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Chemical properties and antioxidative activity of glycated α-lactalbumin with a rare sugar, D-allose, by Maillard reaction

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Abstract

The Maillard reaction (MR) is an ubiquitous reaction of condensation of a reducing sugar with amino groups of protein, which may improve the functional and/or biological properties of foods. The present study was carried out to determine the degree of α-lactalbumin (α-LA) conjugation with a rare sugar (D-allose, All) and two alimentary sugars (D-fructose, Fru; D-glucose, Glc) through MR and the extent to which these reactions could convey antioxidant activity to α-LA. The MR was generated in dry state at 50 °C and 55% relative humidity for up to 48 h. The results showed that the conjugation rate and fluorescence development of α-LA modified with All were faster than that of those modified with Glc and Fru, respectively. Furthermore, α-LA modified with All exhibited the highest tetrazolium salt (XTT) reducibility and radical cation ($ABTS^{\cdot+}$)-scavenging activity. There was a good correlation between the fluorescence temporal patterns and biochemical activity of the different sugar-protein conjugates. Thus, the protein glycated with All could be used in formulated food as a functional ingredient, with a strong antioxidant activity, for scavenging free radicals and delaying deterioration due to oxidation.

Keywords: α-Lactalbumin; D-allose; Antioxidant activity; Maillard reaction

1. Introduction

Maillard reaction products (MRPs), formed as a consequence of heat treatment or prolonged storage, were reported to have functional and/or biological activities. It was suggested that MRPs generally exhibited significant antioxidant properties and antimutagenic characteristics (Anese, Pittia, & Nicoli, 1993; Eichner, 1981; Nicoli, Anese, Parpinel, Franceschi, & Lerici, 1997; Yun & Tsai, 1993). Lipid oxidation rates were significantly slowed when MRPs were added or formed during heating (Bressa, Tesson, Dalla Rosa, Sensidoni, & Yubaro, 1996; Severini & Pittia, 1994). MRPs could exhibit antioxidant activities in both model lipid and food, inhibit the oxidative degradation of natural organic compounds, and improve the oxidative stability of food products (McGookin & Augustin, 1991; Namiki, 1990; Wijewickreme & Kitts, 1997; Wijewickreme, Krejpcio, & Kitts, 1999). Thus, MRPs could be expected to have significant antioxidant potential for use in food systems as functional ingredients.

Recently, rare sugars have attracted a great deal of attention, mainly concentrated on their role for a variety of uses, such as potential inhibitors of various glycosidases (Muniruzzaman et al., 1996), low-calorie carbohydrate sweeteners and bulking agents (Levin, Zehner, Sanders, & Beadle, 1995; Livesey & Brown, 1996; Matsuo, Suzuki, Hashiguchi, & Izumori, 2002), enhancing heat-induced gelling behaviour (Sun, Hayakawa, & Izumori, 2004), and improving clinical effects as immunosuppressants on allogenic orthotopic liver

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transplantation in rats (Hossain et al., 2000). Arnold et al. reported that D-allose (All), a rare aldo-hexose, substantially inhibited segmented neutrophil production and lowered platelet counts without other detrimental clinical effects (US pat. No. 5620960, 1997). Furthermore, they suggested that All might be used in the treatment of myeloid leukemia. However, All is not abundant in nature, and is difficult to prepare by chemical methods. Its biological activities have not been studied very well. In recent years, Bhuiyan, Itami, Rokui, Katayama, and Izumori (1998) reported an enzymatic method of All production from D-psicose (Psi) that acted as a raw material, using L-rhamnose isomerase. Furthermore, Takeshita, Suga, Takada, and Izumori (2000) reported an improved method for the large-scale production of Psi from Fru using D-tagatose 3-epimerase. These findings provide the foundation for rare sugar study and utilization of their biological activities.

Increasing interest has been directed towards the utilization of normal food constituents with antioxidative properties, and MRPs are widely present in foods. It is well known that MRPs from some alimentary sugars possess antioxidant activities; it is also interesting to know whether or not the rare sugar, All, conveys antioxidant activity to protein by non-enzymatic glycation. Here the bovine α-lactalbumin (α-LA) serves as a model protein for studies of glycated protein because it is a low molecular weight globular protein (14,179 Da) with ε-amino groups of lysine residues. A small protein is highly amenable to direct analysis by mass spectrometry for defining covalent modifications (Humeny, Kislinger, Becker, & Pischetsrieder, 2002). Nacka, Chobert, Burova, Léonil, and Haertlé (1998) reported that glycation of α-LA was more pronounced and faster than that of other milk proteins.

