

Development of Improved Process with Treatment of Cellulase for Isolation of Ampelopsin from Dried Fruits of *Ampelopsis grossedentata*

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The commercial method for isolation of ampelopsin, one of the most common flavonoids isolated from the plant species *Ampelopsis grossedentata*, is a simple hydrothermal extraction at high temperature. To develop an improved process to isolate ampelopsin, the effects of treatment of cellulase on hydrolysis of the dried fruit of *A. grossedentata* were investigated. The treatment of cellulase was found to decrease the temperature and time for hydrolysis of the dried fruit of *A. grossedentata*. The conditions of the filter press and continuous flow centrifuge for removal of insoluble materials from the hydrolysate of the dried fruit of *A. grossedentata* were optimized. The recovery yield of ampelopsin from the dried fruits of *A. grossedentata* was 39.4%, as determined by HPLC chromatographic analysis. A safe and economical process at low temperature with treatment of cellulase for the isolation of ampelopsin was developed in this study.

Keywords: Ampelopsin; Isolation; Cellulase; *Ampelopsis grossedentata*

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INTRODUCTION

Ampelopsin (3,5,7,3',4',5'-hexahydroxyl 2,3 dihydrogen flavanonol) is one of the most common flavonoids isolated from the plant species *Ampelopsis grossedentata*, known as rattan tea in China (Woo *et al.* 2012). Ampelopsin is known to have a broad range of functional compounds that have antioxidant, antitumor, and hepatoprotective effects (Kou and Chen 2012; Ye *et al.* 2015). The commercial method for the isolation of ampelopsin from *A. grossedentata* is simple hydrothermal extraction at temperature of approximately 100 °C for 24 h (Zhong *et al.* 2014). Because of this high temperature, the development of an improved process is needed for safety and economical aspects (Yoo *et al.* 2006).

Enzymatic saccharification of lignocellulosic materials can be accomplished through a complex reaction that involves three different types of cellulases: endoglucanase (carboxymethylcellulase), exocellobiohydrolase (avicelase), and β -glucosidase (Jo *et al.* 2008; Lee *et al.* 2008). Rice hulls, as lignocellulosic materials, have been hydrolyzed for production of fermentable sugars by commercial cellulases, in which the major cellulase was carboxymethylcellulase (CMCase) (Wei *et al.* 2009, 2010). In this study, cellulases were used for hydrolysis of the dried fruits of *A. grossedentata* to develop a safe and economical process for the enhanced recovery yield of ampelopsin (Lee *et al.* 2010).

Simple procedures involving liquid-liquid extraction, synthetic adsorbent treatment, and low-pressure chromatography have been investigated for recovery of ampelopsin (Yoo *et al.* 2006). In recent years, membrane technology has been widely used

for isolation and/or purification of functional materials (Yun *et al.* 2015). However, membranes made of polymeric structures have some disadvantages for stable operation. Polymeric membranes cannot be utilized at elevated temperatures or drastic chemical conditions (Kujawski *et al.* 2016). To overcome their disadvantages, numerous studies and development of inorganic membranes such as ceramic membranes have been performed recently (Yun *et al.* 2015). Due to their excellent chemical resistance to inorganic acids and oxidants, the tolerance to high temperatures and pressures, and longer life span, application of ceramic membranes has increased over the last decades (Loganathan *et al.* 2015; Ramakrishnan *et al.* 2015). Ceramic ultrafiltration for the enhanced recovery of ampelopsin was applied in this study (Gringer *et al.* 2015).

EXPERIMENTAL

Materials

The dried fruits of *A. grossedentata* were purchased from a domestic company (Korea Biosolution Ltd. Co., Busan, Korea). They were air-dried in an oven at 70 °C for 12 h before being milled in a hammer mill; particles smaller than 40 mesh were collected for further use in experiments (Wei *et al.* 2009).

