Lignin, an active component in the corn silk water extract, inhibits glycation

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Article

Abstract

The formation of advanced glycation end products (AGEs) is associated with aging and diabetic complications such as neuropathy, retinopathy, and nephropathy. Thus, the suppression of AGE formation could prevent and/or treat their related disorders. Corn silk is used as a traditional medicine for the prevention of diabetic complications and treatment of edema in Japan and China. Previous studies revealed the anti-glycation activity of flavonoids in the methanolic extract of corn silk. The anti-glycation activity of the corn silk water extract was higher than that of the methanolic extract; however, the active components of the water extract remained unidentified. In this study, we explored the components showing anti-glycation activity in the corn silk water extract and elucidated their structural characteristics. Remarkable anti-glycation activity was observed in the > 3 kDa fraction. Reversed-phase HPLC analysis of this fraction showed broad peaks characteristic of high-molecular-weight polyphenols. Decomposition reactions did not provide evidence of condensed or acid-hydrolyzable tannins. Therefore, polyphenols contained in the corn silk water extract were considered to be lignins. The ^{1}H- and ^{13}C-NMR and IR spectra of the > 3 kDa fraction were in agreement with the values reported for lignins. Consequently, we concluded that lignin is one of the active components against glycation in the corn silk water extract.

Introduction

Reducing sugars such as glucose can undergo several non-enzymatically reactions with the amino groups in proteins, lipids, and nucleic acids, forming Schiff bases and Amadori products to produce advanced glycation end products (AGEs). The process of AGE formation is known as glycation. Some AGEs, including N^{ε}-(carboxymethyl) lysine (CML) and pentosidine, have been detected in the lesions of patients with diabetes [1]. AGE formation affects the function and half-life of proteins and induces protein denaturation and irreversible damage. Moreover, AGE formations associated with the development of diabetic complications, such as neurological, cardiovascular, and renal dysfunctions [2, 3]. Therefore, inhibitors of AGE formation have recently received increasing attention as potential therapeutic agents.

Currently, many glycation inhibitors derived from synthetic and natural compounds have been reported [4]. Aminoguanidine, a hydrazine-mimetic compound, is a well-known synthetic glycation inhibitor, whereas the flavonoids such as rutin and luteolin are natural glycation inhibitors. Fruits, vegetables, and beverages are major sources of naturally occurring flavonoids and are relevant for the discovery of new glycation inhibitors [5]. For example, corn silk contains vitamins, proteins, sodium salts, volatile oils, steroids, saponins, tannins, and flavonoids. Corn silk refers to the stigmas of corn (*Zea mays* L., Gramineae) and has been used in folk medicine as a diuretic and for the prevention of diabetic complications in Japan and China [6–8].

In our previous study, we observed the anti-glycation activity of corn silk. In the corn silk methanolic extract, we isolated and identified several flavone *C*-glycosides with an anti-glycation activity comparable to that of aminoguanidine [8]. Furthermore, we revealed that the corn silk water extract has effects on

streptozotocin-induced diabetic nephropathy in rats [9]. However, the active components in the hot water extract remained unidentified. In this study, we aimed to elucidate the structure of the active components that inhibit glycation in the water extract of corn silk, as this herbal extract has great potential as a therapeutic agent to prevent and alleviate diabetic complications and dysfunctions caused by aging.

Results And Discussion

First, we examined the inhibitory effect of the corn silk water extract on AGE formation using a specific enzyme-linked immunosorbent assay (ELISA). The inhibitory activity (%) was 100% at a concentration of 25 μg/mL. To explore the active components of corn silk, its aqueous extract was separated into two fractions based on molecular size using a Centricon Plus-70 (3 kDa, Merck Millipore Ltd., Darmstadt, Germany). The inhibitory activities of these fractions were confirmed using an AGE-specific ELISA. The > 3 kDa and < 3 kDa fractions at 100 μg/mL showed inhibitory activities of 79% and 71.1%, respectively. In comparison, the inhibitory activity of aminoguanidine (positive control) was 96.1% at 10 mM. As the > 3 kDa fraction showed a slightly stronger inhibitory activity than the < 3 kDa fraction, we only used the > 3 kDa fraction in the following tests.

To identify the active components in the corn silk water extract, the > 3 kDa fraction was analyzed using a reversed phase (RP)-HPLC system equipped with a UV detector set at 254 nm. The chromatogram of the > 3 kDa fraction showed a broad peak, similar to a hump (Fig. 1). This pattern has been reported in polyphenol analyses using HPLC [10, 11], which suggested that the > 3 kDa fraction contained high-molecular-weight polyphenols.