The main purpose of this study was to investigate to what extent any antioxidative activity was formed in α-LA glycated with a rare aldo-hexose, All, compared to Fru and Glc as reference sugars. Antioxidative activities were evaluated by using tetrazolium salt (XTT) reducibility and radical cation ($ABTS^{\cdot+}$)-scavenging activity. The chemical characteristics of MRPs were important factors for evaluating antioxidant activity. Therefore, the formation of covalent conjugates and fluorescent substance, due to non-enzymatic glycation, were also characterized in order to gain more insight of the development of these beneficial properties for human health.

2. Materials and methods

2.1. Materials

D-Allose was obtained from Kagawa Rare Sugar Cluster (Lot No. KAI001-09-011, Japan). α-Lactalbumin (from bovine milk, Type 1, Lot No. 12K7048), $K_2S_2O_8$ and XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5carboxanilide] were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Trolox (6-hydrox-2,5,7,8-tetramethylchroman-2-carboxylic aid) was from Aldrich Chemical Co., ABTS [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)], menadione (2-methyl-1,4-naphthoquinone) crotonaldehyde, OPA (*o*-Phthalaldehyde), D-glucose and D-fructose were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals used in this study were of analytical grade.

2.2. Preparation of glycated samples

Glycation was performed with a rare sugar (All) and two alimentary sugars (Fru and Glc), respectively. α-LA was dissolved in 10 mM carbonate buffer (pH 9.0) at a protein concentration of 5% (w/v) with the sugars (16.4% of the protein dry weight), that is, the molar ratio is about 1:13 of protein to sugar (approximate 1:1 ratio of sugar carbonyl to free amino groups). The solutions were lyophilized. The mixed samples were incubated in the dry state at 50 °C and 55% relative humidity (RH) for up to 48 h (an interval of 12 h) in an incubator (LHL-113, Espect Co., Japan). For these procedures, control experiments were carried out with no added sugars, and three separate experiments were conducted.

2.3. Determination of free amino groups

The quantity of free amino groups was determined by the OPA method (Fayle et al., 2001). To a 50 μl aliquot of samples, containing 2 mg/ml α-LA protein, was added 1 ml of OPA reagent. The absorbance was read at 340 nm after a minimal delay of 2 min at room temperature. A calibration curve was obtained by using 0.25–2.00 mM glycine as a standard. The protein content of all samples in the present study was determined by the Lowry method (1951).

2.4. MALDI–TOF–MS

Matrix-assisted laser desorption/ionization time of flight (MALDI–TOF) analysis was performed on an Autoflex (Bruker Daltonics, Germary) mass spectrometer operated in a linear, positive ion mode with N_2 laser. The samples (100 pmol/μl) were diluted 1:10 in TA solution (0.1% trifluoroacetic acid with 33% acetonitrile). One microlitre of sample solution was mixed with 4 μl of matrix solution (a saturated solution of sinapinic acid in TA solution). An aliquot (1 μl) of this mixture was spotted onto a stainless steel target, air-dried, and then subjected to mass determination. Exter-

nal calibration was performed by the use of a standard protein mixture.

2.5. Determination of fluorescence intensity

The fluorescence intensity (FI) of glycated α-LA was measured at 350/420 nm (excitation/emission) using a F-2500 spectrofluorometer (Hitachi, Co. Japan). The sample was dissolved in 10 mM phosphate buffer (pH 7.0) at a protein concentration of 2 mg/ml. FI was expressed as relative FI in arbitrary units (AU).

2.6. Assay of tetrazolium salt XTT reducibility

The assay was performed in a 96-well microtitre plate according to the method described by Shimamura, Takamori, Ukeda, Nagata, and Sawamura (2000). XTT solution was prepared daily with 0.2 M potassium phosphate buffer (pH 7.0) containing menadione at the saturation level. Each well contained 40 μl of samples (5%). Then 60 μl of 0.5 mM XTT solution was added into the well. After it was mixed for 15 s, the difference in the absorbance between 490 and 570 nm (as the reference) was read on a microplate reader (Model 550, Bio-Rad Co., Japan) as the absorbance at 0 min. Again, after 20 min at room temperature, the absorbance difference was read, and the increase in the absorbance difference was calculated as the XTT reducibility of the sample.

