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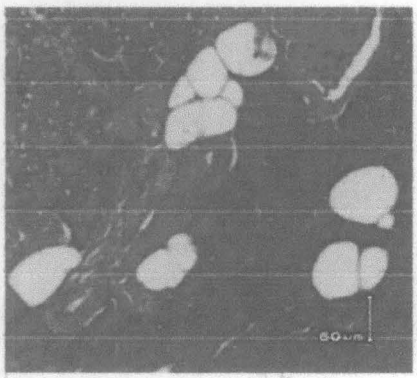

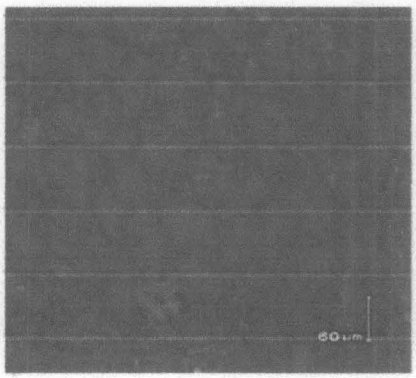

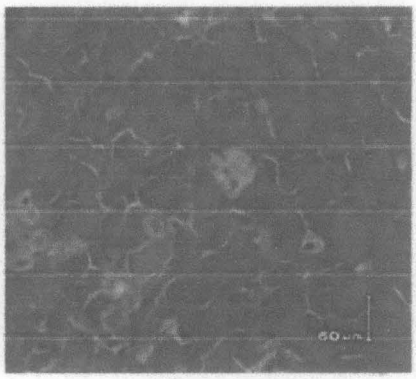

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Hypoglycemic Effect of Mahogany (*Swietenia macrophylla* King) Bark Extracts in Alloxan-induced Diabetic Rats

Syamsul Falah, Mega Safithri, Takeshi Katayama, and Toshisada Suzuki

Abstract

In this study, in vivo hypoglycemic activity of mahogany (*Swietenia macrophylla*) bark extracts was evaluated against alloxan-induced diabetic rats. The hypoglycemic effect was compared to that of standard glibenclamide. Oral administration of hot water and methanol extracts at a dose of 250 mg/kg body weight for thirteen days of daily treatment to diabetic rats was found to possess significant dose dependant hypoglycemic effect in diabetic rats. It less active than that of glibenclamide at dose of 3.22 mg/kg. However, the hot water extract showed significant hypoglycemic activity compared to that standard drug. Phytochemical analysis of hot water and methanol extracts has shown posistive test for the presence of alkaloids, flavonoids, tannins, saponins, dan terpenoids. Histopathological studies of pancreas revealed its significant effect of β-cell count. Therefore, the hot water extract could serve as good adjuvant to other oral hypoglycemic agents and seems to be promising for the development of phytomedicines for diabetes mellitus.

Key words: *Swietenia macrophylla,* bark extract, hypoglycemic activity, alloxan-induced diabetic rats.

Introduction

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels (Bowman and Russel 2001). Statistical projections mentioned that number of diabetics in the world will increase from 151 million in the year 2000 up to 221 million in the year 2010, and hence it become Indonesia is the fourth in the highest number of diabetics in the world after India, China, and USA (King *et al.* 1998; Boyle *et al.* 2001; Zimmet *et al.* 2001).

Traditional medicinal plants have been employed successfully by the local communities since long time to treat diabetes without adverse effects. Researches in traditional medicine for appropriate hypoglycemic agents have been focused on plants due to traditional medicine gives better treatments than drugs (Rates 2001). Seeds of mahogany (*Swietenia macrophylla*) have been used for treatment of diabetes as a folk medicine in Indonesia (Kadota *et al.* 1990). The seed also have been used for leishmaniasis and abortion medicine by an Amazonian Bolivian ethnic group (Bourdy *et al.* 2000) and for treatment of hypertension and malaria (Kadota *et al.* 1990). However, bioactivities from the bark have not been investigated extensively. The bark of mahogany, collected from Indonesia, contain flavonoids with high antioxidant activity, namely swietemacrophyllanin, catechin, and epichatechin (Falah *et al.* 2008). In this study, the hypoglycemic effect of mahogany bark extracts were evaluated, and phytochemicals compounds of the extracts were examined. The effect of hot water and methanol extracts were evaluated on diabetic rats and its effects were compared with glibenclamide, a standard hypoglycemic agent.

Materials and Methods

Plant Material

Mahogany bark was collected from Sumedang, Indonesia since March 2009. A dried bark powder of mahogany (500 g) were boiled in 1 liter of water for 4 h to give a hot water extract (29 g). The extract was filtered with filter paper (Whatman, no. 1) and evaporated with rotary evaporator at 60°C, and the crude extract was used in biological assay. Another 3000 g of the dried bark powder was extracted with acetone for 48 h at room temperature to give acetone extract (237 g), and then the residue was extracted again by methanol to yield methanol extract (184 g). The acetone was used for extraction of non polar substances, i.e. fatty acid, wax. The methanol extract was evaporated, and the extract was used in biological assay.

Chemicals and Drugs

The solvents were of analytical grade and purchased from Merck, Germany. Alloxan and glibenclamide were obtained from Sigma Chemical, USA and Daonil Aventis Pharmacy, USA, respectively. All other chemicals were of analytical grade.

Qualitative Phytochemical Analyses (Harborne 1987)

Alkaloid Test. The hot water and methanol extracts of 0.1 g each were added with 3 mL of chloroform and 3 drops of ammonia. The chloroform fraction was separated and acidified with 10 drops of H_2SO_4 2M. The H_2SO_4 fractions were taken and added separately with Dragendof, Meyer, and Wagner reagents. The alkaloids content was indicated by white precipitant upon addition of Meyer reagent, orange precipitant upon Dragendorf reagent, and brown precipitant upon addition of Wagner reagent.

Saponin Test. The extracts of 0.1 g were added with 2 mL of H_2O and heated for 5 min. The mixtures were cooled down, stirred up until foamy appearance can be observed to indicate the presence of saponin.

Flavonoid Test. The extracts of 0.1 g were soaked with 2 mL of 30% methanol and heated. The filtrates were added with 1 drop of concentrated H_2SO_4. The presence of flavonoid was indicated by the formation of red pigment.

Phenolic Hydroquinone Test. The extracts of 0.1 g were soaked with 2 mL of 30% methanol, heated and filtered. The filtrates were added with 1 drop of NaOH 10%(b/v). The presence of phenolic hydroquinone was indicated by the formation of red color.

Triterpenoid Test. The extracts of 0.1 g were added with 2 mL of 30% ethanol, heated and filtered. The filtrates were evaporated and then diethyl ether was added. The Lieberman Burchard reagent (3 drops of acetic acid anhydride and 1 drop of concentrated H_2SO_4) was added to the ether layer. The presence of triterpenoid was indicated by the formation of reddish-violet pigment.

Tannin Test. The extracts of 0.1 g were added with 2 mL of H_2O and heated for several minutes. The mixtures were filtered and the filtrates were added with $FeCl_3$ 1% (b/v). The presence of tannin was indicated by the formation of dark-blue or greenish-black color.

Animals

Male Sprague-Dawley rats of 3 weeks old were obtained from The National Agency of Drug and Food Control of Indonesia. They were fed with a standard laboratory diet and allowed food and water ad libitum for an acclimatization periods of 2 weeks prior to experiments. The animals were divided into five groups of seven each and housed individually during the experimental period.

