Food Chemistry 112 (2009) 350–353

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Protein glycation inhibitory activity of wheat bran feruloyl oligosaccharides

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ARTICLE INFO

Article history:
Received 4 January 2008
Received in revised form 20 April 2008
Accepted 22 May 2008

Keywords:
Feruloyl oligosaccharides
Glycation
Protein
Wheat bran

ABSTRACT

Protein glycation is believed to play an important role in the development of long-term disorders associated with diabetes. Water-soluble feruloyl oligosaccharides (FOs) from wheat bran, the ferulic acid esters of oligosaccharides, have been reported as natural antioxidants. The present work assesses the chelating activity of FOs and their inhibition of protein glycation in a bovine serum albumin (BSA)/glucose system, using fluorescence spectroscopy and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). FOs exhibited an effective ferrous ion chelating activity, and quenched the fluorescence intensity of glycated BSA in a dose-dependent manner with 64.0% of inhibition at 1.0 mg/ml. Further, the formation of advanced glycation end products in the tested system was significantly decreased by FOs, as shown by SDS–PAGE. These data indicate that FOs might be beneficial as glycation inhibitors under specified conditions.

1. Introduction

Wheat bran, as an important by-product of the cereal, is produced worldwide in enormous quantities and recognized as a good source of dietary fibre. Ferulic acid (4-hydroxy-3-methoxycinnamic acid), the main phenolic acid of the primary cell walls of monocots, constitutes about 0.5% (w/w) of wheat bran (Kroon, Faulds, Ryden, Robertson, & Williamson, 1997). Free ferulic acid is a good antioxidant, since it forms a resonance-stabilized phenoxy radical, terminates free radical chain reactions, and inhibits lipid peroxidation in rat liver microsomal membranes (Graft, 1992; Trombino et al., 2004). It has been reported that ferulic acid has other physiological functions, including antimicrobial, anti-inflammatory, anti-thrombotic, and anti-cancer activities (Ou & Kwok, 2004). Ferulic acid in wheat bran cell wall is covalently bound to arabinoxylan *via* the acetylation of acidic groups with the primary hydroxyl at the C5 position of α-L-arabinofuranosyl residues (Hatfield, Ralph, & Grabber, 1999). Many studies have been performed on the isolation of feruloyl oligosaccharides (FOs) from Gramineae by mild acid hydrolysis or by treatment with a mixture of polysaccharide hydrolyzing enzymes, such as the fungal hydrolase, Driselase (Ishii, 1997). The FOs prepared so far from graminaceous cell walls have shown good consistency of structure. The α-L-arabinofuranosyl residues are attached to *O*-3 positions of β-1, 4-linked D-xylan and are substituted at position *O*-5 with a feruloyl group (Yoshida-Shimokawa, Yoshida, Kakegawa, & Ishii, 2001). The isolation of these FOs has allowed a better understanding of the plant cell wall structures. Furthermore, interest in these oligosaccharides is motivated by their diverse biological activities and their functional applications. FOs possess *in vitro* antioxidant activity against free radical-induced oxidative damage in normal rat erythrocytes, and stimulate *in vitro* growth of *Bifidobacterium bifidum* (Yuan, Wang, & Yao, 2005; Yuan, Wang, Yao, & Chen, 2005). More interestingly, antioxidative activities of FOs were stronger than that of free ferulic acid in a microsomal lipid peroxidation system and a low density lipoprotein oxidation system (Katapodis et al., 2003; Ohta, Yamasaki, Egashira, & Sanada, 1994). *In vivo*, FOs are suitable antioxidants for protection against oxidative damage in diabetic rats (Ou et al., 2007). Also, Rondini et al. (2004) reported that bound ferulic acid from wheat bran was more bioavailable than was the free compound in rat.

