (5%, w/v) in 50 mM phosphate buffer (pH 7.5) was incubated at 50°C, and the resulting brown color was monitored on a spectrophotometer at 420 nm, using a 1 cm light path.

Modification of amino group. A mixture of glycine or protein and a sugar in 100 mM phosphate buffer (pH 7.5) or the same buffer containing 10% ethanol (in the case of wheat proteins) was dried under reduced pressure for 12 h at room temperature, and then incubated over saturated NaOH (relative humidity of 7.6% at 30°C for 12–48 h). The extent of modification was calculated by the determination of a free amino group with trinitrobenzenesulfonic acid (TNBS) reagent.¹²⁾ In the case of wheat proteins, a free amino group was determined by TNBS reagent after the addition of sulfuric acid.¹³⁾

The molecular weight of the modified or unmodified lysozyme were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using egg albumin, triosephosphate isomerase and chicken egg-white lysozyme as the molecular markers or time of flight mass spectrometry (TOF-MS) on Voyager-DE PRO (PE Biosystems).

Assay of lysozyme. The activity of lysozyme on glycolchitin was measured by the method of Yamada *et al.*⁹⁾ One unit of activity is defined as the amount of the enzyme that decreases the absorbance of 0.001 at 420 nm/min, using a light path of 1 cm. Lytic activity of the enzyme was determined as follows: The suspension (2 mL, A_{450} = 1.0) of *M. lysodeikticus* cells in 50 mM buffers and 25 μ L of enzyme were incubated at 25°C for 20 min. The turbidity of the mixture was measured at 450 nm, using a light path of 1 cm. One unit is defined as the amount of the enzyme that decreases the absorbance of 0.001 at 450 nm/min.

Heat stability of modified lysozyme. A lysozyme preparation (50 μ g/mL) in 50 mM phosphate buffer (pH 5.1 or 7.8) was incubated at 75°C for 60 min. The remaining activity was then measured by using the lyophilized cells of *M. lysodeikticus* as described above.

Assay of emulsification ability and solubility. Emulsification ability was determined by the method of Aoki *et al.*¹⁴⁾ Solubility was calculated as follows. Twenty mg of the protein specimen was suspended in 1 mL of 10 mM acetate buffer (pH 6.0) at room temperature for 1 h. Then the protein content of the supernatant was measured with Bio-Rad protein assay reagent after centrifugation at $3000 \times g$ for 10 min.

RESULTS AND DISCUSSION

Browning reaction.

The reactivity of 1,5-AF with the amino group was found to be significantly higher than that of glucose or xylose. After incubation of the reaction mixtures of each sugar and glycine at 50°C for 80 min, A_{420} of the mixture of 1,5-AF was 1.8; that of xylose was 0.1, no color with glucose (Fig. 1). Xylose has been reported as the sugar that causes

Fig. 1. Browning of mixtures of sugars and glycine at 50°C, pH 7.5.

The mixture of a sugar (5%, w/v) and glycine (5%, w/v) in 50 mM phosphate buffer (pH 7.5) was incubated at 50°C. ○, 1,5-AF; ●, glucose; □, xylose.

the largest extent of amino-carbonyl reaction among the known reducing sugars¹⁵; thus, 1,5-AF might be the best sugar for this reaction. The Maillard reaction is often used in food processing to bring out the flavor and/or color and to improve the taste of various foods; however, the reaction needs a rather high temperature. The high reactivity property of 1,5-AF prompted us to create a "neoglycoprotein" by the modification of amino groups in some proteins under mild conditions without losing the functional structure and activity of the proteins.

Modification of glycine with 1,5-AF.

To find the best conditions for modification, we tested a sugar's reaction with glycine under various conditions. Forty-five percent of the amino group was modified with 1,5-AF, and the extent of modification with glucose was less than 5% (Fig. 2) at the molar ratio of sugar to glycine, 15 : 1 at 30°C for 48 h. The modification with 1,5-AF was rapid until 24 h, then the reaction seemed to reach plateau. The reason why the reaction almost ceased after 24 h still remains to be clarified.