Thermal Treatment for Hydrolysis of Dried Fruits of *A. grossedentata*

The dried fruits of *A. grossedentata* were hydrolyzed by thermal treatment with or without cellulase (Wei *et al.* 2010). The temperatures for hydrolytic reaction were 40, 50, 60, 70, and 80 °C. The cellulases used in this study were purified from *Bacillus amyloliquefaciens* DL-3 (Jo *et al.* 2008; Lee *et al.* 2008), *Bacillus velezensis* A-68 (Kim *et al.* 2013; Gao *et al.* 2014), and *Cellulophaga lytica* LBH-14 (Gao *et al.* 2012, 2013). The activities of cellulases as carboxymethylcellulases were measured and adjusted to 75 U/mL before the experiment (Lee *et al.* 2010).

HPLC Analysis of Ampelopsin

The isolated ampelopsin was analyzed by an AUURA Bio-LC system (SYSBIOLC002A, Berlin Germany) equipped with an AS autosampler, pulsed amperometry gold electrode, and Rheodyne injector. The HPLC system was equipped with a C₁₈ reversed phase column (250 mm x 4.6 mm, 5 μm) in conjunction with a precolumn and a UV-visible detector. The mobile phase was composed of CH₃OH and H₂O (60:40), and pH was adjusted to 3 with phosphoric acid (Zga *et al.* 2009). The size of the injection loop was 20 μL, and the temperature of the heated column was 25 °C (Wei *et al.* 2010).

Filter Press and Continuous Flow Centrifugation

A filter press (Plate & Frame Type Filter Press, Korea Filter Co., Ltd. Seoul Korea) was used for removal of insoluble materials after hydrolysis of the dried fruit *A. grossedentata*. It had two chambers; the filter area and volume of each chamber were 0.11 m² and 1.7 L (Kim *et al.* 2011). A continuous flow centrifuge (Tubular Type Centrifuge, Kokusan Chemical Co. Ltd., Tokyo, Japan) was used for removal of insoluble materials after hydrolysis. It had a bowl with a capacity of 9.0 L and a diameter of 12.2 cm. Its rotational speed and relative centrifugal force were fixed at 12,000 rpm and 11,500 x g, respectively.

Ultrafiltration with Ceramic Membrane

An ultrafiltration (UF) system with tubular ceramic membranes made of titanium dioxide (TiO₂) was supplied by TAMI Industries (Nyons, France). This system consisted of a collecting tank (100 L), a recirculating pump, and the UF membrane inside stainless steel housing. The feed solution flowed along the length of the membrane and was divided into permeate or retentate, which returned to the collecting tank (Gringer *et al.* 2015). The filtration area and maximal pressure of ceramic membrane were 0.35 m² and 10 bars. Two tubular ceramic membranes, with molecular weight cut offs (MWCOs) of 300 and 15 kDa, respectively, were used for recovery of reducing sugars.

Analytical Methods

The chemical compositions of the dried fruit of *A. grossedentata* were determined by the Feed & Food Nutrition Research Center at Pukyong National University in Korea, using the National Renewable Energy Laboratory, Laboratory Analytical Procedure, Technical Reports (NREL/TP) (Dagnino *et al.* 2013). Reducing sugars were determined by the DNS method (Miller 1959). Ampelopsin (dihydromyricetin as commercial name) was purchased from Sigma-Aldrich (USA).

RESULTS AND DISCUSSION

Effect of Temperature on Hydrolysis of Dried Fruits of *A. grossedentata*

The effect of temperature on the hydrolysis of the dried fruit of *A. grossedentata* was investigated. As shown in Table 1, the dried fruit of *A. grossedentata* consisted of crude fiber, crude lipid, crude protein, ash, and carbohydrate. Its carbohydrate composition was 57.6%. The concentration of the dried fruit of *A. grossedentata* used for the experiment was 40 g/L. The temperature for thermal hydrolysis ranged from 40 to 80 °C. As shown in Fig. 1, an increase in temperature for thermal hydrolysis resulted in higher conversion rate of reducing sugars. The highest conversion rates of reducing sugars from the dried fruit of *A. grossedentata* and its carbohydrates were 34.2% and 59.4%, respectively.

