ARABINOXYLANS FROM JOB'S TEARS (*COIX LACHRYMA-JOBI* L.) : CHEMICAL, MOLECULAR AND STRUCTURAL CHARACTERIZATION

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Food Technology

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อะราบิโนไซแลนจากลูกเดือย (*Coix lachryma-jobi* L.) : คุณสมบัติทางเคมี โมเลกุลและโครงสร้าง

นางสาวสุภาพร อภิรัตนานุสรณ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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STRUCTURAL CHARACTERIZATION

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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ลูกเดือย (*Coix lachryma-jobi* L.) ที่ผ่านการขัดสี 2 ชนิด คือชนิดเปลือกสีดำและเปลือกสีขาวและรำ นำมาบดและผ่านการกลั่นไหลกลับ (reflux) ของเอทานอลที่มีความเข้มข้นร้อยละ 70 กากที่ได้นำมาสกัดด้วยน้ำที่อุณหภูมิ 75 องศาเซลเซียส และสารละลายด่างโซเดียมไฮดรอกไซด์ที่มีความเข้มข้น 0.5 โมลาร์ ตามลำดับ พบว่าในลูกเดือยขัดสีไม่พบพอลิแซ็กคาไรด์ที่ไม่ใช่สตาร์ช (non-starch polysaccharides) ในส่วนที่สกัดโดยใช้น้ำ (water-extractable fraction) แต่พบในส่วนที่สกัดโดยใช้สารละลายด่าง ส่วนที่สกัดโดยใช้ด่าง (alkali extracts) หรือส่วนที่สกัดไม่ได้ด้วยน้ำ (water-unextractable fraction) จากลูกเดือยขัดสีทั้ง 2 ชนิดมีปริมาณโปรตีนอยู่สูงถึงร้อยละ 47.8 และ 38.4 และมีองค์ประกอบของกรดอะมิโนที่คล้ายคลึงกัน คือมีปริมาณกรดกลูตามิกและเมทไทโอนีนซัลโฟนในปริมาณสูง นอกจากนี้ยังมีปริมาณของเถ้าสูงโดยพบว่าส่วนใหญ่เป็นฟอสฟอรัสและแคลเซียม มอโนแซ็กคาไรด์ที่พบในส่วนที่สกัดไม่ได้ด้วยน้ำจากลูกเดือยเปลือกสีดำประกอบด้วยอะราบิโนสร้อยละ 6.6 ไซโลสร้อยละ 5.3 กูลโคสร้อยละ 4.9 และกรดยูโรนิก ร้อยละ 2.1 ส่วนของลูกเดือยเปลือกสีขาวประกอบด้วยอะราบิโนสร้อยละ 5.1 ไซโลสร้อยละ 4.1 กลูโคสร้อยละ 5.6 และกรดยูโรนิกร้อยละ 1.7 ทั้ง 2 ชนิดพบว่ามีกาแลกโตสและแมนโนสเพียงเล็กน้อย อะราบิโนไซแลนจากลูกเดือยเปลือกสีดำมีน้ำหนักโมเลกุลเฉลี่ย (average molecular weight) 741,000 ดาลตัน ค่าการกระจายน้ำหนักโมเลกุลอยู่ที่ 1.5 (Pd 1.5) ส่วนอะราบิโนไซแลนจากลูกเดือยเปลือกสีขาวมีน้ำหนักโมเลกุลเฉลี่ย 1,449,000 ดาลตัน ค่าการกระจายน้ำหนักโมเลกุลอยู่ที่ 2.6 (Pd 2.6) น้ำหนักโมเลกุลเฉลี่ยของอะราบิโนไซแลนเมื่อผ่านการใช้เอนไซม์โปรติเอส (protease) มีค่าลดลงเหลือ 369,000 ดาลตัน (Pd 2.7) และ 244,000 ดาลตัน (Pd 1.6) ตามลำดับ กิจกรรมพื้นผิว (surface activity) ของส่วนที่สกัดไม่ได้ด้วยน้ำลดลง เมื่อน้ำหนักโมเลกุลเฉลี่ยและความเข้มข้นของโปรตีนลดลง เนื่องมาจากการย่อยสลายโดยเอนไซม์ (enzymatic hydrolysis) อัตราส่วนอะราบิโนสต่อไซโลสของลูกเดือยเปลือกสีดำและสีขาวมีค่า 1.27 และ 1.25 แสดงว่าอะราบิโนไซแลนในเนื้อเมล็ดลูกเดือยมีกิ่งก้านสูง การวิเคราะห์ด้วยวิธีเมธิลเลชั่นและนิวเคลียร์แมกเนติกเรโซแนนซ์พบว่า โครงสร้างของอะราบิโนไซแลนประกอบด้วยสายหลักของไซแลน (xylan) ที่เชื่อมต่อกันด้วยพันธะ β (1, 4) ของ ไซโลส โดยมี α-L อะราบิโนส 1 โมเลกุล ต่อเป็นกิ่งก้านในปริมาณสูง โดยส่วนใหญ่เกาะอยู่ที่พันธะตรงตำแหน่ง O-3 และส่วนหนึ่งเกาะอยู่

ที่พันธะตรงตำแหน่ง O-2 และ O-3 ของไซโลส

ส่วนที่สกัดโดยใช้น้ำและส่วนที่สกัดไม่ได้ด้วยน้ำจากรำของลูกเดือยเปลือกสีดำ และสีขาว ประกอบด้วยโปรตีนและเถ้าสูง และมีปริมาณกรดยูโรนิกส่วนหนึ่งเช่นเดียวกับส่วนที่สกัดไม่ได้ด้วยน้ำจากเนื้อเมล็ดลูกเดือย องค์ประกอบของมอโนแซ็กคาไรด์จากรำลูกเดือยทั้ง 2 ชนิด แตกต่างกันเล็กน้อย ส่วนที่สกัดโดยใช้น้ำและส่วนที่สกัดไม่ได้ด้วยน้ำจากรำลูกเดือยเปลือกสีดำ มีปริมาณพอลิแซ็กคาไรด์ร้อยละ 22.2 และ 34.1 สูงกว่าส่วนที่ได้จากรำลูกเดือยเปลือกสีขาว ซึ่งมีปริมาณพอลิแซ็กคาไรด์ร้อยละ 17.0 และ 24.0 ตามลำดับ อัตราส่วนอะราบิโนสต่อไซโลสในรำลูกเดือยอยู่ระหว่าง 0.93-1.19 ซึ่งมีค่าน้อยกว่าในเนื้อเมล็ด ส่วนที่สกัดโดยใช้น้ำจากรำลูกเดือยทั้ง 2 ชนิด ประกอบด้วยอะราบิโนไซแลนและอะราบิโนกาแลกแตน ส่วนที่สกัดไม่ได้ด้วยน้ำพบเพียงอะราบิโนไซแลนเท่านั้น อะราบิโนไซแลนในส่วนที่สกัดไม่ได้ด้วยน้ำจากรำลูกเดือยเปลือกสีดำและสีขาว มีน้ำหนักโมเลกุลเฉลี่ย 382,000 ดาลตัน (Pd 1.3) และ 488,000 ดาลตัน (Pd 1.1) ซึ่งมีค่าน้อยกว่าที่พบในเนื้อเมล็ดลูกเดือย ส่วนที่สกัดไม่ได้ด้วยน้ำจากเนื้อเมล็ดลูกเดือยทั้ง 2 ชนิดที่ความเข้มข้นสูง คือร้อยละ 20 แสดงพฤติกรรมการไหลแบบนิวโตเนียน (Newtonian flow behavior) ส่วนที่สกัดโดยใช้น้ำ และส่วนที่สกัดไม่ได้ด้วยน้ำจากรำลูกเดือยแสดงพฤติกรรมการไหลแบบนิวโตเนียนที่ความเข้มข้นร้อยละ 20 เช่นกัน

สาขาวิชา เทคโนโลยีอาหาร
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ลายมือชื่อนักศึกษา_____
ลายมือชื่ออาจารย์ที่ปรึกษา_____
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SUPAPORN APIRATTANANUSORN : ARABINOXYLANS FROM JOB'S TEARS (*COIX LACHRYMA-JOBI* L.) : CHEMICAL, MOLECULAR AND STRUCTURAL CHARACTERIZATION. THESIS ADVISOR : ASST. PROF. SUNANTA TONGTA, Ph.D. 128 PP.