In general, the molecular weight of high-molecular-weight polyphenols is difficult to determine because their structures are unspecified, and their sizes are large and wide. Therefore, we attempted to determine molecular weight of the > 3k Da fraction by gel permeation chromatography (GPC). The GPC analysis showed that the average molecular weight of the > 3 kDa fraction was 225,749 Da (Fig. 2). Subsequently, we performed phloroglucinol and benzyl mercaptan degradations, which are widely used for the structural analysis of condensed tannins [12–15]. Flavan-3-ol units specific to condensed tannins were not detected, and hydrolysable tannins, such as ellagic and gallic acids [16], were not observed in the acid hydrolysis experiments. According to these results, the active compounds of the corn silk water extract were neither condensed nor hydrolysable tannins.

The ^{1}H-NMR spectrum of the > 3 kDa fraction (Fig. 3) showed signals between 6.5 and 7.3 ppm, which were attributed to aromatic protons. The strong signals observed between 4.0 and 3.0 ppm might be originated from methoxyl protons. Signals attributed to aliphatic moieties were presented between 0.5 and 1.5 ppm. The ^{13}C-NMR spectrum of the > 3 kDa fraction (Fig. 4) revealed signals at 23 and 56 ppm, which were attributed to sp^3 aliphatic carbons. In particular, the signal at 56 ppm was considered to be methoxyl carbon. Sugar-derived signals were also observed at 70–80 ppm. In addition, signals at 115, 130, and 145 ppm, attributed to sp^2 aromatic carbons, and signals at 173 and 175 ppm, attributed to carbonyls, were observed in the ^{13}C-NMR spectrum. These spectral data suggested that phenolic

components were present in large amounts in the > 3 kDa fraction. Moreover, these NMR spectra suggested that the phenolic polymer compounds were lignins [17]. Therefore, we aimed to confirm the presence of lignin in the > 3 kDa fraction.

In the Fourier transform infrared spectroscopy (FT-IR) spectra, the band at 3370 cm^{-1} was engendered by the hydroxyl group. The band at approximately 2925 cm^{-1} was assigned to the C-H stretching vibration of methyl and methane. The signal at 1640 cm^{-1} was attributed to the conjugated carbonyl group, and the regions at 1567 cm^{-1} and 1550 cm^{-1} represented the characteristic absorption of the benzene skeleton. The band at 1246 cm^{-1} was typical of the guaiacyl unit, and the band at 1036 cm^{-1} was attributed to the stretching vibration of the ether bond in polysaccharides (Fig. 5). These results were consistent with the reported lignin spectrum [17]. As the hydrolysis of lignin releases vanillin and sugars [18], the acid hydrolysate prepared from the > 3 kDa fraction was analyzed by liquid chromatography (LC)-MS to detect vanillin (Fig. 6). In addition, glucose and xylose were identified using HPLC (Fig. 7), confirming the presence of lignin in the > 3 kDa fraction.

In summary, we determined that the > 3 kDa fraction of the corn silk water extract contains lignin, which is a phenolic polymer compound. Polyphenols such as tannins were previously considered as the inhibitory active compounds against the glycation in corn silk. However, this study revealed for the first time that the active component of the corn silk water extract is lignin. Therefore, our results can promote the application of corn silk as a material for functional foods, by specifying the characteristic constituents of corn silk and suggesting a basis for quality control.

Experimental Procedures

Plant material

Corn silk (Lot: 293011) was purchased from Kojima Kampo Co., Ltd. (Osaka, Japan). The specimens were maintained at the Laboratory of Natural Products and Phytochemistry, Department of Pharmaceutical Sciences, Faculty of Pharmacy and Pharmaceutical Sciences, Josai University.

Extraction

Dried corn silk (500 g) was extracted using H_2O for 24 h at room temperature. The extract was filtered through a paper filter and lyophilized to obtain a freeze-dried residue (19.7 g).

Ultrafiltration

The freeze-dried residue (560 mg) was dissolved in H_2O (280 mL) and centrifuged using a Centricon Plus-70 (3 kDa, Merck Millipore Ltd.) at 2000 × g for 60 min. The yields of the resultant fractions with molecular weights > 3 kDa and < 3 kDa were 208.8 mg and 288.8 mg, respectively.