Table 1. Chemical Compositions of Dried Fruits of *A. grossedentata*

Component	Content (%)
Crude fiber	10.6 ± 1.3
Crude lipid	1.7 ± 0.0
Crude protein	8.6 ± 0.1
Ash	2.6 ± 0.2
Carbohydrate	57.6 ± 0.6
Moisture	18.9 ± 0.4
Sum	100.0

Effect of Cellulase on Hydrolysis of Dried Fruits of *A. grossedentata*

The effect of cellulase on the hydrolysis of the dried fruit of *A. grossedentata* was investigated. The cellulase produced by *B. amyloliquefaciens* DL-3 was in this experiment, and its CMCase activity in the reaction mixture was adjusted to be 75 U/mL. As shown in

Fig. 2, an increase in temperature with treatment of cellulase for thermal hydrolysis also resulted in higher conversion rate of reducing sugars.

The highest conversion rates of reducing sugars from the dried fruit of *A. grossedentata* and its carbohydrates were 33.1% and 57.5%, respectively.

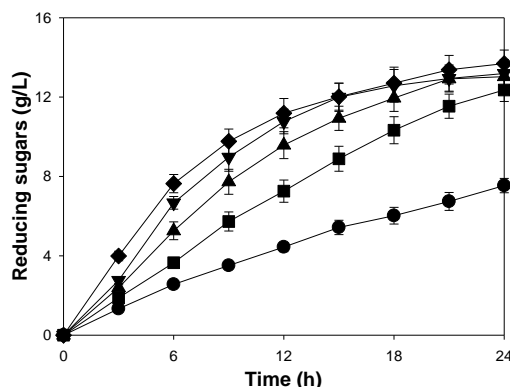


Fig. 1. Effect of temperature on hydrolysis of dried fruits of *A. grossedentata* without treatment of cellulase (●; 40 °C, ■; 50 °C, ▲; 60 °C, ▼; 70 °C, and ◆; 80 °C)

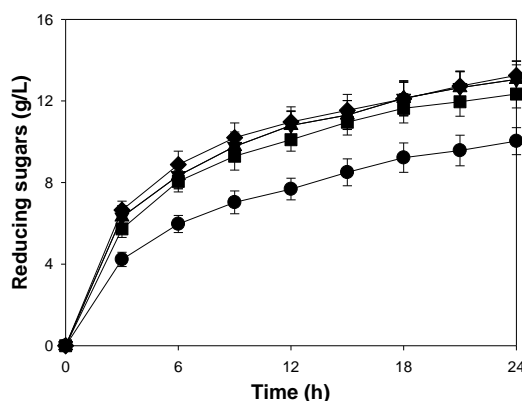


Fig. 2. Effect of temperature on hydrolysis of dried fruits of *A. grossedentata* with treatment of cellulase (●; 40 °C, ■; 50 °C, ▲; 60 °C, ▼; 70 °C, and ◆; 80 °C)

The preferred substrate for production of cellulases by *B. amyloliquefaciens* DL-3 and *B. velezensis* A-68 was rice hulls (Kim *et al.* 2013; Gao *et al.* 2014), whereas that by *C. lytica* LBH-14 was rice bran (Gao *et al.* 2012, 2013). The conversion rates of reducing sugars without treatment of cellulase were compared with those with treatment of cellulase. As shown in Fig. 3, the final conversion rates of reducing sugars without treatment of cellulase after 24 h were similar to those with treatment of cellulase. However, the time and temperature to reach certain conversion rates of reducing sugars with treatment of cellulase were shorter and lower than those without treatment.

Comparison of Various Cellulases for Hydrolysis

The hydrolytic abilities of cellulases produced by *B. amyloliquefaciens* DL-3, *B. velezensis* A-68, and *C. lytica* LBH-14 were compared. The cellulases produced by *Bacillus amyloliquefaciens* DL-3, *Bacillus velezensis* A-68, and *Cellulophaga lytica* LBH-14 were used in this experiment, and their activities of CMCase in the reaction mixture were adjusted to be 75 U/mL. The optimal temperature for the enzymatic reaction of CMCase produced by *B. amyloliquefaciens* DL-3 was 50 °C (Lee *et al.* 2008).