JOB'S TEARS/ARABINOXYLANS/WATER-EXTRACTABLE/WATER-UNEXTRACTABLE/ARABINOGALACTANS

Two types, dark and white husk, of polished Job's tears (*Coix lachryma-jobi* L.) and bran were ground and refluxed with 70% ethanol. The residues were sequentially extracted with water (75 ?C) and 0.5 M NaOH solution. Non-starch polysaccharides were not found in water-extractable (WE) fraction isolated from polished Job's tears but present in alkali extractable fraction. The alkali extracts or water-unextractable (WU) fraction of polished Job's tears from both types contained mainly protein (47.8% and 38.4%) with similar amino acid profiles which were high in glutamic acid and methionine sulfone. In addition, they were rich in ash contents with large amounts of phosphorus and calcium. The results revealed that WU fraction of dark husk Job's tears consisted of 6.6% arabinose, 5.3% xylose, 4.9% glucose, and 2.1% uronic acid, whereas that of white husk Job's tears contained 5.1% arabinose, 4.1% xylose, 5.6% glucose, and 1.7% uronic acid. Minor amounts of galactose and mannose were observed in both extracts. The arabinoxylans from dark husk Job's tears had much lower an average molecular weight (MW) of 741,000 Da with a polydispersity index of 1.5 (Pd 1.5) when compared to that of 1,449,000 Da with a

polydispersity index of 2.6 (Pd 2.6) from white husk Job's tears. The average MW of arabinoxylans reduced after treatment with protease to 369,000 Da (Pd 2.7) and 244,000 Da (Pd 1.6), respectively. The surface activity of WU fraction decreased as the average MW and protein concentration decreased due to enzymatic hydrolysis. The high arabinose/xylose (Ara/Xyl) ratio of 1.27 and 1.25 indicated highly branched WU arabinoxylans extracted from dark and white husk Job's tears from their kernel. With a combination of methylation and NMR analysis, the structural features were elucidated to be (1, 4)-linked β-D-xylan main chain with highly branched side chains, containing a single arabinose. The results showed that α-L arabinofuranosyl residues (Ara*f*) were attached as branched sides mostly at O-3, followed by at both O-2 and O-3 of xylopyranosyl residues (Xyl*p*).

WE and WU fractions isolated from dark husk Job's tears bran (DHB) and white husk Job's tears bran (WHB) contained high protein and ash with some contents of uronic acid similar to the WU fractions obtained from its kernel. Minor differences in monosaccharide components of both Job's tears brans were observed. The WE and WU fractions from DHB had significantly higher in polysaccharides (22.2% and 34.1%) than those from WHB (17.0% and 24.0%, respectively). The degree of branching (Ara/Xyl) of arabinoxylans from Job's tears bran ranged between 0.93 and 1.19 which was slightly lower than its kernel. The WE fraction from two Job's tears brans contained arabinoxylans and arabinogalactans whereas the WU fraction contained only arabinoxylans with an average MW of 382,000 Da (Pd 1.3) and 488,000 Da (Pd 1.1) for DHB and WHB respectively, which were lower than those from kernel. The WU fractions from the two types of kernel exhibited

Newtonian flow behavior at high concentration (20% w/v). The WE and WU fractions from brans also exhibited Newtonian flow behavior (20% w/v).

School of Food Technology

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LIST OF ABBREVIATIONS

Araf	=	arabinofuranosyl residues
AE	=	alkali extract
Ara/Xyl	=	arabinose to xylose
COSY	=	$^{1}H/^{1}H$ correlation spectroscopy
^{13}C NMR	=	carbon 13 nuclear magnetic resonance
D_2O	=	deuterium oxide
Da	=	dalton
DF	=	dietary fiber
DHB	=	dark husk Job's tears bran
DMSO	=	dimethylsulfoxide
d*n*/d*c*	=	refractive index increment
G'	=	storage modulus
G"	=	loss modulus
GC-MS	=	gas chromatography-mass spectrometry
GPC	=	gel permeation chromatography
HCl	=	hydrochloric acid
HMBC	=	long-range heteronuclear correlation spectrum
HMQC	=	heteronuclear Multiple Quantum Coherence
HPAEC	=	high performance anion exchange chromatography
HPLC	=	high performance liquid chromatography
HPSEC	=	high performance size exclusion chromatography

LIST OF ABBREVIATIONS (Continued)

^{1}H NMR	=	proton nuclear magnetic resonance
LS	=	light scattering
MALLS	=	multi-angle laser light scattering
MW	=	molecular weight
$NaBD_4$	=	sodium borodeuteride
$NaBH_4$	=	sodium borohydride
NaN_3	=	sodium azide
$NaNO_3$	=	sodium nitrate
NaOH	=	sodium hydroxide
NMR	=	nuclear magnetic resonance
NSP	=	non-starch polysaccharides
$(NH_4)_2SO_4$	=	ammonium sulfate
Pa	=	pascal
PAD	=	pulsed amperometric detection
Pd	=	polydispersity index
PMAA	=	partially methylated alditol acetates
RALLS	=	right angle laser light scattering
Rg	=	radius of gyration
RI	=	refractive index
S	=	second
SCFA	=	short chain fatty acids
SEC	=	size exclusion chromatography

LIST OF ABBREVIATIONS (Continued)

TFA	=	trifluoroacetic acid
TMS	=	tetramethylsilane
TOCSY	=	$^{1}H/^{1}H$ correlation spectroscopy
UV	=	ultraviolet
WE	=	water-extractable
WHB	=	white husk Job's tears bran
WU	=	water-unextractable
w/v	=	weight per volume
Xylp	=	xylopyranosyl residues
[η]	=	intrinsic viscosity

CHAPTER I

INTRODUCTION

Cereal grains are good sources of food carbohydrate present particularly in kernels or endosperms that generally are consumed by people. Cereals additionally provide a variety of amino acids, vitamins, minerals and non-nutritive substances. In recent years, considerable attention to cereals for health benefit has been focus through provision of non-starch polysaccharides (NSP). Examples of such NSP mostly present in cereals are β-glucans and arabinoxylans, which show potential to reduce cholesterol level in blood system (Wood, 2002; Lopez et al., 1999).

Coix lachryma-jobi L., commonly named Job's tears, is one of the local cereals which has been widely cultivated in many Asia countries such as Philippine, Burma, China, Sri Lanka and Thailand (Duke, 1983; D.A. Bender and A.E. Bender, 1999). Job's tears seed is pear-shaped, around 5 mm in diameter, covered by a hard shiny dark brown to gray-black hull (Li and Corke, 1999). Job's tears is added in soups and broths in form of flour or whole (dehulled) grain and the fermented grain is used for beers and wines making (Duke, 1983; Li and Cork, 1999). This plant can be used for forage and fodder and the bran can be used as a substitute for wheat bran in feeding poultry (Purseglove, 1972). In Thailand, the conventional dessert made from Job's tears mixed with sugar and coconut milk has long been known and a non-dairy drink from Job’s tears has been recently launched to the market as alternatives for health benefit in Thailand.

