Master's Thesis of Science in Agriculture

Characterization of Novel Astragalin Galactosides Synthesized by β-Galactosidase from *Bacillus circulans*

β-Galactosidase를 이용한 신규 아스트라갈린 배당체의 특성 연구

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Songhee Han

Department of International Agricultural Technology
Graduate School of International Agricultural Technology
Seoul National University

Abstract

Characterization of Novel Astragalin Galactosides Synthesized by β-Galactosidase from *Bacillus circulans*

Songhee Han

Department of International Agricultural Technology

The Graduate School

Seoul National University

Astragalin (kaempferol-3-O-β-D-glucopyranoside) is a kind of flavonoid known to have anti-oxidant, anti-HIV, anti-allergic, and anti-inflammatory effects and low solubility in water. In this study, novel astragalin galactosides (Ast-Gals) were enzymatically synthesized with β-galactosidase from *Bacillus circulans.* Reaction conditions were optimized to maximize the conversion yield of astragalin. Purified Ast-Gal1 (11.6% of Ast used, w/w) and Ast-Gal2 (6.7% of Ast used, w/w) were obtained by medium pressure chromatography (MPLC) with silica C18 column and open column packed with Sephadex LH-20. The structures of Ast-Gal1 and Ast-Gal2 were identified by nuclear magnetic resonance (NMR) to be kaempferol-3-O-β-D-glucopyranosyl-(1→6)-O-β-D-galactopyranoside and kaempferol-3-O-β-D-glucopyranosyl-(1→6)-O-β-D-galactopyranosyl-(1→4)-O-β-D-galactopyranoside, respectively. The

water solubility of Ast, Ast-Gal1, and Ast-Gal2 were 28.2 mg/L, 38,300 mg/L, and 38,800 mg/L, respectively. The antioxidant activities (The half maximal scavenging capacity, SC_{50}) of Ast, Ast-Gal1, and Ast-Gal2 based on ABTS•+ radical scavenging activity were 5.05 μM, 6.54 μM, and 4.91 μM, respectively. The angiotensin converting enzyme inhibition activities (ACE, the half maximal inhibitory capacity, IC_{50}) of Ast, Ast-Gal1, and Ast-Gal2 were 171 μM, 191 μM, and 138 μM, respectively.

Keywords : Astragalin, Transgalactosylation, β-Galactosidase, Astragalin galactosides

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Introduction

1. Flavonoid and astragalin

Flavonoid is a ternary phenolic compound which can observed on plants. It can be classified into several different groups of flavanonols, flavones, flavonols, flavans, and anthocyanidins, as the location of its hydroxyl groups. These chemicals are widely distributed in the leaves, seeds, barks and flowers of plants to protect themselves against ultraviolet light, pathogens, and herbivores.

Flavonoid has variety of functional properties. Flavonoid has been known to an antioxidant compound [1] which also has biological and pharmacological activities such as anti-microbial, anti-allergic, anti-inflammatory, and anti-cancer. However, these activities are less effective for *in vivo* because of their low absorption ability in the small intestine [2]. In this reason, studying of improvement on its absorption and metabolism in human body has become the important issue.

Kaempferol is tetrahydroxyflavone, which has hydroxyl groups on its C-3, C-5, and C-7 of the A ring and C-4′ of the B ring. The natural form of kaempferol is an uronic acid conjugate or a glycosylated form likewise kaempferol-3-glucuronide, kaempferol-3-glucoside, or kaempferol-3-(6-malonyl)-glucoside. Kaempferol and its gly cosides exist in endive [3], persimmon leaves [4], black and white mulberry [5, 6], hwangqi (*Astragalus membranaceus*) [7], or green tea.

Kamepferol-3-O-β-D-glucopyranoside (astragalin), one of the natural form of kaempferol, has anti-oxidant [5], anti-HIV, anti-allergic [4], and anti-inflammatory activities likewise with other flavonoids in preclinical studies. Although it already has one glucosyl unit in the C-3 position of its C ring, astragalin is also known for water-immiscible and low absorption ability compound [3]. In this reason, astragalin was contiuously studied to improve its properties.

2. Glycosylation and acceptor reaction

Transglycosylation catalyzed by enzymes has been used to improve the physicochemical properties (such as water solubility and oxidative stability) of various compounds to enter cells throughout sodium-glucose co-transporter 1 (SGLT1). Acceptor reaction is an enzymatic transglycosylation which is caused to aglycon attached glyco-oligomer. Aglycon, also called as an acceptor, could be a broad range of compounds that have more than one hydroxyl group or carboxyl group including saccharides, polyphenols, flavonoids, protein, lipids, and other organic molecules. In this reaction, a small sugar, which also called 'donor', is broken by the enzyme and its glycosyl residue is attached on acceptor.

Glycosylation of flavonoid is a constant issue for improving its bioavailability because of previous research that glycosides of flavonoid could be absorbed as its own form before hydrolyzing [9, 10]. Until now, only astragalin glucosides are alternatives that can overcome demerits of astragalin. Kim, Kang et al. [11] have reported that astragalin glucosides have higher MMP1 inhibition, melanogenesis inhibition and antioxidant activity. Especially, it has been reported that astragalin glucosides, but not astragalin, can inhibit fructose transporter (GLUT5) [12].

3. β-Galactosidase

β-Galactosidase (EC 3.2.1.23) is the enzyme which catalyzes hydrolysis and transgalactosylation of β-glycosidic bond between galactose and organic compounds [13]. This enzyme is profusely utilized in the dairy industry to hydrolyze lactose from milk for targeting lactose intolerance patients. In addition, it can produce various sizes of galacto-oligosaccharides (GalOS) [14-16] which is as alternative sweetener and can be used as prebiotics. Also, GalOS is already known as the functional compound which can improve the immune system of infant body.

β-Galactosidase originated from *Bacillus circulans* has high transgalactosylation ratio [17]. In this reason, this enzyme is already commercialized for synthesizing GalOS. Among β-galactosidases, β-galactosidases from Bacillus circulans have been a subject of investigation because of their high transgalactosylation activities compared to other β-glactosidases [15, 17-19]. They have four isoforms: β-gal-A (189 kDa), β-gal-B (154 kDa), β-gal-C (134 kDa), and β-gal-D (91 kDa). They contribute to galactooligosaccharides synthesis with different productivities [20]. The transferase activity of β-galactosidase from *Bacillus circulans* has been applied to the synthesis of lactosucrose [21], acetyl-lactosamine [22], and galactosylated derivates [23, 24]. This enzyme is an industrial enzyme. It is commercially available at food grade with trade name of Biolacta FN5.

4. Purpose of this study

As the summary, transgalactosylation of astragalin could be a solution not only to improve water solubility but also to find out new functional properties. These properties could be related with immune system because of its GalOS residue and astragalin, either.

In this study, novel astragalin galactosides were synthesized for the first time with lactose as the galactose unit donor and β-galactosidase from *Bacillus circulans*. The effects of reaction factors on the conversion yield of astragalin to Ast-Gals products were optimized using response surface methodology (RSM) and central composite design (CCD). After transgalactosylation, function-improved materials were obtained from purification process. The structures and biological functions of Ast-Gals were determined.

Materials and Methods

1. Transgalactosylation of astragalin

The commercial β-galactosidase produced from *Bacillus circulans* (Lactazyme-B^{TM}, Genofocus, Daejeon, Korea) was used. Astragalin was provided by Amore Pacific Corporation (Yongin, Korea). Astragalin acceptor reaction mixture contained 3 U/mL β-galactosidase, 300 mM lactose, and 50 mM astragalin in 10% (v/v) DMSO and 20 mM potassium phosphate buffer (pH 6.0). The reaction was incubated at 60℃ for 12 h. One μL of reaction mixtures were spotted onto silica gel $60F_{254}$TLC plate (Merck, Darmstadt, Germany) and developed in nitromethane: n-propyl alcohol: water (2: 5: 1.5, v/v/v) solvent. Astragalin and Ast-Gals on TLC plate were observed under UV 254nm light or visualized by dipping the plate into a solvent mixture of 0.5 (w/v) N-(1-naphthyl) ethylenediamine dihydrochloride and 5% (w/v) sulfuric acid in methanol followed by heating at 125℃ for 10 min. Ast-Gals were analyzed with HPLC (Waters, Millford, Massachusetts, USA) and Matrix-assisted laser desorption/ionization-Time of flight-mass spectrometry (MALDI-TOF-MS) Voyager DE-STR (AB SCIEX, Foster City, CA, USA) [11]. The mass spectra were obtained in the positive reflector mode as described previously with 2,5-dihydroxybenzoic acid (DHB) matrix. The astragalin conversion yields were calculated as follow:

Ast conversion (%) = (1 *Amount of astragalin remainded in the reaction mixture (mM)* ÷ *Initial amount of astragalin in the mixture* (mM))×100

Individual Ast-Gal yield (%) = (A*mount of individual Ast-Gal obtained* (mg)÷ *Total loaded astragalin* (mg))×100

2. Optimization of acceptor reaction using RSM

The central composite design (CCD) of RSM software program (Design Expert 10.0.3, Stat-Ease, Minneapolis, USA) was used to optimize Ast-Gals production with following three variables: lactose concentration (300-500 mM), astragalin concentration (30-70 mM), and enzyme concentration (1.5-4.5 U/mL). Twenty runs of the experiment were carried out with six replications at the central point, which were utilized in the fitting of a second-order response surface [25] (**Table 2**). Statistical and mathematical analysises of the results were performed interactions using Design-Expert 10.0.3 to determine the effects of variables. Three-dimensional surface plots were drawn to determine the effects of independent variables on response. Correlation between variables and the response was determined using a quadratic model of a second-order polynomial as shown below:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j$$

where Y represented the predicted response; β_0, β_i, β_{ii}, and β_{ij} were the regression coefficients for intercept, linearity, square, and interaction, respectively, and X_i and X_j were the independent coded variables. The significance of the model was evaluated by determination of R^2 and adjusted R^2 coefficients. An experiment was also conducted to confirm the predicted optimum response using the selected optimum values of the three variables. The amount of astragalin converted to Ast-Gals was calculated using AlphaEaseFC 4.0 program (Alpha Inotech, San Leandro, CA, USA) with astragalin as standard (**Figure 1**).

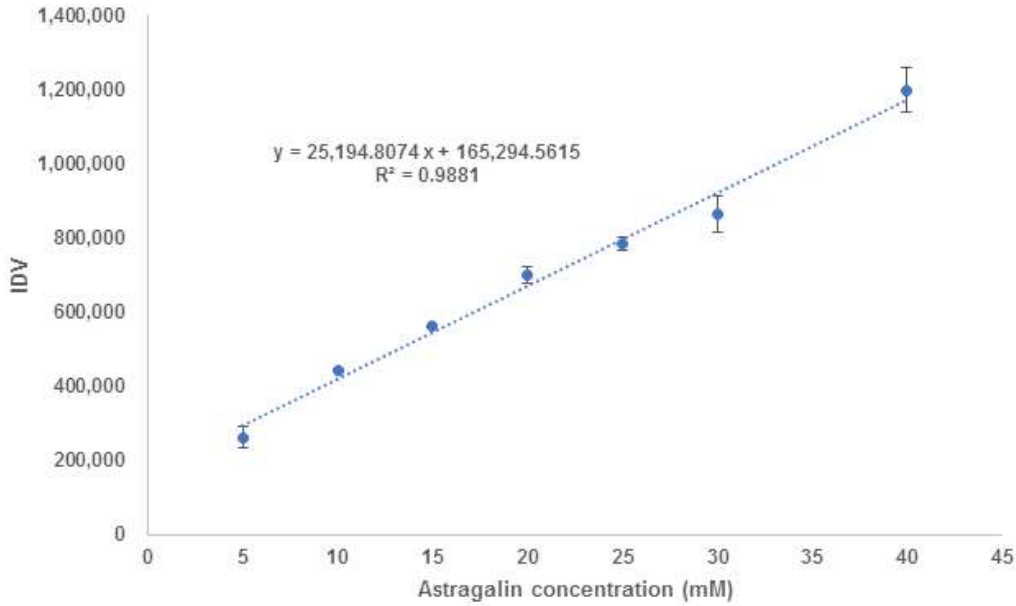


Figure 1. Astragalin standard curve determined by TLC and AlphaEaseFC 4.0.

3. Purification of Ast-Gals

Astragalin acceptor reaction mixture (100 mL) containing 400 mM lactose, 50 mM astragalin, and 3 U/mL β-galactosidase in 10% (v/v) DMSO and 20 mM poassium phosphate buffer (pH 6.0) was incubated at 60°C for 12 h. The reaction mixture was analyzed by TLC method as described above.

To remove saccharides, reaction mixtures were subjected to purification using medium pressure liquid chromatography (MPLC) (PURIFLASH 430, Interchim, Montluçon, France) with two series-connected C18 column (IR-50C18/175G, 36 mm 224 mm, 153 mL CV_0, 50 μm, PURIFLASH, Interchim, Montluçon, France) at flow rate of 30 mL/min. The detection was achieved at 254 nm using UV-detector. Saccharides and DMSO were eluted with 100% water. Then Ast-Gals elution was performed using acetonitrile with gradient [0-100% (v/v)]. Ast-Gals mixture was eluted at 60-80% acetonitrile. Fractions containing Ast-Gals were collected and then evaporated to remove solvent at 60℃ with low pressure.

Ast-Gals were then loaded into Sephadex LH-20 (3.4 cm × 118.5 cm) (GE Healthcare, Little Chalfont, UK) gravity column with 0-100% (v/v) ethanol gradient in order to separate each Ast-Gals. Each fraction was collected and confirmed by TLC. The purity of each Ast-Gal was confirmed by HPLC (Waters, Millford, Massachusetts, USA). The Analytical conditions were based on Kim's conditions [12] with slight modification: SunFire C_{18} Column (Waters, Inc. Massachusetts, USA); solvent, ethanol: water with gradient (70-20% (v/v)); flow rate, 0.5 mL/min; injection, 20 μL; PDA detector, 200-600 nm; and UV detector, 254 nm.