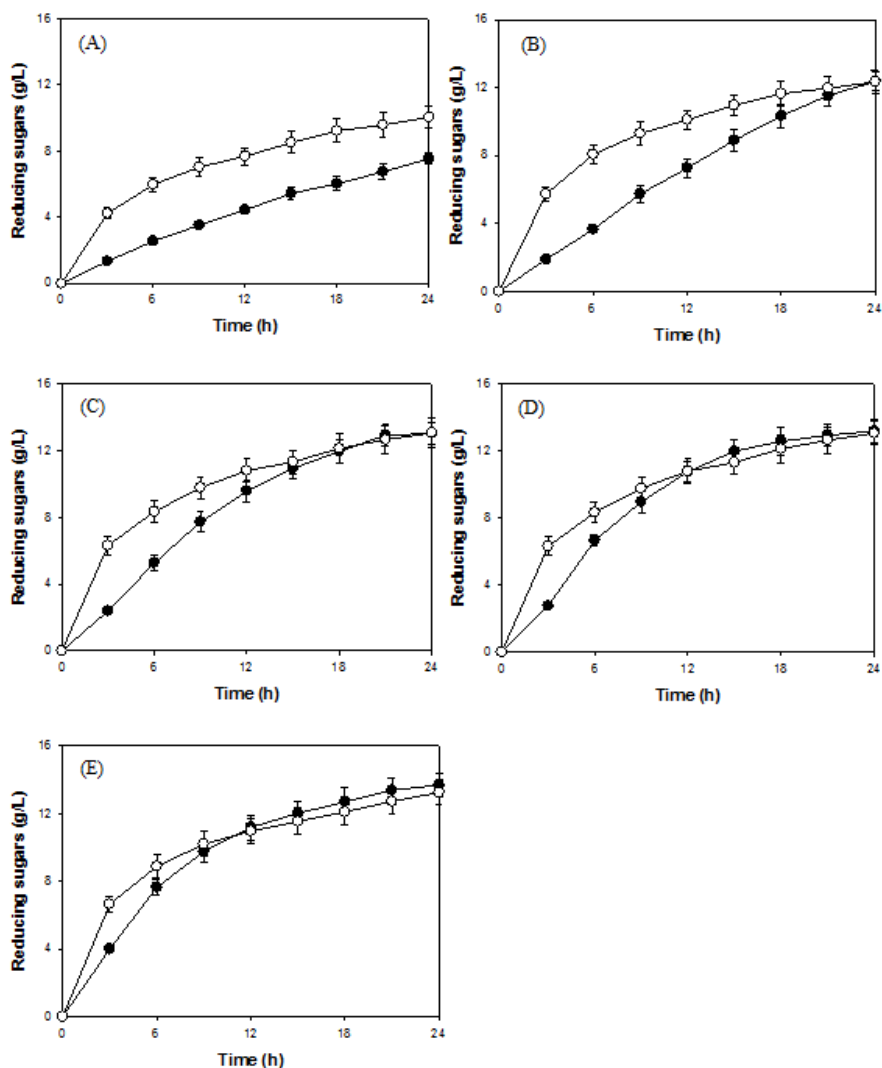


Fig. 3. Comparison in hydrolysis of dried fruits of *A. grossedentata* without (●) and with treatment of cellulase (○) at various temperatures; reaction temperature at (A) 40 °C, (B) 50 °C, (C) 60 °C, (D) 70 °C, and (E) 80 °C

The concentrations of the dried fruits of *A. grossedentata* ranged from 40 to 120 g/L. The reaction temperature and time were 50 °C and 12 h. As shown in Fig. 4, the conversion rate of reducing sugars with treatment of cellulase produced by *B. amyloliquefaciens* DL-3 was higher than those by *B. velezensis* A-68 and *C. lytica* LBH-14. As shown in Fig. 5, the maximal amount of reducing sugars was obtained when the concentration of dried fruits was 120 g/L. However, the highest conversion rate of reducing sugars was obtained when it was 40 g/L. Based on the conversion rate and production of reducing sugars, the concentration of the dried fruits of *A. grossedentata* and reaction time were set at 60 g/L and 6 h, respectively, for the following experiments.