Job's tears has long been used in traditional Chinese medicine and as a nourishing cereal. The consumption of Job's tears has been demonstrated to have a number of beneficial biomedical functions such as antitumor, anti-allergic, and fibrinolytic activities (Chang, Huang, and Hung, 2003; Check, and K'Ombut, 1995; Hsu, B-F. Lin, J-Y. Lin, Kuo, and Chaing, 2003; C-C. Kuo, Shih, Y-H. Kuo, and Chiang, 2001; Numata, Yamamoto, Moribayashi, and Yamada, 1994). More recently, animal and human clinical trials showed that consumption of Job's tears in various forms may potentially improve lipid metabolism, thus decreasing the risk of heart diseases. However, the active components and associated mechanism of action are not clear (Tsai, Yang, Su, and Chen, 1998; Yu, Chang, Liu, and Tsai, 2004), although most studies pointed to the two components, water soluble polysaccharides (Yu, Lu, M-T. Chiang, and W. Chiang, 2005) and oil (Huang, M.T. Chiang, Yao, and W. Chiang, 2005). Yu et al. (2005) reported that water soluble polysaccharide enriched fraction of Job's tears lowered the serum total cholesterol, LDL cholesterol levels, and triglyceride in hamsters. Water soluble β-glucans from cereals such as oats and barleys have been well known to have such hypolipidemic effects (Wood, 2002). Similarly, arabinoxylans from corn bran was also reported to reduce cholesterol level (Lopez et al., 1999). There is currently very limited information on the NSP from Job's tears.

It is, therefore, important to investigate the chemical components of NSP in Job's tears, elucidate their molecular and structural characteristics as well as chemical and physical properties. The results may lead to understanding of its biological properties.

1.1 Research objectives

1. To study the chemical composition of NSP from polished Job's tears seed and bran.
2. To elucidate molecular weight (MW) distribution and structural features of NSP.
3. To determine the physical properties of NSP.

1.2 Research hypothesis

1. Job's tears is composed of significant NSP in its kernel and bran.
2. The MW distribution and structural features of NSP can be identified.
3. The physical properties of NSP are revealed.

1.3 Scope and limitation of the study

NSP of Job's tears will be extracted and the chemical composition will be analyzed. The molecular size distribution of NSP will be analyzed by High Performance Size Exclusion Chromatography (HPSEC). The molecular structural features of NSP will be identified by High Performance Anion Exchange Chromatography (HPAEC), methylation analysis and Nuclear Magnetic Resonance (NMR) techniques. The physical properties of NSP will be investigated.

1.4 Location of research

1. Suranaree University of Technology, Thailand

2. Agriculture and Agri-Food Canada, Food Research Program, Guelph, Ontario, Canada

3. University of Guelph, Ontario, Canada

1.5 Expected results

1. To understand chemical composition and structural characteristics of NSP in Job's tears.
2. To understand the physical properties of NSP in Job's tears.

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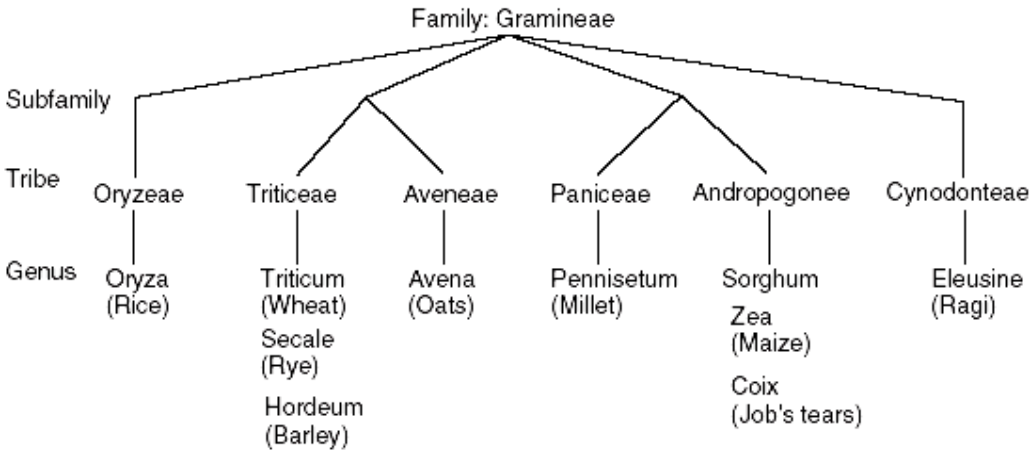
CHAPTER II

LITERATURE REVIEW

2.1 *Coix lachryma-jobi* L.

Coix lachryma-jobi L., commonly named Job's tears, coix, or adlay is a type of millet wild grass, which is a relative of maize in the tribe Andropogoneae. It is a grain crop, annual leafy grass with 1 to 3 m height in Gramineae/Poaceae family (Figure 2.1) (Encyclopedia Britannica, 1997). There are a number of varieties according to size and seed structure cultivated in many parts of Africa and Asia as well as North America. It is likely that it was first domesticated in the region of Indo-China with sufficient rain and soil of reasonable fertility (D.A. Bender and A.E. Bender, 1999; Encyclopedia Britannica, 1997). It looks like tear-shaped bead hard shell and is around 8-12 mm long that encloses the seed kernel with grey in colour and also possibly ivory, pale blue, rose, brown or black (Purseglove, 1972). The seed of Job's tears is classified into 2 forms, seed of cultivated varieties characterized by a soft shell and seed of wild varieties characterized by a hard shell (Arora, 1977). The former one is widely used for food and feed but the latter one is mostly used for ornamental purposes and made into rosaries. The edible form is known as 'adlay' in the Philippines and as 'ma yuen' in China. The hulled grain is edible in the same way as cereal foods and can be ground into flour (Purseglove, 1972). Job's tears is also classified into 4 types according to varieties (Vacharotayan, Jan-orn, Cheaorn, Titatarn, and Kingkaew, 1982, quoted in Pornkitprasarn, 1987):



a) Taxonomy of the Gramineae family

Source: Shewry, Tatham, and Kasarda (1992), quoted in McKevith (2004)

b) Job's tears

Source: Matz (1969)

Figure 2.1 Appearance of Job's tears

1. *C. lachryma-jobi* L. var. typical

2. *C. lachryma-jobi* L. var. stenocarpa Stapf.

3. *C. lachryma-jobi* L. var. monilifer Stapf.

4. *C. lachryma-jobi* L. var. ma-yuen Stapf.

In Thailand, commercial Job's tears named *C. lachryma-jobi* L. var. ma-yuen has been thoroughly grown for consumption. Regarding specific characteristics and compositions, *C. lachryma-jobi* L. var. ma-yuen can be referred to two types, glutinous and non-glutinous (Vacharotayan et al., 1982, quoted in Pornkitprasarn, 1987). According to its good flavor and texture, the glutinous type has been mostly grown in Thailand with the content of low amylose around 10% (Pornkitprasarn, 1987).