Inhibition of AGE formation

Bovine serum albumin (4 mg/mL) was incubated with 200 mM glucose in the presence or absence of extract (25 μg/mL) or fraction (100 μg/mL) in 0.1 M phosphate buffer (pH 7.4) for 7 days at 37 °C. After incubation, the level of generated AGEs (CML) was measured using an AGE-specific ELISA based on the method described by Suzuki et al. [19]. Inhibition was calculated as follows: inhibition (%) = [1-(As-Ab)/(Ac-Ab)] × 100, where As is the level of AGEs of the incubated mixture with sample, Ab is the level of AGEs of the incubated mixture without sample and glucose as blank control, and Ac is the level of AGEs of the incubated mixture without sample as a positive control.

RP-HPLC analysis of the > 3 kDa fraction

RP-HPLC assays were performed using a Shimadzu Prominence HPLC system (Shimadzu Co., Kyoto, Japan) composed of a pump equipped with a degasser, autosampler, column oven, UV detector (detection wavelength set at 254 nm), and evaporative light scattering detector (ELSD)-LT II (Shimadzu Co.). The ELSD (Shimadzu Co.) parameters were set as follows: drift tube temperature, 40 °C and pressure, 300 kPa. Data were recorded using LabSolutions software (Shimadzu Co.). HPLC assays were conducted using a Senshu Pak ODS column (5 μm, 250 × 4.6 mm i.d., Senshu Scientific Co., Ltd., Tokyo, Japan). The sample injection volume, flow rate, and column temperature were 10 μL, 1 mL/min, and 40 °C, respectively. Mobile phases A (H_2O) and B (90% aqueous MeCN) followed a linear gradient: 5% solvent B for 3 min, 5-100% B over 15 min, and 100% B for 15 min. Samples were filtered through a 0.45-μm Minisart Syringe Filter (Sartorius Stedim Biotech GmbH, Goettingen, Germany) before HPLC analysis.

NMR spectra measurements of the > 3 kDa fraction

One-dimensional NMR spectra were recorded using an Agilent 400MR-vnmrs 400 spectrometer (Agilent Technologies, Inc., Santa Clara, CA, USA; 400 MHz for ^{1}H, 100 MHz for ^{13}C) at room temperature with solvent signals as internal references. All chemical shifts (δ) were given in ppm, and the sample was dissolved in D_2O: acetone-d_6 (1:1).

GPC analysis of the > 3 kDa fraction

The GPC system was composed of an SSC-3461 HPLC pump (Senshu Scientific Co., Ltd., Suginami-ku, Tokyo, Japan), SSC-2320 column oven, and ERC-7517 RI detector (IDEX Health & Science, Oak Harbor, WA, USA). GPC assays were conducted using a TSK-gel Super AW 4000 column (150 × 6.0 mm i.d., Tosoh Co., Tokyo, Minato-ku, Japan). The mobile phase was *N*, *N*-dimethylformamide with 0.5% of ammonium formate solution (3 mol/L). The flow rate was 0.6 mL/min. The temperature was maintained at 40 °C. Shodex Standard SM-105 (polystyrene standards, Showa Denko K. K., Minato-ku, Tokyo, Japan) was used for GPC assays. GPC data were analyzed using μ7 Data Station software (System Instruments Co., Ltd., Hachioji, Tokyo, Japan).

Acid hydrolysis of the > 3 kDa fraction

The > 3 kDa fraction (50 mg) was dissolved in 0.5 N H_2SO_4 (10 mL) and heated under reflux for 5 h at 105 °C with magnetic stirring. The mixture was neutralized with Amberlite IRA96SB and lyophilized. The sugars generated by acid hydrolysis were identified by HPLC using standard markers of glucose, xylose, arabinose, sucrose, rhamnose, and fructose, as described in the following section. Aglycones were extracted with ethyl acetate and analyzed by LC-MS.

Sugar analysis

HPLC analysis of sugars was conducted using a Unison UK-Amino column (3 μm, 250 × 3 mm i.d., Imtakt Co., Kyoto, Japan). The sample injection volume was 2 μL, flow rate was 0.6 mL/min, and column temperature was maintained at 60 °C. Mobile phases A (H_2O) and B (MeCN) followed a linear gradient: 90% solvent B for 6 min, 90-75% B over 20 min, and 75% B for 25 min.