In living organisms, proteins may be susceptible to modification by glucose or other reducing sugars through the non-enzymatic glycation reaction. This reaction finally produces advanced glycation end-products (AGEs). The proposed reaction pathway for AGEs production in a protein–glucose reaction system is subdivided simply into three main stages. First, the glycation process is initiated by the reaction of glucose with free amino groups of proteins to form Schiff bases. Secondly, the formed Schiff bases undergo rearrangement to form early, reversible Amadori products. Finally, the Amadori products subsequently degrade into α-dicarbonyl compounds, deoxyglucosones. These compounds react with amino groups of proteins to form cross-linked, yellow-brown, fluorescent, insoluble, irreversible compounds, usually called AGEs (Hayase, Shibuya, Sato, & Yamamoto, 1996; Yim, Kang, Hah, Chock, & Yim, 1995). The accumulation of AGEs in the body leads to structural and functional modifications of tissue proteins. There is emerging evidence that protein glycation is implicated in the aging process, as well as the pathogenesis of the complications of diabetes

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doi:10.1016/j.foodchem.2008.05.072

(retinopathy, neuropathy, nephropathy and atherosclerosis) and Alzheimer's disease (Lapolla, Traldi, & Fedele, 2005). Oxidative reactions are now known to participate in the process of AGEs formation, so antioxidants and/or radical-scavengers may prevent this event (Fu et al., 1994). There have been many reports on the inhibitory activities of some phenolic compounds in plant against non-enzymatic glycation of proteins (Kim & Kim, 2003; Wu & Yen, 2005). Therefore, it has been suggested that plants might offer a new source of glycation inhibition agents.

To the best of our knowledge, there is, so far, no report on inhibition of protein glycation by water-soluble FOs from wheat bran insoluble dietary fibre. In the present study, the main purpose was to evaluate the inhibitory activity of FOs on protein glycation in a bovine serum albumin (BSA)/glucose system using fluorescence spectroscopy and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

2. Materials and methods

2.1. Materials

FOs were supplied by Dr. Xiaoping Yuan. Bovine serum albumin (fraction V) (BSA), ferulic acid and electrophoresis reagents were purchased from Sigma Company. All other chemicals and solvents used were of analytical grade.

2.2. Measurement of metal ion-chelating activity

The ferrous ion-chelating capacity of FOs was measured according to the method of Yamaguchi, Ariga, Yoshimura, and Nakazawa (2000) with minor modification. Disodium ethylenediaminetetraacetate (EDTA) was used as a reference material. Briefly, 0.25 ml of $FeSO_4$ solution (1 mM) and an equal volume of test sample solution at different concentrations (0.05–1.0 mg/ml) were mixed; 1 ml of Tris–HCl buffer (pH 7.4) and 2,2′-bipyridyl solution (0.1% in 0.2 M HCl), were added to the mixture, respectively, together with 0.4 ml of 10% (w/v) hydroxylamine–HCl and 2.5 ml of ethanol. The control contained all the reaction reagents except FOs or EDTA. The reaction mixture was adjusted to a final volume of 5 ml with water. After shaking well, the mixture was incubated for 10 min at room temperature. The absorbance was determined at 522 nm with a UV-1000 UV/VIS recording spectrophotometer (Rayleigh Analytical Instruments, Beijing, China). A lower absorbance of the reaction mixture indicated a higher ferrous ion-chelating ability. The capability to chelate the ferrous iron was calculated by the following Eq. (1):

$$\text{Chelating effect}(\%) = (1 - A_{\text{Sample522nm}}/A_{\text{Control522nm}}) \times 100 \quad (1)$$

All determinations were performed in triplicate.

2.3. In vitro glycation of BSA

The procedure of protein and glucose reaction followed was that of Luo, Yuan, Yamazaki, Sasaki, and Oka (2001) and was slightly modified. FOs, BSA (bovine serum albumin, fraction V, 10 mg/ml) and glucose (500 mM) were dissolved in 100 mM potassium phosphate buffer (pH 7.4). Ferulic acid was poorly soluble in aqueous solution at pH 7.4. Therefore, its preparation was in alkaline solution and then the pH was adjusted to 7.4 immediately before use. FOs (1 ml) with different concentrations (0.1–1.0 mg/ml) and 1 ml of ferulic acid (0.25 mg/ml) were preincubated with 1 ml of BSA for 30 min at room temperature. After that, the mixture was mixed with 1 ml of glucose and incubated for 40 h at 55 °C. The tested compounds were replaced with 1 ml of potassium phosphate buffer (100 mM, pH 7.4) in the control. An equal volume of 10% (w/v) trichloroacetic acid (TCA) was added to the reaction mixture and the precipitate was collected by centrifugation (5000*g*, 10 min). The precipitate was washed twice with 5% of ice-cold TCA and dissolved in the same potassium phosphate buffer for further analysis. All incubations were performed under sterile conditions. A small drop of chloroform was added to the solution, and the corks were moistened with toluene to inhibit bacterial growth.

