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Study on the purification and chemical compositions of tea glycoprotein

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Abstract

In this paper, improvement in the method for purifying glycoprotein from green tea (*Camellia sinensis*) was described; some properties and chemical compositions of tea glycoprotein (TGP) were determined by HPGPC, FT-IR, GC–MS technologies. Compared to existing methods, a more compatible method for purifying TGP was proposed. This method was faster, simpler, more effective and easier to be extended to the industrial production than the method that used in our previous work. The molecular weight of TGP was 126,513 Da using HPGPC. GC–MS analysis of TGP showed that TGP was composed of seven kinds of monosaccharides, namely ribose, rhamnose, arabinose, xylose, mannose, glucose, galactose in molar ratios of 1.71:5.88:13.70:1.99:1.00:1.84:33.75. Eighteen amino acids were identified in TGP by amino acid analysis. The FT-IR spectrum of the TGP revealed also typical characteristics of polysaccharides, protein and uronic acid.

Keywords: Green tea (*Camellia sinensis*); Tea glycoprotein; Molecular weight; Monosaccharide composition; Amino acid composition; FT-IR

1. Introduction

A glycoprotein is a glycoconjugate in which a protein carries one or more carbohydrate chains covalently attached to a polypeptide backbone, usually via N- or O-linkages (Ajit et al., 1997). In nature glycoproteins have a wide variety of biological functions, such as maintenance of protein conformation and stability, surface or intracellular recognition, cell adhesion, processing and diversion of protein, and so on. These functions may be dependent on either the protein part or the carbohydrate part or on both (Chen & Wang, 1997; Sara & Jos, 1997; Vliegenthart, 1998). The fast development of biological and chemical technologies during the last 20 years has lead to more and more in-depth understanding on bioactivities of the glycoproteins, structure of glycoproteins and their relationship. It was found that glycoproteins have various important bioactivities in many aspects, such as anti-tumor, anti-inflammation, anti-virus, lowering blood sugar, anti-caducity, anti-coagulant, etc. (Ajit et al., 1997; Paul, 1997; Yoshida, 2001).

Tea (*Camellia sinensis*) is one of the first set of medicinal herbs documented in ancient Chinese medicinal literature. Supposedly originated from China, tea was discovered to be an antidote for poisonous herbs by a great herbalist, Shen Nong, about 4700 years ago, when he tested hundreds of unknown herbs in search of plants with medicinal applications. At the same time, lower grade green tea was traditionally used to cure diabetics in East Asia, especially in China and Japan. To date, tea, particularly green tea, has been reported to have a broad range of therapeutic and nutritional values in lowering blood sugar, blood lipids, blood pressure, slowing heartbeat, anti-blood coagulation, anti-tumor, anti-HIV, protecting blood phase, enhancing human non-specific immunity (Marcel & Chi, 2004; Richard & Denis, 2004; Yan, Wang, Wang, Lu, & Wang, 1998). Previous researches have demonstrated that the tea glycoconjugate, especially tea glycoprotein (TGP) is one of the active ingredients responsible for many of the observed bioactivities (Chen, Zhang, & Xie, 2004; Chen, Zhang, & Xie, 2005; Nie, Xie, & Wang, 2005; Nie, Xie, Zhou, & Cao, 2006; Wang, Wang, Li, & Zhao, 2001; Xie

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& Nie, 2006). Several reports have indicated that tea glycoconjugates from different types of tea exhibit different properties and chemical components, and the molecular weight (M_w) of tea glycoconjugates is one of the major important factors responsible for their activities (Chen et al., 2005; Nie et al., 2005; Zhou, Xie, Nie, & Wang, 2004). However, full utilizations of glycoconjugates have been hampered for many various hurdles, one of which was lack of simple methods for its isolation and purification. Although several methods for purification of tea glycoprotein have been reported, all of them are rather labor costing and time consuming (Chen et al., 2005; Nie et al., 2005). The aim of this study was to improve the conditions for purifying tea glycoprotein from green tea (*C. sinensis*), and to further determine some of its properties and chemical compositions, such as molecular weight, amino acid and monosaccharide compositions and so on. The results of this study would become the good foundation for the further research on the relationships between the structure and bioactivity of tea glycoprotein.