The degree of modification increased with the increase in the reaction tested from 20 to 50°C (Fig. 3A). It is noteworthy that the reaction proceeded even at a low temperature of 20°C; thus, this modification can be applicable for use with materials unstable when heated. The 1,5-AF/glycine ratios of 20 to 30 seemed enough for the more than 40% of glycosylation when operated at

Fig. 2. Modification of glycine with 1,5-AF or glucose.

The mixtures with the molar ratio of sugars to glycine, 15 : 1, were lyophilized and incubated at 30°C under 7.6% relative humidity. ●, 1,5-AF; ○, glucose.

Fig. 3. Modification of glycine with 1,5-AF.

(A) Temperature dependence for the reaction at the molar ratio of 1,5-AF to glycine, 15 : 1. (B) Molar ratio dependence of 1,5-AF to glycine on the reaction at 30°C for 48 h.

30°C for 48 h (Fig. 3B). From these results, we chose the sugar/amino-group ratio of 30 and a reaction temperature of 30°C for further studies. When 1,5-AF was incubated as above without lysine, the amount of the sugar was decreased to 50% after 48 h. This could be one reason why a large excess amount of 1,5-AF was needed for the reaction. The glycosylation with 1,5-AF proceeded in a solid state even under mild conditions, but it did not proceed as well in a diluted buffer solution (data not shown). 1,5-AF exists in a hydrate form in an aqueous solution, but in dimeric forms in non-aqueous solution.¹⁶ Thus, it is probable that the different forms of 1,5-AF have different reactivity and products. The analyses of the resulting products remain for as a future study.

Modification of lysozyme.

Under the above conditions, 35% of the free

amino groups in lysozyme were modified after incubation for 144 h at 30°C (Fig. 4). We prepared two lysozyme preparations, of which 10 and 35% of the free amino groups were abolished (termed as 10- and 35-lysozyme, respectively). These were dissolved in water, and the free 1,5-AF was removed by repeated dialysis against water.

Both preparations showed two bands (14 and 28 kDa) on SDS-PAGE (Fig. 5), and one band was found in a control preparation (c-lysozyme) that was treated as above without 1,5-AF. The band width of 14 kDa in 35-lysozyme was broader than the c-lysozyme band, suggesting that the prepara-

Fig. 4. Modification of lysozyme with 1,5-AF.

The mixture of lysozyme (1 mg/mL) and 1,5-AF (2.5 mg/mL) in 100 mM phosphate buffer (pH 7.5) was lyophilized and incubated at 30°C under 7.6% relative humidity.

Fig. 5. SDS-PAGE of modified lysozyme.

Proteins were stained by Coomassie Brilliant Blue R250. Lane 1, M_r markers; lanes 2, 3 and 4, 35-, 10-, and c-lysozyme, respectively. Protein standards: egg albumin (45.0 kDa), triosephosphate isomerase (26.6 kDa), and chicken egg-white lysozyme (14.3 kDa).

tion was a mixture of different M_r because of the different extent of glycosylation. The band at the position of 28 kDa in both preparations seemed to be a dimer protein that might emerge with cross-linking. By a quantitative analysis of the each band, the ratios of the amount of 14 to 28 kDa were determined to be 5 : 1 and 3.5 : 1 in regard to 10- and 35-lysozyme, respectively, showing that the ratio of the amount of 28 kDa increased as the reaction proceeded. Yeboah *et al.* reported that a polymerization of bovine serum albumin was induced by modification with glucose¹⁷⁾ possibly due to the dicarbonic acid which was produced by the oxidation of glucose.

To analyze the molecular weights of the modified lysozyme in detail, 35-lysozyme was analyzed by TOF-MS. The molecular weight of the control lysozyme preparation was 14,265, and this result certified that no degradation or cross-linking of lysozyme had occurred during incubation in the absence of 1,5-AF. The spectrum of 35-lysozyme showed 3 peaks (Fig. 6, inset), and a main peak around 14 kDa had a very wide distribution that consisted of several peaks. We could find a very small peak at 14,265, whose value showed M_r of a native enzyme, and several small peaks were found at every 144 interval. The most abundant molecular species seemed to be the protein conjugated with 4 sugars (Fig. 6). This number seemed larger than an expected value of 2 or 3, since lysozyme has 6 lysyl residues and an N-terminal residue, and the extent of modification was 35%. One possibility would be the modification of arginine residues in lysozyme as suggested by Yeboah *et al.*¹⁸⁾ on the occasion of lysozyme modification with glucose. Further experiments will be needed to account clearly for this discrepancy.

