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Original article

Conventional evaluation of glycation with plate reader and its correlation with CML formation

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Abstract

The Maillard reaction is a reaction between the carbonyl group of reducing sugars and the amino group of amino acids, which proceeds not only in foods but also in the body. In the early stage of this reaction, Amadori rearrangement products are formed, followed by oxidation and condensation reactions, and finally forming advanced glycation end products (AGEs). In this study, AGEs, which are still difficult to quantify, were simply measured using a plate reader and compared with an ELISA using an antibody against N^{ε}-(carboxymethyl) lysine (CML). It was evaluated how the formation of these AGEs changed with different types (ribose, fructose, glucose) and concentrations (5 mM, 30 mM) of carbohydrates that are predicted to alter AGE formation. In the results, changes were observed depending on the incubation period and the type of carbohydrate. Furthermore, the effect was more pronounced with higher concentrations of carbohydrates. The correlation between the fluorescence measurement and the CML measurement using antibodies was confirmed, suggesting that the plate reader method can be used to evaluate glycation conveniently.

KEY WORDS: advanced glycation end products (AGEs), glycation, fluorescence, N^{ε}-(carboxymethyl) lysine (CML)

Introduction

A variety of sugars, amino acids, peptides, and proteins are present in foods and the body. The Maillard reaction, a browning reaction, occurs when the carbonyl group of a sugar reacts with the amino group of an amino acid. This reaction can be divided into early and late stage reactions. In the early stage, the carbonyl group and the amino group dehydrate and condense to form Amadori rearrangement products (Amadori compounds) *via* Schiff bases, from which various dicarbonyl compounds are also formed. These Amadori compounds are relatively stable, but in the late stage, advanced glycation end-products (AGEs) are formed by oxidation and dehydration reactions. Factors that affect the progress of this reaction include the type of sugar and amino acids, and temperature [1)].

The Maillard reaction occurs not only in the heating of food but also *in vivo*, and its progression is slower than in the cooking of food, but in diabetic patients, the progression accelerates and is faster than healthy subjects. This is because the Maillard reaction progresses more rapidly in diabetics due to higher blood glucose levels than in healthy subjects, and also because carbonyl compounds formed by metabolic pathways and inflammatory reactions rapidly generate AGEs *in vivo* [2)].

Some AGEs are fluorescent, and they are generally detected at an excitation wavelength of 370 nm and a fluorescence wavelength of 440 nm [3)]. Methods for assessing AGE content include measuring fluorescence intensity, enzyme-linked immune-sorbent assay (ELISA) using anti-AGE antibodies, and instrumental analysis, however, AGE measurement by instrumental analysis is still difficult in many cases because of multiple pretreatment steps.

In this study, we evaluated AGEs by fluorescence intensity using a microplate reader and compared it with ELISA using antibodies against CML [4,5)], which is known to be formed by oxidation reactions. In addition, we compared the effects of the type and concentration of sugars known to affect AGE formation by fluorescence and ELISA. Since

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fluorescent AGEs and non-fluorescent CML are measured in different ways, different methods are currently used when evaluating the formation or inhibition of these AGEs. In this study, we examined whether there was a correlation between fluorescent AGE and CML formation when the conditions for AGE formation were varied.

Methods

Sample preparation

Bovine serum albumin (BSA) (2 mg/mL) (Sigma, Darmstadt, Germany) was added to glucose, fructose (Kanto Chemical, Tokyo, Japan) or ribose (Wako, Osaka, Japan) in phosphate buffer saline (PBS) so that the concentration of sugar was 5 mM or 30 mM. After incubation at 37 °C for 0, 3, 7, and 14 days, the samples were stored at −20 °C. AGEs were measured by the fluorescence method and ELISA at a later date. Three samples of each sugar, concentration, and incubation time were prepared.

Fluorescence measurement

Each incubated sample was diluted 10-fold with distilled water, and 200 µL were added to a 96-well plate (Tecan Greiner 96 Flat Transparent, Greiner Bio-One, Frickenhausen, Germany). The fluorescence was measured with a fluorescence plate reader (Tecan infinite M PLEX, Tecan Japan, Kanagawa, Japan) at an excitation wavelength of 370 nm and a fluorescence wavelength of 470 nm.