2. Materials and methods

2.1. Materials and reagents

Tea leaves were sampled from Shangyou County of Jiangxi Province, China; D-ribose (Rib), L-rhamnose (Rha), D-arabinose (Ara), L-fucose (Fuc), D-xylose (Xyl), D-mannose (Man), D-glucose (Glc), D-galactose (Gal), D-fructose (Fru), D-galacturonic acid (GalA) and bovine serum albumin (BSA) were purchased from Sigma (USA); M_w standards of dextrans were obtained from Pharmacia Biotech (Sweden). The dextran standards consisted of T-2000, T-70, T-40 and T-10 with weight-averaged molecular masses of 2,000,000, 70,000, 40,000 and 10,000, respectively; All other reagents were of analytical pure grade, purchased from Shanghai Chemical Reagent (China); Ultra-pure water (>18 MΩ).

2.2. Apparatus

GC–MS system is an Agilent 6890N gas chromatograph/5973 mass selective detector with an Agilent 7694E auto-sampling apparatus and a HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm). The HPLC apparatus is a Waters dual gradient chromatographic system and was equipped with two 515 HPLC pumps, a Waters 2410 Refractive Index (RI) Detector and a Waters 2487 Dual wavelength Absorbance Detector. Purification system is an Amersham Pharmacia Biotech's ÄKTA Purifier 100 system and was equipped with a P-900 series pump, Monitor UV-900, Monitor pH/C-900, Mixer M-925, a complete set of motor valves, Fraction Collector Frac-950, Autosampler A-900 and an Analog/Digital Converter AD-900 connecting a Shimadzu's 10A Refractive Index Detector. Amino Acid Analyzer is a Hitachi L-8800 Amino Acid Auto Analyzer. Fourier-transform infrared spectrometer is a Nicolet 5700 FT-IR spectrometer with an OMNIC workstation.

Other major equipments were used, including a Milli-Q^{50} water purification system (Millipore, USA), an ALPHA 1–2 freezing drying apparatus (Martin Christ, Germany), a TDL-5A centrifuge (Shanghai Anting Scientific Instrument Factory, China), a RE-52A rotary evaporator (Shanghai Yarong Biochemistry Instrument Factory, China), and a TU-1900 Ultraviolet–Visible Spectrophotometer (Beijing Purkinje General Instrument Co. Ltd., China).

2.3. Purification of tea glycoprotein (TGP)

The tea samples were pre-extracted with ten volumes of 80% ethanol at room temperature to remove small molecules and then filtered and dried at 50 °C. The crude glycoconjugates were extracted with twice twenty volumes of water at 50 °C for 4 h under continuous stirring. The two extracts were combined, centrifuged (4800 r/min, 15 min) and concentrated by rotary evaporation under reduced pressure (50 °C, 50 r/min, −0.095 Mpa). The concentrate was repeatedly extracted with Sevag reagent ($CHCl_3$–*n*BuOH with v/v = 4:1) (50 mL × 3) to remove free proteins (Navarini et al., 1999). After removing the Sevag reagent, the water phase was dialyzed against running tap water for two days, and then against distilled water for an additional day. The non-dialysate was poured into four volumes of absolute ethanol and kept at 4 °C overnight in a refrigerator to precipitate crude glycoconjugates. The above ethanol precipitation step was repeated three times before further purification as described below.