Experimental Design

All the rats (*Sprague dawley* albino male rats) were randomly divided into the five groups.

Group A: Normal rats administered NaCl 0.9% by intraperitoneal and orally aquades 1 ml daily for 13 days.

Group B: Diabetic control rats administered alloxan 150 mg/kg by intraperitoneal and orally aquades 1 ml daily for 13 days.

Group C: Diabetic rats administered standard drug glibenclamide (3.22 mg/kg, orally) daily for 13 days.

Group D: Diabetic rats administered hot water extract (250 mg/kg, orally) daily for 13 days.

Group E: Diabetic rats administered methanol extract (250 mg/kg, orally) daily for 13 days.

Alloxan was injected to all rat groups on 1st day. Treatment with the extracts and glibenclamide was started 48 h after alloxan injection. Blood samples were obtained from the tail vein in fasting rats for 18 h and blood glucose levels were measured using an electronic glucometer (Miles Inc, USA). Fasting blood glucose and body weight were measured on 1st, 3rd, and 15th days.

Statistical Analysis

All the values of body weight and fasting blood sugar were expressed as mean ± standard error of mean (S.E.M) and analyzed for ANOVA and Duncan's t-test. Differences between groups were considered significant at $P < 0.05$.

Histopathological Studies

All the animals were sacrificed on 15th day by cervical dislocation. Pancreases were excised, isolated, and were subjected to histopathological studies and microscopical finding were noted. The pancreas tissues were removed immediately and washed with ice-cooled saline, and then fixed in 10% of neutral formalin. The sections stained in haemetoxylin and aeosin and mounted were observed under microscope.

Results and Discussion

Phytochemicals assay of hot water and methanol extracts of mahogany bark revealed the presence of the flavonoids, tannins, triterpenoids, saponins and alkaloids (Table 1). Phytochemical compounds such as, flavonoids, triterpenoids, alkaloids, and phenolics are known to be bioactive antidiabetic principles (Nagappa *et al.* 2003; Battu *et al.* 2007; Safithri and Fahma 2008).

The effect of treatment on rat body weight on 1st day showed that rats body weight in all groups did not differ significantly ($P<0.05$) (Table 2). Rats body weight decreased on 3rd day after alloxan (B, D, and E group) induction; the highest degradation occurred at group D (4.4% from body weight of 1st day). However, body weight degradation on 3rd day in B, D, and E group did not different significantly with A and C group ($P<0.05$). On 15th day, the rats body weight was measured to evaluate the effect of hot water and methanol extracts of mahogany bark which orally administered at the dose of 250 mg/kg body weight (D and E group). During 13 days treatment (from 3rd day till 15th day), hot water and methanol extracts reduced the body weight by 8.54% and 7.36%, respectively. D and E groups did not differ with B and C group ($P<0.05$). It means that mahogany bark extracts reduced rat body weight the same as B and C group. Body weight reduction was also indicated by aqueous extract of *Terminalia catappa* at the dose of 42 mg/kg for 12 days treatment in alloxan-induced diabetes up to 63.09% (Nagappa *et al.* 2003) and decoction of *Piper crocatum* of 322 mg/kg for 10 days treatment in alloxan-induced diabetes up to 17.28% (Safithri and Fahma 2008). The decrease of body weight in diabetes is due to

continuous excretion of glucose and glycogen synthesis (Defronzo *et al.* 1992).

Measurement of blood glucose level was carried out on the 1st, 3rd, and 15th day to observe the effect of aquades, glibenclamide, and mahogany bark extracts orally administration and induction of NaCl or alloxan. The induction influences blood glucose rats during experiment. On 1st day (before treatment), rats blood glucose in all groups resulted not significant different ($P<0.05$) (Table 3) and performed at normal range (60~110 mg/dl). However, after NaCl and alloxan induction (on 3rd day), rats blood glucose increased. Induction of alloxan (150 mg/kg) (B, C, D, and E group) increased blood glucose up to 0.5~1.0 folds. Blood glucose levels data showed B group has the highest increasing by 150.9%. Increasing of blood glucose rats on 3rd day after induction of alloxan, showed significantly different ($P<0.05$) with A group (Table 3). Rats blood sugar was reduced up to 45.73% and 25.80% in thirteen days after treated with hot water and methanol extracts at a dose of 250 mg/kg. It is less active than that of glibenclamide which reduced blood sugar level by 48.42%. It was indicated that the hot water extract showed significant antihyperglycemic activity as compared to that of standard drug.

Antihyperglycemic activity from decoction of *P. crocatum* at 322 mg/kg body weight reduced blood glucose level up to 10.46% after ten days given to diabetic rat. The extract contained flavonoids, alkaloids, and tannin (Safithri and Fahma 2008). Blood glucose reduction up to 3.68% occured from alcoholic extract of Chinese squash (*Benincasa hispida*) at 200 mg/kg after 24 h given to diabetic mice. The extract contained alkaloids, flavonoids, saponins, and steroids (Battu *et al.* 2007). The alcohol extract of gopher plant (*Euphorbia leucophyllum*) at 500 mg/kg in diabetic mice showed that it possessed an antihyperglycemic activity to reduce blood glucose up to 21.54% after 24 h the extract was given (Satyanarayana *et al.* 2006).

Table 1. Phytochemical constitutes of mahogany bark.

Test	Hot water extract	Methanol extract
Alkaloids	+	+
Flavonoids	+	+
Saponins	+	+
Triterpenoids	+	+
Tannins	+	+

(+) Positive (–) Negative

Table 2. The effect of 13 days treatment with extract of mahogany bark on body weight.

Group No	Body weight (g)		
	Day 1	Day 3	Day 15
A	380.6^{ab}	385.6^{ab}	389.6^{a}
B	367.6^{abc}	357.6^{abc}	352.8^{abc}
C	367.2^{abc}	370.0^{abc}	360.8^{abc}
D	372.4^{ab}	356.0^{abc}	325.6^{c}
E	379.6^{ab}	369.2^{abc}	342.0^{bc}

The same letter(s) indicated not significant different on $P<0.05$. The groups refer to Table 1.

Table 3. The effect of 13 days treatment with extract of mahogany bark on blood glucose level.

Group No	Average blood glucose level (mg/dl)		
	Day 1	Day 3	Day 15
A	71.0^{a}	91.6^{a}	84.6^{a}
B	66.4^{a}	166.6^{b}	103.6^{ab}
C	75.4^{a}	177.6^{b}	91.6^{a}
D	81.2^{a}	173.2^{b}	94.0^{a}
E	81.8^{a}	118.6^{b}	88.0^{a}

The same letter(s) indicated not significant different ($P<0.05$) . The groups refer to Table 1.

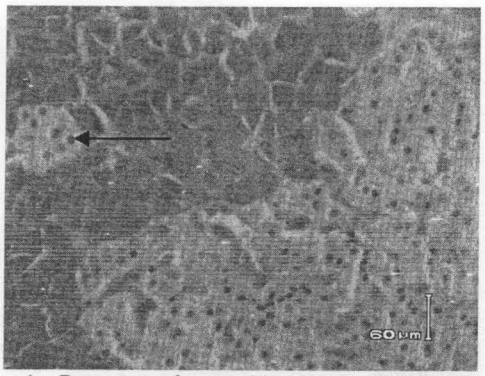

Figure 1. Pancreas of normal health rat, H & E staining (x100). The islet of langerhands is shown by arrow.