Per 100 g, Job's tears seed is reported to consist of 380 calories, 11.2 g water, 15.4 g protein, 6.2 g fat, 65.3 g total carbohydrate, 0.8 g fiber, 1.9 g ash, 25 mg Ca, 435 mg P, 5.0 mg Fe, 0 µg beta-carotene equivalent, 0.28 mg thiamine, 0.19 mg riboflavin, 4.3 mg niacin, and 0 mg ascorbic acid (Duke, 1983). Sisawad and Chatket (1985) reported that Job's tears seed was composed of moisture, protein, lipid, carbohydrate, fiber, and ash 10.77, 14.00, 6.05, 66.62, 0.60 and 1.92%, respectively. According to Juntana (1986), Job's tears contained 10.83 % moisture, 13.05 g protein, 5.45 g fat, 68.60 g carbohydrate, 0.36 g fiber and 1.3 g ash and Job's tears flour contained 2.25% amylose. Pornkitprasarn (1987) found that coarse Job's tears flour consisted of 8.86 % moisture, 15.18% protein, 5.52% fat, 77.52% carbohydrate, 0.25% fiber and 1.53% ash, 56.68% starch and Job's tears starch contained 10.85% amylose. The major protein component in Job's tears seed is a prolamin called coixin which is rich in proline and leucine but poor in lysine. Job's tears also contains

albumins and globulins and other residual proteins (Ottoboni, Leite, Targon, Crozier, and Arruda, 1990).

Job's tears has long been used in traditional Chinese medicine and as a nourishing cereal. The consumption of Job's tears has been demonstrated to have a number of beneficial biomedical functions such as antitumor, anti-allergic and fibrinolytic activities (Chang, Huang, and Hung, 2003; Check, and K'Ombut, 1995; Hsu, B-F. Lin, J-Y. Lin, Kuo, and Chaing, 2003; C-C. Kuo, Shih, Y-H. Kuo, and Chiang, 2001; Numata, Yamamoto, Moribayashi, and Yamada, 1994). Ukita and Tanimura (1961) identified that the antitumor component in the Job's tears seeds was contributed to coixenolide. The recent report studied by Numata et al. (1994) discovered that an acetone extract of Job's tears seeds attributed to an acidic fraction had antitumor activity in vivo test. This acidic fraction was composed of 4 free fatty acids; palmitic, steric, oleic and linoleic acids. Nagoa, Otsuka, Kohda, Sato, and Yamasaki (1985) reported that benzoxazinones isolated from Job's tears seed showed anti-inflammatory activity. In addition, Takahashi, Konno, and Hikino (1986) revealed that coixan A, B and C isolated form Job's tears seeds possessed hypoglycemic activity in rats. Hsu et al. (2003) found that the dehulled Job's tears methanolic extract exhibited the most capacity to reduce anti-OVA IgE which might be beneficial for the treatment of allergic disorders. The Job's tears hull methanolic extract exhibited greatest antioxidant capacity, followed by the other parts of Job's tears seed; testa, bran, and polished grain. Six specific compounds were found to be strongly responsible for antioxidant activity; coniferyl alcohol, syringic acid, ferulic acid, syringaresinol, 4-ketopinoresinol and a new ligan, mayuenolide. These six phenolic compounds also had different degree of antioxidant effects (Kuo, et al.,

2002). In addition, it has been recently found that a methanolic extract of Job's tears exerted an antiproliferative effect on lung cancer cells by inducing cell cycle arrest and apoptosis and also found that tumor growth and cancer growth *in vivo* were inhibited by methanolic extract (Chang et al., 2003; Hung and Chang, 2003). It was reported that more than 270,000 patients in China were taken with a neutral lipid extract from endosperm of Job's tears, named "Kanglaite", which has proven effective against malignant tumors. Therefore, the Chinese Government has endorsed Kanglaite as a treatment for cancers of the lung, liver, stomach, and breast. However, it seems that it has been unclear how Kanglaite works (Gibbs, 1998; Ho, 2003). W. Chiang, Cheng, M-T. Chiang, and Chung (2000) demonstrated that the content of short chain fatty acids increased in cecum and feces of rats fed with 20-40% Job's tears. More recently, Yu, Lu, M-T. Chiang, and W. Chiang (2005) reported that water soluble polysaccharide enriched fraction of Job's tears lowered the level of serum total cholesterol, LDL cholesterol and triglyceride in hamsters. Kim et al. (2004) also reported that the levels of triglyceride, total-cholesterol and leptin in blood serum of rats were significantly decreased when they were fed by crude water extract of Job's tears.

2.2 Proteins and minerals in cereals

Basically, protein molecules consist of the linked chains of amino acid peptide bonds between the carboxyl (COOH) group of one amino acid and the alpha-amino (NH_2) group of the next. The peptide chains of protein may be linked together by neighborhood of disulphide bonds from cystine residues. In addition, the polypeptides may be coiled in spirals, with hydrogen bonds linking the protruding

side chains. The properties of proteins vary considerably due to their amino acid composition and arrangement.

Based on their solubility, proteins have been classified into four groups according to Osborne as followings (Hoseney, 1986).

1. Albumins are proteins soluble in water. They are coagulated while heated. Their solubility is not affected by some extent of salt concentrations. The classic example of this type is protein in egg white.

2. Globulins are proteins insoluble in water but soluble in dilute salt solutions and insoluble at high salt concentrations. These proteins exhibit the phenomenal salting in and salting out.

3. Prolamins are proteins soluble in 70% ethyl alcohol.

4. Glutelins are proteins soluble in dilute acids or bases.

In general, there are other proteins not classified into any of groups as mentioned above. These proteins are considered as residual proteins which remain behind. Some cereals containing differences in the amounts of protein group are summarized in Table 2.1. The endosperm of Job's tears comprises three fractions. They are albumins and globulins (shown in Table 2.1 as salt-soluble), prolamin (ethanol-soluble) mainly coixin, and residual proteins that were unextractable in dilute neither salt nor alcohol (Ottoboni et al., 1990).

The eighteen various amino acids are found in the cereal proteins in which glutamic acid and proline are of predominantly presence (Kent, 1983). The proportion and order of amino acids along the protein chains give them the specific characteristics. The composition of flour from several cereals is summarized in Table 2.2. Most cereals are good sources of a number of minerals. In general, the

Table 2.1 Solubility distribution of the endosperm proteins of cereals (% of total protein)

Solubility fraction	Hard red spring wheat	Durum wheat	Triticale	Rye	Barley	Maize	Oats	Rice	Sorghum	Job's tears[1]
Water-soluble	11.9	12.2	26.4	34.3	na[2]	11.0-11.7	13.1	2.7-4.6	} 9.0-16.2	na
Salt-soluble	5.2	4.7	6.5	10.7	12.5-17.1	12.4-22.0	29.0	3.7-6.4		2.7
Ethanol-soluble	28.5	40.7	24.4	19.0	} 56.2	33.9	} 63.9	1.8-2.9	na	10.8
Acetic or formic acid soluble	16.6	18.3	17.3	9.4				} 78.0	na	na
Residue protein	34.0	23.2	19.0	20.6	31.3	36.8	13.0		na	86.5

Source: Simmonds and Orth (1973)

[1] From Ottoboni et al. (1990)

[2] Data not available

Table 2.2 Composition of flour from various cereals

	Wheat	Oat	Barley	Rice	Corn (dry milling)	Sorghum (refined)
Protein	11.3	12.9	10.2	6.7	7.1	9.5
Fat	1.0	7.2	1.7	0.4	1.3	1.0
Carbohydrate	71.5	66.6	76.9	80.1	77.5	na[1]
Crude fiber	0.12	1.0	0.7	0.3	0.9	1.0
Ash	0.66	1.8	1.2	0.5	0.6	0.8
Moisture	na	9.3	10.0	12	12.6	na

Source: Adapted from Kent (1983)

[1] Data not available

majority of the minerals and vitamins are located in the aleurone layer (outer layer of endosperm). However, this part of endosperm is removed as bran during milling, commonly together with other compounds such as seed coat and nucellar epidermis (Hoseney, 1986). The content of ash in flour, occasionally, depends on how much the aleurone layer is included in the flour. Those cereals consist of the phosphate and sulphate complexes with potassium, magnesium, and calcium as salts. For example, the potassium phosphate present in wheat is probably in terms of KH_2PO_4 and K_2HPO_4 (Kent, 1983). Similar to other cereals, Job's tears provides a high phosphorous and potassium (Table 2.3). The majority of phosphorous in cereal has been reported in the form of inositol hexaphosphoric acid, known as phytic acid, which accounts for approximately 60-90% of the total seed phosphorus (Lolas, Palamidis, and Markakis, 1976). It was thought that the reactive phosphate groups

attached to the inositol ring of phytic acid chelate polycations of other minerals, and then decreasing them from being absorbed in the intestinal tract. However, it was indicated that phytase, an esterase enzyme present in cereal itself, could convert phytic acid to inositol and free phosphoric acid during processing (Hoseney, 1986).