2.4. Fluorescence measurement

The fluorescence of the glycated BSA was measured at an excitation wavelength of 337 nm and emission wavelengths ranging from 350 nm to 550 nm, using a HITACHI 650-60 spectrofluorometer. To standardize the instrument, a preparation of control BSA solution, which was also incubated under the same conditions as for *in vitro* glycation of BSA but without glucose, was used to adjust the meter to zero fluorescence.

2.5. SDS–PAGE

SDS–PAGE was performed according to the method of Laemmli (1970) with 4% stacking and 10% separating gels. One aliquot of glycated BSA solution was mixed with an equal volume of SDS sample buffer (20 mg/ml SDS, 30% glycerol, 0.25 M Tris–HCl buffer, pH 6.8) and boiled for 3 min. Fifteen microliters of the mixed solution were put on the gel and the separating gel was run at a constant current of 20 mA for about 3 h. After electrophoresis, the gel was fixed with 60% ethanol, followed by Coomassie brilliant blue R-250 staining.

2.6. Statistical analysis

Data were reported as the means ± standard deviation of triplicate determinations. Data were analyzed by an analysis of variance ($P < 0.05$) and the means separated by Duncan's multiple range test. Results were processed by Statistica (version 6.0).

3. Results and discussion

3.1. Chelating activity

It has been reported that autoxidation of monosaccharides, such as glucose and fructose, under the influence of transition metal ions can lead to ketoaldehyde and H_2O_2 formation. The ketoaldehydes can in turn react with amino groups of proteins, forming ketoimines. These ketoimines may finally lead to AGEs formations. The metal-catalyzed AGEs formation is also inhibited by the metal-chelating agents (Sajthlal, Chithra, & Chanddrakasan, 1998). As metal chelation is one of the important properties of antioxidants, in the present study, the chelating activity of ferrous ion by FOs was estimated by 2,2′-bipyridyl competition assay. FOs interfered with the formation of ferrous and bipyridyl complex, suggesting that they have chelating activity and capture ferrous ion before bipyridyl. It can be seen that there is a significant increase of ferrous ion-chelating activity over the concentration range (0.05–1.0 mg/ml) of FOs in Fig. 1. Although FOs had an overall lower chelating effect than had EDTA, a maximum chelating effect (64.1%) was evident at a concentration of 0.5 mg/ml FOs. Ferrous ion-chelating capacity was significant since it reduced the concentration of the catalysing transition metal in the Fenton reaction and lipid peroxidation (Singh & Rajini, 2004). Reduction of Fe^{2+} concentration in the Fenton reaction would protect against oxidative damage. Wheat bran extracts enriched in phenolic compounds have been reported to inhibit iron-induced lipid oxidation (Baublis, Decker, & Clydesdale, 2000), and to complex with and stabilize transition metal ions (Zhou, Su, & Yu, 2004). In fact, the phenoxide groups

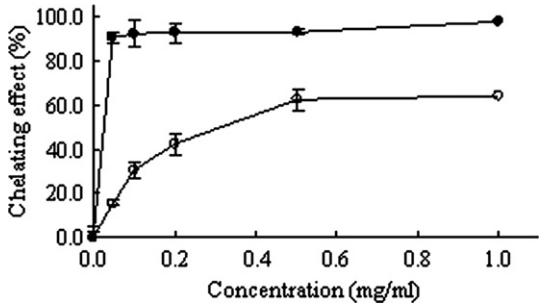

Fig. 1. Fe^{2+}-chelating activity of FOs and EDTA with various concentrations. Values are means ± SD of three determinations. ●, EDTA; ○, FOs.

of deprotonated phenolic compounds possess a high charge density, enabling them to bind suitably charged cations such as transition metal ions.