4. Structure analysis of Ast-Gals using NMR

Ten mg of each purified Ast-Gal was dissolved in DMSO-d_6 (Sigma Aldrich, St. Louis, USA) and placed into 5 mm PABBO BB/19F-1H/D Z-GRD Probe. Nuclear magnetic resonance (NMR) spectra were obtained using AVANCE 500 (Bruker Co., Billerica, US) operated at 500 MHz for ^{1}H and ^{13}C at 25℃ at the National Center for Inter-University Research Faculties (NCIRF) of Seoul National University (Seoul, Korea).

Linkages between astragalin and galactosyl residue and their anomeric carbons were evaluated using homonuclear correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) [12].

5. Pharmacological characterization of novel astragalin galactosides

5.1. Water solubility

Astragalin, purified Ast-Gal1, and Ast-Gal2 were dissolved in water as saturated solutions at 25℃ and then filtered with 0.45 μm syringe filter after centrifugation. The concentrations of astragalin, Ast-Gal1, and Ast-Gal2 in aqueous solutions were determined by TLC and Spot Denso Tool of AlphaEaseFC Imaging Software (version 4.0.0, Alpha Innotech Co., San Leandro, USA) using linear standard curves (**Figure 1**).

5.2. ABTS·+ radical scavenging activity

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) (Sigma Aldrich, St. Louis, USA) radical scavenging activity of astragalin and Ast-Gals were determined according to the methods of Re et al. [26] with some modifications. Ionized ABTS (ABTS·+) mixture solution was prepared by mixing 7 mM of ABTS and 2.34 mM of potassium persulfate (K_2S_2O8). The mixture was then placed in the dark at room temperature for 8 h. Working solution was diluted from this mixture to reach A_{734} at 0.70 ± 0.02. For radical scavenging activity analyzing, 5 μL of different concentration sample in 10% (v/v) DMSO and 245 μL working solution were mixed and incubated at room temperature for 8 min. The absorbance value was measured at wavelength of 734 nm on a VersaMAX ELISA Microplate Reader (Molecular devices, California, USA).

6-Hydroxy-2,5,7,8-tetramethylchroman−2-carboxylic acid (trolox) was used as a positive control.

5.3. ACE inhibitory activity

Rabbit angiotensin converting enzyme (ACE), hippuryl-L-histidyl-L-Leucine (HHL), and O-phthaldialdehyde (OPA) were purchased from Sigma. The assay method was followed that of Li et al. [27] with slight modifications. Briefly, the enzyme reaction mixture (350 μL) containing 0.5 mU/mL ACE, 1.5 mM HHL, 2-50 μM inhibitor in 1% (v/v) DMSO (Ast or Ast-Gal), and 0.4 M NaCl in 0.1 M sodium borate (pH 8.3) was incubated at 37℃ for 60 min. The reaction was stopped by adding 2 M NaOH. After adding 6.2 mM OPA at 25°C, inhibition activities were measured for 60 min in relative fluorescent units (RFU) with an excitation wavelength of 365 nm and an emission wavelength of 460 nm with SpectraMax M3 Microplate Reader (Molecular Devices, Sunnyvale, USA).

Results and Discussion

1. Synthesis of novel Ast-Gals using β-galactosidase from *Bacillus circulans*

Using β-galactosidase from *Bacillus circulans,* transgalactosylation products of astragalin are shown in **Figure 2**. After removing DMSO and saccharides (glucose, lactose, galactooligosaccharides) by MPLC, Ast-Gals containing mono- to penta-galactosyl residues in the reaction products were detected via MALDI-TOF-MS analysis (**Figure 3**). Based on MALDI-TOF-MS analysis, Ast-Gal1 containing a galactosyl unit was observed at m/z 633 $(M + Na)^+$, Ast-Gal2 containing two attached galactosyl units was observed at m/z 795 $(M + Na)^+$, Ast-Gal3 containing three attached galactosyl units was observed at m/z 957 $(M + Na)^+$, Ast-Gal4 containing four attached galactosyl units was observed at m/x 1119 $(M + Na)^+$, and Ast-Gal5 containing five attached galactosyl units was observed at m/x 1281 $(M + Na)^+$.

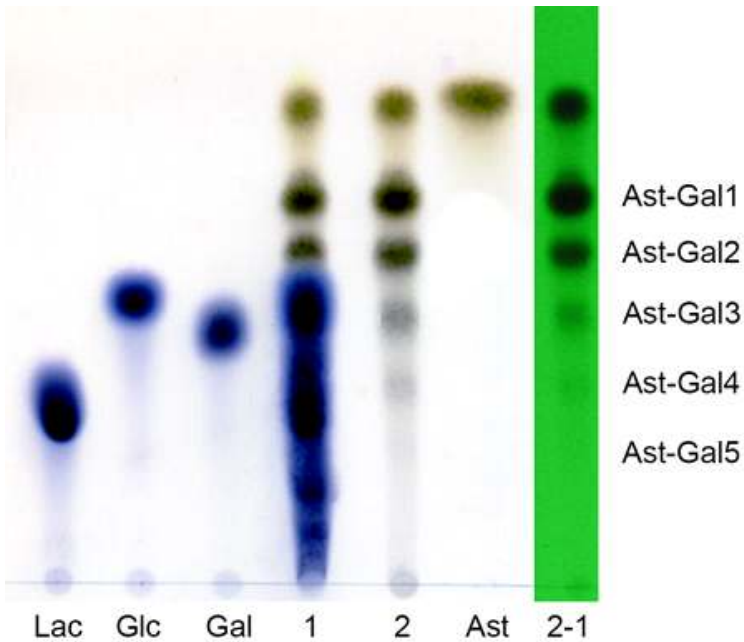


Figure 2. Ast-Gals observed by thin layer chromatography. Lac: lactose 100 mM; Glc: glucose 100 mM; Gal: galactose 100 mM; lane 1: acceptor reaction products; lane 2: galactosylation products after removing saccharides via MPLC C18 column; Ast, astragalin 50mM; lane2-1: Ast-Gals detected by 254nm UV light.

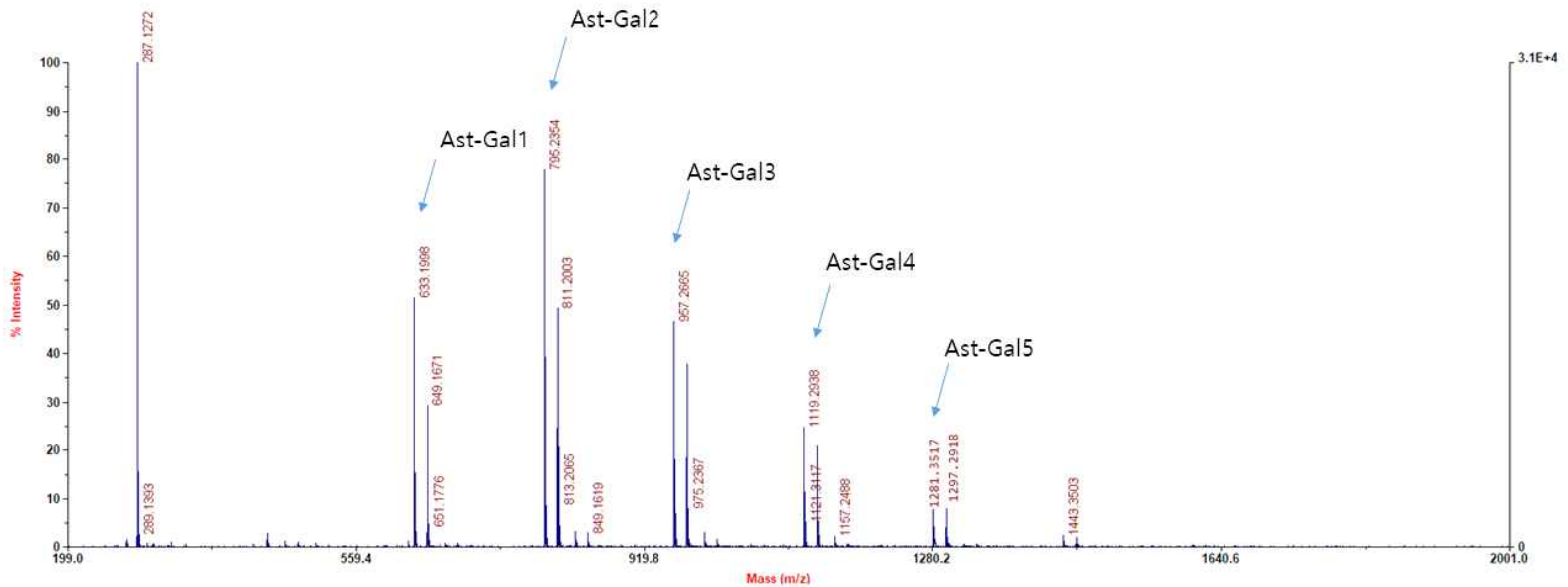


Figure 3. MALDI-TOF-MS spectra of Ast-Gals reaction mixture.

2. Optimization of Ast-Gals synthesis by RSM

Response surface methodology (RSM) is a statistical technique for modelling and optimizing with multiple variables. It can be used to determine the optimum process conditions by combining experimental designs with interpolation by first- or second- order polynomial equations in a sequential testing procedure [25]. In this study, RSM was progressed with following three independent variables: lactose concentration (300-500 mM), astragalin concentration (30-70 mM), and enzyme concentration (1.5-4.5 U/mL). The predicted and actual astragalin conversion yields are summarized in **Table 1**. The 3D response surface and 2D contour plots of independent variables with respect to the response are shown in Figure 4 and results of ANOVA (Analysis of variance) are shown in **Table 2**. Transgalactosylation products of astragalin were mostly affected ($p < 0.05$) by enzyme concentration (X_3), followed by lactose concentration (X_1) ($p = 0.058$) and astragalin concentration (X_2) ($p = 0.389$). X_2^2 and X_3^2 were significantly affected on the production yield at the level of $p < 0.05$, whereas the remaining quadratic parameters (X_1^2) and all interaction quadratic parameters (X_1X_2, X_1X_3, and X_2X_3) were insignificant ($p > 0.1$). The experimental data had a determination coefficient (R^2) of 0.8638 and adjusted coefficient (Adj R^2) of 0.9539, meaning that the calculated model was able to explain 86.38% and 95.39% of results, respectively. This indicated that the model used to fit the independent response variables was significant ($p < 0.001$). The amount of astragalin converted into Ast-Gals was expressed with the following regression equation:

$$Y = (-47.471 + 0.0928X_1 + 1.156X_2 + 10.040X_3 - 0.000114X_1X_2 - 0.000125X_2X_3 + 0.0429X_1X_3 - 0.0000941X_1^2 - 0.00604X_2^2 - 1.596X_3^2) / X_2 \quad 100$$

Where Y was astragalin conversion to product (%), X_1 was lactose concentration for acceptor reaction (mM), X_2 was astragalin concentration (mM), and X_3 was reacted enzyme concentration (U/mL).

The predicted maximum astragalin conversion to Ast-Gals was 76.7% at 435.4 mM lactose, 39.2 mM astragalin, and 3.1 U/mL of enzyme. To validate the predicted astragalin conversion yield, an experiment was conducted using the above conditions.

Table 1. Running condition for astragalin acceptor reaction and astragalin conversion

Run No.	Independent variables			Astragalin conversion (%)	
	X_1	X_2	X_3	Predicted	Actual
1	500	30	4.5	71.0	73.6
2	500	70	4.5	69.8	72.5
3	300	70	4.5	67.2	62.0
4	500	30	1.5	54.1	66.3
5	400	50	5.52	66.7	70.2
6	400	16.36	3	54.1	52.4
7	400	83.64	3	61.7	62.0
8	400	50	3	74.1	71.1
9	400	50	0.48	40.9	37.3
10	568.18	50	3	72.6	67.6
11	300	30	1.5	44.9	38.4
12	400	50	3	74.1	72.3
13	300	30	4.5	62.0	62.3
14	500	70	1.5	55.2	55.1
15	300	70	1.5	52.5	54.5
16	400	50	3	74.1	75.7
17	400	50	3	74.1	79.1

X_1, the concentration of lactose;

X_2, the concentration of astragalin;

X_3, the concentration of enzyme.