The final precipitate from above was re-dissolved in water, followed by dialysis against distilled water for one day. The non-dialysate was applied to a prepacked Hiload 26/60 Superdex 200 prep grade column (separations in two ranges from M_r 10,000 up to 600,000 (globular proteins) and from M_r 1000 up to 100,000 (dextrans)) with the ÄKTA Purifier 100 system and was eluted with water at a flow rate of 2.6 mL min^{-1} monitored online by Monitor UV-900 at 280 nm and Shimadzu's 10A Refractive Index Detector. The eluent was collected as 10 mL fractional aliquots, and each fraction was analyzed by the anthrone–sulfuric acid reaction (Fu, Xie, Nie, Zhou, & Wang, 2001) in which the absorptions at 620 nm (for polysaccharide) and 280 nm (for protein) were monitored, then tea glycoprotein was collected, concentrated, dialyzed and lyophilized.

2.4. Determination of the molecular weight

Ten milligrams of TGP was dissolved in 5 mL 0.1 mol L^{-1} NaCl solution, applied to a Waters HPLC system equipped with an Ultrahydrogel™-500 (300 × 7.8 mm) gel-filtration chromatographic column, maintained at a temperature of 35 °C, eluted with 0.1 mol L^{-1} NaCl solution at a flow rate of 0.6 mL min^{-1} and detected by a Waters 2410 Refractive Index (RI) Detector. In the

estimation of the molecular weight of the TGP by high performance gel-permeation chromatography (HPGPC), dextran standards (M_w 2,000,000, 70,000, 40,000 and 10,000, Sigma) and glucose (M_w 180, Sigma) were used to calibrate the column and establish a standard curve (Nie et al., 2005).

2.5. Determination of polysaccharide, protein and uronic acid content

The polysaccharide content was determined by the anthrone–sulfuric acid reaction, using glucose as standard (Fu et al., 2001). The protein content was determined by the Lowry–Folin assay, using bovine serum albumin (BSA) as standard (Ronald et al., 2005). The uronic acid content was tested by Sulfuric acid–carbazole method using galacturonic acid as standard (Bitter & Muir, 1962).

2.6. Spectroscopic methods

Ultraviolet–visible spectra of TGP were recorded with a TU-1900 Ultraviolet–Visible Spectrophotometer. Fourier-transform infrared spectra of TGP were recorded with a Nicolet 5700 FT-IR spectrometer in the range 4000–400 cm^{-1}, using KBr disks method (Wang et al., 2004).

2.7. Monosaccharide composition analysis of the TGP

TGP was hydrolyzed in 2 M trifluoroacetic acid (TFA) for 6 h at 100 °C in a sealed glass tube. The residual acid was removed under vacuum and then the hydrolyzates were converted to acetylated aldononitrile derivatives according to conventional protocols and analyzed by an Agilent 6890N GC–MS system with a HP-5MS capillary column (30 m × 0.25 mm × 0.25 μm). Helium was used as the carrier gas at a constant rate of 1 mL min^{-1}. The oven conditions included an initial temperature of 50 °C and an initial time of 5 min, 15 °C/min to 170 °C, and finally 10 °C/min to 210 °C and held at this temperature for 15 min. The inlet temperature was kept to be constant at 250 °C, and the MS transfer line was set at 300 °C. As references, following monosaccharides were converted to their acetylated derivatives and analyzed: ribose, rhamnose, arabinose, fucose, xylose, mannose, glucose, galactose and fructose (Zhou et al., 2004).

2.8. Amino acids analysis

The TGP was hydrolyzed in vacuo at 110 °C in 6 M HCL for 24 h. The amino acids, except for tryptophan, were determined with a Hitachi L-8800 Amino Acid Auto Analyzer. For the analysis of tryptophan, the TGP was hydrolyzed in 4.2 N barium hydroxide, including 3% thiodiethylene glycol, in a sealed tube at 110 °C for 12 h and then the tryptophan was tested as described previously (Fu, Tian, Cai, Liu, & Li, 2007).