2.7. Determination of ABTS radical-scavenging activity

The ABTS radical cation method (Pellegrini, Ying, & Rice-Evans, 1999) was used to evaluate the free radical-scavenging effect of MRPs. $ABTS^{\cdot+}$ was formed by adding $K_2S_2O_8$ to ABTS. The $ABTS^{\cdot+}$ stock solution was diluted with 10 mM phosphate buffer (pH 7.4) to a final absorbance of the control of 0.7 ± 0.02 at 734 nm. Scavenging activity of MRPs was calculated by determining the percentage of decolorization at room temperature exactly 20 s after the initial mixing and up to 6 min. Because of a gradual decrease in absorbance of the working $ABTS^{\cdot+}$ solution (without the sample added), appropriate solvent blanks were run in each measurement.

2.8. Calculation of trolox equivalent antioxidant capacity

A calibration curve was prepared with different concentrations (standard range of 0–20 μM, final concentration) of trolox. Trolox equivalent antioxidant capacity (TEAC) can be assigned to all samples able to scavenge the $ABTS^{\cdot+}$ by comparing their scavenging capacity to that of trolox, a water-soluble vitamin E analogue.

All determinations were carried out at least three times, and in triplicate. Mean values were plotted for all Figures, and standard deviation was calculated using data of each replicate.

3. Results and discussion

3.1. Change of free amino groups

Glycation of proteins occurs by a chemical reaction of reducing sugars with free amino groups in proteins to form the Schiff's base linkage (Singh, Barden, Mori, & Beilin, 2001). To assay amino group availability, the quantities of free amino groups in α-LA incubated in the presence and absence (heated control) of reducing sugar for up to 48 h were measured using the OPA modified method (Fig. 1). α-LA contains 13 potential reactive amino groups, including one terminal α-NH_2 and 12 ε-NH_2 of lysine residues. Since no amino groups were modified on native α-LA, all results were reported relative to 100% of amino groups of native sample. In the heated control samples, the free amino groups did not significantly change for up to 48 h of incubation as compared to native sample. However, the free amino groups significantly decreased in all glycated-protein samples incubated for up to 24 h, in which 56.8%, 46.9% and 15.3% of 13 amino groups were modified by All, Glc and Fru, respectively. After 24 h of incubation, the free amino groups appeared to decrease slightly. The results clearly showed a higher reactivity of All, compared with two alimentary sugars, in undergoing condensation reactions with amines under these conditions.

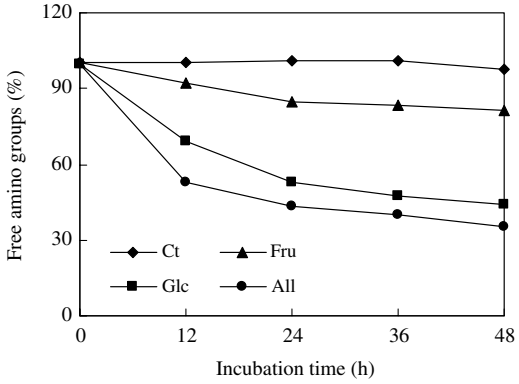

Fig. 1. Change of free amino groups of α-LA alone (Ct) and glycated α-LA with three reducing sugars (Fru, Glc and All) at 50 °C and 55% RH over 48 h of incubation period, as determined by the OPA assay. The protein concentration was 2 mg/ml. All results were determined at least three times and in triplicate.

3.2. Characteristics of sugar-protein conjugates

Mass spectrometry, providing accurate relative molecular mass values (M), has been used to qualitatively study the formation of sugar-protein conjugates. In this study, the confirmation of the covalent addition of the reducing sugar to α-LA was obtained by applying MALDI–TOF–MS. The molecular ion of native α-LA produced a mass peak of 14,179 ± 2.6 Da (not shown). The mass spectra of control samples, incubated for up to 48 h, were almost identical to that of native protein, confirming that the heat treatment could not affect the molecular integrity of protein in the absence of reducing sugar. However, the initial treated samples observed in the presence of reducing sugars (after 12 h of incubation) significantly exhibited a mass shift toward high molecular weight (not shown). The MALDI–TOF–MS spectrum of α-LA samples incubated for 24 h became more complex, indicating that the extent of modification further increased. After heat treatment for 24 h, the mass spectrum exhibited less alteration.