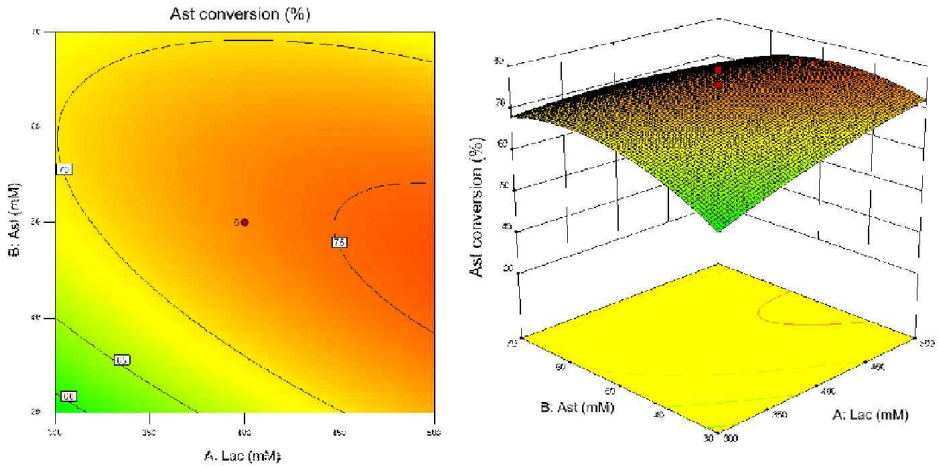
Table 2. Results of two-way analysis of variance (ANOVA)

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Squares	F Statistic	p-value Prob > F
Model	12601.82	9	245.36	7.05	< 0.05
A-Lactose	159.01	1	159.01	4.57	0.06
B-Astragalin	28.26	1	28.26	0.81	0.39
C-Enzyme	909.30	1	909.30	26.11	< 0.05
AB	98.70	1	98.70	2.83	0.12
AC	5.61	1	5.61	0.16	0.70
BC	4.96	1	4.96	0.42	0.71
A^2	30.93	1	30.93	0.89	0.37
B^2	440.86	1	440.86	12.66	< 0.05
C^2	656.76	1	656.76	18.86	< 0.05
Error	48.35	5	9.67		
Total	13202.86	19			

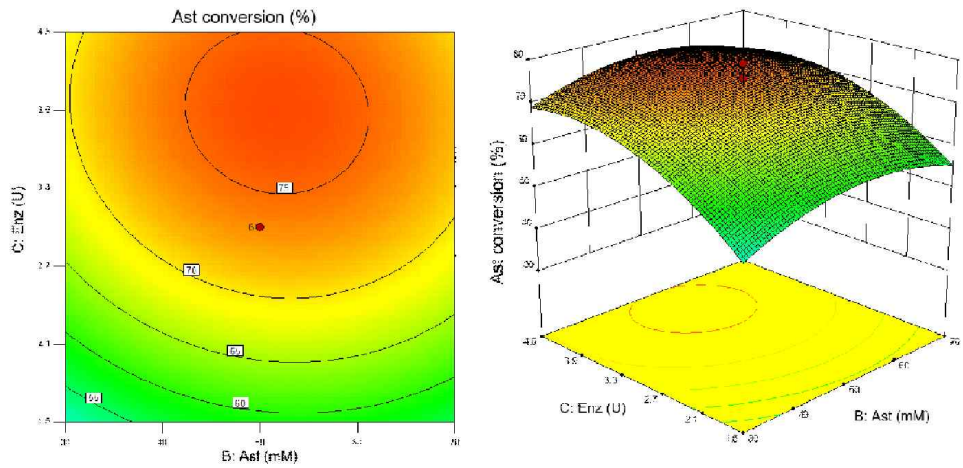
$R^2 = 0.8643$

Adj $R^2 = 0.9539$

(A)


Ast conversion (%)
B: Ast (mM)
A: Lac (mM)
Ast conversion (%)
B: Ast (mM)
A: Lac (mM)

(B)


Ast conversion (%)
C: Enz (U)
B: Ast (mM)
Ast conversion (%)
C: Enz (U)
B: Ast (mM)

(C)

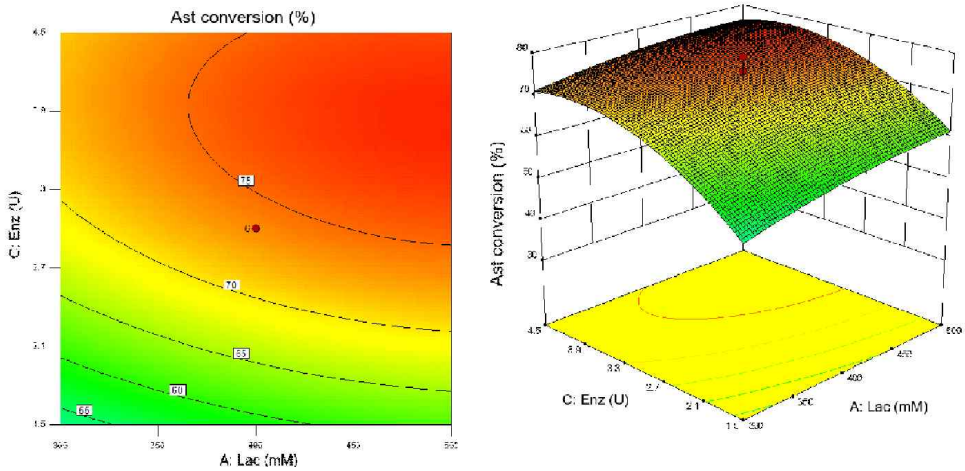


Figure 4. Response surface plot and contour plot.

Astragalin concentration vs. lactose concentration (A), lactose concentration vs. enzyme concentration (B), and enzyme concentration vs. Astragalin concentration (C).

3. Purification of Ast-Gals

Saccharide removed Ast-Gals mixture from MPLC and refined Ast-Gal1 and Ast-Gal2 through Sephadex LH-20 resin were obtained (**Figure 5**). Each compound was determined with high performance liquid chromatography (HPLC) detected by pulsed amperometric detection (PAD) (**Figure 6**). Ast-Gal1 and Ast-Gal2 were determined as a single peak with high purity. The amount of refined Ast-Gal1 and Ast-Gal2 were 520 mg (11.6%, w/w of consumed astragalin) and 300 mg (6.7%, w/w of consumed astragalin), respectively (**Table 3**).

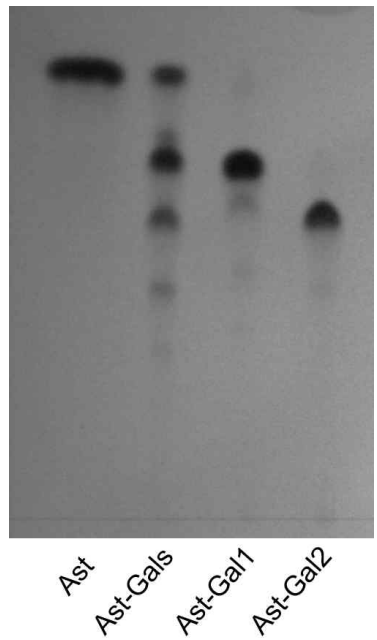


Figure 5. Refined Ast-Gal1 and Ast-Gal2 through out Sephadex LH-20 resin.

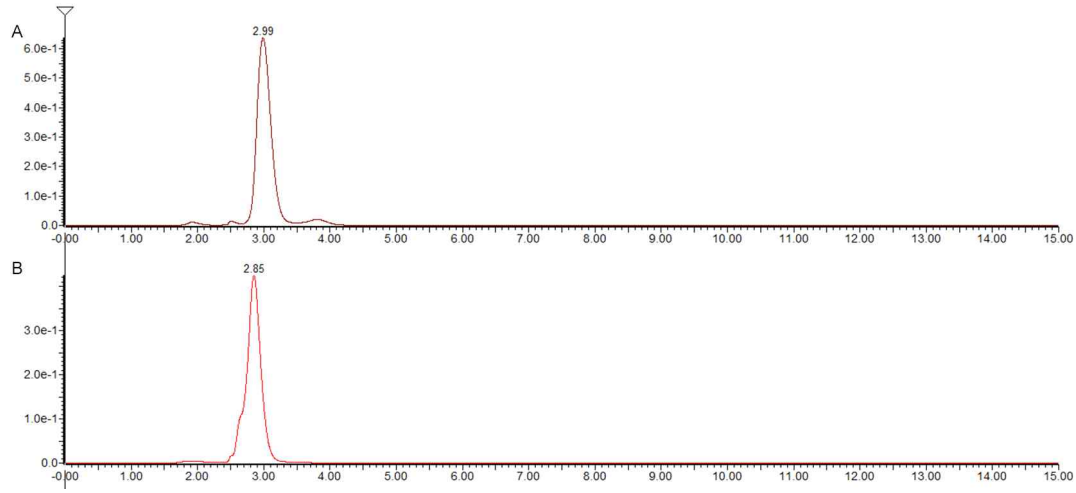


Figure 6. HPLC chromatogram of (A) Ast-Gal1 and (B) Ast-Gal2.

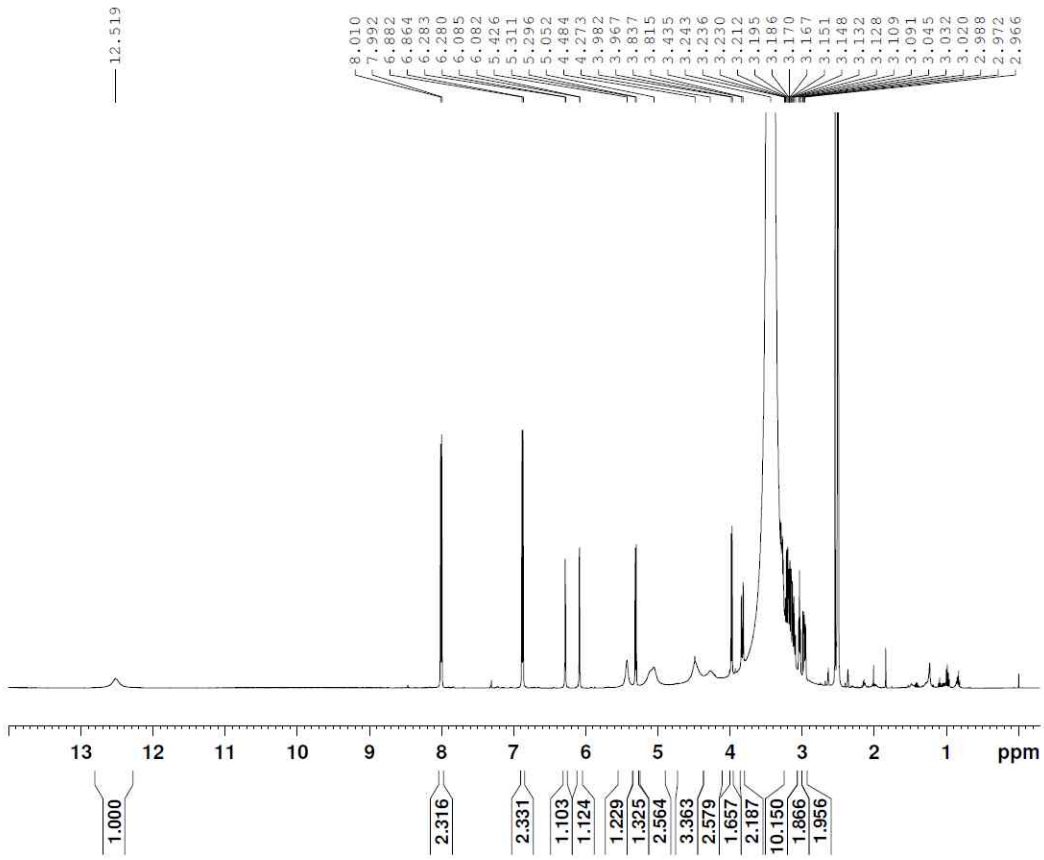
Table 3. Purification table of Ast-Gals through out column chromatography

Step	Total Products (mg)	Recover (%)	Yield (%)	
			Ast-Gal1	Ast-Gal2
Initial	4500	100	-	-
MPLC	4000	88.9	-	-
Gravity column	2200	48.9	11.6	6.7

4. Structural determination by NMR analysis

Structures of Ast-Gals were identified by NMR (^{1}H, ^{13}C, COSY, HSQC, and HMBC) (**Figure 7** and **Figure 8**). Results are summarized in **Table 3**. The molecular ions of Ast-Gal1 were observed at m/z 633 $(M + Na)^{+}$, indicating that one galactosyl residue was attached to astragalin. A doublet signal at 3.97 ppm (J = 7.5 Hz, H-1‴) was assigned to the anomeric proton, indicating that one galactose unit was β-linked with astragalin. There are some carbon signals identical to those of astragalin except for the following signals: at 76.33 ppm to C-2", at 74.16 ppm to C-5", and at 67.80 ppm to C-6" (**Table 4**). According to these results, the structure of Ast-Gal1 could be assigned as kaempferol-3-O-β-D-glucopyranosyl-(1→6)-β-D- galactopyranoside (**Figure 9A**). The molecular ions of Ast-Gal2 were observed at m/z 795 $(M + Na)^{+}$, indicating that two galactosyl residues were attached to astragalin. Two doublet signals at 3.97 ppm (J = 7.5 Hz, H-1‴) and 4.17 ppm (J = 7.5 Hz, H-1⁗) were assigned to anomeric proton, indicating that both galactosyl units were β-linked with astragalin. In Table 4, Ast-Gal2 showed the significant chemical shift change at C-6" (67.54 ppm, +6.7) indicating that the first galactose (3.97 ppm H-1‴) was attached to C-6" of astragalin same as the Ast-Gal1 structure. Additionally, the other anomeric proton (4.17 ppm, H-1⁗) of the second galactose was correlated with C-4‴ (78.55, +10.51) of the first galactose in HMBC data. From these results, the structure of Ast-Gal2 could be assigned as kaempferol-3-O-β-D-glucopyranosyl-(1→6)-β-D-galactopyranosyl-(1→4)-β-D-galactopyranoside (**Figure 9B**).