3.2. Fluorescence spectrum of glycated BSA

The non-enzymatic reaction occurring between reducing sugars, such as glucose and amino groups of proteins results in the Maillard reaction, or non-enzymatic glycation of proteins. Free amino groups of proteins react initially with reducing sugar to form Schiff's bases, which then covalently bond to form Amadori rearrangement products. These Amadori products undergo a rearrangement reaction to form a heterogeneous group of protein-bound moieties with specific fluorescence called AGEs. It is well known that the non-enzymatic glycation of albumin occurs in biological tissues. Serum albumin is most abundant in mammalian plasma. It has been the subject of many investigations because of its easy isolation in large quantities, its high stability and its solubility. BSA usually undergoes marked reversible changes in conformation under non-physiological conditions. The fluorescence intensity of AGEs was highly increased through incubation of BSA with glucose. Therefore, in the present study, BSA was chosen as the model protein for the formation of AGEs through *in vitro* glycation reaction in a glucose–BSA system. The measurement of fluorescence intensity of AGEs was employed for investigation of the inhibitory effects of FOs in glycation. As depicted in Fig. 2, FOs inhibited the fluorescence formation at different concentrations, and a significant decrease of the fluorescence intensities of AGEs with increase of FOs concentrations was observed. The fluorescence intensity was quenched by nearly 64.0% at 1.0 mg/ml of FOs compared with the control.

Interestingly, it can be seen from Fig. 2 that the fluorescence emission wavelengths of AGEs have obvious red shifts in the glucose–BSA system in the presence of FOs, more significant at higher FOs concentrations. When the concentration of FOs is 1.0 mg/ml, the fluorescence emission wavelength of AGEs has been shifted about 36 nm. The red shift indicated that some amino acid residues in proteins have been brought to a more hydrophilic environment.

Ferulic acid possesses electron-donating groups on the benzene ring (3-methoxy, and more importantly, 4-hydroxyl) that can form a resonance-stabilized phenoxy radical, enabling it to act as a natural antioxidant. Therefore, we speculated that the inhibitory activity of FOs against glucose-mediated protein glycation in this study might be related to the free radical-scavenging activity of the ferulic acid moiety in them. As shown in Fig. 3, a significant decrease of the fluorescence intensity of AGEs at 0.25 mg/ml of ferulic acid concentration was observed, and the fluorescence intensity was quenched by 54.0% compared with the control.

3.3. Analysis of SDS–PAGE for AGEs

AGEs are a heterogeneous group of high-molecular weight aggregates stabilized by non-reducible cross-linking components with characteristic fluorescence spectra (Shaw & Crabbe, 1994). In this study, SDS–PAGE was employed to evaluate the formation of AGEs by incubation of BSA with glucose in the tested condition. In the SDS–PAGE profile, as shown in Fig. 4, a very clear band (lane C) with higher relative molecular weight (M_r) than that of the original BSA (lane D) was observed, which is indicated by an arrow. The M_r was estimated to be almost twice that of the original BSA by comparison with molecular weight markers (lane S) and was thus determined to be dimeric BSA. The polymerized protein bands in lanes A and B (for ferulic acid and FOs, respectively) are very weak, which is similar to that of non-glucose-treated BSA in lane D. The results indicate that FOs and ferulic acid suppressed the formation of such a band. This demonstrated that the cross-linked protein aggregates with higher molecular weight, based on the rearrangements, were formed in the BSA glycation induced by glucose.

FOs strongly quenched the fluorescence of the glycated BSA induced by glucose and obviously suppressed the formation of a dimeric BSA under *in vitro* conditions by the analysis of fluorescence and SDS–PAGE, respectively. The results indicate that FOs effectively

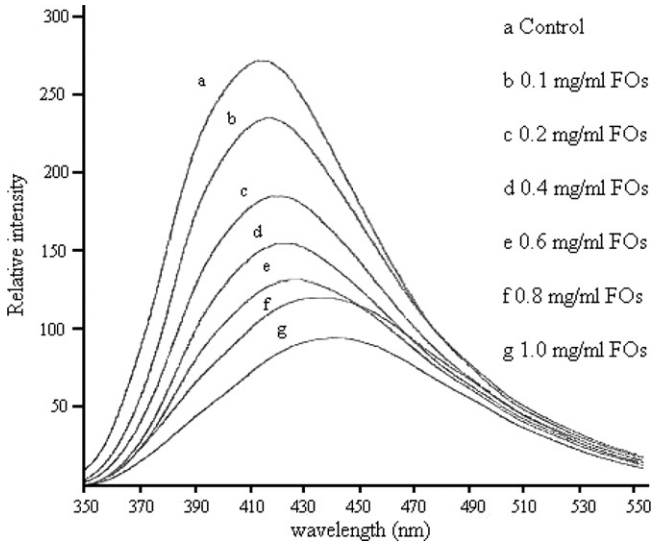

Fig. 2. Fluorescence-quenched spectrum of AGEs by FOs with different concentrations.