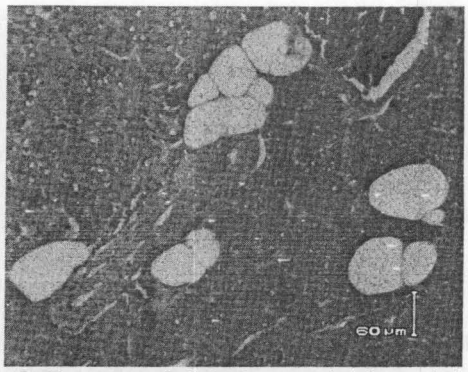

Figure 2. Pancreas of diabetic control (alloxan-induced diabetic) rat, H & E staining (x100). The islet of langerhands couldn't be found.

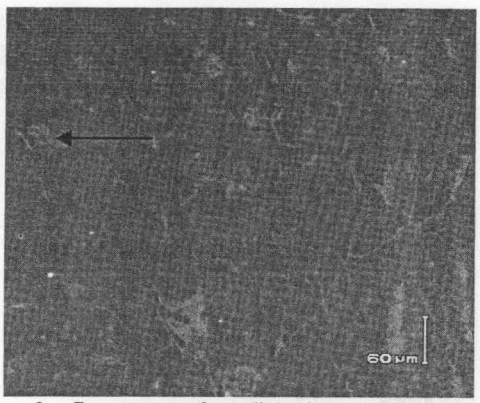

Figure 3. Pancreas of diabetic rat treated with Glibenclamide 0.25 mg/kg body wt, H & E staining (x100). The islet of langerhans is shown by arrow.

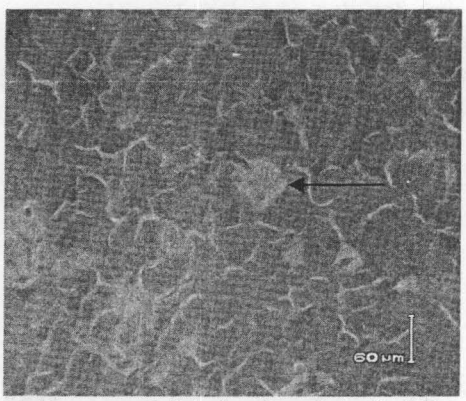

Figure 4. Pancreas of diabetic rat treated with 250 mg/kg body wt hot water extract, H & E staining (x200). The islet of langerhans is shown by arrow.

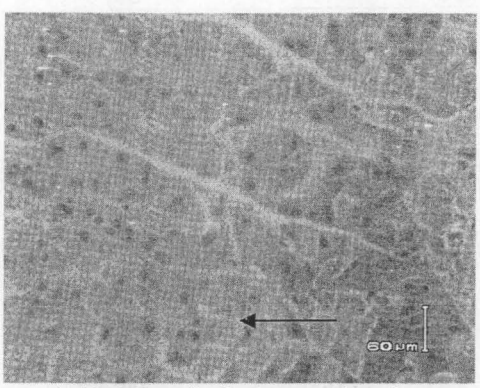

Figure 5. Pancreas of diabetic rat treated with 250 mg/kg body wt methanol extract, H & E staining (x200). The islet of langerhands is shown by arrow.

The histological sections of the pancreas, tissues were observed to know the effect of extract of mahogany bark in alloxan diabetic rats. The cellular integrity and architecture were intact in the A group. Besides that, in group A there was no specific abnormalities, and easy to find the islets of Langerhans (Figure 1). Pancreatic sections stained with hematoxylin and eosin (H & E) showed that alloxan caused fat necrosis; acinar cell necrosis, and hemorrhage and the islet of Langerhans cannot be found (Figure 2). However, administration of glibenclamide at the dose of 3.22 mg/kg/day orally in alloxan diabetic rats showed no necrosis. The size and the number of islets of Langerhans is smaller and fewer than normal group, respectively (Figure 3). Meanwhile, administration of hot water extract of mahogany bark (250 mg/kg/day, orally) in alloxan diabetic rats showed fat necrosis, acinar cell necrosis, the number

and size of islets of Langerhans smaller than normal group (Figure 4). Furthermore, administration of methanol extract of mahogany bark (250 mg/kg/day, orally) in alloxan diabetic rats showed necrosis and easy to be found the islets of Langerhans (Figure 5).

In this study, the pancreatic ß cells were destroyed with the help of alloxan. Alloxan is one of the usual substances used for the induction of diabetes mellitus apart from streptozotocin. Alloxan has a destructive effect on the ß cells of the islets of Langerhans (Szkdelski 2001). The alloxan produce permanent hyperglycemia by selective destruction of the ß cells of the islets of Langerhans are in agreement with those of Singh and Gupta (2007a). The histopathological study of diabetic treated with the extracts indicated the increasing of volume density of islets and percentage of ß cells, in the diabetic rats that received the extracts, which may be a sign of regeneration. Signs of regeneration of ß cells, potentiation of insulin secretion from surviving ß cells of the islets of Langerhans and decrease of blood glucose have been reported following consumption of some plant extracts (Yadav *et al*. 2008; Singh and Gupta 2007b). Hot water and methanol extracts of mahogany bark may have some chemical components that exert regenerative effects on ß cells, stimulate these cells to produce more insulin (pancreatotrophic action) or may have some insulinlike substances. Induction of regenerative stimulus in diabetic state triggers pancreatic regenerative processes, thereby restoring functional activities of the pancreas (Adewole and Ojewole 2007). A higher dose of the extract has a greater restorative effect on the islet cells of diabetic rats than a lower dose of extract.

Conclusions

The hot water and methanol extracts of mahogany bark contained flavonoids, tannins, triterpenoids, sapcnins and alkaloids. Oral administration of hot water and methanol extract at a dose of 250mg/kg for thirteen days of daily treatment led to reduce blood sugar level by 45.73% and 25.80%, respectively. It is less active than that of glibenclamide which reduce blood sugar level by 48.42%. However, the hot water extract showed significant antihyperglycemic activity as compared to that of standard drug. Histophatological study indicated the extracts exert regenerative effect on ß cells, stimulate to produce more insulin. Further research is needed to explore different mechanisms to reduce blood glucose levels.

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Precise Structure of Acidic Polysaccharide Present in *Salvia* Hydrogels

Rike Yudianti, Myrtha Karina, Masahiro Sakamoto, and Jun-ichi Azuma

Abstract

Precise structures of acidic ß-(1,4)-xylan in the hydrogels from three species of *Salvia* (S. *miltiorrhiza* (SM), *S. sclarea* (SS) and *S. viridis* (SV)) were characterized. SS and SV contained two different acidic residues (4-*O*-methylglucuronic acid (MeGlcA) and glucuronic acid (GlcA)) substituted at O-2 of ß-(1,4)-linked xylopyranose residues, whereas MeGlcA is absent in SM. Molar ratios of xylose to uronic acid are 2.0 : 1.0 (SM), 1.7 : 1.0 (SS), 1.4 : 1.0 (SV). Distribution of acidic residues in the ß-(1,4)-xylan chains was analyzed by Matrix-Assisted Laser Desorption/Ionization (MALDI)/Time of Flight (TOF) mass spectroscopy after reduction and partial hydrolysis. The results showed that many series of ions appeared as sodium adducts $[M+Na]^+$, indicating that uronic acid residues are randomly and mixed distributed in xylo-oligosaccharide chains in the SS and SV xylans. All species showed presence of oligosaccharides in ranges of *m/z* 833.3~2561.2 (SM), 657.2~1655.5 (SS) and 731.2~1421.5 (SV). Acidic residues in SS and SV are distributed in shorter xylo-oligosaccharides than those in SM, although complicated substituted profiles with MeGlcA and GlcA were similarly detected in SS and SV. Presence of long free xylan chains in the SM oligosaccharides supported lower number of substituent in its xylan backbone.