Table 2.3 Mineral composition of cereals (mg/100g, dry weight)

	Rye	Wheat	Barley (kernel)	Corn	Oats (kernel)	Rice (kernel)	Job's tears (kernel)[1]
Phosphorous	380	410	400	310	400	290	425
Potassium	520	580	600	330	380	120	352
Calcium	70	60	80	30	66	67	70
Magnesium	130	180	130	140	120	47	238
Iron	9	6	-	2	4	6	8.7
Copper	0.9	0.8	-	0.2	5	0.4	Trace
Manganese	7.5	5.5	-	0.6	4	2	Trace
Zinc	3.4	4.4	-	-	-	1.2-2.1	5.5
Sodium	3.1	4.6	-	-	-	2.2-5.1	-

Source: Bock (1991)

[1] From ยุพดี สิทธิบุศย์ (2526)

2.3 Non-starch polysaccharides (NSP)

Although starch is the most abundant cereal polysaccharides and is a major food reserve providing a bulk nutrient and energy source, the other carbohydrate component currently under an attention is non-starch polysaccharides (NSP)

considered as a key chemical component of dietary fiber. NSP are classified into soluble and insoluble dietary fiber. Both types of them show health benefits to humans, where soluble fibers delay gastric emptying, increase transit time (slower movement) through the intestine, and decrease nutrient (e.g. glucose) absorption. On the other aspect, insoluble fibers decrease intestinal transit time and increase fecal bulk (Russell and Bass, 1985).

Soluble fibers are generally fermented by the large intestine microflora and then increase a production of short chain fatty acids (SCFA) which help mineral solubility (Lopez et al., 1999). In addition, it has been proved that they have no adverse affects on mineral absorption (Debon and Tester, 2001). It is indicated that soluble NSP are able to reduce cholesterol and control blood sugar level (Spiller, 1993). The exact mechanisms of soluble fibers to beneficial effects are unclear. However, it is possibly due to their following activities; inhibition of fat digestion and absorption, interaction with bile acid or cholesterol with a consequently increased loss by excretion, enhancement of fermentation products e.g. SCFA in colon, and reduction of the rate of digestion and absorption, then affecting insulin and hormone secretion (Edwards and Parrett, 1996; Camire, Zhao, and Violette, 1993). Coronary heart disease is a major cause of death in humans and one of the risk factors is an increase in serum cholesterol, especially low density liproprotein (LDL) cholesterol and lipids (Shepherd and Packard, 1992; Anderson and Tietyen-Clark, 1986). Many studies have been demonstrated the ability of soluble fibers to reduce plasma cholesterol, a specific health benefit for hypercholesterolemic patients (Kahlon, 2003; Lopez et al., 1999; Al-Othman, Al-Shagrawi, Hewedy, and Hamdi, 1998; Sudheesh, and Vijayalakshmi, 1999; Wang, Onnagawa, Yoshie, and Suzuki, 2001), while this

ability is not shown in insoluble fibers (Carter, Hardman, Heitman, and Cameron, 1998; Anderson and Tietyen-Clark, 1986). Effective soluble NSP in cereals have been studied such as arabinoxylans in corn bran (Lopez et al., 1999), β-glucans in oats (Kalra and Jood, 2000) and barleys (Delaney et al., 2003). β-glucans and arabinoxylans are components of the cell walls of endosperm that can be classified on the basis of solubility into water-extractable and water-unextractable fractions. Arabinoxylans are the main water soluble NSP in wheats, and ryes, while they are β-glucans in oats and barleys (Cui, 2001). However, the major component of NSP in cereals is water-unextractable esterified to an extent of diferulic acid bridges between individual macromolecules (Gubler, Ashford, Bacic, Blakeney, and Stone, 1985) and bound to other cell wall components such as lignin (Jeffries, 1990), thus resulting in insolubility. To solubilize NSP in aqueous solution, alkali such as sodium hydroxide solution is applied to release the ester bond of the crosslink (Gubler, et.al, 1985; Cui, 2001). In addition, non-covalent links between unsubstituted xylose residues (Andrewartha, Phillips, and Stone, 1979) or unsubstututed xylose and cellulose (McNeil, Alberheim, Taiz, and Jones, 1975) may cause the arabinoxylans insoluble.

Arabinoxylans are built up of pentose sugars named pentosans, mostly arabinose and xylose residues, and are therefore often referred to as arabinoxylans (Åman and Westerlund, 1996). Wheat flour comprises both water-soluble and water-insoluble arabinoxyalns with 25% and 75%, respectively (Gruppen, Marseille, Voragen, Hamer, and Pilnik, 1989). The content of arabinoxylans varies based on the varieties and alternative methods of extraction. When wheat flour is extracted by cold water, for example, the total yield of water-soluble arabinoxylans in wheat is 1.0-1.5% (Hoseney, 1986). After making dough and washing by 0.1 M sodium chloride,

the level of soluble arabinoxylans is 0.5% with minor protein contents as low as 0.6% (Hoffmann, Roza, Maat, Kamerling, and Vliegenthar, 1991). According to Dervilly-Pinel, Rimsten, Saulnier, Andersson, and Åman (2001), when pearled flours of wheat, barley, and triticale were extracted by warm water, the arabinoxyans showed in the range of 0.2-0.7%, while those of 2.1% was reported in rye. It has been reported that the existence of arabinoxylans in wheat flour are effective on dough formation (Wang, 2003). Early studies were found that arabinoxylans were important for the quality of baking products. For example, removal of water soluble pentosans from wheat flour reduced the loaf volume of the bread while the addition of 2% water soluble endosperm pentosans increased the loaf volume by 30-45% with improving all uniformity of texture (Hoseney, 1984). Biliaderis, Izydorczyk and Rattan (1995) found that after 7 day storage period, crumbs of breads added with arabinoxylans consistently reduced the starch retrogradation and staling event as the moisture content increased. In rye grain, it was composed of 0.74% of water soluble arabinoxylans which indicated 72% arabinoxylans (Bengtsson and Åman, 1990). Similar to wheat, the water-unextractable arabinoxylans, of which 60-70% of rye is the major constituent (Pettersson and Åman, 1987; Saini and Henry, 1989). The pentosan content of rye is about twice as high as that of wheat, so it has much more effect on dough formation.

Few clinical studies have been done in arabinoxylans. It has been proposed that whole-grain rye contains remarkable amounts of soluble arabinoxylans, which seem to have positive health effects similar to the β-glucan in oats (Kujala, 2005). Recently, the study by Lopez et al. (1999) was found that soluble arabinoxylans from corn bran remarkably reduced cholesterol absorption in rats, enhanced mineral

absorption and stimulated the production of SCFA. There was no effect on the level of HDL, while the level of LDL and triglyceride were reduced.