ELISA

The incubated samples were diluted to 1 µg/mL of BSA using PBS and added to a 96 well plate (Clear Flat-Bottom Immuno Nonsterile 96-Well Plates, Thermo, Massachusetts, USA), and CML was measured according to the standard method [6]. In practice, the antigen was kept refrigerated for one day, then blocked with 0.5 % gelatin (Sigma, Darmstadt, Germany), and 100 µL of monoclonal anti-CML antibody (0.5 µg/mL) was added to the plate as the primary antibody and stored at room temperature for one hour. Then, 100 µL of horseradish peroxidase-conjugated secondary antibody, Goat antibody Mouse IgG (KPL, Milford, MA, USA), was added to the plate and stored at room temperature for one hour, followed by color development using *o*-phenylenediamine dihydrochloride (OPD; Wako, Osaka, Japan). After 6 minutes of color development, the reaction was stopped with 2 M sulfuric acid, followed by measuring the absorbance with Tecan infinite M PLEX at 492 nm.

Statistical analysis

Bonferroni's multiple analysis was performed using the free statistical software EZR (Easy R) [7] and a significance test was performed.

Result

Comparison of AGE formation by fluorescence

The results of fluorescence measurements showed that the formation of fluorescent AGEs increased more in the pentose, ribose, than in the hexose, glucose and fructose, and that the increase was concentration- and time-dependent (***Fig. 1-a, b***). Furthermore, fructose showed higher fluorescence intensity than glucose in the same hexose. As a result of multiple testing by Bonferroni for each sample on day 14, there was a significant difference in the fluorescence intensity between ribose and fructose, ribose and glucose, and fructose and glucose groups at 5 mM concentration; at 30 mM concentration, the fluorescence intensity of ribose was significantly higher than that of fructose and glucose.

Comparison of CML formation

At a sugar concentration of 5 mM, CML formation increased more in the pentose ribose than in the hexose glucose and fructose, and the same result was obtained at 30 mM (***Fig. 2-a, b***). The results of Bonferroni's multiple tests in each sample on day 14 showed that there was a significant difference in CML formation between ribose and fructose, ribose and glucose at 5 mM, and the same significant difference was also observed at 30 mM.

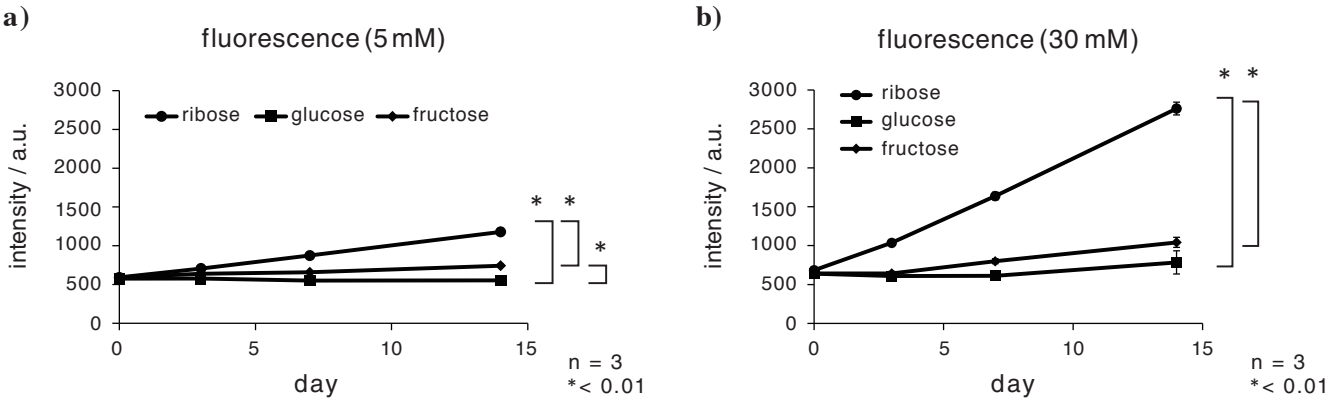

Fig. 1. **Measurement of fluorescent AGEs in fluorescence assay.**

a) Fluorescence AGE intensity of 5 mM ribose, 5 mM glucose, 5 mM fructose; **b)** Fluorescence AGE intesnsity of 30 mM ribose, 30 mM glucose, 30 mM fructose. Bar indicates standard deviation. * $p < 0.01$ by Bonferroni's multiple test, n = 3, performed on each sample at day 14 using EZR software. AGEs, advanced glycation end products.