The mass spectra of the α-LA derivatives, with different reducing sugars for 24 h of incubation as an example, illustrated the modified degree (Fig. 2). As the condensation of a hexose molecule leads to a mass increase of 162 Da, the number of hexose residues on the proteins can easily be calculated by comparing mass difference (ΔM) between glycated and native protein. In case of α-LA modified with Fru, the spectrum showed two main peaks at m/z 14,341.5 (ΔM = 162.5) and 14,502.3 (ΔM = 323.3). In addition, an intact peak at m/z 14,179 Da, as well as two minor peaks at m/z 14,665.5 (ΔM = 486.5) and 14,827.6 Da (ΔM = 648.6) were also detected. These mass species, in turn, could account for 0–4 molecules of Fru condensing on one α-LA molecule. The mass change observed here (162 Da per reducing hexose unit) is consistent with those previously reported for the glycation of protein (Lapolla, Fedele, Garbeglio, & Martano, 2000). In the cases of Glc- and All-α-LA conjugates, the mass spectra showed that the intact α-LA peak at m/z 14,179 Da had disappeared, which suggested that all α-LA molecules had been modified by two such reducing sugars (Fig. 2). Furthermore, the reaction of α-LA with Glc and All resulted in the higher-molecular mass species, corresponding to different numbers of sugar molecules condensing on α-LA, in the ranges from 2 to 10 and from 3 to 11 for Glc- and All-protein, respectively. The main peaks at m/z 15,153.2 (ΔM = 974.2) and 15,478.4 (ΔM = 1299.4 Da) were shown in modified proteins with Glc and All, respectively, which accounted for a corresponding addition of about 6 molecules of Glc and 8 molecules of All. The results based on the mass spectrometry were in agreement with those obtained by OPA analysis.

3.3. Development of FI

The progress of FI of glycated protein with different reducing sugars is shown in Fig. 3. The results showed that control samples appeared to have no formation of fluorescent substance over 48 h of the incubation period at 50 °C and 55% RH, whereas the glycated protein exhibited significant FI. The rate of fluorescent development in the All-glycated protein was found to be highest among the three sugars. The Glc-glycated protein was at the second fastest rate, but its rate was closer to the rate of the Fru-glycated protein than that of the All-glycated protein. A correlation between the length of incubation period and FI of glycated proteins was quite evident, with longer incubation treatment resulting in a more intense fluorescence. In our previous study, glycated ovalbumin, with a rare ketohexose, D-psicose, also yielded very high FI (50 AU), which was about twice that of Glc-ovalbumin (26 AU), and Fru-ovalbumin, which displayed intermediate FI (39 AU) at 55 °C and 65% RH

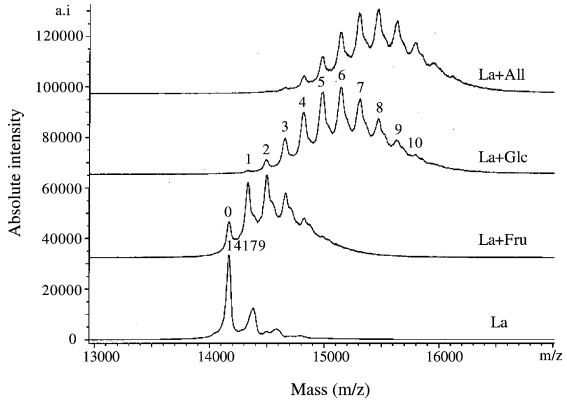

Fig. 2. MALDI-TOF-MS spectra of control (Ct) and glycated α-LA with Fru, Glc and All, respectively, at 50 °C and 55% RH for 24 h of incubation. The peak number means the number of sugar molecules condensing on α-LA.

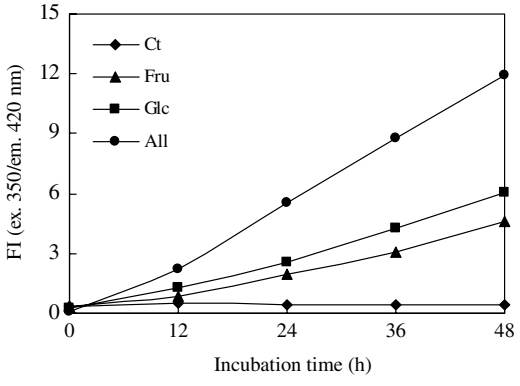

Fig. 3. Change in fluorescence intensity of α-LA glycated with three reducing sugars over 48 h of incubation period. The protein concentration was 2 mg/ml. All results were determined at least three times and in triplicate.

after 2 days of incubation (Sun et al., 2004). In the present investigation, we explored the effect of glycation on chemical and functional properties by use of a rare sugar (All) and control sugars (Glc and Fru). Glc and Fru were selected as control sugar reactants due to the difference in aldose and ketose structures. In general, aldoses are considered to be more reactive than ketoses, because of their more electrophilic carbonyl groups (Yeboah, Alli, & Yaylayan, 1999). However, the ketose sugar Fru is found to brown more quickly than the aldose isomer Glc, because it has a high concentration of acyclic forms in aqueous solutions (Jing & Kitts, 2002). Our previous results showed that Fru was more reactive in browning, fluorescence and protein cross-linking than Glc during incubation of ovalbumin at 55 °C and 65% RH. In contrast, the rate of fluorescence development in glycation of α-LA with Glc was higher than that with Fru at 50 °C and 55% RH, suggesting that different sugars might produce different reaction rates, amounts and types of MRPs in different reaction conditions.