To examine the alteration of function upon the modification, the activities of the 35- and c-lysozyme on glycolchitin were compared. The apparent shift of pH-dependent activity profile of the modified lysozyme could be expected, since the positive charges of the protein surface had been neutralized by conjugation of the neutral sugar. The optimum pH values of the 35- and c-lysozyme were 4.5 and 5.0, respectively. The relative specific activity of 35-lysozyme was 95% of the ac-

Fig. 6. TOF-MS spectrum of 35-lysozyme.

Arrow indicates the m/z value of c-lysozyme. The insert shows the spectrum of the wide range of m/z .

Fig. 7. pH dependence of the activity of modified lysozyme.

(A) Glycolchitin as a substrate. The pHs were adjusted with acetate buffer (final conc., 50 mM). The specific activity of c-lysozyme at pH 4.5 was taken as 100%. (B) Freeze dried *M. lysodeikticus* as a substrate. The specific activity of c-lysozyme at pH 7.8 was taken as 100%. The pHs were adjusted with phosphate buffer (final conc., 50 mM). ●, 35-lysozyme; ▲, 10-lysozyme; ◇, c-lysozyme.

tivity of c-lysozyme (Fig. 7A) at maximum pH. These results indicated that the glycosylation inflicted almost no damage on the catalytic activity and structure of lysozyme, and suggested that the lysozyme dimer kept its own activity.

Although the enzymatic activity on soluble glycolchitin indicates the catalytic efficiency of the enzyme, the action on the bacterial cell wall is practically important to evaluate the effect of the lysozyme modification. The pH-activity profiles of the control and modified enzymes on lyophilized

cells of *M. lysodeikticus* are shown in Fig. 7B. Although the pH profiles of c- and 10-lysozyme were almost the same, that of 35-lysozyme was completely different. The optimum pH value of 35-lysozyme was pH 6.0, and the relative activity of 35-lysozyme was 130% of that of c-lysozyme at this pH. Similar results have been reported on the modification of lysozyme with acetic anhydride¹⁹⁾ and monomethoxypolyethylene.²⁰⁾ The positive charges on the surface of lysozyme are suggested not to be essential for its catalytic action on β -1,4-*N*-acetyl-glucosaminidyl linkage of substrate, but essential for the interaction of the enzyme with the negatively charged cell wall of bacteria. The adsorption of the enzyme to the cell by an electrostatic interaction seems to be important for the cell-lytic activity, and the decrease in this activity might be explained by the disappearance of such forces because of the modification of NH_2 residues.

A modification of lysine for alteration of properties of a protein by chemicals often introduces insolubility to a target protein and requires severe conditions for a protein to maintain its physiological activity. The coupling of monomethoxypolyethylene to the NH_2 group²⁰⁾ seems advantageous because the reaction proceeds at a low temperature; however, the chemical synthesis of the com-

pound might be laborious. In consideration of the application of the modified lysozyme in the preservation of acidic foods, the modification by 1,5-anhydro-D-fructose should be advantageous over those modified by chemicals, since the sugar is a natural and soluble material.

The heat stability of the 35- and c-lysozyme were evaluated by the incubation of the enzymes at 75°C for 60 min. These reactions were tested at pH 5.1 and 7.8. The lytic activities of heated 35-lysozyme decreased to 45% (pH 5.1) and 14% (pH 7.8) of the values before heating, respectively. When c-lysozyme was heated under the same conditions, the activities decreased to 91% (pH 5.1) and 55% (pH 7.8). These suggested that the modification possibly introduced some distortion of the structure due to the hydrophilic nature of the sugars and/or the disappearance of negative charges.

Modification of wheat proteins with 1,5-AF.