(A)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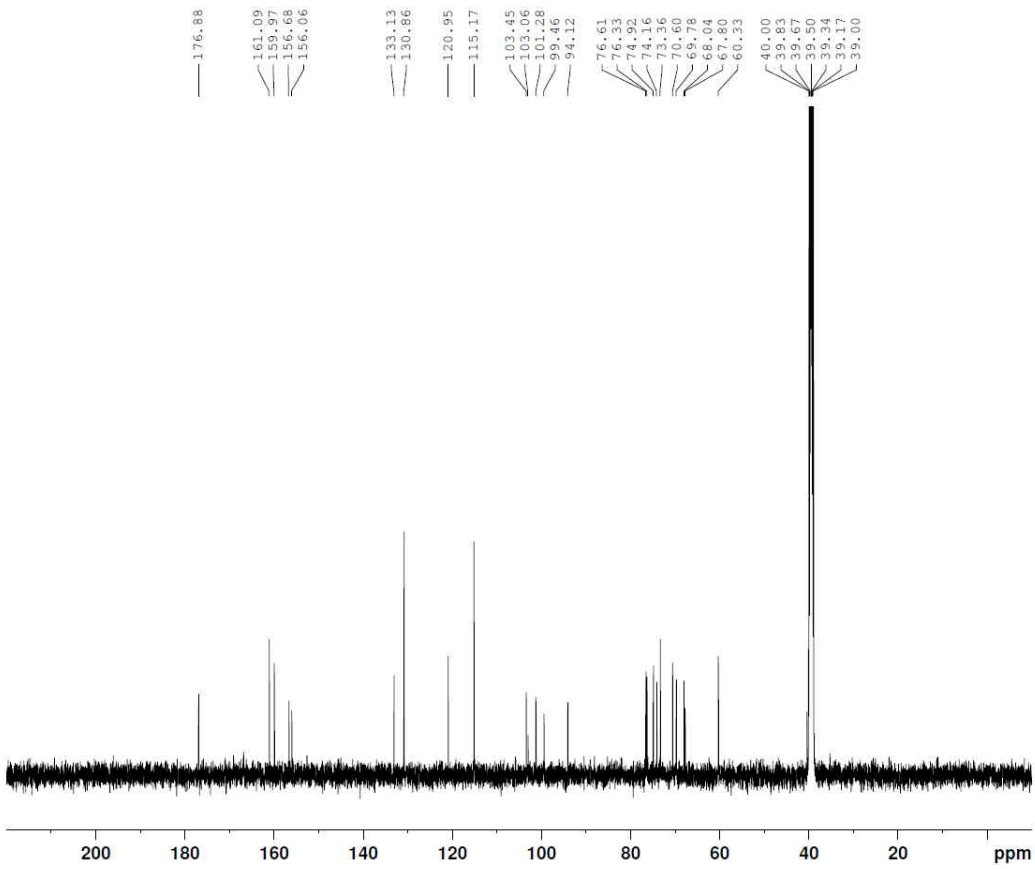
6.864
6.283
6.280
6.085
6.082
5.426
5.311
5.296
5.052
4.484
4.273
3.982
3.967
3.837
3.815
3.435
3.243
3.236
3.230
3.212
3.195
3.186
3.170
3.167
3.151
3.148
3.132
3.128
3.109
3.091
3.045
3.032
3.020
2.988
2.972
2.966
13
12
11
10
9
8
7
6
5
4
3
2
1
ppm
1.000
2.316
2.331
1.103
1.124
1.229
1.325
2.564
3.363
2.579
1.657
2.187
10.150
1.866
1.956

(B)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133.13
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120.95
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103.45
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99.46
94.12
76.61
76.33
74.92
74.16
73.36
70.60
69.78
68.04
67.80
60.33
40.00
39.83
39.67
39.50
39.34
39.17
39.00
200
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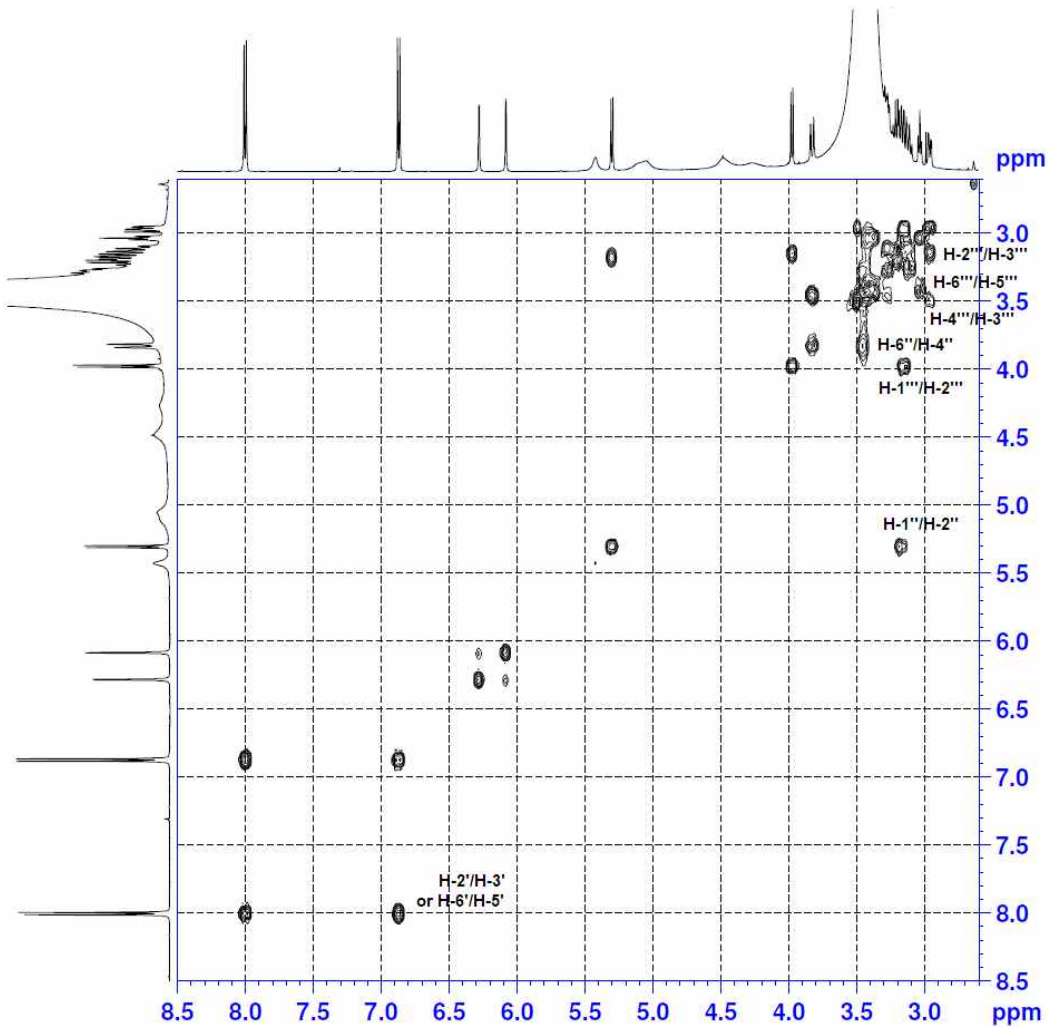
160
140
120
100
80
60
40
20
ppm

(C)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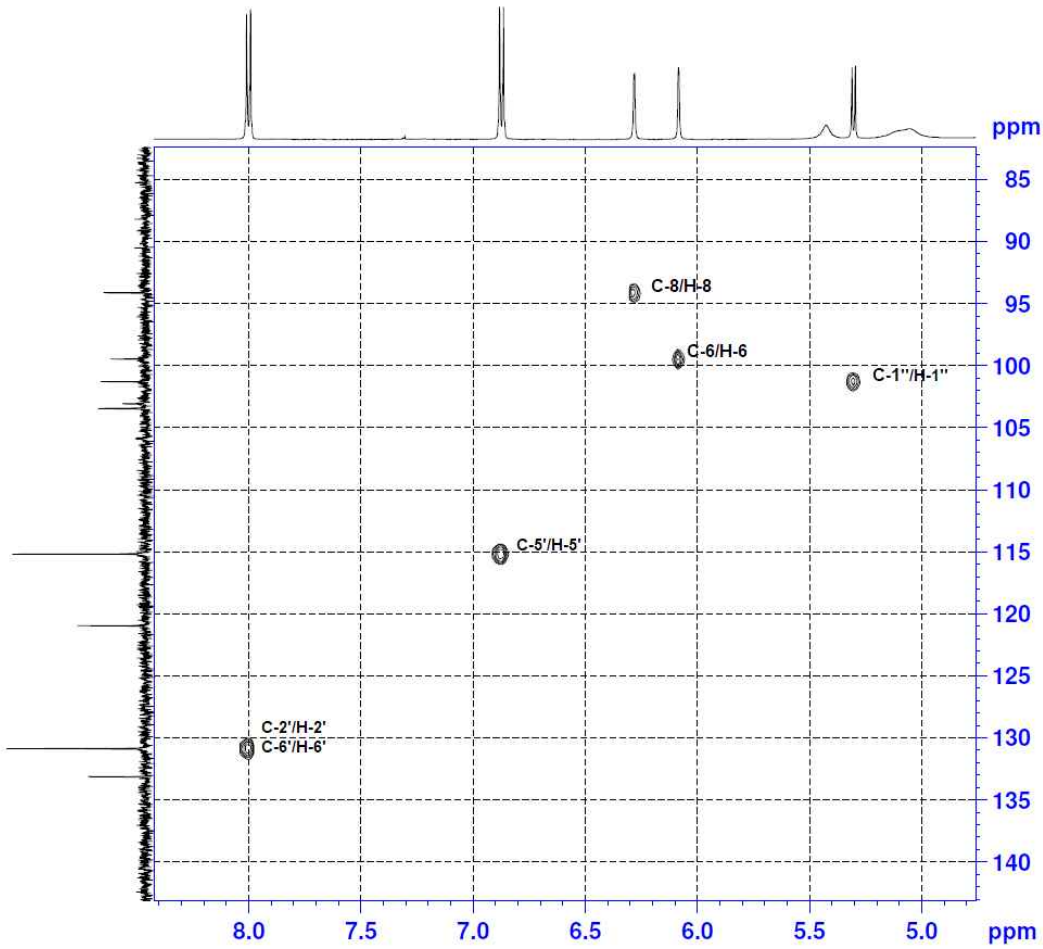
3.5
4.0
4.5
5.0
5.5
6.0
6.5
7.0
7.5
8.0
8.5
H-2'''/H-3'''
H-6'''/H-5'''
H-4'''/H-3'''
H-6''/H-4''
H-1'''/H-2'''
H-1''/H-2''
H-2'/H-3'
or H-6'/H-5'
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
ppm

(D)


ppm
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90
95
100
105
110
115
120
125
130
135
140
C-8/H-8
C-6/H-6
C-1''/H-1''
C-5'/H-5'
C-2'/H-2'
C-6'/H-6'
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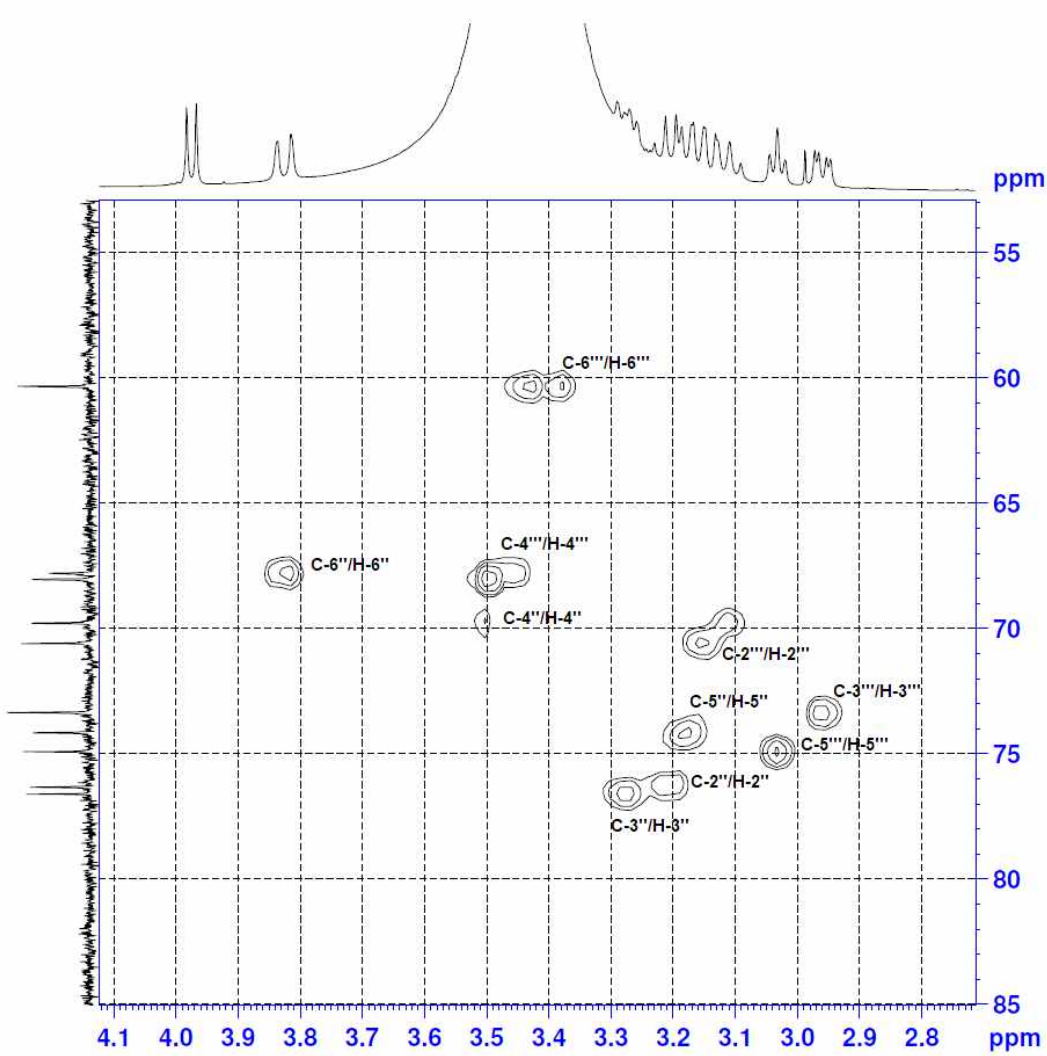
7.5
7.0
6.5
6.0
5.5
5.0
ppm

(E)


ppm
55
60
65
70
75
80
85
C-6'''/H-6'''
C-6''/H-6''
C-4'''/H-4'''
C-4''/H-4''
C-2'''/H-2'''
C-3'''/H-3'''
C-5''/H-5''
C-5'''/H-5'''
C-2''/H-2''
C-3''/H-3''
4.1
4.0
3.9
3.8
3.7
3.6
3.5
3.4
3.3
3.2
3.1
3.0
2.9
2.8
ppm