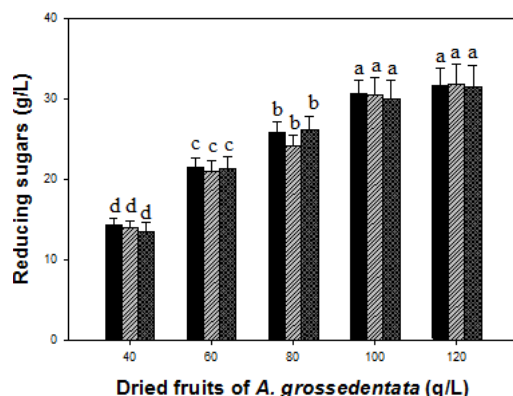


Fig. 4. Comparison of cellulases produced by *B. amyloliquefaciens* DL-3 (■), *B. velezensis* A-68 (▨), and *C. lytica* LBH-14 (▩) in hydrolysis of dried fruits of *A. grossedentata* (Different letters above bars mean that each value with a different letter is significantly different at $p < 0.05$)

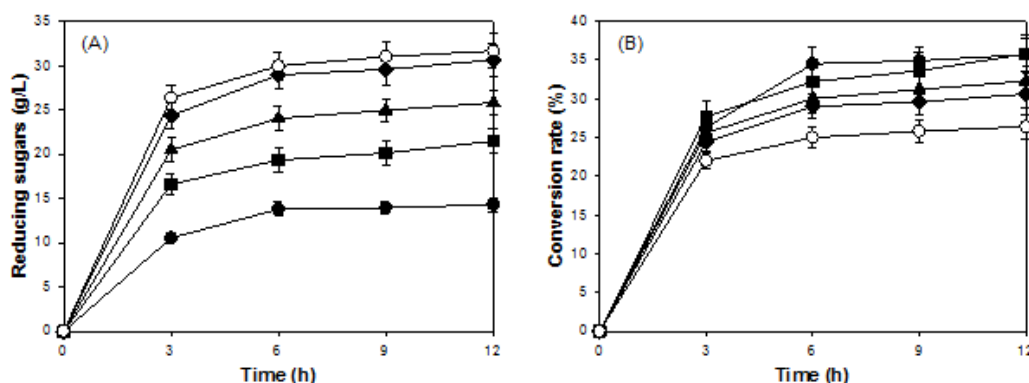


Fig. 5. Effect of amount of dried fruit of *A. grossedentata* on its hydrolysis with treatment of cellulase produced by *B. amyloliquefaciens* DL-3 (●; 40 g/L, ■; 60 g/L, ▲; 80 g/L, ◆; 100 g/L, and ○; 120 g/L); (A) reducing sugars and (B) conversion rate

Effect of Diatomite on Removal of Insoluble Materials after Hydrolysis

Filtration for isolation is widely used in many areas of industries (Lihong *et al.* 2011). Diatomite as a filter aid is needed to improve the efficiency of filtration operation (Du *et al.* 2011). The effect of diatomite concentration in the filter press on removal of insoluble materials after thermal hydrolysis with treatment of cellulase was investigated. The concentration of the dried fruits of *A. grossedentata* for thermal hydrolysis was 60 g/L. The reaction temperature and time were 50 °C and 6 h. The final concentration of diatomite mixed with the hydrolyzed fruits of *A. grossedentata* ranged from 0.0 to 10%. The volume of reaction mixture for thermal hydrolysis was 60 L. As shown in Fig. 6, higher concentration of diatomite resulted in more removal of insoluble materials. However, recovery yield of reducing sugars in the filtrate decreased with increasing concentration of diatomite. The optimal concentration of diatomite was set at 3.0% based on removal of insoluble materials and recovery yield of reducing sugars. Statistical analyses of Duncan's multiple range test were conducted using the Statistical Program for Social Science (SPSS version 12, SPSS Co., Chicago, USA).

Effect of Flow Rate on Removal of Insoluble Materials after Hydrolysis

Continuous flow centrifugation (CFC) offers recoveries equivalent to filtration with improved economy and ease of use (Higgins *et al.* 2003). The effects of flow rate of CFC on removal of insoluble materials after thermal hydrolysis with treatment of cellulase were investigated. The flow rate into the continuous flow centrifuge ranged from 0.0 to 10.0 L/min. As shown in Fig. 7, a lower flow rate of the continuous flow centrifuge resulted in more removal of insoluble materials. The highest removal rate was 82.6% when the flow rate was 2 L/min, at which the removal of insoluble materials and the recovery rate of reducing sugars were 86.8% and 99.8%, respectively.