Twenty five percent and 22% of amino groups in gliadin and glutenin, respectively, were modified with 1,5-AF by the reactions at 30°C for 48 h. The number of modified amino groups in these proteins was less than that of lysozyme possibly due to the insoluble nature of these proteins. In the case of gliadin, the modification brought a 56% and 70% increase in the solubility and emulsification ability, respectively, as expected. However, almost the same solubility and emulsification ability as those of native protein were observed when the modified glutenin was tested. Glutenin and gliadin are major proteins in wheat flour and give specific properties such as expansibility of the dough,²¹⁾ so that the addition of the modified gliadin may give new properties to the mixed wheat flour.

In this study, the availability of 1,5-AF as a modifier of the amino group of food proteins was revealed by the lysozyme and wheat protein modifications in a model experiment. The modification of a protein with 1,5-AF causes the loss of the positive charge of the surface of the target, and the alteration of the physical properties of the modified proteins can be expected. The merits of 1,5-AF as a modifier for food proteins would be that the reaction proceeds under mild conditions and that 1,5-AF is an edible and soluble sugar. The

1,5-AF glycosylation of food proteins may result in improvements in the other characteristics, such as stability and resistance against protease.

REFERENCES

- 1) M.A. Baute, R. Baute and G. Deffieux: Fungal enzymic activity degrading 1,4- α -D-glucans to 1,5-D-anhydrofructose. *Phytochemistry*, **27**, 3401–3403 (1988).
- 2) A. Broberg, L. Kenne and M. Pedersen: Analysis of 1,5-anhydro-D-fructose, microthecin, and 4-deoxyglycero-hexo-2,3-diulose in algae using gas chromatography-mass spectrometry in selected ion monitoring mode. *Anal. Biochem.*, **268**, 35–42 (1999).
- 3) S. Kametani, Y. Shiga and H. Akanuma: Hepatic production of 1,5-anhydrofructose and 1,5-anhydroglucitol in rat by the third glycogenolytic pathway. *Eur. J. Biochem.*, **242**, 832–838 (1996).
- 4) S. Yu, L. Kenne and M. Pedersen: α -1,4-Glucan lyase, a new class of starch/glycogen degrading enzyme. I. Efficient purification and characterization from red seaweeds. *Biochim. Biophys. Acta*, **1156**, 313–320 (1993).
- 5) S. Yu, C. E. Olsen and J. Marcussen: Methods for the assay of 1,5-anhydro-D-fructose and α -1,4-glucan lyase. *Carbohydr. Res.*, **305**, 73–82 (1998).
- 6) M. Fujisue, K. Yoshinaga, K. Muroya, J. Abe and S. Hizukuri: Preparation and antioxidative activity of 1,5-anhydro-D-fructose. *J. Appl. Glycosci.*, **46**, 439–444 (1999).
- 7) K. Yoshinaga, M. Fujisue, J. Abe, Y. Takeda and S. Hizukuri: Specific and sensitive determination methods for 1,5-anhydro-D-fructose. *J. Appl. Glycosci.*, **48**, 139–142 (2001).
- 8) Y. Kato, T. Aoki, N. Kato, R. Nakamura and T. Matsuda: Modification of ovalbumin with glucose 6-phosphate by amino-carbonyl reaction: Improvement of protein heat stability and emulsifying activity. *J. Agric. Food Chem.*, **43**, 301–305 (1995).
- 9) K. Takahashi, X. F. Lou, Y. Ishii and M. Hattori: Lysozyme-glucose stearic acid monoester conjugate formed through the maillard reaction as an antibacterial emulsifier. *J. Agric. Food Chem.*, **48**, 2044–2049 (2000).
- 10) H. Yamada and T. Imoto: A convenient synthesis of glycolchitin, a substrate of lysozyme. *Carbohydr. Res.*, **92**, 160–162 (1981).
- 11) S. Antes and H. Wieser: Effect of high and low molecular weight glutenin subunits on rheological dough properties and bread making quality of wheat. *Cereal Chem.*, **78**, 157–159 (2001).
- 12) R. Fields: The measurement of amino group in protein