3. Results and discussion

3.1. Purification of the TGP

Ordinarily, existing methods for purifying glycoconjugates were complicated. Although several methods for purification of tea glycoprotein were reported, all of them are rather labor costing and time consuming (Chen et al., 2005; Nie et al., 2005). In this study, ÄKTA purifier100 system was employed to purify tea glycoprotein. But ÄKTA purifier100 system was mainly designed to purify proteins and nucleic acids with UV detector and pH/C detector. Considering no UV absorption for polysaccharides, RI Detector was connected after UV detector in order to detect the polysaccharides. After Hiload 26/60 Superdex 200 prep grade column separation, chromatograms were recorded automatically using both UV and RI Detectors (Figs. 1 and 2). The operations of data collection were driven by UNICORN software which guarantees quick, simple communications between systems and users, and meets the stringent control and data handling procedures of modern laboratories. As shown in Figs. 1 and 2, the two components both appeared at the same retention time. At the same time, the separated fractions were collected as 10 mL fractions by the automated fraction collector, and UV/VIS absorptions at 620 nm for

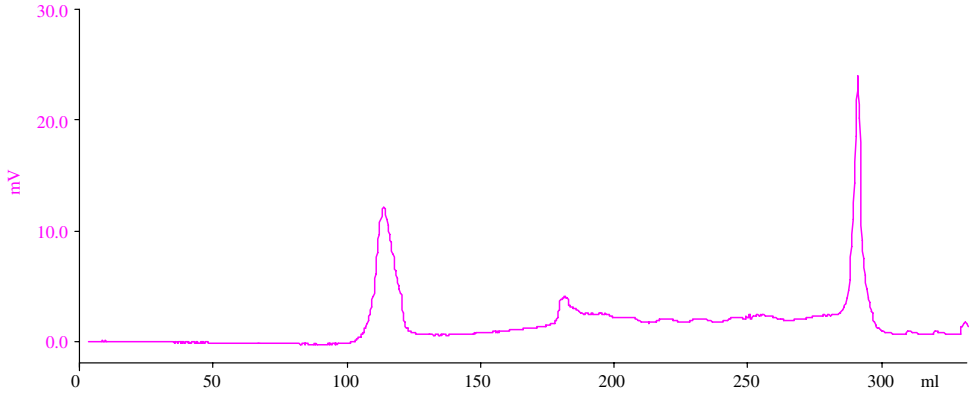

Fig. 1. The RI chromatogram of ÄKTA purifier100.

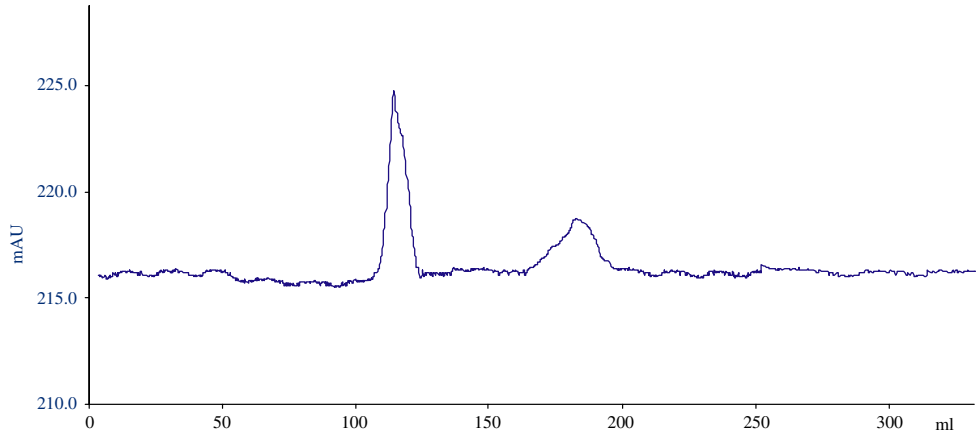

Fig. 2. The UV (280 nm) chromatogram of ÄKTA purifier100.