Key words: *Salvia*, hydrogel, MALDI/MS, 4-*O*-methylglucuronic acid, glucuronic acid, xylan.

Introduction

Development of new utilization method of bio-based materials for replacement of materials based on fossil resources is one of the tasks of "Green Chemistry". Utilization of cellulosic materials is a key step to achieve the final goal. We have been interested in cellulosic hydrogels produced from nutlets of three different *Salvias* (*S. miltiorriza* (SM), *S. sclarea* (SS), *S. viridis* (SV)) (Yudianti *et al.* 2009a; 2009b; 2009c). As the importance of natural resource increased, characterization of the hydrogels is becoming a challenging work to access new field of technology. These hydrogels contain acidic polysaccharides which contain about 25~30% of uronic acid (Yudianti *et al.* 2005; 2007). High xylose content (above 88% of neutral sugars) in all acidic polysaccharides indicated that framework of acidic fraction was composed of xylan. Carboxyl groups (COOH) of acidic residues substituted in xylan backbone promote electrostatic repulsion and calcium bridge formation which contribute hydrogel formation (Yudianti *et al.* 2009a; 2009b; 2009c), suggesting importance of acidic polysaccharides in these *Salvia* hydrogels.

On account of chemical properties of the *Salvia* hydrogels, only a few preliminary research works were published (Weber *et al.* 1991; Lin *et al.* 1994). Weber *et al.* (1991) reported carbohydrate contents in the nutlets of three *Salvia* species (*S. columbariae*, *S. carduacea* and *S. hispanica*) as 35.4~43.8 % (Weber *et al.* 1991). Lin *et al.* (1994) isolated an acidic xylan consisting of xylose : glucose : 4-*O*-methylglucuronic acid in a molar ratio of 2 : 1 : 1 from the hydrogels produced from three *Salvia* species (*S. hispanica*, *S. columbariae* and *S. polystachya)* (Lin and Daniel 1994). They also succeeded to isolate an aldobiouronic acid, 2-*O*-(4-*O*-methyl-α-D-glucopyranosiduronic acid)-D-xylose, from this xylan. So far no chemical properties have been characterized on account of the present *Salvia* hydrogels. Because chemical properties are basically important for further characterization of their physicochemical properties, in this paper we intended to focus on the precise chemical properties of their acidic polysaccharides. These *Salvia* species were selected as the starting materials because of differences in sugar composition and commercial availability of their nutlets.

Recently, MALDI-TOF/MS spectroscopy has been widely used for structural analysis of high molecular weight compounds such as synthetic polymer (Choi *et al.* 2007; Janiak and Blank 2006), protein (Taranenko *et al.* 2003), several oligosaccharides containing acidic residues (Hsu *et al.* 2007) in fruit xylan (Reis *et al.* 2002; 2003), hardwood and softwood xylans (Jacob *et al.* 2001). This approach has been desirably applied to structural analysis of other type of polysaccharides. Deep analysis of acidic xylan as oligosaccharides conducted by MALDI-TOF/MS will make a great contribution to the characterization of the type of substituent and degree of substituent pattern distributed in ß-(1,4)-xylopyranose backbone.

In this paper, precise structural characterization of the acidic xylans present in the hydrogels from three species of *Salvias* was carried out by thorough carbohydrate analysis including reduction of uronic acid carboxyl groups followed by methylation analysis and MALDI-TOF/MAS analysis of oligosaccharides obtained by partial acid hydrolysis.

Materials and Methods

Isolation of Hydrogel from *Salvia* Nutlets

Nutlets of *three species of Salvias* (*S. miltiorrhiza*, *S. sclarea* and *S. viridis) in Lamiaceae family* used as origin of hydrogels were purchased on March, 2009, from Richters

Co., Ontario, Canada. After soaking in water, the hydrogels expanded out from exocarp layer of seeds were isolated by treatment of electric mixer for 7 sec and subsequently filtrated through 180 μm screen.

Chemical Analysis

Alkaline-soluble portion of each hydrogel was recovered by extraction with 17.5% sodium hydroxyde solution containing 3% of boric acid and separated into neutral and acidic fraction by Anion Exchange Chromatography on Toyopearl DEAE-650M. Adsorbed fractions were recovered by a linear gradient elution of sodium chloride to 1.2 M in 5.0 mM sodium phosphate buffer, pH 6.8.

Reduction of uronic acid in the acidic fraction was performed following the procedure described by Taylor and Conrad (1972). The acidic fraction (50 mg) was dissolved in 10 mL of distilled water. *N*-cyclohexyl-*N'*-(2-morpholinoethyl) carbodiimide-methyl-p-toluenesulfonate (CMC) (250 mg) was added under maintaining pH at 4.75 with 0.1 N hydrochloric-acid for 1 h by automatic titrator, Hiranuma COM-1600. After stirring for 1 h, 1 g of sodium borohydride ($NaBH_4$) was added portion wisely under maintaining pH at 7.0 with 1.0 N hydrochloric acid for 1 h. The reduced material was recovered by dialysis against water, concentrated by vacuum evaporator and finally freeze-dried. The process was repeated once again.

For partial acid hydrolysis of the reduced acidic xylans, each polysaccharide (100 mg) was hydrolyzed with 0.1 N sulfuric acid (50 mL) for 2 h at 100°C. After cooling down, the hydrolyzed solution was neutralized with barium carbonate, filtrated to remove barium sulfate and subsequently treated with joint columns of Dowex 50x8 (H^+ form) and Dowex 1x8 (acetate form). Eluted solution containing neutral partially degraded materials was concentrated to a small volume by evaporator. Finally, all degraded materials were recovered by freeze drier.

MALDI-TOF/MS spectroscopy and MALDI-TOF/TOF MS including collision induced dissociation (CID) was done by Ultraflex III (Bruker Daltonics Co.) equipped with Smart Beam (YAG laser, 355 nm) and 2,5-dihydroxybenzoic acid (DHB) was used as the matrix. For CID argon was used as the collision gas at 8 kV.

Molecular Weight Analysis

Analysis of Molecular Weight of alkali soluble portion was estimated by Size Exclusion Chromatography on a column of YMC-Pack Diol-300 S-5 (8.0 mm x 50.0 cm) using 5.0 mM sodium phosphate buffer, pH 6.8, containing 0.1 M sodium chloride as an elution solvent at 0.6 mL/min. Elution was monitored by refractive index detector (TOSO RI-8) and recorded by Waters 741 Data Module.

Methylation Analysis

Permethylation of polysaccharides was carried out according to the Hakomori method (Hakamori 1964). The permethylated polysaccharides were subjected to two step hydrolysis by treatment with 90% formic acid for 2 h at 100°C and 0.5 N sulfuric acid for 12 h at 100°C. After neutralization with barium carbonate, the hydrolyzate was reduced with sodium borohydride and acetylated with mixture of acetic anhydride and pyridine (1:1, v/v). The resulting mixture of partially methylated alditol acetates was analyzed by GC/MS with a Shimadzu Parvum 2 (70 eV) using a column of CBP-1 (0.25 μm, 0.25 mm × 25 m) and a linear temperature gradient from 140°C to 220°C at 2°C/min.