LC-MS analysis of aglycons liberated by acid hydrolysis

LC-MS was performed using an SCL-40 system (Nexera series, Shimadzu Co.) composed of a pump equipped with a degasser, autosampler, column oven, SPD-40 UV detector (wavelength set at 254 nm), and LCMS-8050 triple quadrupole mass spectrometer. Data were recorded using LabSolutions software (Shimadzu Co.). HPLC assays were performed using an X Bridge C18 column (5 μm, 150 × 2.1 mm i.d., Waters Co., Milford, MA, USA). The sample injection volume was 1 μL, flow rate was 0.2 mL/min, and column temperature was maintained at 40 °C. Mobile phases A (H_2O) and B (MeCN) followed a linear gradient: 5% solvent B for 3 min, 5-20% B over 10 min, and 100% B for 33 min. Mass spectra were recorded in ESI (negative ionization scan mode) between m/z 100 and 600 using the following conditions: nebulizing gas flow, 3.0 L/min; drying gas flow, 10 L/min; heating gas flow, 10 L/min; desolvation line temperature, 250 °C; block heater temperature, 400 °C; and interface temperature, 300 °C. The vanillin signal (m/z 151, [M-H]⁻) was confirmed on an extracted ion chromatogram.

FT-IR

Water extraction was characterized by a JASCO FT/IR-410 (JASCO Corporation, Tokyo, Japan) using the KBr tablet method (resolution: 4 cm⁻¹, measurement wavenumber range: 400-4000 cm⁻¹). Background correction was performed using a single KBr tablet.

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

The authors are grateful to Ms. Takahashi and Mr. Miyazawa at Senshu Scientific Co., Ltd. for their assistance with the GPC assays. We are also indebted to Ms. Rino Tachikawa for her assistance with the FT-IR spectra measurements.

Author contributions

A. S.; carried out all experiments and wrote this manuscript. Y. I.; contributed to the IR spectra measurement and data analysis. R. S.; supervision, project administration. All authors have read and agreed to published version of the manuscript.

Competing interest

The authors declare that they have no conflict of interest.

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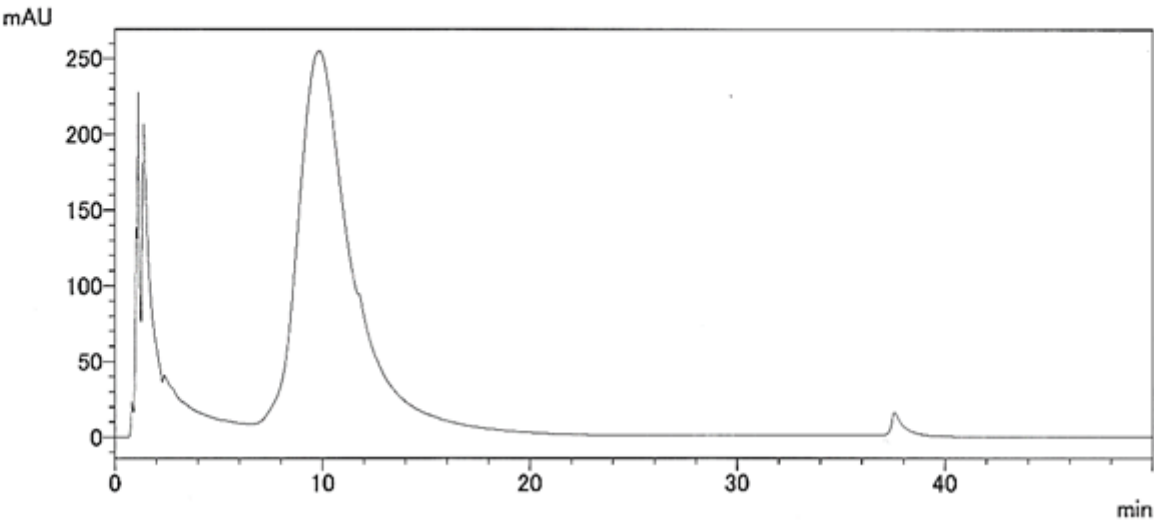
Figures