(F)

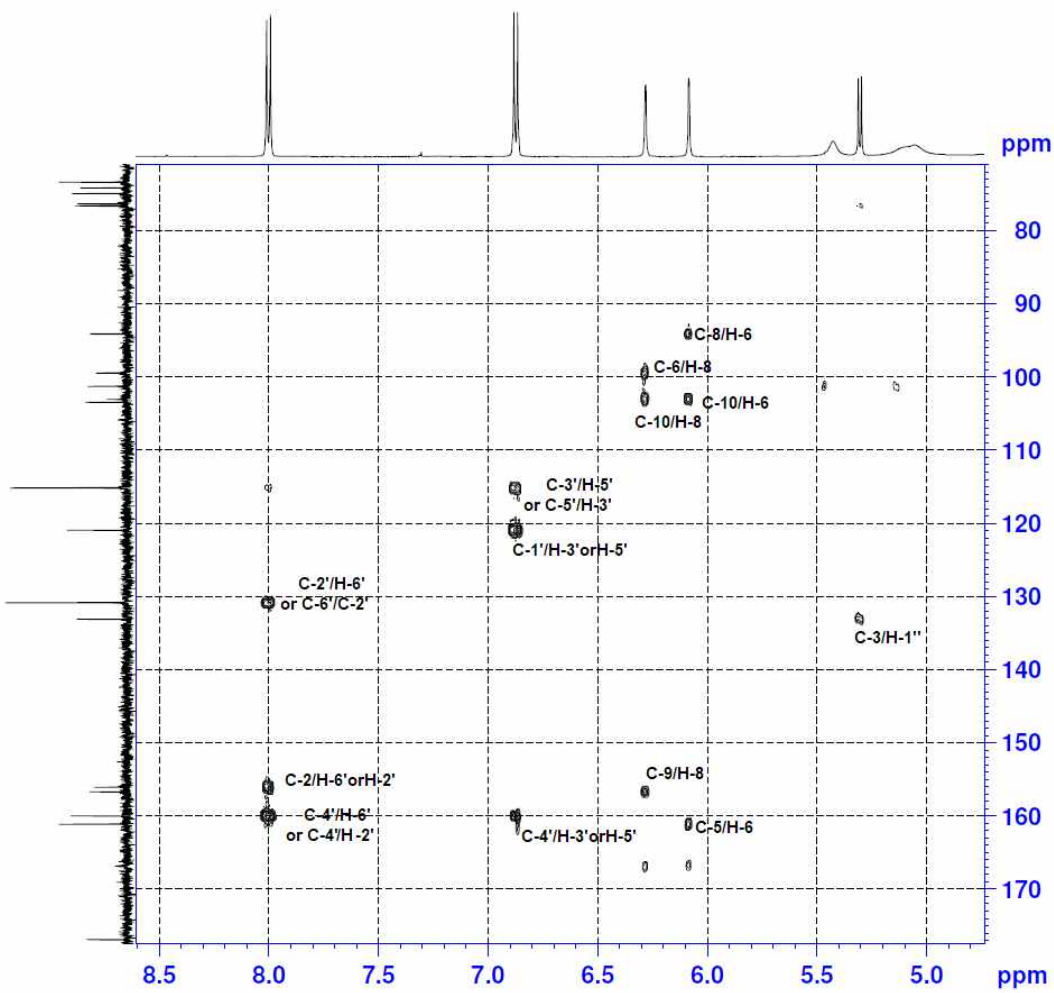
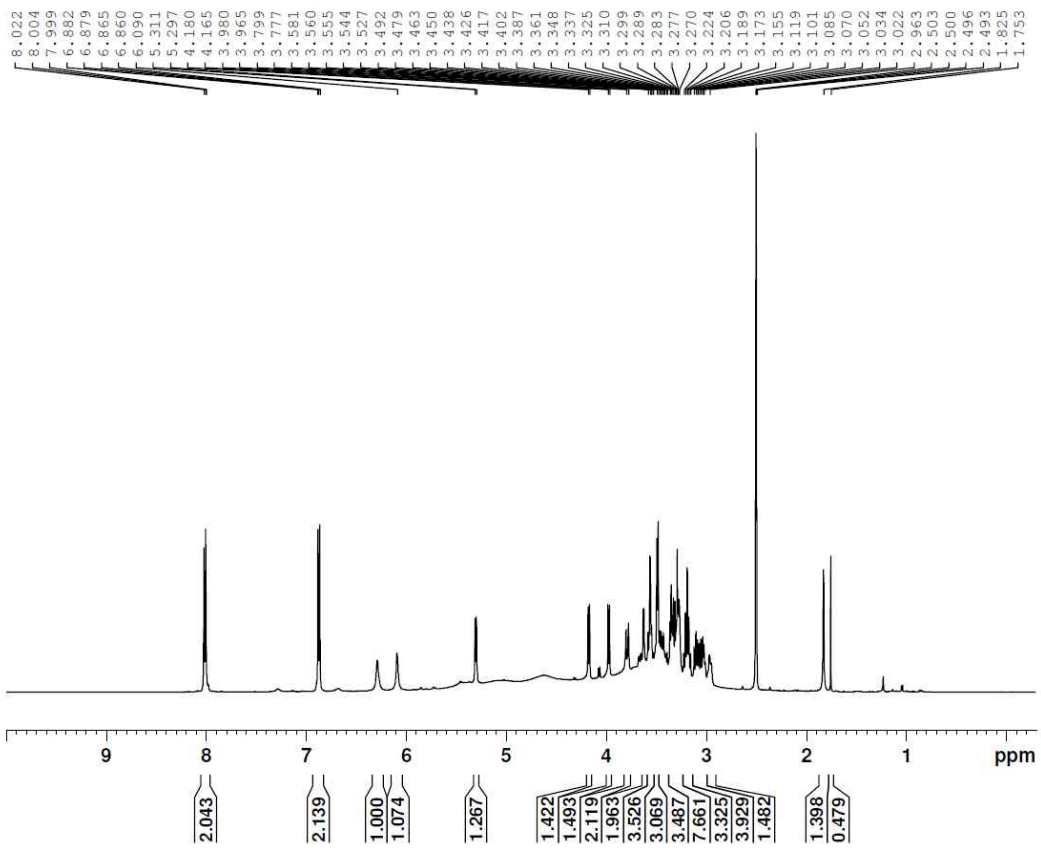


Figure 7. 500 Hz NMR spectra of Ast-Gal1. ^{1}H (A), ^{13}C (B), COSY (C), HSQC (D,E), and HMBC (F)

(A)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6.865
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6.090
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5.297
4.180
4.165
3.980
3.965
3.799
3.777
3.581
3.560
3.555
3.544
3.527
3.492
3.479
3.463
3.450
3.438
3.426
3.417
3.402
3.387
3.361
3.348
3.337
3.325
3.310
3.299
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3.283
3.277
3.270
3.224
3.206
3.189
3.173
3.155
3.119
3.101
3.085
3.070
3.052
3.034
3.022
2.963
2.503
2.500
2.496
2.493
1.825
1.753
9
8
7
6
5
4
3
2
1
ppm
2.043
2.139
1.000
1.074
1.267
1.422
1.493
2.119
1.963
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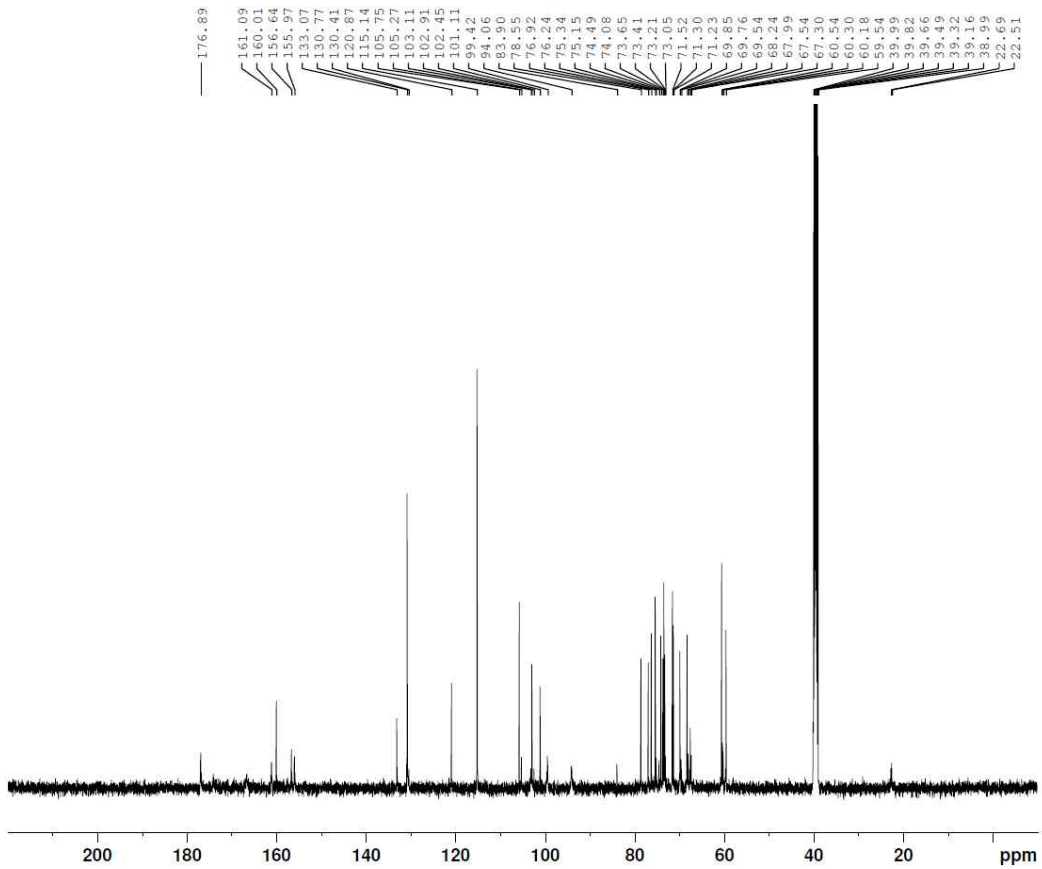
3.069
3.487
7.661
3.325
3.929
1.482
1.398
0.479

(B)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155.97
133.07
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130.41
120.87
115.14
105.75
105.27
103.11
102.91
102.45
101.11
99.42
94.06
83.90
78.55
76.92
76.24
75.34
75.15
74.49
74.08
73.65
73.41
73.21
73.05
71.52
71.30
71.23
69.85
69.76
69.54
68.24
67.99
67.54
67.30
60.54
60.30
60.18
59.54
39.99
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39.66
39.49
39.32
39.16
38.99
22.69
22.51
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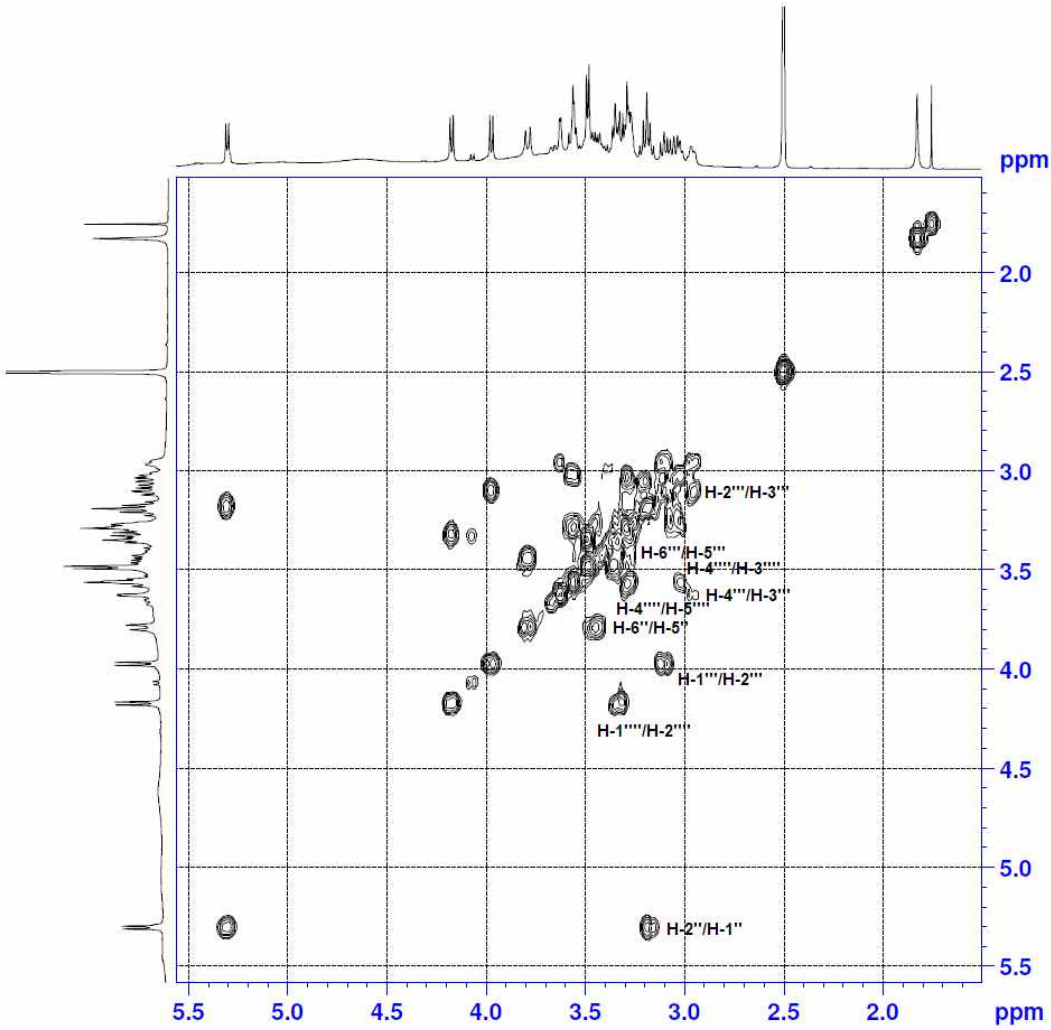
160
140
120
100
80
60
40
20
ppm