2.4 Extraction and compositions of non-starch polysaccharides (NSP)

Non-starch polysaccharides (NSP) from cereals are linkaged or non-covalent linkaged together with cellulose and lignin in the plant cell wall. NSP can be isolated by water (Bengtsson and Åman, 1990; Izydorzyk, Macri, and MacGregor, 1998a) and by alkali extraction (Cui, Wood, Weisz, and Beer, 1999; Nilsson, Saulnier, Andersson and Åman, 1996). The extracts are categorized into two different types, water-extractable and water-unextractable, respectively. The extractability of these polysaccharides is based on the conformational aggregation, the covalent ester bonds between ferulic acid and other components such as lignin (Scalbert, Monties, Lallemand, Guittet, and Rolando, 1985), the degree, and substitution pattern of arabinoses at side chain, and nature of physical entanglement (Girhammar, Nakamura, and Nair, 1986). Hydroxyl ions are known to cause the swelling of cellulose, hydrolysis of ester linkages, and disruption of intermolecular hydrogen bonds between cellulose and hemicellulose, thus dissolving a portion of hemicellulosic material into solution. Higher concentrations of alkali and higher temperatures result in higher yields of extraction due to a disruption of stronger linkages; however, this circumstance apparently causes the depolymerization of NSP (Bergmans, Beldman, Gruppen, and Voragen, 1996; Cui, 2001).

Solution of sodium hydroxide was commonly used to extract arabinoxylans in many cereals (Annison, Choct, and Cheetham, 1992; Cui et al., 1999; Ebringerova, Hromadkova, Burchard, Dolega, and Vorwerg, 1994). The addition of sodium

borohydride ($NaBH_4$ 0.5%) was probably required in order to prevent degradation of NSP by peeling reactions (Höije, Gröndahl, Tømmeraas, and Gatenholm, 2005; McNeil et al., 1975). Recently, saturated barium hydroxide has been selected to extract water-unextractable materials in some cereals in order to reduce the contamination of β-glucan (Izydorczyk, Macri, and MacGregor, 1998b; Verbruggen, Beldman, and Voragen, 1995), because Ba^{+} forms insoluble complexes with β-glucans (D'Appolonia and MacArthur, 1975).

The approach to study water-soluble NSP was employed by Izydorczyk et al. (1998a). Water-soluble NSP were extracted from barley by a sequential treatment with water at 40°C and 65°C. The polysaccharides were extracted with water to seed ratio of 3:1 at 40°C for 30 min. The aqueous extract was separated from the insoluble residue by centrifugation. The extract was brought to 95 °C to denaturize proteins. The denatured proteins were then removed by filtration using celite as a filter aid and further treated by adsorption on Vega clay to remove the protein residue. Porcine pancreas α-amylase was added in the extract to digest starch contaminants. The extract was incubated and then dialyzed against distilled water until the dialysate was free of sugar. The enzyme was inactivated by heating at 95 °C and removed by centrifugation. Finally, the purified extract was freeze-dried. The insoluble residue was continued to extracting with water at 65 °C for 90 min., mixed with thermostable α-amylase and then centrifuged. The aqueous extract was purified in the same manner as described above used for 40 °C extract. Finally, the yields obtained from the extract at 40 °C and 65 °C were 1.4 and 1.3% (w/w), respectively, in which arabinoxylans of the 40 °C extract was present 16%, higher than 6% of the 65 °C extract (based on the

amount of arabinose and xylose). The extract at 40 °C was composed of 82.5% glucose, 8.9% xylose and 7.0% arabinose whereas the extract at 65 °C contained 93.3% glucose, 3.3% xylose and 2.5% arabinose. Only minor of mannose and galactose were found in either fraction. When the purified extracts were dissolved in phosphate buffer and fractionated by ammonium sulfate ($(NH_4)_2SO_4$) saturation, the results showed that β-glucans from both extracts were most fractionated by precipitation with 35% $(NH_4)_2SO_4$, while arabinoxylans were most dissolved in the supernatant. Fractions precipitated at higher saturation levels of $(NH_4)_2SO_4$ (>45%) contained progressively more arabinoxylans and less β-glucans.

Westerlund, Anderson and Åman (1993) studied the procedure to give β-glucans and arabinoxylans from milled oat fractions. The lipophilic components in oat was removed and the endogenous β-glucanase was inactivated by a mixture of hot isopropanol and petroleum ether. An extraction with 90% ethanol was used to further remove polar substances such as low molecular weight sugars and some proteins. The starch was degraded by treatment with a thermally stable α-amylase. The supernatant from hot water extraction was treated with pancreatine to decrease protein content, and then the NSP mixture was precipitated in 60% ethanol. The supernatant was discarded and the precipitate was dissolved in water. The NSP were further fractionated by precipitation with 20% $(NH_4)_2SO_4$. The β-glucans and arabinoxylans were obtained from the precipitate and the supernatant, respectively. An alternative method to inactivate endogenous enzyme by heating at 130 °C for 24 hr was applied to durum wheat before extraction with water at 30 °C (Roels, Collado, Loosveld, Grobet, and Delcour, 1999). After centrifugation, the supernatants were incubated

with α-amylase at 90 °C for 4 hr, cooled, and centrifuged. The arabinoxylans in the resulting supernatant were precipitated from the solution by stepwise addition of ethanol to a final concentration of 65%. Only small levels of galactose and glucose were present in the water-extractable material. The arabinoxylan fraction consisted of 27.4% arabinose, 44.2% xylose, 19.8% ash, and 4.8% proteins.

The extraction and purification procedure used to obtain arabinoxylans from rye was reported by Bengtsson and Åman (1990). Rye meal was ground and refluxed with 90% ethanol to remove low molecular weight compounds and to inactivate endogenous enzymes. The insoluble residue was isolated and washed with the 90% ethanol and then extracted with water at 40 °C for 90 min. In the middle of this treatment, the slurry was homogenized for 2 min to improve extractability. About 500 ml of water extract was precipitated with 200 g $(NH_4)_2SO_4$ to give the precipitate. The precipitate was then dissolved in water to further purify and precipitated with 67% ethanol to obtain a crude arabinoxylans. Similarly, Cyran, Courtin, and Delcour (2003) reported that after refluxing under 90% ethanol, the inactivated-enzymatic rye flour was extracted by a sequential extraction at 4 ºC, 40 ºC, and 100 ºC. The crude extract was hydrolyzed with α-amylase, amyloglucosidase, and proteinase to obtain purified extract. The results showed that the consecutive extraction substantially influenced the extraction of water-extractable arabinoxylans, which the majority of arabinoxylans was recovered in the extract at 4 ºC. Higher protein content (16-21%) of the 100 ºC fractions was determined than those (4-5% and 7-10%) of the 4 ºC and 40 ºC fractions. The results suggested that arabinoxylans were freely extractable at low temperature (62-66%) while the rest required higher temperatures.

The water-unextractable NSP can be extracted under alkaline condition. Storsley, Izydorczyk, You, Biliaderis, and Rossnagel (2003) sequentially extracted NSP from inactivated hull-less barley samples with water and alkaline solutions. It was found that the majority of fractions extracted by saturated barium hydroxide and 1 M sodium hydroxide from hull-less barley was arabinoxylans considerably different from those of fractions extracted with water, which mostly were β-glucans. The alkali-extractable fractions were significantly high in protein content (24-35%), although the extensive purification procedures were applied.