Results and Discussion

Carbohydrate Compositional Analysis of *Salvia* Hydrogels

The results of carbohydrate compositional analyses summarized in Table 1 showed that the SV hydrogel contained the lowest hemicellulose (63.8%) and the highest cellulose (36.2%). Conversely, the SM hydrogel contained the highest hemicellulose (81.2%) and the lowest cellulose (18.75%). Intermediate cellulose (25.6%) and hemicellulose (74.3%) contents were observed in the SS hydrogel.

Hemicellulosic polysaccharides were further fractionated into neutral and acidic fractions by Anion Exchange Chromatography with a linear gradient elution of sodium chloride up to 1.2 M. Acidic fraction adsorbed on a column was recovered at 0.6 M sodium chloride in all hydrogels. The proportion of the acidic fraction decreased from 43.5 (SM) to 17.6% (SV). The molecular weights of the isolated fractions were estimated to be in the order of 10^5~10^6 as shown in Table 1. Carbohydrate compositions of all isolated polysaccharides were listed in Table 2. Generally, galactose and xylose are major sugars except glucose in the native hydrogels and hemicellulosic polysaccharides. Neutral sugar analysis of the separated fractions, however, revealed that xylose was the major neutral sugar in the acidic fraction (89.2~93.0%), while localized distribution of galactose (29.2~53.5%) together with arabinose and glucose was observed in the neutral fraction. Uronic acids present in the hydrogels were further analyzed by comparison of the neutral sugar compositional data given before and after reduction. The results showed that both SS and SV hydrogels contained mixtures of glucuronic acid (GlcA) and 4-O-methylglucuronic acid (MeGlcA), recovered as glucose (Glc) and 4-O-methylglucose (MeGlc), respectively. However, only GlcA was detected in the SM hydrogel. Molar ratios of xylose to MeGlcA to GlcA were estimated as in 2.1 : 0.0 : 1.0 (SM), 5.2 : 1.0 : 2.2 (SS) and 4.3 : 1.0 : 1.9 (SV), respectively, corresponding to molar ratios of xylose to uronic acid could be 2.0 : 1.0 (SM), 1.7 : 1.0 (SS) and 1.4 : 1.0 (SV), respectively. Presence of MeGlcA as 4-O-methylglucitol pentaacetate was confirmed by GC/MS analysis.

Table 1. Carbohydrate composition of hydrogels and fractionated fractions of *Salvia* hydrogels.

Salvia spp.	Contents of cellulose and hemicellulose in hydrogels (%)		Composition of hemicellulose (%)		Molecular weight (x 10^{-5})	
	Cellulose	Hemicellulose	Neutral fraction	Acidic fraction	Neutral fraction	Acidic fraction
S. miltiorrhiza	18.7	81.2	56.6	43.5	10.7	7.9
S. sclarea	25.6	74.3	77.9	22.1	13.6	12.7
S. viridis	36.2	63.8	82.4	17.6	13.6	16.7

Table 2. Carbohydrate composition of polysaccharides isolated from *Salvia* hydrogels (SM : *S. miltiorrhiza*, SS : *S. sclarea*, SV : *S. viridis*).

Polysaccharides	*Salvia* spp.	Relative neutral sugar composition (%)						Increment after reduction	
		Ara	Rha	Gal	Glc	Xyl	Man	MeGlc	Glc
Native	SM	7.0	2.7	8.5	27.4	51.9	2.4	-	-
	SS	1.6	0.8	23.1	40.4	34.1	0.0	-	-
	SV	0.6	0.4	27.2	53.8	18.0	0.0	-	-
Hemicellulose	SM	7.3	2.4	8.5	9.7	72.0	0.0	-	-
	SS	1.0	1.3	30.6	18.2	48.8	0.0	-	-
	SV	0.5	0.3	41.4	31.3	23.2	3.4	-	-
Neutral fraction	SM	19.3	0.0	29.6	37.7	8.8	5.4	-	-
	SS	1.5	0.0	59.1	37.5	1.3	0.6	-	-
	SV	0.5	0.0	53.5	44.5	0.7	0.9	-	-
Acidic fraction	SM	3.6	3.8	2.1	1.3	89.2	0.0	-	-
	SS	1.2	2.2	4.5	0.9	91.2	0.0	-	-
	SV	0.8	1.0	13.1	8.4	93.0	0.0	-	-
Reduced acidic fraction	SM	1.4	2.4	2.8	0.5	62.6	0.0	-	30.5
	SS	0.5	3.1	4.2	0.4	57.2	0.0	10.9	23.6
	SV	0.2	0.9	4.1	2.0	55.2	0.0	12.9	24.5

(Ara : arabinose ; Rha : rhamnose; Gal : galactose; Glc : glucose; Xyl : xylose ; Man : mannose ; MeGlc : 4-*O*-methylglucopyranose)

Methylation Analysis of Reduced Acidic Polysaccharides

The results of methylation analysis of the reduced acidic polysaccharides were listed in Table 3. The overall profiles agreed with presence of a common backbone structure of (1,4)-linked xylans. Similarity of the amount of 2,3,4,6-tetra-*O*-methylated glucopyranose residues to that of 3-*O*-methylated xylopyranose residues indicates attachment of all glucose derivatives at *O*-2 of xylopyranose residues, confirming the presence of (1,4)-linked xylan with highly substitution at *O*-2 positions. Ratios of substituted (3-*O*-methylated xylopyranose) to unsubtituted xylopyranose (2,3-di-*O*-methylated xylopyranose) residues in xylan backbone are 1.0:1.75 (SM), 1.0:1.0 (SS) and 1.2:1.0 (SV) respectively. The results presented above indicate that the xylan backbones in the acidic polysaccharides of all hydrogels, SM, SS and SV, had abnormally high substitution with uronic acids, confirming the previous results of Lin dan Daniel (1994) . The present results indicate for the first time that the xylans in the SS and SV hydrogels have mixed substitutions with MeGlcA and GlcA, while GlcA was exclusively substituted in the SM hydrogel.

Table 3. Mode of linkages present in the acidic fractions of hemicellulosic polysaccharides of *Salvia* hydrogels after reduction.