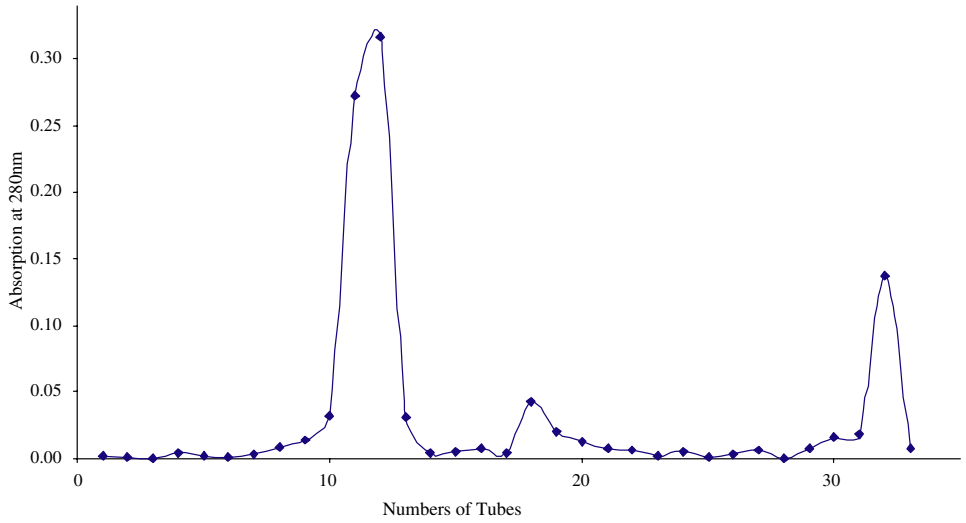

Fig. 3. Each tube assay results at 620 nm.

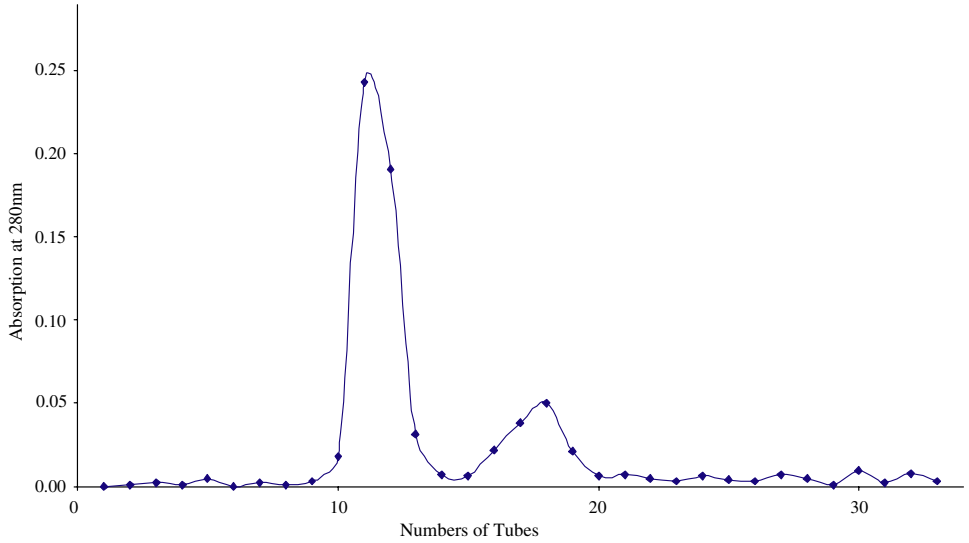

Fig. 4. Each tube assay results at 280 nm.

polysaccharides, and protein absorption at 280 nm were measured for each fraction. The data were re-plotted as reconstructed chromatograms as shown in Figs. 3 and 4. The reconstructed chromatograms (Figs. 3 and 4) were nearly superimposable with the recorded ones (Figs. 1 and 2), respectively. It was further confirmed that the fraction was likely the eluted tea glycoprotein material. The fractions were collected, concentrated, dried, and designated as TGP fraction. The results demonstrated that the ÄKTA purifier100 system is more compatible for the purification of TGP and this method is faster, simpler, more effective and easier to be extended to the industrial production than the method that used in our previous work (Nie et al., 2005).