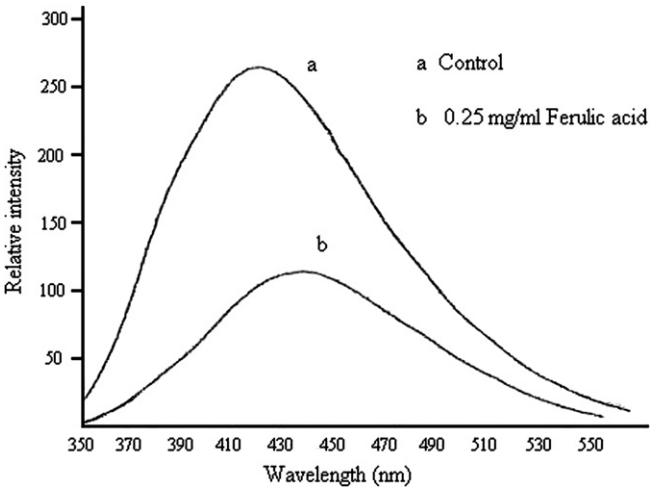

Fig. 3. Fluorescence-quenched spectrum of AGEs by ferulic acid.

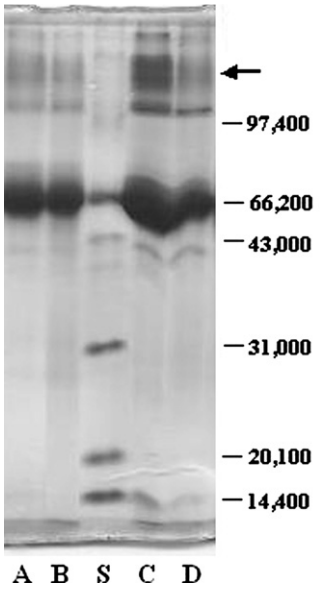

Fig. 4. Effects of FOs on glycated protein using SDS–PAGE. A. Ferulic acid (0.25 mg/ml), B. FOs (1.0 mg/ml), S. standard protein, C. reaction products between glucose and BSA, D. BSA.

inhibited the formation of AGEs in the BSA/glucose system. It has been suggested that free radical formation is involved in the non-enzymatic glycation process, and transition metals accelerate the formation of Amadori compounds *via* Schiff's base (Hayase et al., 1996; Yim et al., 1995). Thus, free radical-scavengers are effective for glycation inhibition (Oya, Osawa, & Kawakishi, 1997). In our previous study, it was reported that FOs could scavenge DPPH free radicals and inhibited the oxidative hemolysis of rat erythrocytes induced by AAPH under *in vitro* conditions (Yuan et al., 2005). FOs also exhibited an effective ferrous ion-chelating activity in the present study. Therefore, the chelating activity and free radical-scavenging activity of FOs were presumed to contribute to the mechanism of glycation inhibition. Experiments with diabetic rats showed that ferulic acid increased the activities of endogenous antioxidants, such as glutathione, superoxide dismutase and catalase, thereby neutralizing free radicals which, in diabetics, are primary causes of accelerated tissue damage (Balasubashini, Rukkumani, Viswanathan, & Menon, 2004).

In conclusion, our investigation had shown that FOs could effectively exhibit a ferrous ion-chelating activity and inhibit non-enzymatic glycation of protein in a glucose/BSA system under *in vitro* conditions. The mechanism of the activities of FOs may be attributable to the ferulic acid moiety in them. The potent glycation inhibitory and antioxidative activities of FOs suggest their possible beneficial roles in the prevention of glycation-associated diseases.

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