3.4. XTT reducibility of glycated proteins

Reducing activity of MRPs formed from α-LA with three reducing sugars was determined by XTT assay (Fig. 4). All glycated α-LA showed various degrees of XTT reducibility, depending on the duration of incubation. In contrast, it was shown that there was not significant variation in XTT reducibility of control samples incubated over 48 h. So we speculated that MRPs derived from glycated-protein, but not the protein itself, took part in their reducing activity against XTT. On the other hand, intensity of XTT reducibility, induced by glycation, also depended on the sugar used for modification. Among MRPs derived from three reducing sugars, MRPs modified with All exhibited the highest XTT reducibility, which implied that protein modified with such a rare sugar might have greater reducing power than that modified with the two alimentary sugars. Recently, XTT assay has been reported to be applicable for estimating, not only the extent of MR, but also the biological activity, such as DNA strand breakage, of reaction products derived from glycated protein with reducing sugars (Shimamura et al., 2000; Shimamura, Ukeda, & Sawamura, 2000). In the present study, it was shown that notable differences in FI between the rare sugar and alimentary sugars reflected variations in biochemical activities in reference to reduced XTT activities. At the same time, the result showed also a good correlation ($R^2 = 0.94$) between XTT reducibility and FI, which suggested that XTT assay might be an important linkage between the extent of MR and the reducibility of MRPs.

3.5. Processing of $ABTS^{\cdot+}$-scavenging activity

The ABTS method gives a measure of the antioxidant activity of MRPs by measuring the reduction of the radical cation as the percentage inhibition of absorbance at 734 nm. Fig. 5 shows the effect of control and glycated samples (at 14 μM, final concentration) on the radical-scavenging activity with the duration of incubation. The $ABTS^{\cdot+}$-scavenging activity was determined at 1 min after the initial mixing. Radical-scavenging activities of control samples over 48 h of incubation were only ~10.9%, showing a weak radical-scavenging activity of protein. In contrast, the glycated protein showed a marked scavenging effect. In the case of glycated α-LA with All, the highest radical-scavenging activity, with ~53.5% over 48 h of incubation, was shown. α-LA modified with Fru and Glc had about ~22.5% and ~29.0% of radical-scavenging activity, respectively. The results indicated that glycation could induce an antioxidant activity to the protein, the intensity of which depended on the duration of incubation and reducing sugar used

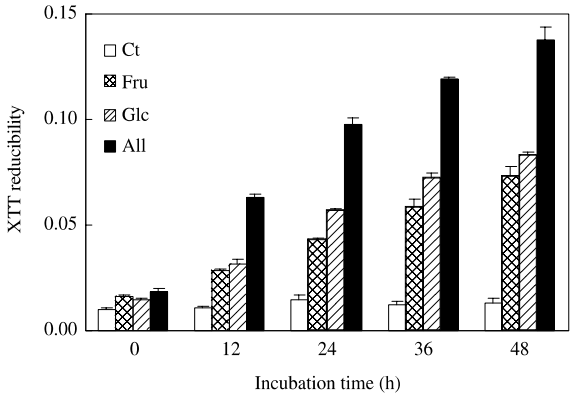

Fig. 4. XTT reducibility of control (Ct) and glycated α-LA (at 5% of protein concentration) with different reducing sugars (Fru, Glc and All) and different incubation periods. Data shown are mean values ± SD of three complete sets of experiments ($n = 9$).