3.2. Homogeneity and molecular weight of TGP

The TGP was eluted as a single symmetrical peak, as determined by HPGPC, which indicated that TGP was homogeneous. The molecular weight of the TGP was calculated as 126,513 Da, according to the calibration curve with standard dextrans and glucose (Figs. 5 and 6).

3.3. Spectroscopic characteristics

The UV spectrum of the TGP was shown in Fig. 7. An intense peak at 209 nm was the characteristic band of double bonds or triple bonds. However, the peak at 279 nm should be attributed to protein. The IR spectrum of the TGP was shown in Fig. 8. It exhibited a broadly stretched intense peak at around 3418.3 cm^{-1} which was the characteristic absorption of hydroxyl group and two weak C–H bands at around 2914 cm^{-1} and 2811 cm^{-1}. The relatively strong absorption peak at around 1650 cm^{-1} and some weak ones from 1400 cm^{-1} to 1200 cm^{-1} were also the characteristic IR absorptions of polysaccharide. The IR absorptions at 1087.4 $cm^{-1}$and

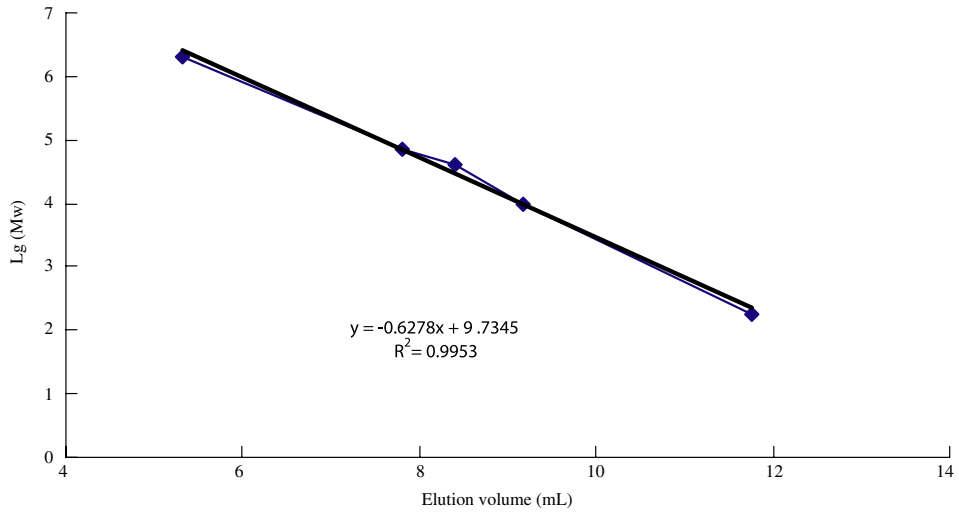

Fig. 5. The GPC calibration curve of dextran standards.

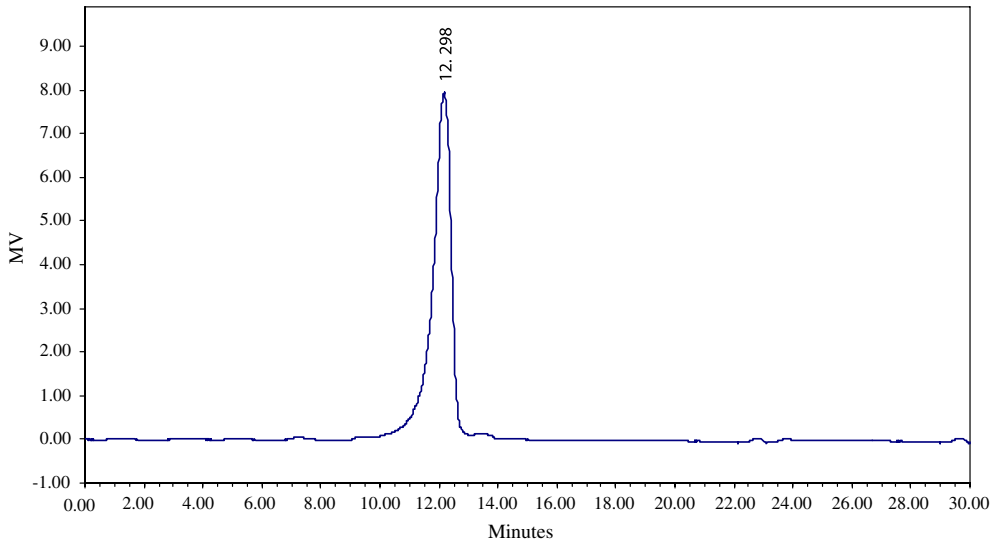

Fig. 6. The RI chromatogram of TGP purified from green tea sampled from Shangyou County.