To investigate alkaline NSP extracts from wheat bran, similarly as flour, preprocessed wheat bran was refluxed with ethanol (70%) at 70 ºC to remove the ethanol soluble materials and inactivated enzyme (Cui et al., 1999). The ethanol extracted residue was subjected to α-amylase to hydrolyze starch. The destarched samples were subsequently extracted with sodium hydroxide solutions, and the alkaline extracts were neutralized with 2 N hydrochloric acid. After centrifugation, the supernatant was precipitated by ethanol (~65%) to give NSP. The NSP contained 5% ash, 6% proteins, 19% arabinose, 58% xylose, and 23% glucose with only a trace amount of galactose. From the delipidated, destarched, depectinated, and delignified rye bran, arabinoxylan-protein complex was isolated by extraction with 5% sodium hydroxide (Ebringerová, Hromádková, and Berth, 1994). The extract contained 1.1% of protein and consisted of arabinose and xylose in the molar ratio 97:100 with minor amounts of glucose and galactose. The uronic acid which represented 3.1% of the polysaccharides was present mainly as D-glucuronic acid and its 4-O-methyl derivatives which were indicative of acidic xylans known to occur in bran tissues

(Wilkie, 1979). No phenolic substances such as ferulic acid were found, probably due to a cleavage of the ester bond during the alkaline extraction step.

2.5 Structural features of arabinoxylans

2.5.1 Molecular weight (MW) distribution

The apparent molecular weights distribution of water soluble arabinoxylans from eight wheat varieties calculated from the Mark-Houwink equation ranged from 134,700 to 204,600 (Rattan, Izydorczyk, and Billaderis, 1994). The range of limiting viscosities was 3.60 to 5.48 dl/g which is known to relate to molecular weight and chain conformation of these macromolecules. All arabinoxylans exhibited a rather broad MW distribution as revealed by the gel filtration profiles. Dervilly, Saulnier, Roger, and Thibault (2000) reported the value of MW of 300,000 and a polydispersity index of 1.6 for wheat water soluble arabinoxylans determined by high performance size exclusion chromatography (SEC) using a multiangle laser light scattering. The differences probably arise from the fact that MW values are determined on very polydisperse arabinoxylans that need to be fractionated. The intrinsic viscosity was similar to that found by Rattan et al. (1994). For barium hydroxide extractable wheat arabinoxylans, the weight average MW of 850,000 was reported as measured by laser light scattering (Gruppen, Hamer, and Voragen, 1992). The MW of soluble arabinoxylans in rye flour was 2-3 times greater than those found in wheat flour (Meuser and Suckown, 1986). Girhammar and Nair (1992) found that the MW of rye arabinoxylans by utilizing gel permeation chromatography (GPC) was estimated between 519,000 and 770,000 compared to the range of 219,000 and 255,000 for wheat. Supportably, water soluble pentosans of rye were determined by gel

chromatography and reported that pentosan material of rye had a higher average molecular weight than that of wheat (Vinkx, Nieuwenhove, and Delcour, 1991). Moreover, polydispersity of water-extractable rye arabinoxylans was around 2, showing that there was a large span of MW distributed between 150,000-650,000 with an average intrinsic viscosity of 2.72 dL/g, as determined by light scattering and viscosity detectors (Nilsson, R. Andersson, R.E. Andersson, Autio, and Åman, 2000). Recently, it was found that MW values for water-extractable and alkali-extractable arabinoxylans obtained from rye flour by GPC were much higher than 788,000 of calibrated pullulan standards (Cyran et al., 2003; Cyran, Courtin, and Delcour, 2004). Maes and Delcour (2002) reported the presence of two arabinoxylan populations, which had low apparent MW of 20,000 and less than 5,000 for water-extractable and MW of 100,000-120,000 and 5,000-10,000 for akali-extractable arabinoxylans in wheat bran determined by GPC. The MW distribution revealed the molecular heterogeneity of water- and alkali-extractable arabinoxylans in wheat bran. Most of extractable material from rye bran had an apparent MW between 10,000-1,000,000 with a weight average of 315,000 and a polydispersity index of 2.33 (Ebringerová, et al., 1994). As examined by SEC with laser light scattering, a high weight average MW of hull-less barley arabinoxylan extraction with saturated barium hydroxide reported by Storsley et al. (2003) was estimated between 640,000 and 2,220,000.

2.5.2 Structural features of arabinoxylans

Arabinoxylans are constituents of cell walls from cereal grains (Fincher and Stone, 1986). The structure of arabinoxylans have been commonly shown to contain a linear backbone of β-(1, 4) linked D-xylopyranoxyl units which are unsubstituted

(uXyl) or substutituted with the side chains of single α-L-arabinofuranoxyl units predominantly on the O-2 (2mXyl), O-3 (3mXyl) and/or both of O-2 and O-3 (dXyl) (Izydorczyk and Biliaderis, 1995; Vinkx, Stenvens, Gruppen, Grobet, and Delcour, 1995; McNeil et al., 1975), as shown in Figure 2.2. The substitution of some ferulic acids esterified at O-5 of arabinose was also reported with respect to the source of plant cell wall materials (Gubler et al., 1985).

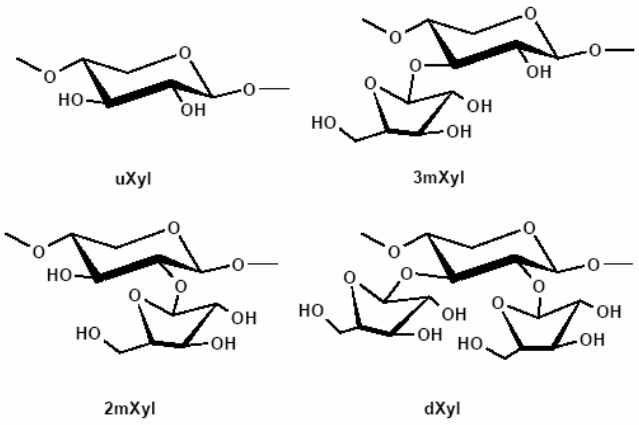


Figure 2.2 Main structural elements of arabinoxylans: (1, 4)-linked β-D xylopyranose residue (uXyl), (1, 3, 4)-linked β-D xylopyranose residue (3mXyl), (1, 2, 4)-linked β-D xylopyranose residue (2mXyl), and (1, 2, 3, 4)-linked β-D xylopyranose residue (dXyl) substituted with terminal α-L-arabinofuranose residues

Source: Andersson and Åman (2001)

Although arabinoxylans from various cereals share the same basic chemical structure composed of a β-D-xylan backbone branched with mono- or di arabinose, the degree of substitution of the xylan backbone are different and can be defined as the ratio of arabinose to xylose (Ara/Xyl). Apart from this, the manner of substitution such as the relative proportions and sequence of the various linkages between these two sugars, the position at which was substituted, and the presence of other substituents are also distinctive (Izydorczyk and Biliaderis, 1995).