Comparison between the formation of fluorescence AGEs and CML

The correlation between fluorescent AGE intensity and CML formation was evaluated using samples incubated for 14 days. A strong correlation was observed for ribose and fructose at both 5 mM and 30 mM (***Fig. 3-a, b, e, f***). A stronger correlation was noted for higher concentrations of ribose and fructose. In contrast, no correlation was observed for glucose (***Fig. 3-c, d***).

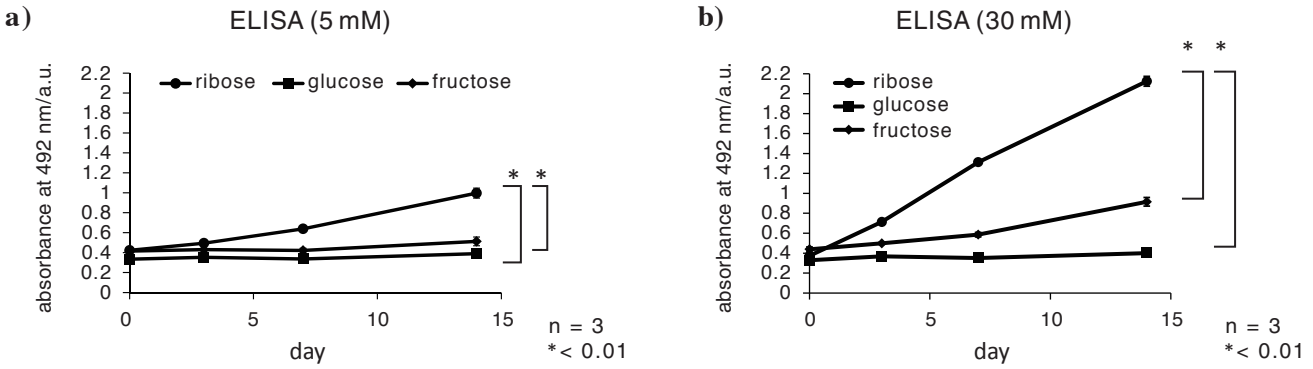

Fig. 2. **CML measurement in ELISA.**

a) CML formation of 5 mM ribose, 5 mM glucose and 5 mM fructose; **b)** CML formation of 30 mM ribose, 30 mM glucose and 30 mM fructose. Bar indicates standard deviation. * $p < 0.01$ by Bonferroni's multiple test, n = 3, performed on each sample at day 14 using EZR software. CML, N^{ε}-(carboxymethyl) lysine; ELISA, enzyme-linked immuno sorbent assay.