(C)


ppm
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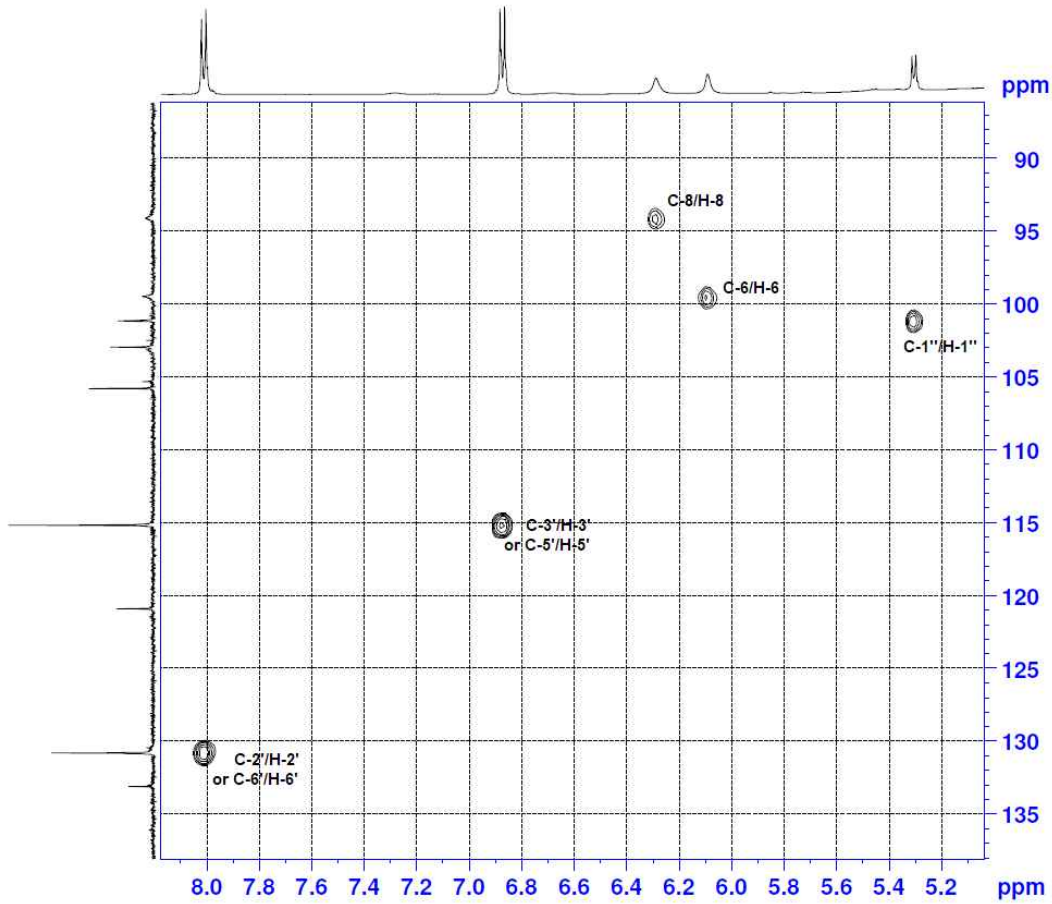
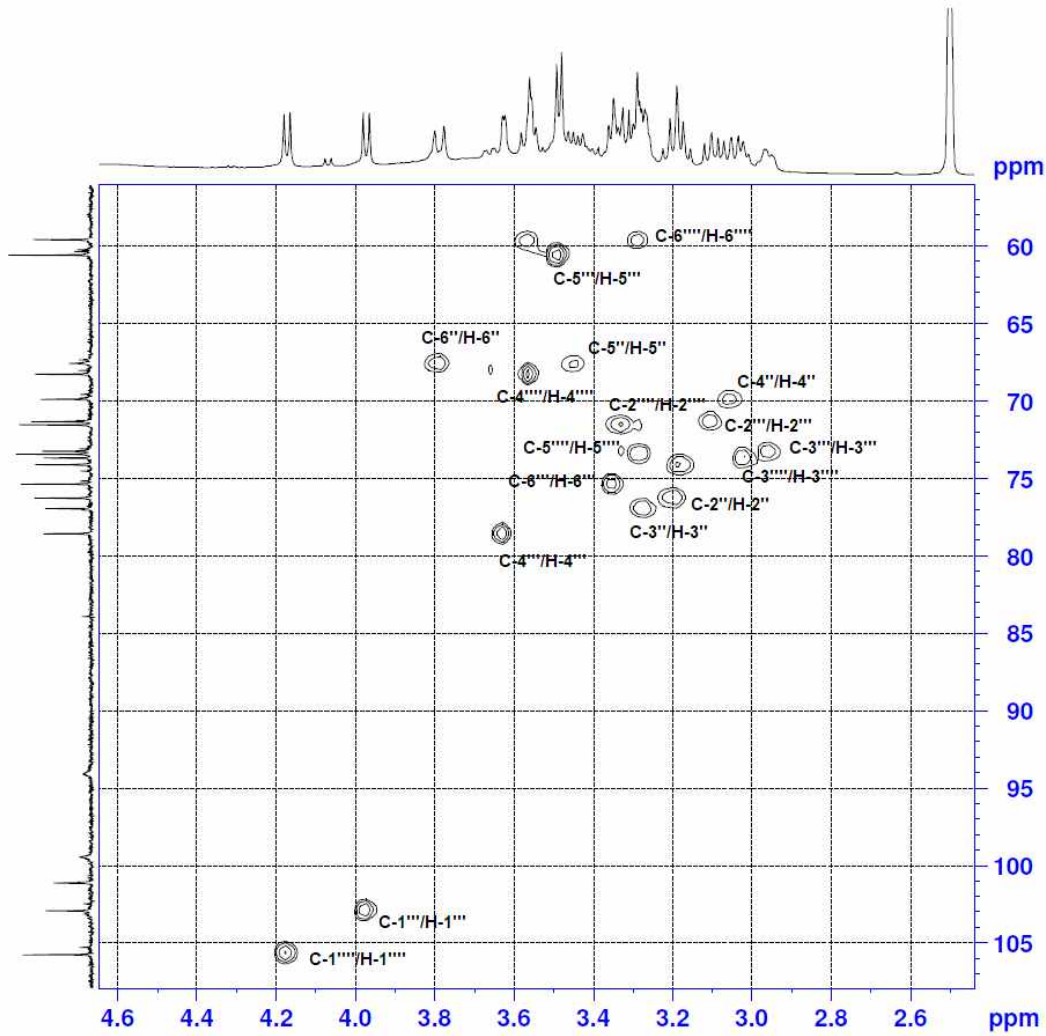
2.5
3.0
3.5
4.0
4.5
5.0
5.5
H-2'''/H-3'''
H-6''''/H-5''''
H-4''''/H-3''''
H-4'''/H-3'''
H-4''''/H-5''''
H-6''/H-5''
H-1'''/H-2'''
H-1''''/H-2''''
H-2''/H-1''
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
ppm

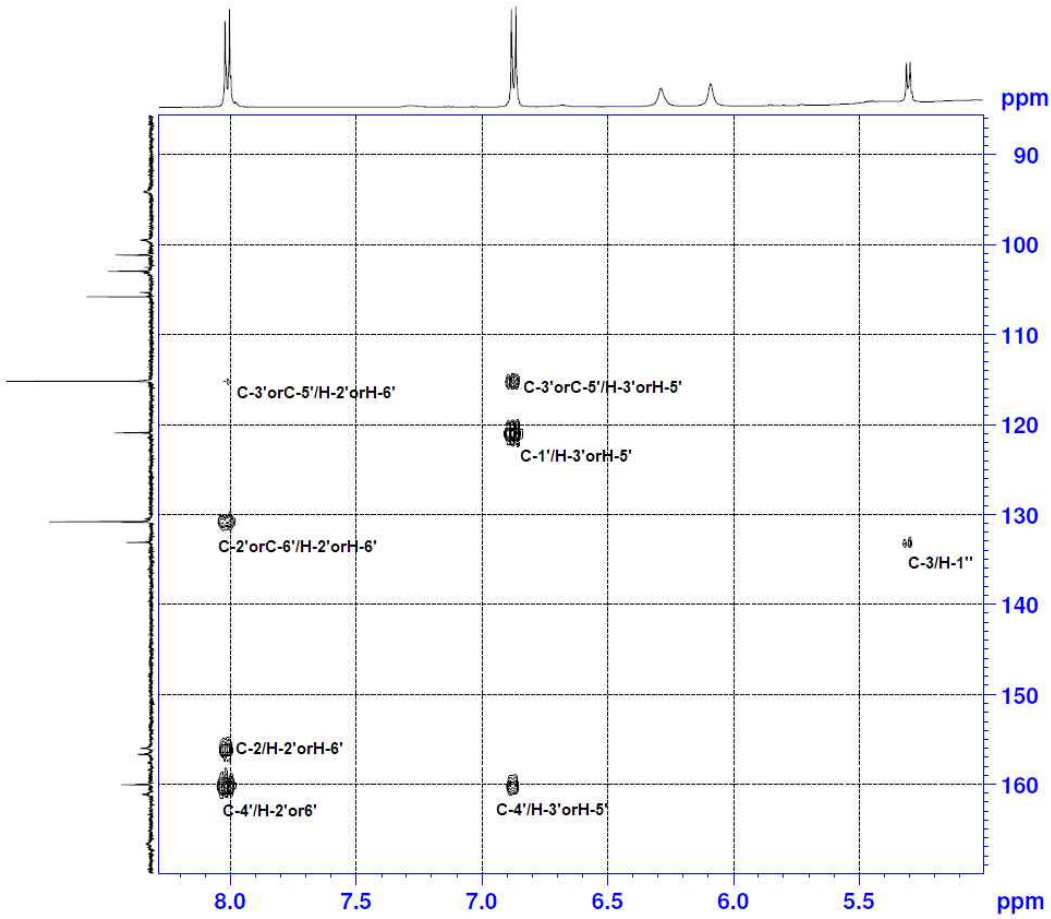
(D)


ppm
90
95
100
105
110
115
120
125
130
135
C-8/H-8
C-6/H-6
C-1''/H-1''
C-3'/H-3'
or C-5'/H-5'
C-2'/H-2'
or C-6'/H-6'
8.0 7.8 7.6 7.4 7.2 7.0 6.8 6.6 6.4 6.2 6.0 5.8 5.6 5.4 5.2
ppm

(E)


ppm
C-6''''/H-6''''
C-5'''/H-5'''
C-6''/H-6''
C-5''/H-5''
C-4''/H-4''
C-4''''/H-4''''
C-2''''/H-2''''
C-2'''/H-2'''
C-5''''/H-5''''
C-3'''/H-3'''
C-6'''/H-6'''
C-3''''/H-3''''
C-2''/H-2''
C-3''/H-3''
C-4'''/H-4'''
C-1'''/H-1'''
C-1''''/H-1''''
60
65
70
75
80
85
90
95
100
105
4.6
4.4
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
2.6
ppm

(F)


ppm
90
100
110
120
130
140
150
160
C-3'orC-5'/H-2'orH-6'
C-3'orC-5'/H-3'orH-5'
C-1'/H-3'orH-5'
C-2'orC-6'/H-2'orH-6'
C-3/H-1''
C-2/H-2'orH-6'
C-4'/H-2'or6'
C-4'/H-3'orH-5'
8.0
7.5
7.0
6.5
6.0
5.5
ppm

(G)

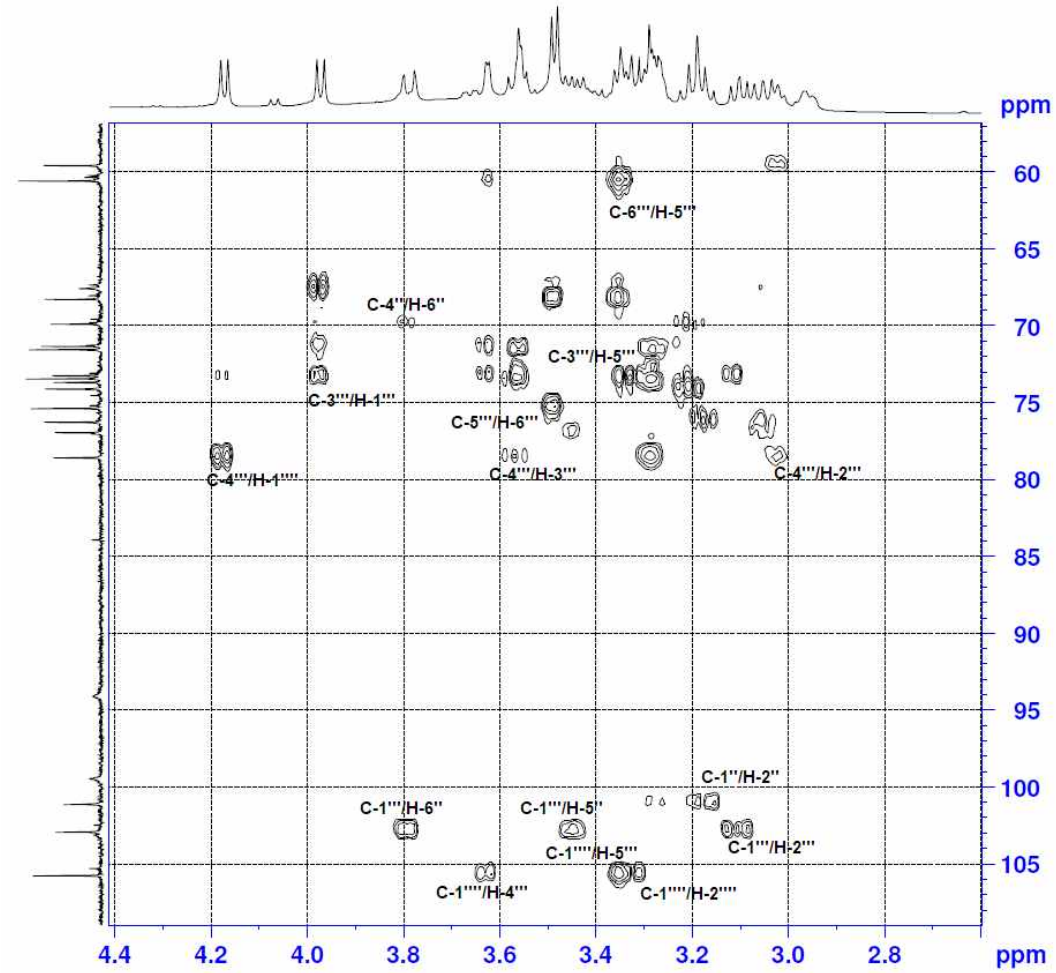


Figure 8. 500 Hz NMR spectra of Ast-Gal2. ^{1}H (A), ^{13}C (B), COSY (C), HSQC (D,E), and HMBC (F.G)