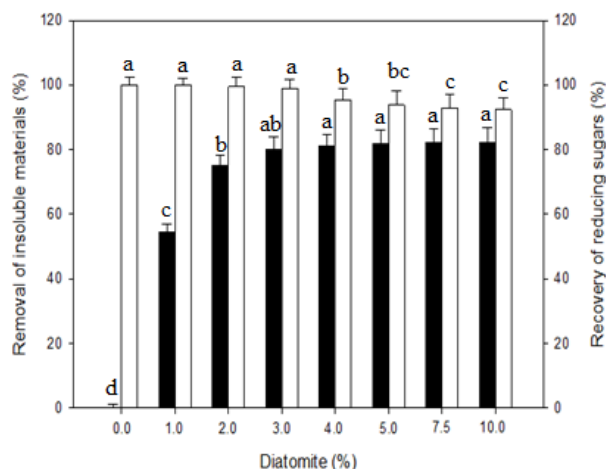


Fig. 6. Effect of diatomite on removal of insoluble materials after hydrolysis of dried fruits of *A. grossedentata* (■; removal of insoluble materials and □; recovery yield)

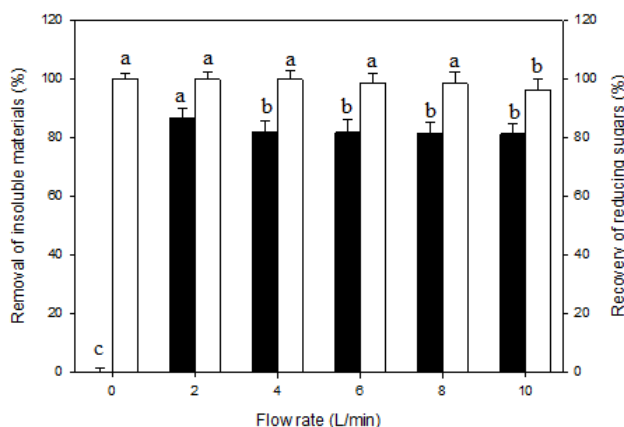


Fig. 7. Effect of flow rate on removal of insoluble materials after hydrolysis of dried fruits of *A. grossedentata* (■; removal of insoluble materials and □; recovery yield)

Recovery of Ampelopsin using Ultrafiltration System

The filtrate after removal of insoluble materials using the filter press and continuous flow centrifuge was applied in an ultrafiltration system to isolate ampelopsin. The filtrate was first divided by the membrane with the molecular weight cut off (MWCO) of 300 kDa. The permeate with molecular weight less than 300 kDa was divided by the membrane with the 15-kDa MWCO. As shown in Table 2, the concentrations of reducing sugars and ampelopsin in the permeate after filtration with the membrane with MWCO of 15 kDa were 13.3 and 0.23 g/L, respectively. The recovery yields of reducing sugars and ampelopsin

from the dried fruits of *A. grossedentata* were 33.9% and 39.5%, respectively. The recovery yield of ampelopsin throughout the ceramic filtration was relative to that of reducing sugars. The molecular weights of major reducing sugars seemed to be similar to that of ampelopsin.

Table 2. Recovery Yields of Reducing Sugars and Ampelopsin using Ceramic Ultrafiltration (UF) System

Step	MWCO	Volume (L)	Reducing sugars (g/L)	Recovery of reducing sugars (%)	Ampelopsin (g/L)	Recovery of ampelopsin (%)
Before UF		48.2	15.9	100	0.24	100
1 st UF	≥ 300 KD	6.5	15.6	13.2	0.18	10.5
	≤ 300 KD	36.5	16.1	76.8	0.24	77.2
2 nd UF of ≤ 300 KD	≥ 15 KD	12.2	13.9	22.1	0.20	21.1
	≤ 15 KD	19.5	13.3	33.9	0.23	39.5

The HPLC chromatograms of the hydrolysate of dried fruits of *A. grossedentata* with cellulase treatment and the ampelopsin isolated from the hydrolysate using an ultrafiltration system after the filter press and continuous flow centrifugation are shown in Fig. 8. The relatively large peak between 3.0 and 3.4 min can be attributed to the absorption peak of ampelopsin. The peak of ampelopsin was identified by comparison of chromatographic retention time with that of the ampelopsin standard (Zhong *et al.* 2014).