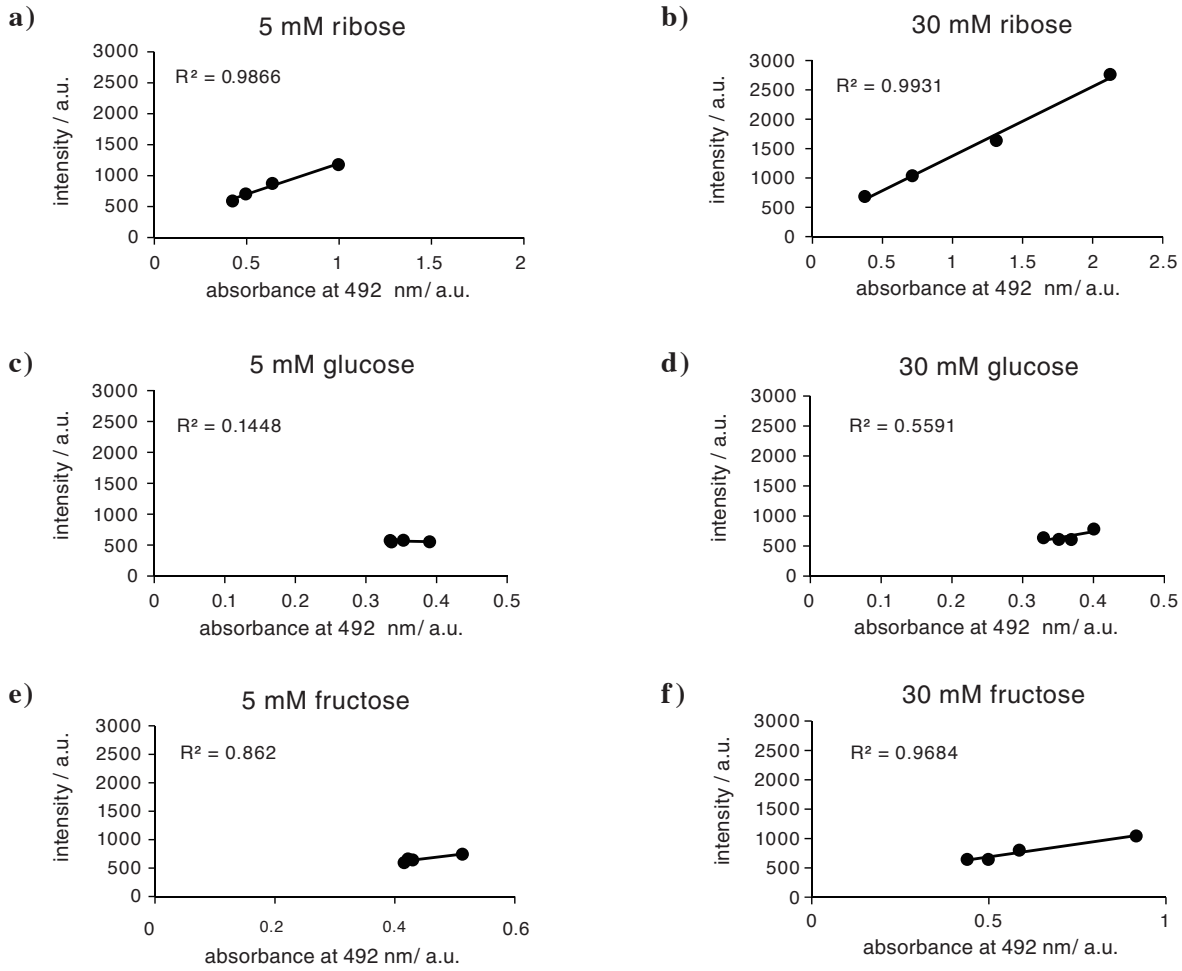

Fig. 3. **Correlation between results of fluorescent AGEs and CML.**

a) 5 mM ribose; **b)** 30 mM ribose; **c)** 5 mM glucose; **d)** 30 mM glucose; **e)** 5 mM fructose; **f)** 30 mM fructose. Samples incubated for 14 days with each sugar and concentration shown in **Fig. 1** and **Fig. 2** were used for evaluation. AGEs, advanced glycation end products; CML, N^{ε}-(carboxymethyl) lysine.

Discussion

When measuring AGEs, it is common to use HPLC equipped with a fluorescent detector or mass spectrometry. However, the maintenance cost of the equipment is necessary and sample pretreatment is required in multiple steps. In contrast, the use of a plate reader made it possible to measure multiple samples of fluorescent AGEs at once with low cost and in a simple manner.

Since the fluorescence intensity increases with protein glycation, it is common practice to evaluate AGE content using a fluorescent detector. However, the conventional fluorescent measurement requires transferring about 3 mL of sample to a cuvette and measuring it with a fluorescent detector. In the present study, we measured the fluorescent intensity using 0.2 mL of the 0.2 mg/mL reaction solution, and confirmed that the fluorescence intensity increased in a time-dependent manner, suggesting that AGEs could be evaluated in the plate reader. The correlation between results of the fluorescence intensity and the ELISA using anti-CML antibody was also confirmed, indicating that the present method can evaluate AGE formation in a more convenient manner than the conventional method. This method is expected to be useful in the search for inhibitors of AGE formation.

Fluorescence measurements showed that AGE formation was accelerated depending on sugar concentration and reaction time, and that AGE formation was increased in pentose rather than in hexose. Results by ELISA showed that CML formation increased in a concentration- and time-dependent manner for ribose, glucose, and fructose. That is to say, as with fluorescent AGEs, CML formation increased in pentose rather than in hexose.

Our data showed that the higher the sugar concentration, the stronger the correlation. Therefore, it is highly likely that fluorescent AGEs correlate with the amount of CML formation in hyperglycemic conditions such as diabetes. Furthermore N^{δ}-(5-hydro-5-methyl-4-imidazolone-2-yl)-ornithine (MG-H1) was also tested by ELISA using antibodies, but not much change was observed (data not shown).

In summary, it was confirmed that fluorescent AGEs can be easily evaluated in a 96-well plate reader and correlated with CML formation. This method will be useful in the search for inhibitors of AGEs formation, which requires the measurement of a large number of samples.

Conflicts of Interest

All authors state that they have no conflicts of interest.

Acknowledgments

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