- and peptides. *Biochem. J.*, **124**, 581–590 (1971).
- 13) V. Tropini, J.P. Lens, W.J. Mulder and F. Silvestre: Cross-linking of wheat gluten using a water-soluble carboimide. *Cereal Chem.*, **77**, 333–338 (2000).
 - 14) T. Aoki, Y. Hiidome, K. Kitahata, Y. Sugino, H.R. Ibrahim and Y. Kato: Improvement of heat stability and emulsifying activity of ovalbumin by conjugation with glucuronic acid through the Maillard reaction. *Food Res. Int.*, **32**, 129–133 (1999).
 - 15) R.M. Cleale, 4th, T.J. Klopfenstein, R.A. Britton, L. D. Satterlee and S.R. Lowry: Induced non-enzymatic browning of soybean meal. I. Effect of factors controlling non-enzymatic browning on *in vitro* ammonia release. *J. Anim. Sci.*, **65**, 1312–1318 (1987).
 - 16) S.M. Andersen, I. Lundt and J. Marcussen: 1,5-Anhydro-D-fructose: stereoselective conversions to 1,5-anhydroalditols and deoxy/amino substituted analogues. *J. Carbohydr. Chem.*, **19**, 717–725 (2000).
 - 17) F.K. Yeboah, I. Alli and V.A. Yaylayan: Reactive of D-glucose and D-fructose during glycation of bovine serum albumin. *J. Agric. Food Chem.*, **47**, 3164–3172 (1999).
 - 18) F.K. Yeboah, I. Alli, V.A. Yaylayan, Y. Konishi and P. Stefanowicz: Monitoring glycation of lysozyme by electrospray ionization mass spectrometry. *J. Agric. Food Chem.*, **48**, 2766–2774 (2000).
 - 19) N. Yamasaki, K. Hayashi and M. Funatsu: Acetylation of lysozyme part I. Mechanism of lysis by lysozyme. *Agric. Biol. Chem.*, **32**, 64–68 (1968).
 - 20) Y. Nodake and N. Yamasaki: Some properties of a macromolecular conjugate of lysozyme prepared by modification with a monomethoxypolyethylene glycol derivative. *Biosci. Biotechnol. Biochem.*, **64**, 767–774 (2000).
 - 21) W.P. Campbell, C.W. Wrigley, P.J. Cressey and C.R. Slack: Statistical correlation between quality attributes and grain-protein for 71 hexaploid wheats used as breeding parents. *Cereal Chem.*, **64**, 293–299 (1987).

(Received October 9, 2001; Accepted November 19, 2001)

新しいアミノ基の修飾剤,
1,5-アンヒドロ-D-フルクトースによる
アミノ酸と蛋白質の修飾

吉永一浩, 藤末真実¹, 安部淳一²
竹田靖史², 檜作 進³

鹿児島大学大学院農学研究科
(890-0065 鹿児島市郡元 1-21-24)

¹ 日本澱粉工業株式会社
(891-0196 鹿児島市南栄 3-20)

² 鹿児島大学農学部
(890-0065 鹿児島市郡元 1-21-24)

³ 神戸女子大学家政学部
(654-8585 神戸市須磨区東須磨青山 2-1)

1,5-アンヒドロ-D-フルクトース(1,5-アンヒドロ-D-アラビノ-ヘキソ-2-ウロース, 1,5-AF)はアミノ基とよく反応するため, アミノ化合物の修飾剤としての利用が期待できる. グリシンと1,5-AFを50°C, pH 7.5で反応させた場合の着色は, グルコースやキシロースよりも大きかった. 1,5-AFとグリシンの混合物を凍結乾燥した後, 30°Cで48時間反応させると45%のアミノ基が修飾された. 一方, 同じ条件のグルコースでは5%以下であった. この1,5-AFによる修飾をリゾチームに応用した. 修飾リゾチームの質量分析から1,5-AFが分子内に導入されていることが確かめられた. 修飾リゾチームの最適pHは, グリコールキチンを基質とした場合pH 4.5で未修飾はpH 5.0であった. 一方, *Micrococcus lysodeikticus*の乾燥菌体を基質とした場合は最適pHが修飾によりpH 7.8から6.0に変化した. コムギ蛋白であるグリアジンとグルテニンを同様に修飾したところ, 修飾グリアジンの乳化性と溶解性が向上した.