Fig. 1

Figure 1

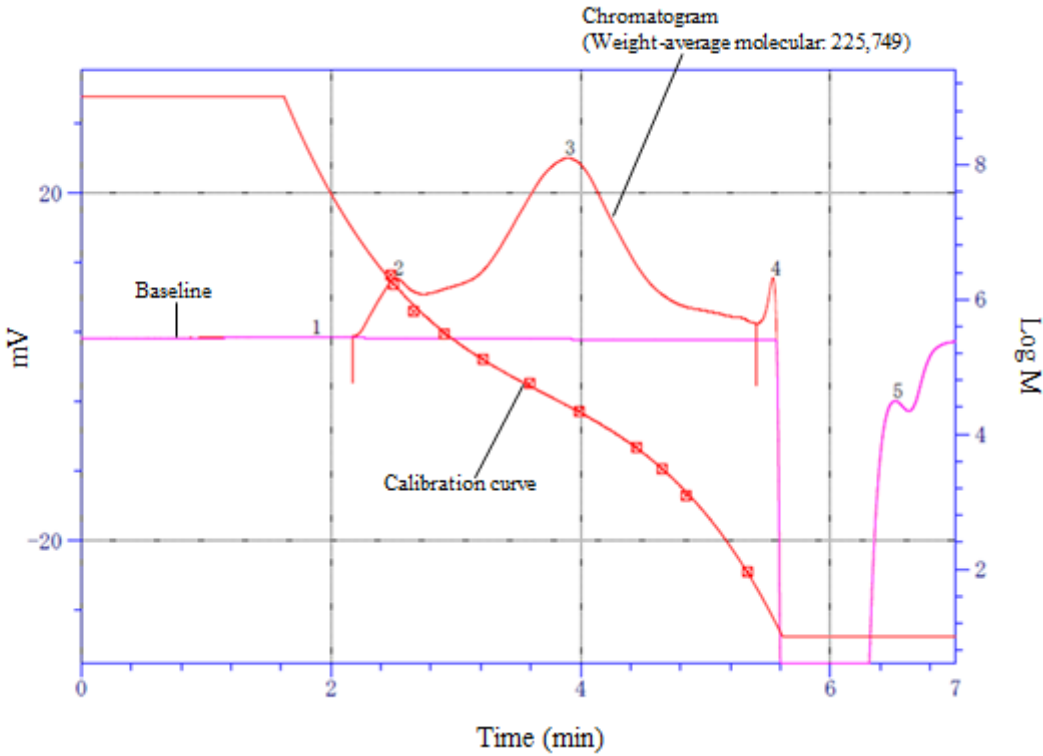HPLC chromatogram of the > 3 kDa fraction of the corn silk water extract

Fig. 2

Figure 2

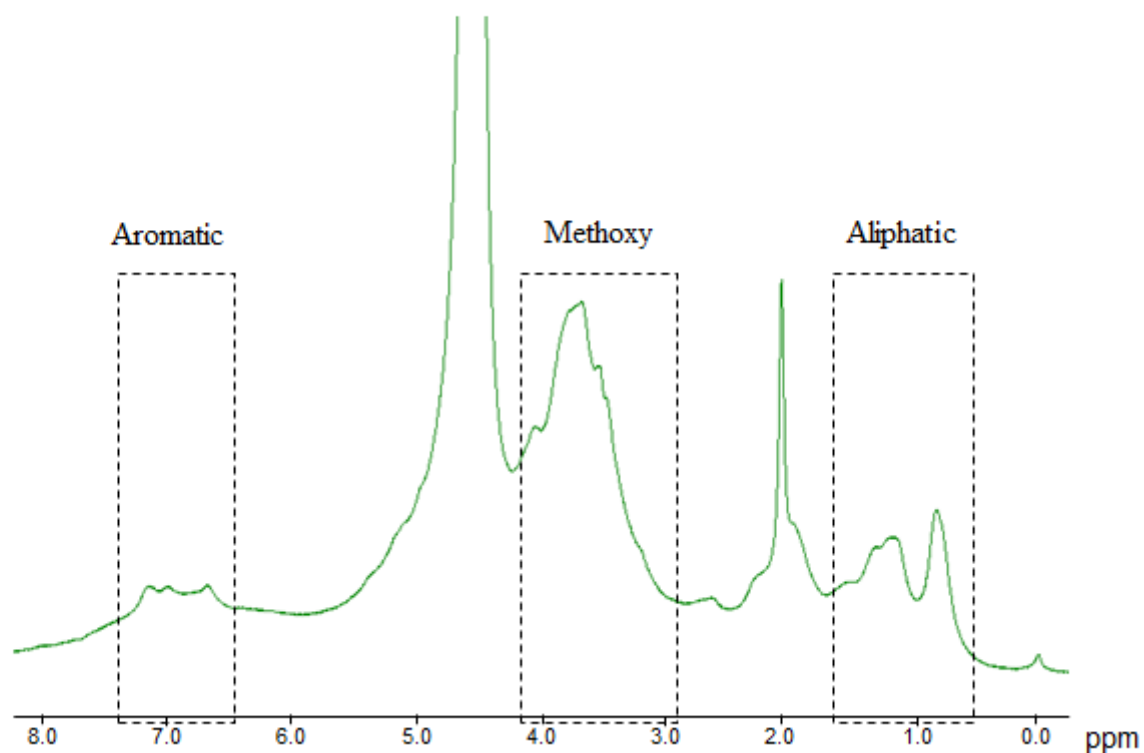GPC Chromatograms of > 3k Da fraction of corn silk water wxtract.