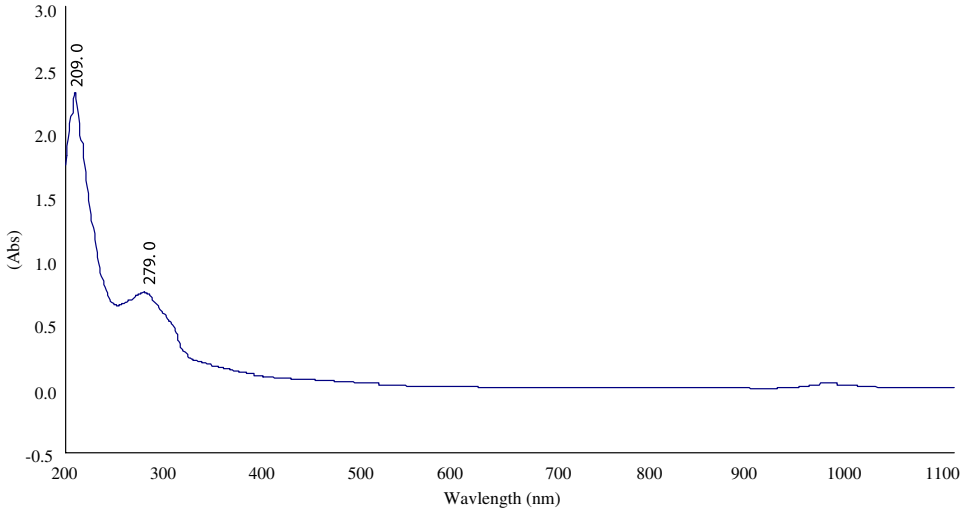

Fig. 7. The UV spectrum of TGP.

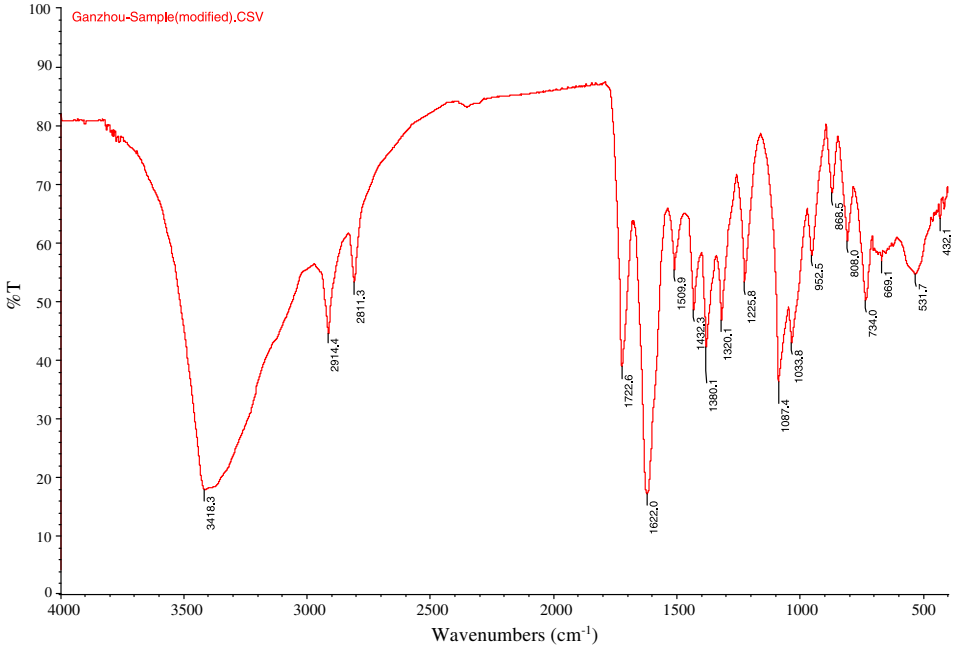

Fig. 8. The IR spectrum of TGP.