Table 4. ^{13}C and ^{1}H NMR data of astragalin and astragalin galactosides (ppm)

	astragalin (δ) (*Kim* et al.)		Ast-Gal1(δ_1)			Ast-Gal2(δ_2)		
C position	δ_C	δ_H	δ_{1C}	δ_1- δ	δ_H	δ_{2C}	δ_2- δ	δ_H
Astragalin								
2	156.43		156.06	-0.37		155.97	-0.46	
3	133.17		133.13	-0.04		133.07	-0.1	
4	177.48		176.88	-0.6		176.87	-0.59	
5	161.25		161.90	0.65		161.09	-0.16	
6	98.77	6.20 (1H, *d*, *J* = 1.8 Hz)	99.46	0.69	6.08 (1H, *d*, *J* = 1.5 Hz)	99.42	0.65	6.09 (1H, *s*)
7	164.34		167.00	2.66		166.08	1.74	
8	93.71	6.68 (1H, *d*, *J* = 1.8 Hz)	94.12	0.41	6.28 (1H, *d*, *J* = 1.5 Hz)	94.06	0.35	6.29 (1H, *s*)
9	156.24		156.68	0.44		156.54	0.3	
10	103.97		103.06	-0.91		102.91	-0.86	
1'	120.93		120.95	0.02		120.87	-0.06	
2', 6'	130.94	8.03 (2H, *d*, *J* = 7.2 Hz)	130.86	-0.08	8.00 (2H, *br.d*, *J* = 9 Hz)	130.77	-0.17	8.01 (2H, *d*)
3', 5'	115.14	7.17 (2H, *d*, *J* = 6.9 Hz)	115.17	0.03	6.87 (2H, *br.d*, *J* = 9 Hz)	115.14	0	6.87 (2H, *d*)
4'	159.98		159.97	-0.01		160.01	0.03	
1''	100.83	5.46 (1H, *d*, *J* = 7.8 Hz)	101.28	0.45	5.30 (1H, *d*, *J* = 7.5 Hz)	101.11	0.28	5.30 (1H, *d*, *J* = 7 Hz)
2''	74.23	3.32 (1H, *m*)	76.33	2.1	3.20 (1H, *m*)	76.24	2.01	3.19 (1H, *m*)
3''	76.42	3.21 (1H, *m*)	76.61	0.19	3.28 (1H, *m*)	76.92	0.5	3.27 (1H, *m*)
4''	69.91	3.17 (1H, *m*)	69.78	-0.13	3.50 (1H, *m*)	69.85	-0.06	3.06 (1H, *m*)
5''	77.56	3.08 (1H, *m*)	74.16	-3.4	3.18 (1H, *m*)	67.31	-10.25	3.45 (1H, *m*)
6''	60.84	3.56, 3.37 (2H, *d*, *J* = 11.4 Hz)	67.80	6.96	3.83 (2H, *br.d*, *J* = 11 Hz)	67.54	6.7	3.79 (1H, *m*)
Gal (-Gal1)								
1'''			103.45		3.97 (1H, *d*, *J* = 7.5 Hz)	102.91	-0.54	3.97 (1H, *d*, *J* = 7.5 Hz)
2'''			70.60		3.13 (1H, *m*)	71.29	0.69	3.10 (1H, *m*)
3'''			73.36		2.97 (1H, *m*)	73.21	-0.15	2.97 (1H, *m*)
4'''			68.04		3.49 (1H, *m*)	78.55	10.51	3.62 (1H, *m*)
5'''			74.92		3.03 (1H, *m*)	75.34	0.42	3.36 (1H, *m*)
6'''			60.33		3.44 (1H, *m*)	60.54	0.21	3.49 (1H, *m*)
Gal (-Gal2)								
1''''						105.75		4.17 (1H, *d*, *J* = 7.5 Hz)
2''''						71.52		3.32 (1H, *m*)
3''''						73.65		3.03 (1H, *m*)
4''''						68.24		3.55 (1H, *m*)
5''''						73.41		3.28 (1H, *m*)
6''''						59.54		3.29 (1H, *m*)

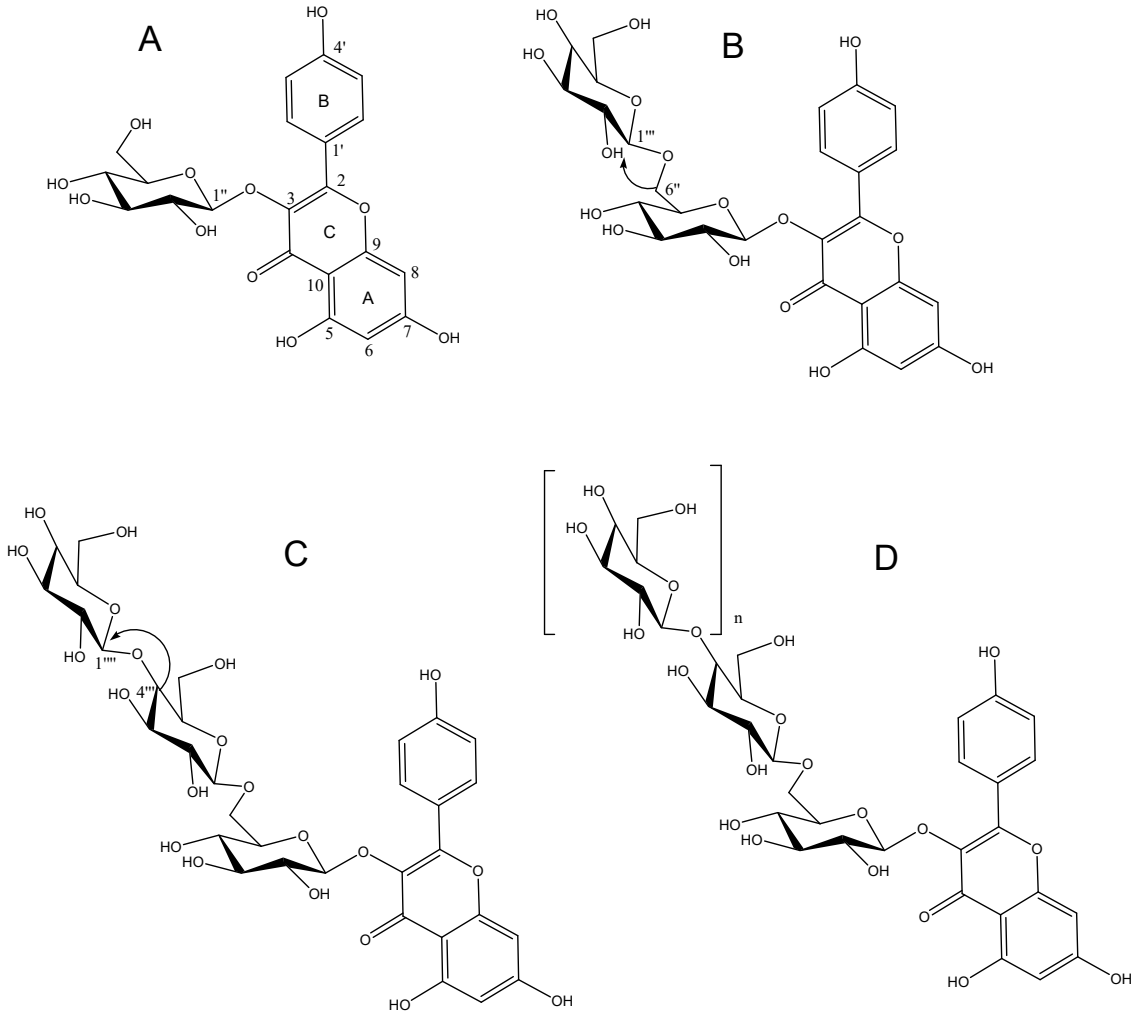


Figure 9. Structures and HMBC correlation of astragalin and its galactosides.

(A) Ast, astragalin;

(B) Ast-Gal1, kaempferol-3-O-β-D-glucopyranosyl-(1→6)-β-D-galactopyranoside,

(C) Ast-Gal2, kaempferol-3-O-β-D-glucopyranosyl-(1→6)-β-D-galactopyranosy-(1→4)-β-D-galactopyranoside,

and (D) Ast-Gal, astragalin galactoside which attached n of galactose, n = 0 ~ 4,

5. Biological characterization of Ast-Gals

5.1. Water solubility

Solubility may play a major role in the therapeutic efficacy of flavonoids. The low solubility of flavonoids in aqueous media and body fluids leads to certain difficulties in creating highly effective medicines. The solubility is one of the major biopharmaceutical characteristics to determine a drug's bioequivalence and the possibility of creating drug forms with effective dose and effective absorption rate [28]. In this study, the water solubilities of astragalin, Ast-Gal1, and Ast-Gal2 at 25℃ were 28.2 mg/L, 38,300 mg/L, and 38,800 mg/L, respectively (**Figure 10**). Ast-Gal1 and Ast-Gal2 had 1,360 and 1,470 times higher water solubility than astragalin.

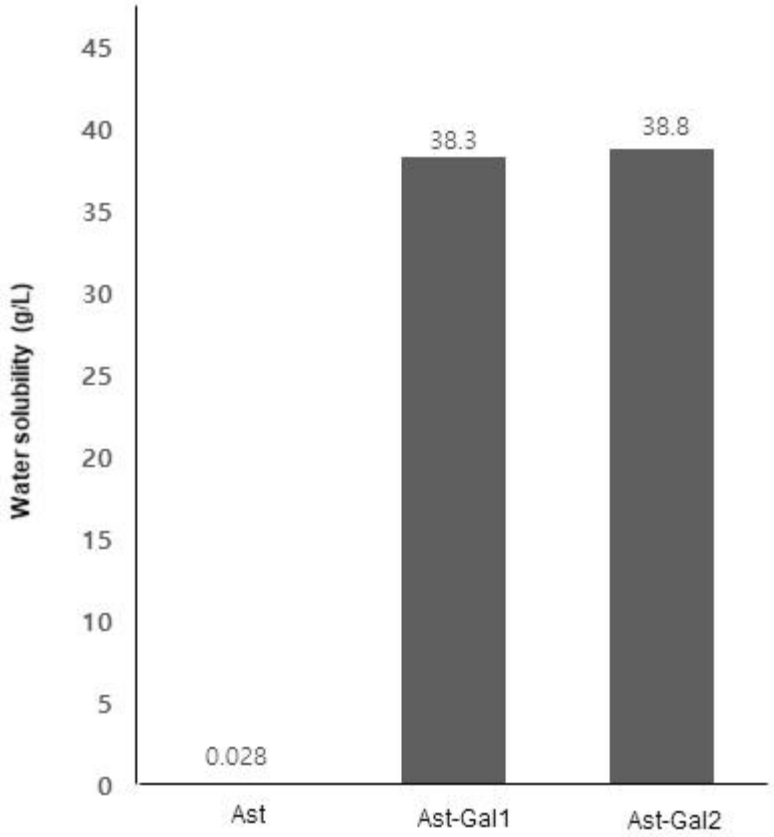


Figure 10. Water solubilities of astragalin (Ast), Ast-Gal1 and Ast-Gal2 at 25℃

5.2. ABTS·+ radical scavenging activity

Oxidative stress plays a major part in the development of chronic and degenerative ailments such as cancer, arthritis, aging, autoimmune disorders, cardiovascular disease, and neuro-degenerative disease [30-33]. The human body has several mechanisms to counteract oxidative stress by producing antioxidants either naturally *in situ* or obtaining them externally from food and/or supplements. Endogenous and exogenous antioxidants act as "free radical scavengers" by preventing and repairing damage caused by reactive oxygen species and reactive nitrogen species, therefore enhancing immune defense and lowering the risk of cancer and degenerative diseases. In this study, the antioxidant activities of astragalin, Ast-Gal1, and Ast-Gal2 were compared by the ABTS·+ radical scavenging method. The antioxidant activity was represented by the half maximal scavenging capacity (SC_{50}) and compared to astragalin. The SC_{50} of astragalin, Ast-Gal1, and Ast-Gal2 were 5.05 μM, 6.54 μM, and 4.91 μM, respectively (**Figure 11** and **Table 5**). There was no statistical difference between each compound. Trolox was used as a positive control and its SC_{50} value was at 16.3 μM.