Fig. 3

Figure 3

^{1}H-NMR spectrum [400 MHz, D_2O: acetone-$d6$ (1:1)] of the > 3 kDa fraction of the corn silk water extract
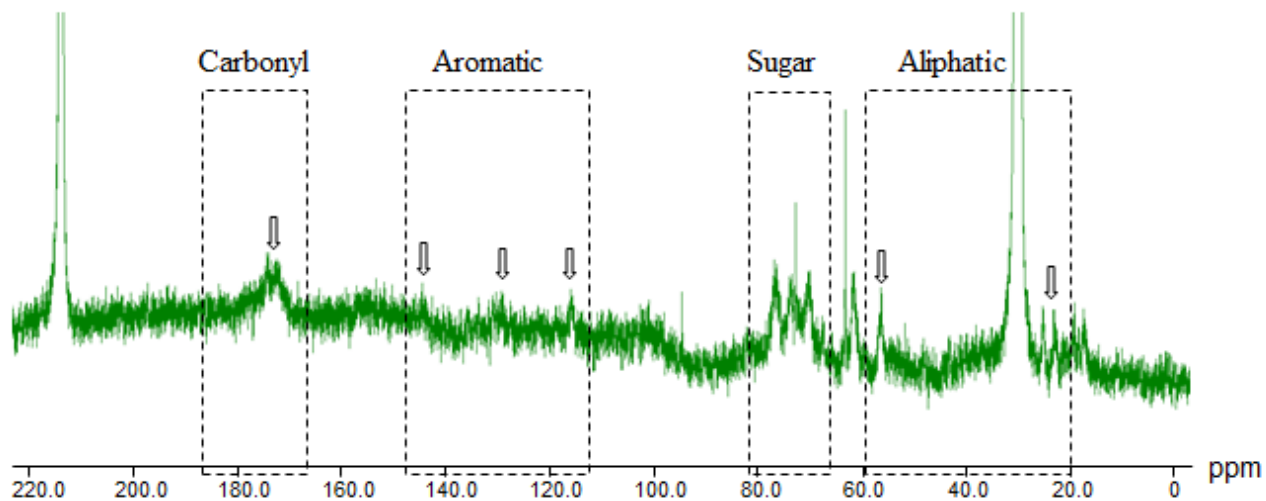
Fig. 4

Figure 4

^{13}C-NMR spectrum [100 MHz, D$_2$O: acetone-*d*6 (1:1)] of the > 3 kDa fraction of the corn silk water extract
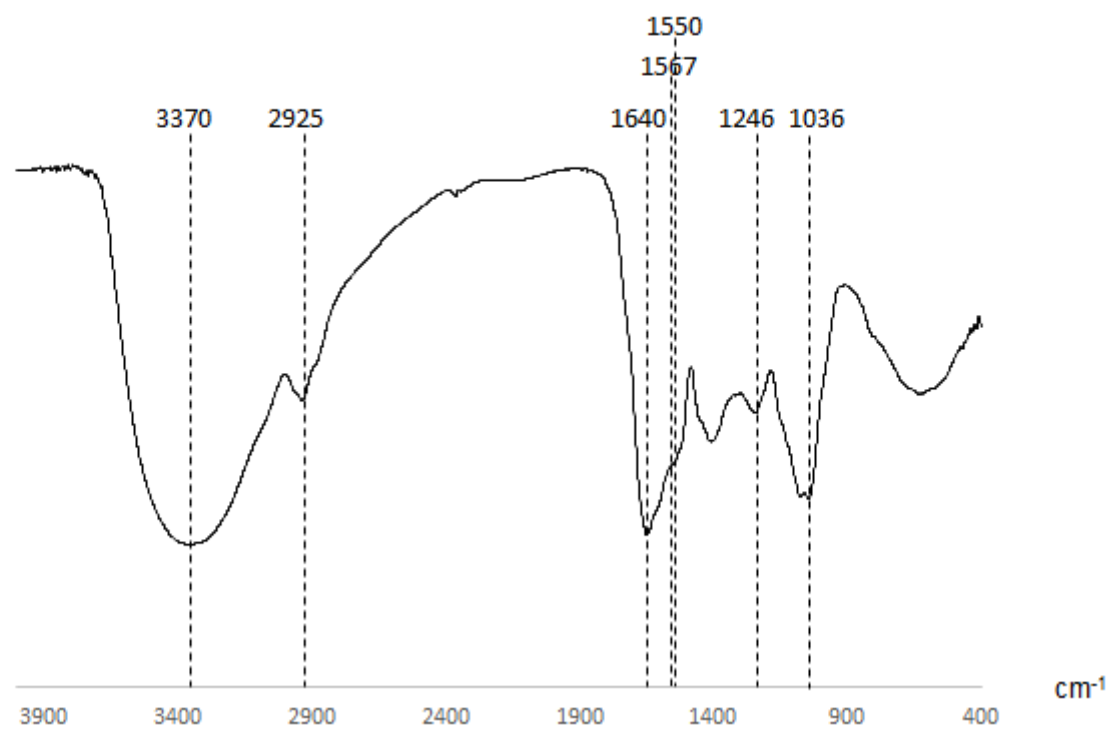
Fig. 5