Origin of the acidic fractions	Methylated sugar	Mode of linkage	%
S. miltiorrhiza	2,3 - Xyl	4)-Xylp-(1	40.2
	2,3,4,6 - Glc	Glcp-(1	25.2
	3 - Xyl	2,4)-Xylp-(1	22.8
	2,3,6 - Glc	4)-Glcp-(1	5.3
	2,3,4 - Xyl	Xylp-(1	6.6
S. sclarea	2,3 - Xyl	4)-Xylp-(1	30.7
	2,3,4,6 - Glc	Glcp-(1	33.6
	3 - Xyl	2,4)-Xylp-(1	31.2
	2,3,6 - Glc	4)-Glcp-(1	1.2
	2,3,4 - Xyl	Xylp-(1	3.3
S. viridis	2,3 - Xyl	4)-Xylp-(1	26.5
	2,3,4,6 - Glc	Glcp-(1	36.9
	3 - Xyl	2,4)-Xylp-(1	34.0
	2,3,6 - Glc	4)-Glcp-(1	0.5
	2,3,4 - Xyl	Xylp-(1	0.8

(Xyl : xylose ; Glc : glucose ; Glcp : glucopyranose ; Xylp : xylopyranose)

MALDI-TOF/TOF MS Analysis of Oligosaccharides Prepared from Reduced Acidic Polysaccharides

Distribution of uronic acid residues along the xylan backbone was examined by partial acid hydrolysis. Because the linkages between xylopyranose and MeGlcA or GlcA substituents are more stable toward acid hydrolysis than ß-(1,4) linkages between xylose residues in xylan backbone, analysis of oligosaccharides prepared from reduced acidic fractions was carried out. Figure 1 showed MALDI mass spectrum of the oligosaccharides prepared from the SV hydrogel in the mass range *m/z* 731.2~1421.5, identified as sodium adducts $[M+Na]^+$. The spectrum exhibited presence of many xylo-oligosaccharides containing MeGlcA and GlcA substituents appeared as MeGlc and Glc, respectively. The five molecular ions at *m/z* 833.3, 965.3, 1097.4, 1229.4 and 1361.5 have two possible structures, neutral xylo-oligosaccharide (X_n) or three MeGlc substituents distributed in xylan backbone (X_n $MeGlc_3$). In order to confirm the possible structure present in the molecular ion, TOF/TOF MS was conducted for each molecular ion. Successive fragmentation of molecular ion at *m/z* 1097.4 occurred with twice losses of *m/z* 176.2 as mono-*O*-methyl glucose residue corresponding to X_4 $MeGlc_2$ at *m/z* 921.3 and X_4 MeGlc at *m/z* 745.3. The structure of this mother ion was deduced as X_4 $MeGlc_3$ indicating condiguous substitution in the (1,4)-linked xylan chain. In the case of molecular ion at *m/z* 1229.4, successive losses of mono-*O*-methyl glucose residue (*m/z* 176.2) yielding fragmentation at *m/z* 1053.3 identified as X_5 $MeGlc_2$ and *m/z* 877.2 as X_5 MeGlc, respectively. From the molecular ion at *m/z* 1361.5, three fragment ions were also generated with a loss of *m/z* 176.2 from the reducing end to form fragment X_6 $MeGlcA_2$ appeared at *m/z* 1185.4, followed by loss of *m/z* 176.2 as fragment X_6 MeGlc at *m/z* 1009.2 and finally loss of *m/z* 176.2 to form X_6 at *m/z* 833.3. These analytical results indicate that the five mother ions have structures strongly corresponded to X_n $MeGlc_3$ (n=3-6), except a molecular ion at *m/z* 833.3 which was identified as X_6. In addition, five abundant molecular ions in the spectrum appeared at *m/z* 775.2, 907.3, 1039.3, 1171.4 and 1303.4. When TOF/TOF MS analysis of these molecular ions was conducted, molecular ion at m/z 1039.3 generated only one fragment ion at *m/z* 863.2 as X_4 Glc with loss of *m/z* 176.2 as mono-*O*-methyl glucose residue. In the case of mass ion at *m/z* 907.3, fragment ions at *m/z* 731.2 as X_4GlcA and 569.2 as X_4 was generated by successive losses of 176.2 as mono-*O*-methyl glucose, followed by 160.2 as glucose residues, respectively. These ions were estimated as X_n Glc MeGlc (n=3~7) with one Glc and one MeGlc residues substituted in irregularly within the seven xylose residues. Most predominant ion appeared at *m/z* 775.3 was deduced as X_3 Glc MeGlc. In a mass range higher than *m/z* 1100, molecular ions at *m/z* 1187.4 (X_6 $MeGlc_2$), 1319.4 (X_7 $MeGlc_2$), 1201.4 (X_5 Glc_2 MeGlc), 1319.4 (X_7 $MeGlc_2$), and 1333.4 (X_6 Glc_2 MeGlc) were dominant with the highest mass at m/z 1421.4 corresponding to X_8 Glc_2.

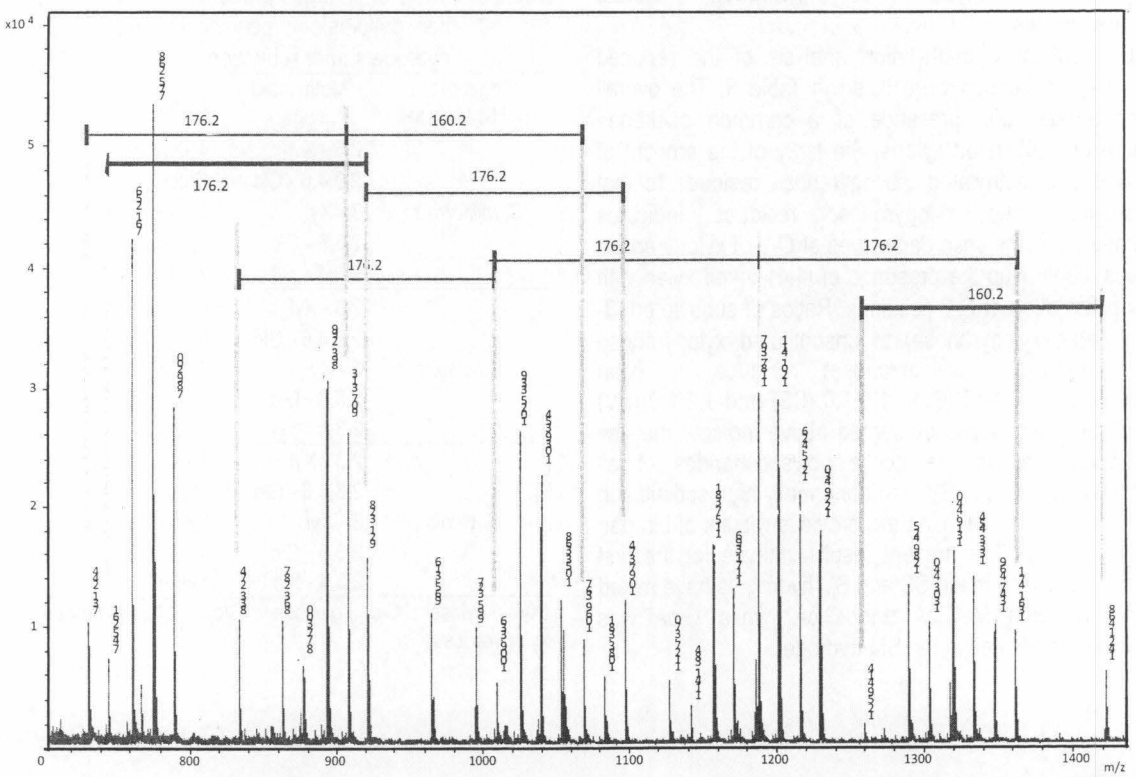

Figure 1. MALDI-MS spectrum of the oligosaccharides obtained by partial hydrolysis of the reduced SV acidic xylan.