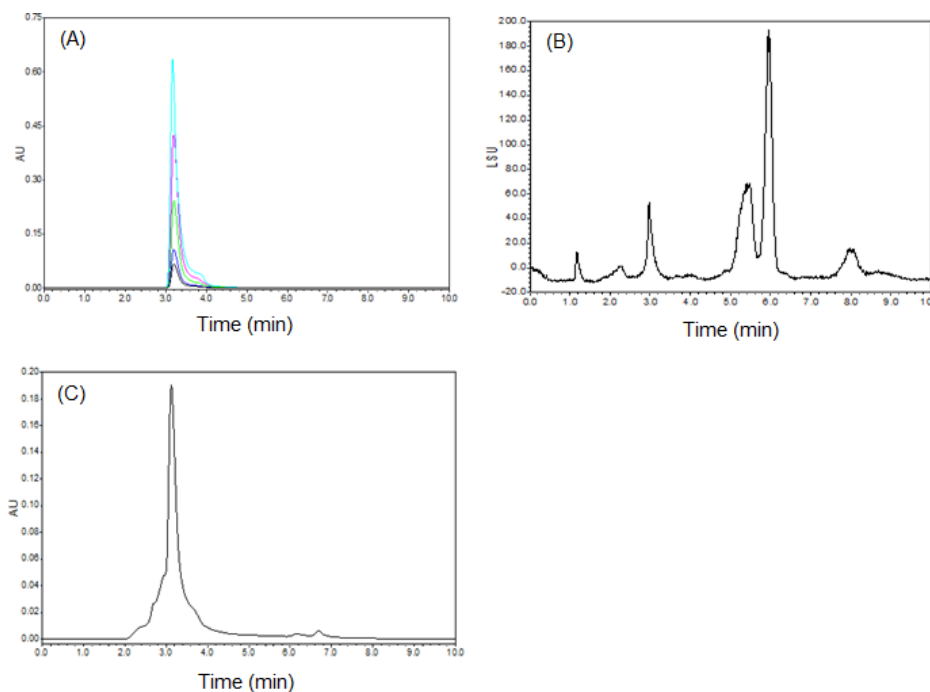


Fig. 8. HPLC analysis of (A) ampelopsin with concentrations ranging from 0.05 to 0.40 mg/L, (B) hydrolysate of dried fruits of *A. grossedentata*, and (C) ampelopsin isolated from hydrolysate

CONCLUSIONS

1. The process developed in this study with treatment of cellulases decreased in temperature and time for hydrolysis of the fruits of *A. grossedentata*. The time and temperature of this process were 6 h and 50 °C whereas those of the conventional method without treatment of cellulases were 24 h and 100 °C.
2. The safe and economic process at lower temperature and shorter time for extraction with treatment of cellulase for isolation of ampelopsin can be directly applied on the industrial scale.
3. The maximal amount of reducing sugars was obtained when the concentration of dried fruits was 120 g/L, whereas the highest conversion rate of reducing sugars was obtained when it was 40 g/L.
4. Higher concentration of diatomite in the filter press resulted in more removal of insoluble materials from the hydrolysate of the fruits of *A. grossedentata*. Lower flow rate of the continuous flow centrifuge also resulted in more removal of insoluble materials. The optimal concentration of diatomite mixed with hydrolysate and flow rate of continuous flow centrifuge were 3.0% and 2 L/min, respectively, based on removal of insoluble materials and recovery yield of reducing sugars.
5. The recovery yields of reducing sugars and ampelopsin from the dried fruits of *A. grossedentata* using the ceramic ultrafiltration system were 33.9% and 39.5%, respectively. The ampelopsin isolated from the dried fruits of *A. grossedentata* was identified with the absorption peak in the HPLC chromatogram.

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