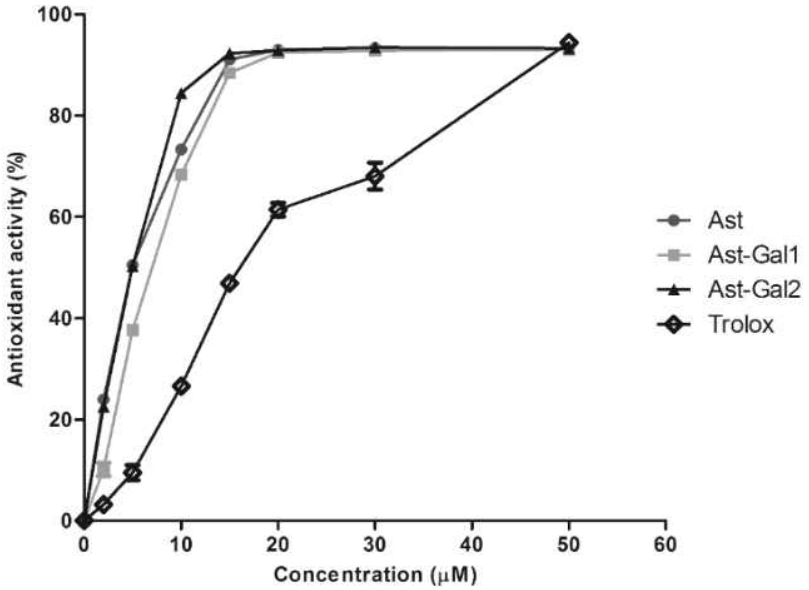


Figure 11. Antioxidant activities of astragalin (Ast) and Ast-Gals.

Table 5. Antioxidant activities (%) of astragalin and Ast-Gals as their concentrations.

Concentration (μM)	Astragalin	Ast-Gal1	Ast-Gal2	Trolox
2	23.9 ± 0.7	10.1 ± 2.3	22.5 ± 0.8	3.2 ± 1.0
4.5	38.9 ± 3.2	30.0 ± 1.2	38.4 ± 1.0	8.0 ± 0.9
5	50.5 ± 1.6	37.7 ± 1.2	50.3 ± 1.1	9.5 ± 2.6
7.5	64.1 ± 0.8	55.9 ± 1.0	75.1 ± 0.6	18.1 ± 1.0
10	73.3 ± 1.3	68.3 ± 1.6	84.5 ± 0.6	26.6 ± 1.4
15	91.0 ± 0.5	88.4 ± 0.4	92.3 ± 0.1	48.9 ± 1.9
20	93.0 ± 0.1	92.4 ± 0.3	92.9 ± 0.2	61.5 ± 2.3
30	93.4 ± 0.1	92.8 ± 0.4	93.5 ± 0.2	68.1 ± 4.6
50	93.1 ± 0.2	93.0 ± 0.1	93.3 ± 0.3	94.4 ± 0.1

5.3. ACE inhibitory activity

The renin-angiotensin-aldosterone system is a key factor in the maintenance of arterial blood pressure. One of its main component is the angiotensin-converting enzyme (ACE) (EC 3.4.15.1) [34] which is a glycosylated zinc-dipeptidyl-carboxypeptidase whose main function is to regulate arterial blood pressure and electrolyte balance through the renin-angiotensin-aldosterone system [35]. ACE inhibitors are widely used for the treatment of cardiovascular disease since they can improve blood pressure, control patients with hypertension, and prolong survival of patients with acute myocardial infarction [36] or congestive heart failure [37]. In this study, the inhibitory activities of astragalin, Ast-Gal1, and Ast-Gal2 against ACE were investigated and represented by the half maximal inhibitory capacity (IC_{50}). IC_{50} of astragalin, Ast-Gal1, and Ast-Gal2 were 171 μM, 191 μM and 138 μM, respectively (**Figure 12** and **Table 6**). Kameda et al. reported that among four flavonoids [astragalin, kaempferol-3-O-(2'-Ogalloyl)-glucoside, isoquercetin, and quercetin-3-O-(2"-O-galloyl)-glucoside] isolated from leaves of persimmon Diospyros kaki, Astragalin showed the strongest inhibitory activity against ACE [38]. Ast-Gal1 showed 1.12 times lower inhibitory activity against ACE in this study. However, Ast-Gal2 showed 1.24 times higher inhibitory activity against ACE than astragalin. From these results, we can conclude that various galactosyl residue attached to C-ring could increase ACE inhibition activity.

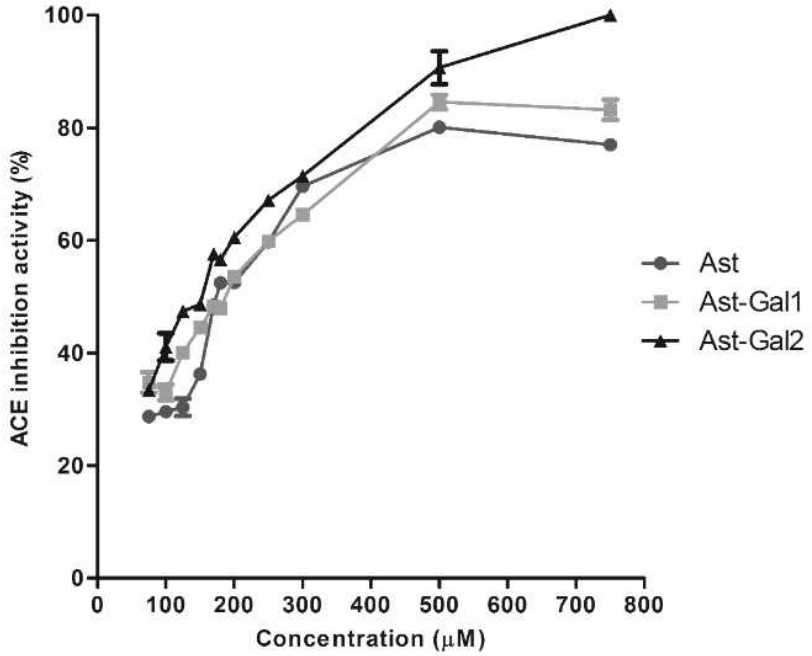


Figure 12. ACE Inhibitory activities of astragalin (Ast) and Ast-Gals

Table 6. ACE Inhibitory activities (%) of astragalin and Ast-Gals as their concentrations.

Concentration (μM)	Astragalin	Ast-Gal1	Ast-Gal2
75	28.7 ± 0.53	34.8 ± 3.11	33.4 ± 1.47
100	29.6 ± 1.82	33.0 ± 2.43	41.1 ± 4.19
125	26.7 ± 1.54	40.1 ± 0.23	47.4 ± 0.43
150	36.3 ± 1.71	44.6 ± 0.22	48.7 ± 0.12
170	48.5 ± 0.79	48.2 ± 0.53	57.6 ± 0.41
180	52.4 ± 1.27	48.0 ± 0.21	56.6 ± 0.08
200	52.5 ± 1.36	53.6 ± 0.31	60.6 ± 0.38
250	59.7 ± 0.33	59.8 ± 0.24	67.2 ± 0.10
300	69.6 ± 0.60	64.5 ± 0.22	71.4 ± 0.37
500	80.1 ± 0.20	84.6 ± 2.20	90.7 ± 5.04
750	77.0 ± 0.22	83.2 ± 3.15	100.9 ± 0.75

Table 7. Biological activities of astragalin and Ast-Gals

Compound	ABTS radical scavenging (SC_{50}) (μM)	ACE inhibition (IC_{50}) (μM)	Water solubility (mg/L)
Astragalin	5.1 ± 1.6	171.0 ± 1.2	28.2 ± 1.2
Ast-Gal1	6.5 ± 0.4	186.0 ± 0.0 +++	38,300 ± 3.5 ***
Ast-Gal2	4.9 ± 1.1	139.0 ± 0.2 ***	38,800 ± 2.8 ***
Trolox	16.0 ± 0.5 +++	ND	ND
α-Tocopherol	20.2 ± 2.0 +++	ND	ND

Mean ± SD

ND: Not determined.

***High function in the significant level of 0.001 (compared with astragalin)

+++Low function in the significant level of 0.001 (compared with astragalin)

Discussion

It has been reported that glycosylation of flavonoids can increase their water solubility [29, 39]. Especially, in case of Ast-Gals, their water solubilities were dramatically increased compared with that of any other glycosides. This might be reason from their structural characteristics. Ast-Gals have two kinds of glycosidic residues itself with different linkages. Their stereochemical characteristics would make unique biofunctional characteristics of these compound.

Antioxidant activities of phenolic compound glycosides could be decreased [25, 40-42], and this tendency depends on the position of bound glycosidic residues. This phenomenon is because their hydroxyl groups, which have potential radical scavenging capacity, were blocked with glycosidic residue during glycosylation. It is expected that a hydroxyl group on C-6" position of astragalin, the galactose bound part of Ast-Gals, has no effect or few effect on ABTS·+ radical scavenging capacity. Also in this study, addition of galactosyl residue on Ast-Gal1 makes the ACE inhibitory activity increased. This tendency could be explained with their structures likewise the reason described about antioxidant above. However for detailed description, the mechanism of ABTS·+ scavenging, ACE inhibition and additional properties of astragalin and Ast-Gals should be studied.

Increase of water solubility and decreaseless of antioxidant, anti-hypertension effect of Ast-Gals could be an important point in bioavailability of animal body. Moreover, high water-soluble functional materials could be involved in commercial products with functional-effective concentration. In this reason, research of their absorption mechanism should be followed by *in vivo* and *in vitro* studies.

One of the main weakness of astragalin is it can be broken in the intestine into kaempferol and glucose so it cannot act its own biofunctional activity in the animal body [2]. For example, glycosides of quercetin appeared different absorption pattern as their glycosidic residue. Quercetin-3-glucoside was degraded 36% in the rat duodenum for 30 min, during quercetin-3-galactoside was degraded 2% [43]. In this reason, the metabolism of Ast-Gals in intestine should be studied in further research to make the

compound usable. Also in further study, production process should be considered in order to make the cost lower. Some approaches to improve the process including research about the substrate which have effective cost more than lactose or the purification process could be contained.

Conclusion

In this study, the synthesis of novel galactosylated astragalins using β-galactosidase from *Bacillus circulans* was successfully carried out for the first time. The optimum conditions for Ast-Gals production were: 435.4 mM lactose, 39.2 mM astragalin, and 3.1 U/mL enzyme based on response surface methodology and central composite design in pH 6.0 at 60℃. Structures of Ast-Gals were identified with MALDI-TOF MS, and 1D (^{1}H and ^{13}C) and 2D (HSQC, COSY, and HMBC) NMR data. Galactosylated astratalin had dramatically improved water solubility. Their water solubility were 1,300 times higher than that of astragalin at 25℃. Antioxidant activities of Ast-Gals were maintained even though they have more than two glycosidic residues. Ast-Gal2 showed higher antioxidant activity and ACE inhibitory activity compared to astragalin. These conspicuous characteristics might be reason from their stereochemical properties. The production optimization of Ast-Gals synthesized with commercial β-galactosidase and its biological characterization could give potential to industrial application of Ast-Gals in a large scale.

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Abstract in Korean 국문초록

베타갈락토시데이즈를 이용하여 합성한 신규 아스트라갈린 배당체의 특성 연구

아스트라갈린은 플라보노이드의 한 종류로, 항산화능 및 항-HIV, 항알레르기, 항염증 효과를 가지고 있으며 수용해도가 매우 낮은 기능성 물질이다. 본 연구에서는 새로운 아스트라갈린 갈락토오스 배당체를 유당을 기질로 하고 *Bacillus circulans* 균주에서 유래한 베타갈락토시데이즈를 활용하여 효소적으로 합성하였다. 아스트라갈린 갈락토오스 배당체가 최대로 생산될 수 있는 반응조건을 RSM을 통하여 확립하였으며, Ast-Gals 산물이 가장 효율적으로 생산될 수 있는 조건은 유당 435.4 mM, 아스트라갈린 39.2 mM, 효소 3.1 U/mL일 때로 76.7%의 생산율을 보였다. 중압크로마토그래피 (MPLC) 실리카 C18 컬럼과 Sephadex LH-20레진 컬럼을 연속적으로 이용하여 Ast-Gal1과 Ast-Gal2를 순수하게 정제하였으며, 그 결과 얻은 물질의 양은 각각 반응에 참여한 아스트라갈린의 11.6% (w/w)와 6.7% (w/w)이었다. Ast-Gal1과 Ast-Gal2의 구조를 핵자기공명분석법 (NMR analysis)을 이용하여 각각 kaempferol-3-O-β-D-glucopyranosyl-(1→6)-O-β-D- galactopyranoside와 kaempferol−3-O-β-D-glucopyranosyl-(1→6)-O-β-D-galactopyranosyl-(1→4)-O-β-D-galactopyranoside로 정의하였다.

아스트라갈린과 Ast-Gal1, Ast-Gal2의 수용해도는 각각 28.2 mg/L, 38,300 mg/L, and 38,800 mg/L로, 아스트라갈린 갈락토오스 배당체는 아스트라갈린에 비하여 수용해도가 1,300배 이상 높게 나타났다. 기능성 물질의 수용해도 증가는 생체활용가능성 뿐만 아니라 산업적활용가능성과 연관되어 있는 중요한 척도이다. 또한 ABTS•+ 라디칼 소거능을 이용하여 확인한 아스트라갈린과 Ast-Gal1, Ast-Gal2의 라디칼 소거 중간값 (The half maximal scavenging capacity, SC_{50})은 각각 5.05 μM, 6.54 μM, 4.91 μM로 물질 간 유의적인 차이가 나타나지 않아 배당체가 형성되었음에도 불구하고 항산화능이 유지되었다. 아스트라갈린과 Ast-Gal1, Ast-Gal2의 안지오텐신전환효소 (ACE) 저해 효과를 확인한 결과, 각 물질의 ACE 억제중간값 (the half maximal inhibitory capacity, IC_{50})은 171 μM, 191 μM, 138 μM로 나타났다.

주제어 : 아스트라갈린, 수용체반응, 베타갈락토시데이즈, 아스트라갈린 갈락토사이드

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