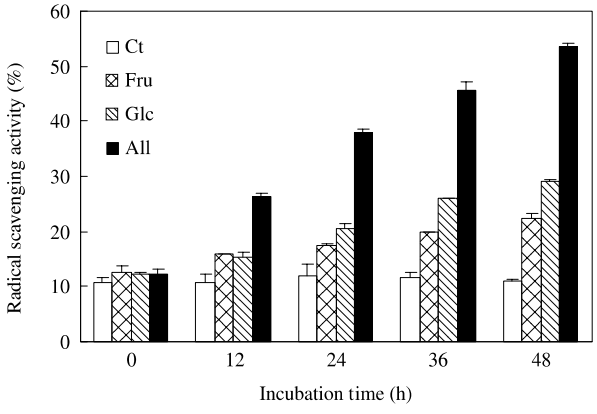

Fig. 5. ABTS radical-scavenging activity (%) of control (Ct) and glycated α-LA (at 14 μM, final concentration) with different reducing sugars (Fru, Glc and All) and different incubation periods. Data shown are mean values ± SD of three complete sets of experiments ($n = 9$).

for modification. The tendency of the radical-scavenging activity also agreed with that of the XTT reducibility; that is, protein modified with such a rare sugar produced relatively higher radical-scavenging capacity and stronger reducing power than that modified with alimentary sugars. Therefore, it is speculated that food ingredients with strong antioxidant potential, which derived from food-grade protein and a rare sugar, might act as new functional foods.

3.6. Influence of covalent linking and fluorescent substance on antioxidant activity

It is known that the extent of MR is important for the explanation of the functional behaviours of MRPs. Firstly it was investigated that the radical-scavenging activity of glycated α-LA over 48 h of incubation versus the number of reactional amino groups (Fig. 6(a)). In the cases of α-LA modified with Fru and Glc, although the quantities of reactional amino groups were significantly different, the scavenging effects showed only minor differences between them. On the other hand, as compared to Glc-α-LA, the degree of α-LA conjugated with All was slightly increased, whereas its scavenging effect was significantly enhanced. The results showed that the scavenging activity did not correspond directly with the interaction initiated between free amino groups in proteins and the carbonyl moiety of reducing sugars. It has been implied that this activity might vary considerably with the nature of reducing sugar used for the modification, in which each sugar produced different amounts and types of MRP compounds (Wijewickreme, Kitts, & Durance, 1997).

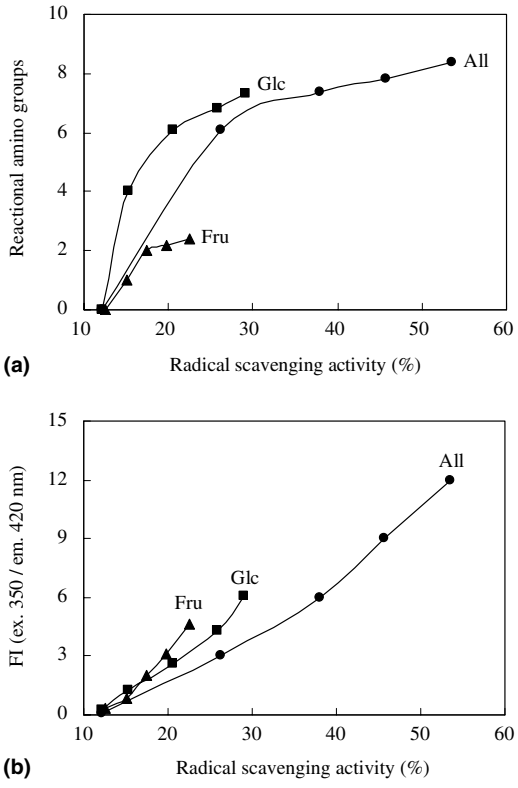

Fig. 6. The effects of reactional amino groups (a) and fluorescence intensity (b) on radical-scavenging activity of MRPs after various durations of incubation (0, 12, 24, 36 and 48 h).

Data of FI and scavenging activities in glycated α-LA over 48 h of incubation were analyzed in order to evaluate whether the fluorescence development, due to MR, was related to the antioxidant activity of glycated protein (Fig. 6(b)). A high positive correlation between FI and antioxidant activity of MRPs was identified with the three reducing sugars used (Fru:$R^2 = 0.976$; Glc: $R^2 = 0.982$; All:$R^2 = 0.979$). It was noteworthy that a lower scavenging activity in incubated samples was shown during the early stages of the MR (after 12 h of incubation) in which there were very low FI values. The initial process of the MR leads to a rather unstable Schiff base which may rearrange to the more stable Amadori and Heyn's products (Hodge, 1953; Yaylayan, 1997). These products are degraded to a large number of compounds summarized as MRPs. The formation of fluorescent substance has frequently been associated with the formation of compounds with antioxidant capacity, suggesting that FI could be a good indirect index for monitoring the formation of MRPs with free radical-scavenging activity (Morales & Jiménez-Pérez, 2001; Nishino, Shibahara-Sone, Kikuchi-Hayakawa, & Ishikawa, 2000). In this sense, the antioxidant activity of MRPs could mainly be attributed to the high molecular weight fluorescent compounds, which were formed in the advanced stages of reaction.