Figure 5

FT-IR spectrum of the > 3 kDa fraction of the corn silk water extract
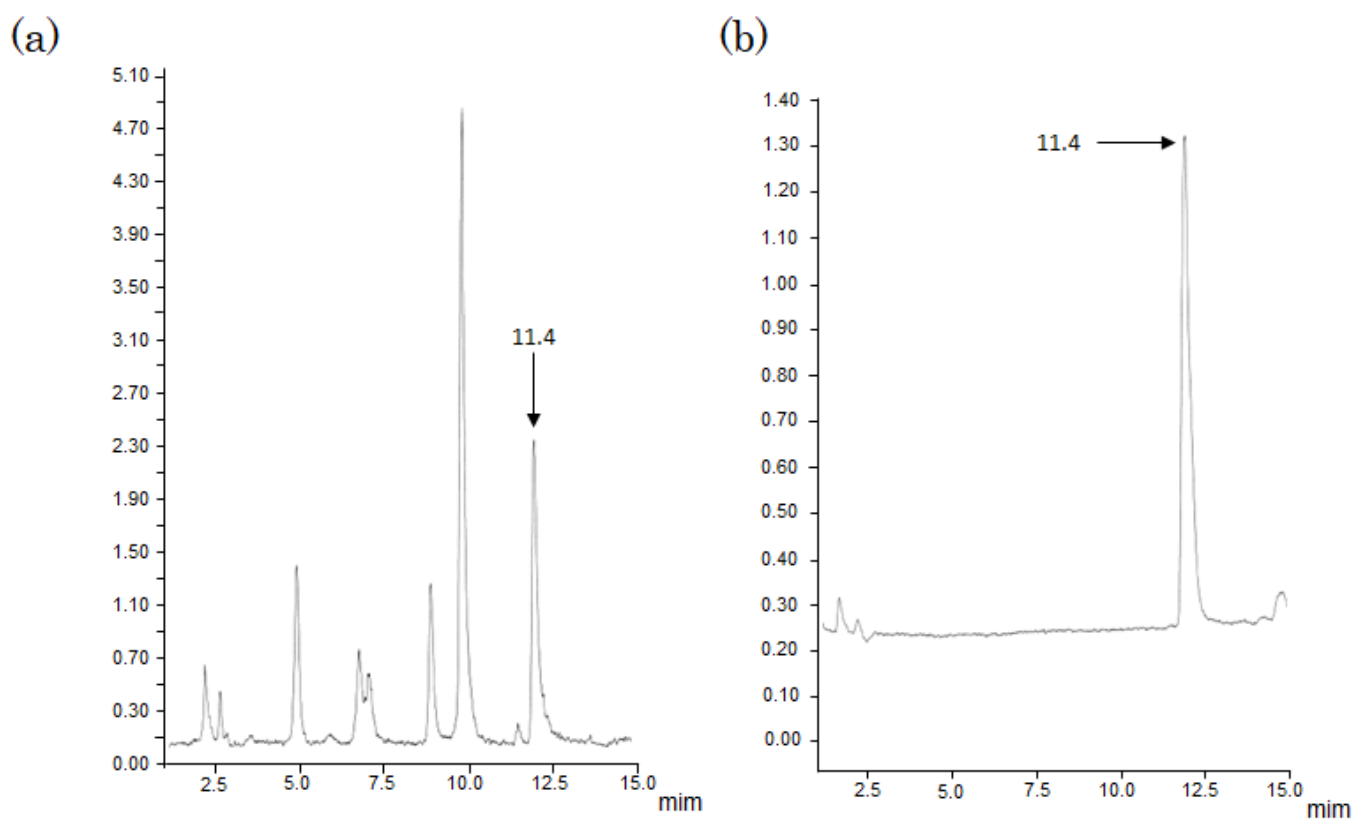
Fig. 6