1033.8 cm^{-1} should be the characteristic absorption bands of pyranglycosides, whereas the absorption bands at 952.5 cm^{-1} and 808.0 cm^{-1} should be assigned to methylene of deoxysugars and mannose, respectively. The relatively strong absorption peak at 1622 cm^{-1} and a weak one at 1510 cm^{-1} were the characteristic IR absorptions of protein. The strong absorption peak at around 1722 cm^{-1} and a weak one at near 1380 cm^{-1} were indicative of the presence of carboxyl groups and carbonyl groups that indicated the characteristic IR absorption of uronic acids. These observations further confirmed that the TGP was composed of polysaccharide, protein and uronic acids.

3.4. Chemical compositions of TGP

The TGP was identified as consisting mainly of polysaccharide, protein and uronic acid by the anthrone–sulfuric acid reaction, the Lowry–Folin assay and Sulfuric acid–carbazole method. The percentages of these components were 65.3%, 8.625% and 25.14%, respectively. Our results

Table 1
The content of amino acids of TGP

Amino acid	Content (%)
Aspartic acid	0.65
Threonine	0.39
Serine	0.47
Glutamic acid	0.76
Glycine	0.56
Alanine	0.43
Cystine	0.21
Valine	0.35
Methionine	0.08
Lysine	0.28
Histidine	0.10
Arginine	0.91
Tyrosine	0.16
Phenylalanine	0.16
Isoleucine	0.27
Leucine	0.42
Proline	0.28
Tryptophan	0.36
Total amino acid	6.83

further showed that the TGP was a glycoprotein containing polysaccharide, protein and uronic acid.

GC–MS analysis of the TGP that had been hydrolyzed and derived showed that the TGP was composed of seven kinds of monosaccharides, namely ribose, rhamnose, arabinose, xylose, mannose, glucose, galactose in molar ratios of 1.71:5.88:13.70:1.99:1.00:1.84:33.75. This result was in good agreement with standard saccharides (ribose, rhamnose, arabinose, fucose, xylose, mannose, glucose, galactose, fructose). The results indicated Gal was the predominant monosaccharides. The content of total amino acid was 6.83% in the TGP (Table 1), in which 18 amino acids were identified. Arginine was the major amino acid (0.91%), followed by Glutamic acid (0.76%), Aspartic acid (0.65%), Glycine (0.56%) and Serine (0.47%).

From the preceding results, we can see the purified tea glycoprotein should be an acidic glycoprotein. The attachment of polysaccharides to protein can occur in different linkages involving a variety of amino acids that have often been found in many known glycoproteins. Recently, it has been suggested that glycosylation can occur in approximately 41 different amino acid–sugar linkages. Well-known ones are *N*- and *O*-glycosylation occurring exclusively through GlcNAc-β-Asn and GalNAc-α-Ser/Thr linkages, respectively. Many examples of such glycosylation have been reported in known glycoproteins mainly from animal sources and recently from recombinant protein expression systems (including plants, fungi, insect and animal cells) (Anumula, 2006; Spiro, 2002). From the chemical compositions of TGP, it could be seen that galactose was the predominant monosaccharides and relatively high contents of serine and threonine were found by amino acid analysis. It was suggested that *O*-glycosidic bonds were involved in binding between protein and carbohydrate.

Further studies will focus on determination of the structure and bioactivity of the purified tea glycoprotein and the relationships between its structure and bioactivity, which has already been underway in our lab.

Acknowledgements

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