3.7. Antioxidant efficiency of glycated protein

To predict the antioxidant efficiency of MRPs, the glycated samples from 48 h of incubation were assessed by TEAC assay as described below.

Fig. 7 illustrates the effect of time on the suppression of the absorption of the ABTS radical cation at 734 nm by addition of trolox and samples. Trolox reacted instantaneously with $ABTS^{\bullet+}$, and then a plateau was rapidly attained. This pattern implied that the reaction was completed within a few seconds for trolox. A closer look at the time course of the $ABTS^{\bullet+}$ consumption in the absorbance assay indicated that MRPs reacted slowly with the $ABTS^{\bullet+}$ and the reaction took several minutes. This kinetic experiment was realized to estimate the time necessary to obtain a relatively stable absorbance of $ABTS^{\bullet+}$ after being in contact with glycated proteins. From the findings, we chose two time-points, at 1 and 6 min, to describe the antioxidant efficiency by TEAC assay. The 1 min TEAC gave a fast reaction, whereas the TEAC at 6 min was chosen because it included the greater part of the slow reaction.

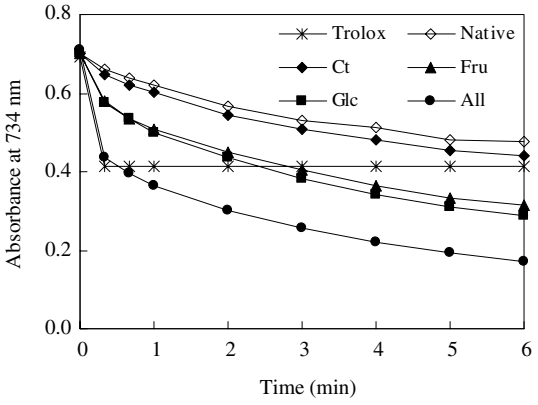

Fig. 7. The effect of time on the suppression of the absorbance of $ABTS^{\cdot+}$ by addition of trolox, native, control (Ct) and glycated α-LA. The final concentrations of trolox and protein samples are 10 and 10.5 μM, respectively. The control and modified α-LA with three reducing sugars (Fru, Glc and All) are incubated for 48 h.

Miller, Diplock, and Rice-Evans (1995) also used 6 min as a function of time for TEAC assay.

The scavenging effect was plotted as a function of concentration in order to determine the TEAC, which could be assessed as a function of time (at 1 and 6 min). As shown in Fig. 8, the concentration-dependent curve obtained at 1 min after initial mixing was plotted as the percentage of scavenging activity. As expected, the increase in scavenging activity in the trolox standard curve was linearly related to the increase in the concentration of trolox. In the case of glycated α-LA, the scavenging activity increased significantly at lower concentration, and then appeared to increase slowly, more than the sample concentration of 15 μM. Since a linear correlation between scavenging effect and concentration was not found in the glycated α-LA, the three concentration-points (7, 10.5 and 14 μM) were selected as function of concentration for MRPs. The TEAC value was assigned by comparing the scavenging activity of MRPs to that of trolox at each specific concentration- and time-point.

The TEAC values of MRPs on the reduction of the absorption measured at 1 and 6 min are depicted in Fig. 9(a) and (b), respectively. The TEAC values of All-α-LA (7.0 μM) were 1.38 ± 0.03 (1 min) and 2.33 ± 0.04 (6 min). Whereas the TEAC values of Fru-α-LA (7.0 μM) were 0.81 ± 0.06 (1 min) and 1.82 ± 0.09 (6 min); the TEAC values of Glc-α-LA (7.0 μM) were 0.82 ± 0.02 (1 min) and 1.87 ± 0.05 (6 min). At the other two concentrations (10.5 and 14.0 μM), the TEAC values of MRPs appeared to decrease, compared to 7.0 μM, in both fast and slow reactions. The results showed that the TEAC, between 1 and 6 min after addition of MRPs, exhibited a significant difference. At the same time, the TEAC of MRPs was concentration-dependent: a higher concentration resulted in a lower TEAC. It can be concluded, therefore, that the TEAC of MRPs has to depend on the concentration and time interval used in the assay.