Figure 6

LC-MS chromatogram. (a) Aglycons liberated by acid hydrolysis. (b) Vanillin
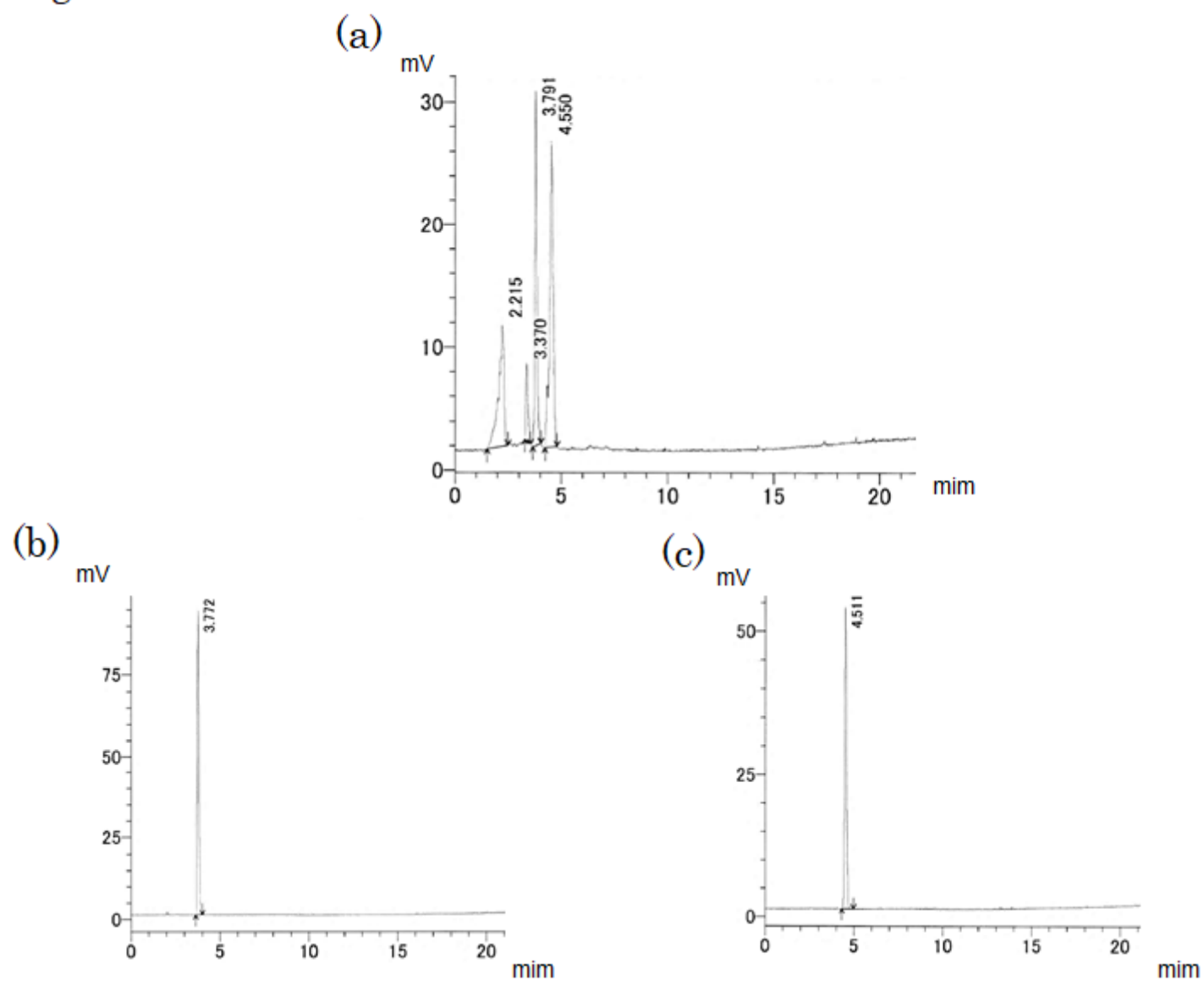
Fig. 7

Figure 7

HPLC analysis of sugars. (a) > 3 kDa fraction of the corn silk water extract. (b) Xylose. (c) Glucose