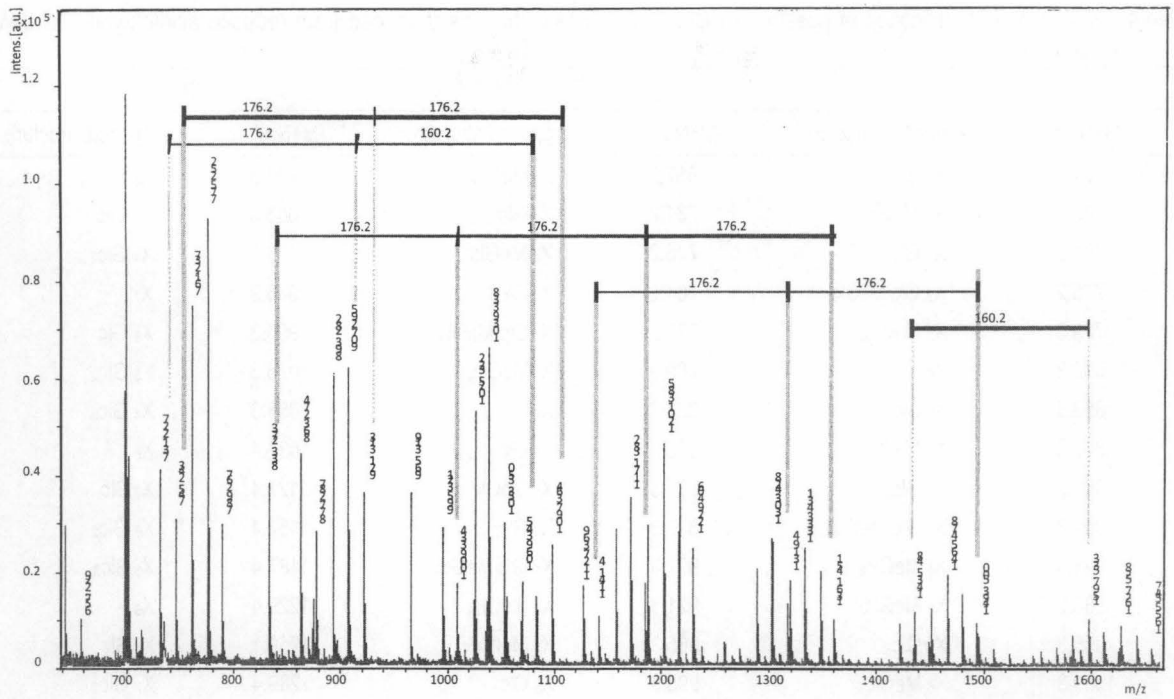

Figure 2. MALDI-MS spectrum of the oligosaccharides obtained by partial hydrolysis of the reduced SS acidic xylan.

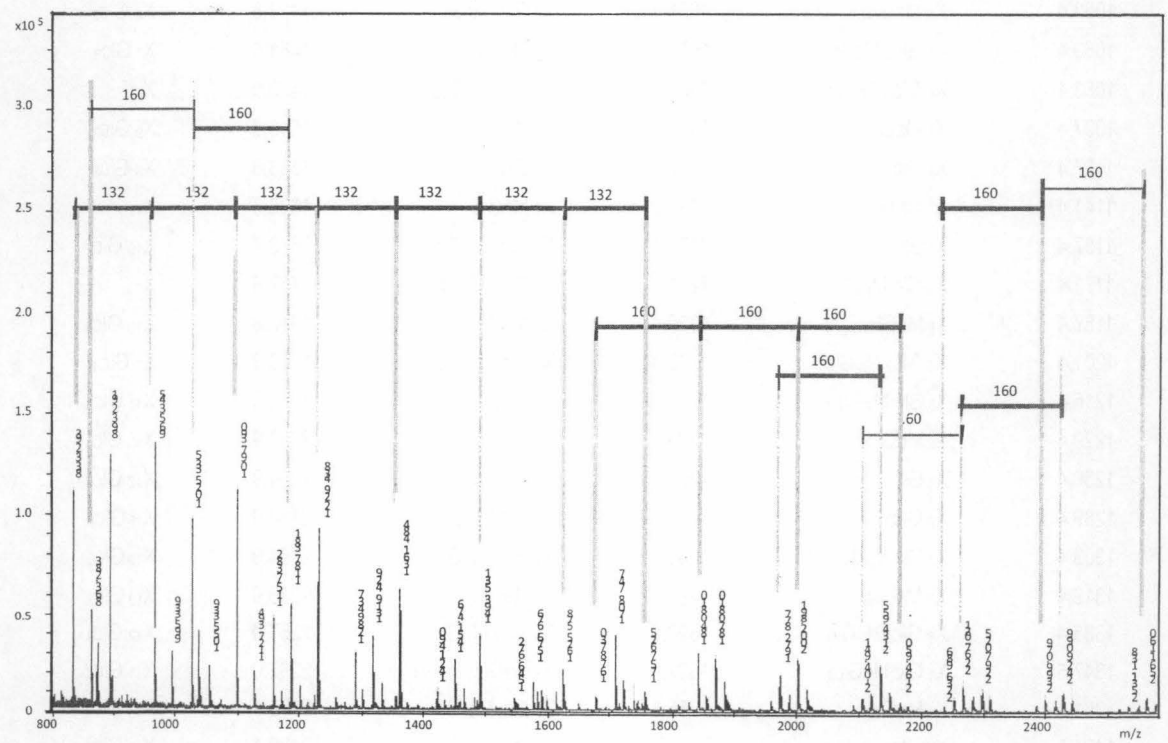

Figure 3. MALDI-MS spectrum of the oligosaccharides obtained by partial hydrolysis of the reduced SM acidic xylan.

Table 4. MALDI-MS identification of possible structures of oligosaccharides produced from reduced acidic xylans in *Salvia* hydrogels.