On the other hand, the TEAC of All-α-LA was significantly higher than that of Fru- and Glc-α-LA at all concentration and time points (Fig. 9). This showed that

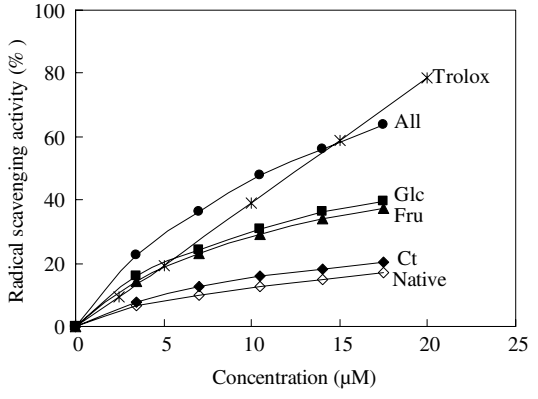

Fig. 8. The effects of concentration of trolox and protein samples on $ABTS^{\cdot+}$-scavenging activity. The extent of decolorization, as percentage scavenging activity of absorbance at 734 nm, is determined at 1 min. The control (Ct) and modified α-LA with three reducing sugars (Fru, Glc and All) are incubated for 48 h. Trolox, as the standard, gives a linear response ($R^2 = 1$) in the concentration range 0–20 μM.

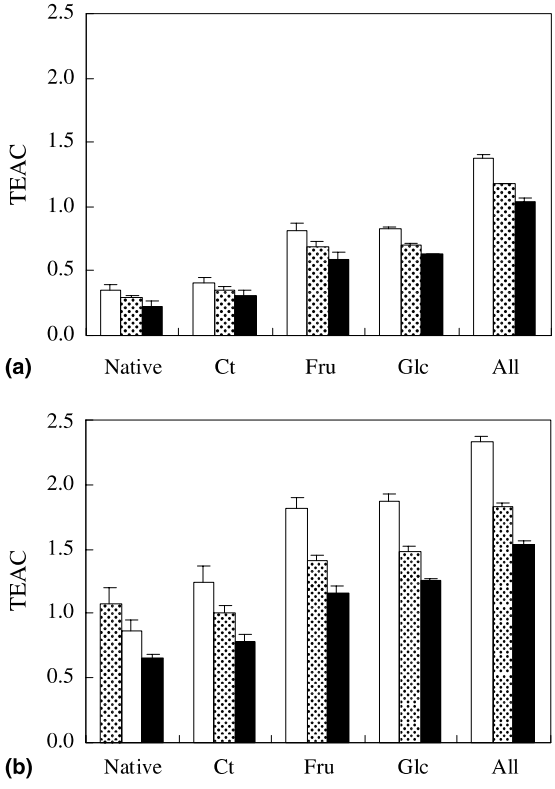

Fig. 9. Trolox equivalent antioxidant capacities (TEAC) of native, control (Ct) and glycated α-LA with three reducing sugars (Fru, Glc and All). The TEAC values were determined at 1 min (a) and 6 min (b). For each sample (incubated for 48 h), columns refer to (from left to right) final concentrations at 7.0, 10.5 and 14.0 μM. Data shown are mean values ± SD of three complete sets of experiments ($n = 9$).

All-α-LA could be qualified as the best antioxidant of the three MRPs by the TEAC assay. The high TEAC value might be explained by the major contribution of MRPs to TEAC. However, it had been found that the TEAC values of heated control and intact samples were time-dependent: a slow reaction (at 6 min) induced the TEAC increase. An explanation was that α-LA itself might have a weak radical-scavenging capacity with a slow reaction. So it was inferred that the TEAC assay for glycated protein could measure the total amount of radical-scavenging effect, formed by both the intact α-LA and new reaction products. It is important in the applicability of the TEAC assay for predicting the antioxidant efficiency of a new antioxidant compound.

4. Conclusion

As compared to alimentary sugars (Fru and Glc), the rare hexose (All) exhibited greater covalent linking and a faster reaction rate in undergoing condensation reactions with amines. Furthermore, the α-LA glycated with All possessed stronger antioxidant properties, including XTT reducibility and $ABTS^{\cdot+}$ radical scavenging activity, and could be qualified as the best antioxidant of the three MRPs with the TEAC assay. In addition, it was obvious that antioxidant activity developed with increased fluorescence of MRPs, the intensity of which depended on the sugar used for the modification and the duration of incubation. For application of an antioxidant activity of protein modified with All as a functional ingredient in food, more research is needed, especially regarding the antioxidative effect of peptides derived from the proteolytic hydrolysis of glycated protein and the antioxidative action of protein hydrolyzates. This is particularly important for human applications.

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