SV		SS		SM	
$[M+Na]^+$	Proposed structure	$[M+Na]^+$	Proposed structure	$[M+Na]^+$	Proposed structure
731.4	X_4 Glc	657.2	X_2 $MeGlc_2$	833.3	X_6
745.3	X_4 MeGlc	731.2	X_4 Glc	863.3	X_5 Glc
761.2	X_3 Glc_2	745.2	X_4 MeGlc	893.3	X_4 Glc_2
775.2	X_3 Glc MeGlc	761.2	X_3 Glc_2	965.3	X_7
789.3	X_3 $MeGlc_2$	775.2	X_3 Glc MeGlc	995.3	X_6 Glc
833.3	X_6	789.3	X_3 $MeGlc_2$	1025.3	X_5 Glc_2
863.3	X_5 Glc	833.3	X_6	1055.3	X_4 Glc_3
877.3	X_5 MeGlc	863.3	X_5 Glc	1097.4	X_8
893.3	X_3 Glc_2	877.3	X_5 MeGlc	1127.4	X_7 Glc
907.3	X_4 Glc MeGlc	893.3	X_4 Glc_2	1157.4	X_6 Glc_2
921.3	X_4 $MeGlc_2$	907.3	X_4 Glc MeGlc	1187.4	X_5 Glc_3
965.3	X_3 $MeGlc_3$	921.3	X_4 $MeGlc_2$	1229.4	X_9
995.3	X_6 Glc	965.3	X_3 $MeGlc_3$	1259.1	X_8 Glc
1009.3	X_6 MeGlc	995.3	X_6 Glc	1289.4	X_7 Glc_2
1025.4	X_5 $MeGlc_2$	1009.3	X_6 MeGlc	1319.4	X_6 Glc_3
1039.3	X_5 Glc MeGlc	1025.3	X_5 Glc_2	1361.5	X_{10}
1053.4	X_5 $MeGlc_2$	1039.3	X_5 Glc MeGlc	1421.5	X_8 Glc_2
1069.4	X_4 Glc_2 MeGlc	1053.3	X_5 $MeGlc_2$	1451.5	X_7 Glc_3
1083.4	X_4 Glc $MeGlc_2$	1069.3	X_4 Glc_2 MeGlc	1493.5	X_{11}
1097.4	X_4 $MeGlc_3$	1097.4	X_4 $MeGlc_3$	1546.7	X_9 Glc_2
1127.4	X_7 Glc	1127.4	X_7 Glc	1583.8	X_8 Glc_3
1141.4	X_7 MeGlc	1141.2	X_7 MeGlc	1625.6	X_{12}
1157.4	X_6 Glc_2	1171.4	X_6 Glc MeGlc	1678.7	X_{10} Glc_2
1171.4	X_6 Glc MeGlc	1201.4	X_5 Glc_2 MeGlc	1757.6	X_{13}
1187.4	X_6 $MeGlc_2$	1229.4	X_5 $MeGlc_3$	1840.8	X_{10} Glc_3
1201.4	X_5 Glc_2 MeGlc	1303.4	X_7 Glc MeGlc	1870.8	X_9 Glc_4
1215.4	X_5 Glc $MeGlc_2$	1319.4	X_7 $MeGlc_2$	1972.8	X_{11} Glc_3
1229.4	X_5 $MeGlc_3$	1333.4	X_6 Glc_2 MeGlc	2002.9	X_{10} Glc_4
1259.4	X_8 Glc	1361.4	X_6 $MeGlc_3$	2104.9	X_{12} Glc_3
1289.4	X_7 Glc_2	1435.5	X_8 Glc MeGlc	2134.9	X_{11} Glc_4
1303.4	X_7 Glc MeGlc	1465.5	X_7 Glc_2 MeGlc	2164.9	X_{10} Glc_5
1319.4	X_7 $MeGlc_2$	1493.5	X_7 $MeGlc_3$	2236.9	X_{13} Glc_3
1333.4	X_6 Glc_2 MeGlc	1597.5	X_8 Glc_2 MeGlc	2267.0	X_{12} Glc_4
1347.5	X_6 Glc $MeGlc_2$	1627.5	X_8 $MeGlc_3$	2297.0	X_{11} Glc_5
1361.5	X_6 $MeGlc_3$	1655.5	X_7 Glc $MeGlc_3$	2399.1	X_{13} Glc_4
1421.5	X_8 Glc_2			2429.1	X_{12} Glc_5
				2531.1	X_{14} Glc_4
				2561.2	X_{13} Glc_5

(SM : *S. miltiorrhiza* ; SS : *S. sclarea* ; SV : *S. viridis* ; X : xylose ; Glc : glucose from glucuronic acid ; MeGlc : 4-*O*-methylglucose from 4-*O*-methylglucuronic acid).

Basically, the structures proposed for the SS oligosaccharide are similar to the oligosaccharides given from SV. As shown in the MALDI mass spectrum of the oligosaccharides derived from SS hydrogel (Figure 2), molecular ions appeared in a range from *m/z* 657.2 to 1655.5. The highest mass at *m/z* 1655.5 generated successive ions at m/z 1493.5, 1319.4 and 1141.2 corresponding to X_7 $MeGlc_3$, X_7 $MeGlc_2$, X_7 MeGlc with losses of glucose residue and mono-*O*-methyl glucose from the reducing end.

MALDI mass spectrum of oligosaccharides given after reduction and partial hydrolysis of the acidic polysaccharide from SM (Figure 3) showed profiles different from the SS and SV hydrogels. The molecular ions were also identified as sodium adducts $[M+Na]^+$ in a mass range of *m/z* 833.3~2561.2 including ions assignable as the xylo-oligosaccharides contained various degrees of Glc residues. Presence of mass ions corresponding to xylo-oligosaccharides (X_n, n=6~13) were detected and mass ion appeared at *m/z* 1625.6 resulted in formation of several fragment ions with successive losses of six xylose residues at *m/z* 1493.5, 1361.4, 1229.4, 1097.4, 965.3 and 833.3. These ions were more abundant than other mass ions in a mass range *m/z* 800~1800. The highest mass ion appeared at *m/z* 2561.2 was proposed to have a structure of X_{13} Glc_5 based on successive generation of fragment ions at *m/z* 2399.1 as X_{13} Glc_4 and 2236.9 as X_{13} Glc_3 with losses of glucose residues. Two fragment ions occurred at *m/z* 2267.4 and 2104.9 were similarly deduced to have structures corresponding to X_{12} Glc_4 and X_{12} Glc_3, respectively, with the losses of successive glucose residues. The ion mass at *m/z* 2429.1 was suggested to have a structure of X_{12} Glc_5. Similar fragmentation also occurred for ions which have mass numbers at *m/z* 2297.4 and 2164.9 with successive losses of glucose residues corresponding to have a possible structure of X_n Glc_5 (n=10~13). A low abundant ion at *m/z* 2164.9 produced fragment ions at *m/z* 2002.9, 1840.8 and 1678.7 with lose of one glucose residue corresponding to oligosaccharides which have structures of $X_{10}Glc_4$, $X_{10}Glc_3$ and $X_{10}Glc_2$, respectively. Other ion at *m/z* 1187.4 generated fragmentation with successive losses of glucose residue to form X_5 Glc_3 (*m/z* 1025.3) and X_5 Glc_2 (*m/z* 863.3). The ion mass at *m/z* 2429.1 was suggested to have a structure of X_{12} Glc_5. Similar fragmentation also occurred for ions which have mass numbers at *m/z* 2297.4 and 2164.9 with successive losses of glucose residues corresponding to have a possible structure of X_n Glc_5 (n=10~13). A low abundant ion at *m/z* 2164.9 produced have fragment ions at m/z 2002.9, 1840.8 and 1678.7 with loss of glucose residue corresponding to oligosaccharides which have structures of $X_{10}Glc_4$, $X_{10}Glc_3$ and $X_{10}Glc_2$, respectively. Other ion at *m/z* 1187.4 generated fragmentation with successive losses of glucose residue to form X_5 Glc_3 (*m/z* 1025.3) and X_5 Glc_2 (*m/z* 863.3). All deduced structures of the oligosaccharides produced from the present *Salvia* hydrogels were listed in Table 4. Presence of random and contiguous substitutions was a new finding for acidic xylans in the *Salvia* hydrogels.

Conclusions

Acidic polysaccharides present in the hydrogels produced from three species of *Salvia* (*S. miltiorrhiza* (SM), *S. sclarea* (SS) and *S. viridis* (SV)) were commonly composed of ß-(1,4)-xylans highly substituted at *O*-2 positions with uronic acid in molar ratios of xylose to uronic acid of 2.1 : 1.0 (SM), 1.7 : 1.0 (SS), 1.4 : 1.0 (SV), respecitively. Mixed substitutions with MeGlcA and GlcA occurred in both of the SS and SV hydrogels, while GlcA was exclusively substituted in the SM hydrogel. The precise chemical composition analysis and MALDI-TOF/TOF MS analyses elucidated random and contiguous substitutions of GlcA and MeGlcA at *O*-2 of xylopyranosyl residues. In addtion, SM oligosaccharides contained higher degree of free xylopyranose residues than those in SS and SV in agreement with the lowest content of uronic acid among three *Salvias*.

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