Arabinoxylans from the endosperm of wheat isolated by water were found to consist of a backbone of (1, 4)-linked β-D-xylopyranosyl residues that were variously uXyl, and 3mXyl or dXly with single α-L-arabinofuranosyl groups (Hoffmann, Kamerling, and Vliegenthart, 1992). In order to study the fine structure of water extracts from wheat endosperm, Izydorczyk and Biliaderis (1994) applied a % graded ammonium sulfate ($(NH_4)_2SO_4$) to isolate several fractions. Fractions precipitated at higher saturation levels of $(NH_4)_2SO_4$ were more highly substituted, as indicated by the higher Ara/Xyl ratios rising from 0.51 to 0.90. The relative amount of xylose residues doubly substituted at O-2 and O-3 positions with arabinose increased from $(NH_4)_2SO_4$ saturation at 55%, 60%, 70%, 80%, and 100%, respectively, whereas the amount of mono- and unsubstituted xylose residues and average MW decreased in the same order. The results suggested the heterogeneity of arabinoxylans that means arabinoxylans have a variation in structure. Bengtsson and Åman (1990) studied water-extractable arabinoxylans from rye grains fractionation on a DEAE-cellulose column. The main fraction eluted with water was composed of arabinose and xylose residues in a ratio of 1: 2.1. Structure contained a main chain of (1, 4)-linked β-D xylopyranosyl residues of which about 50% were substituted at O-3 with terminal α-

L-arabinofuranosyl residues. The quantitatively second fraction was eluted with weak sodium borate and contained arabinose and xylose at a ratio of 1: 1.8. The structural units revealed more branched and double-branched xylose compared to the water eluted-fraction. More recently, the study of heterogeneity of water-extractable arabinoxylans from rye grains by fractionation on a DEAE-cellulose column revealed a low degree of disubstitution of arabinose residues (Nilsson et al., 2000). With increasing $(NH_4)_2SO_4$, 50%, 70%, and 100% respectively, rye water soluble arabinoxylan fractions precipitated with an increasing in the ratio of disubstituted to total branched xyloses, and the Ara/Xyl ratio increased from 0.55 to 1.42. In contrast to wheat arabinoxylans, the MW increased with an increasing in % $(NH_4)_2SO_4$ (Vinkx, Reynaert, Grobet, and Delcour, 1993). The first fraction, precipitating between 25 and 50% $(NH_4)_2SO_4$ had all branched xylose residues substituted at O-3 with arabinose. The second fraction, precipitating between 75 and 100% $(NH_4)_2SO_4$ had xylose residues substituted arabinose at both O-2 and O-3. To investigate the fine structure of water-extratable arabinoxylans from barley, the arabinoxylan extracts were fractionated by using a % graded $(NH_4)_2SO_4$ precipitation (Izydorczyk et al., 1998a). When the purified extracts were dissolved in phosphate buffer and fractionated by a $(NH_4)_2SO_4$ saturation, the results showed that β-glucans were most fractionated by precipitation with 35% $(NH_4)_2SO_4$ while arabinoxylans were most dissolved in the supernatant. Subfractions precipitating at higher saturation levels of $(NH_4)_2SO_4$ (>45%) contained progressively more arabinoxylans and less β-glucans. In general, arabinoxylans obtained at higher concentration of $(NH_4)_2SO_4$ were more highly substituted, as indicated by the higher Ara/Xyl ratios. In addition, methylation analysis, providing an idea of glycosidic linkage arrangement, indicated that an

increase in $(NH_4)_2SO_4$ saturation produced arabinoxylan fractions with decreasing content of un- and monosubstituted xylose residues but increasing amounts of disubstituted units. In addition to O-3 monosubstituted xylose residues, the mass spectra of alditol derivatives also indicated the presence of O-2 monosubstituted residues. The presence of small quantities of arabinan of 100% $(NH_4)_2SO_4$ subfractions also indicated that short arabinan side chains might be present in some arabinoxylans. Although the exact mechanism of salting out neutral polysaccharides, such as arabinoxylans, remains obscure, the study of Izydorczyk and Biliaderis (1992) indicated that fractional precipitation with this agent might be affected by intermolecular alignment and interaction of the unbranched portions between arabinoxylan chains.

The alkali extractable (AE) arabinoxylans from two rye flours were sequential extraction with saturated barium hydroxide solution, water, and 1 M sodium hydroxide solution, respectively and further fractionation of isolated fractions by $(NH_4)_2SO_4$ (Cyran et al., 2004). ^{1}H NMR and sugar analysis of AE subfractions that precipitated at 60% $(NH_4)_2SO_4$ were built up predominantly of uXyl and 3mXyl (55-59% and 32-37%, respectively), with relatively low level of dXyl (6-11%). The AE subfractions obtained at higher saturation levels contained progressively more dXyl residues and simultaneously less uXyl. The highly substituted arabinoxylan populations isolated at 100% saturation from all AE fractions were enriched in both 3mXyl and dXyl.

Early studies of rye arabinoxylans were investigated by Ebringerová, Hromádková, Petráková, and Hricovíni (1990). The arabinoxylans from delipidated, destarched, and depectinated rye bran were isolated by extraction with 1% ammonium

hydroxide. The structural features of rye arabinoxylans were investigated and evaluated by means of methylation analysis and ^{1}H NMR as well as ^{13}C NMR spectroscopy. The polysaccharides were composed of L-arabinose and D-xylose in the molar ratio 7.8: 10 and had MW 36,500 with polydispersity of 1.35. The backbone was shown to comprise (1, 4)-linked β-D-xylopyranose residues, with 41% unsubstituted, ~33% 2- or 3-substituted, and ~26% disubstituted. Mostly, ~80% of arabinose residues was attached to the xylan chain as single units, and the rest were 2-, 3-, and/or 5-linked as short arabinan. Subsequently, arabinoxylan-protein complex was isolated by extraction with 5% sodium hydroxide from the delipidated, destarched, depectinated, and delignified rye bran (Ebringerová et al., 1994). A highly branched arabinoxylans with arabinose residues in mono- and di-substitution represented, as evidenced by methylation analysis and ^{13}C NMR. More than 30% of the arabinose was linked in various positions probably as oligosaccharide side-chains and/or branchd (1, 5)-arabinan. The data also suggested that there were serine-carbohydrate linkages, which probably linked to arabinoxylan chains, and may be cleaved by β-elimination of alkaline treatment. The MW of arabinoxylans was reduced after treatment with pronase which could point out some covalent interactions between protein and arabinoxylans.

2.5.3 Phenolic components and oxidative gelation

Ferulic acid, a major bound phenolic acid, is known to exist ester linked mainly to position O-5 of arabinosyl side chain, and influence arabinoxylan physicochemical properties (Ishii, 1997). Feruloyl polysaccharides can interlink each other and form gel in aqueous solutions in the presence of peroxidase or some

chemicals, as shown in Figure 2.3 (Geisssman and Neukom, 1973; Hoseney and Faubion, 1981; Izydorczyk, Biliaderis, and Bushuk, 1991; Figueroa-Espinoza and Rouau, 1998). The covalent cross-linking of arabinoxylans induces gel formation with specific properties depending on their molecular features. (Rattan et al., 1994; Izydorczyk and Biliaderis, 1995; Dervilly-Pinel et.al., 2001).

The equal amounts of ferulic acid were found in all fractions of wheat arabinoxylans (1.15-1.43 mg/g) investigated by Izydorczyk and Biliaderis (1992). After treatment with an oxidizing agent (peroxidase/H_2O_2) the ability of arabinoxylan fractions to form three-dimentional gel networks was determined by oscillatory testing. It was evident that the ability of arabinoxylans to form gel networks was related to their MW. At any polymer concentration, the storage modulus was much higher for fractions with higher MW. Vinkx et al. (1991) showed that the extract of pentosan-protein fraction from rye and wheat by cold water (6 ˚C) consisted of 20% and 33% protein, 62.5% and 51.8 carbohydrates, and 0.04% and 0.08% ferulic acid, respectively. The oxidative gelation induced by the addition of H_2O_2 (0.39g/L) and peroxidase (0.2 PU/ml) showed that protein probably was not involved in oxidative process. The relative viscosities of wheat and rye pentosan-protein after oxidative gelation as a function of their concentration increased. The authors demonstrated that since the chemical composition of the pentosan-protein fraction of rye and wheat was relatively similar, the much higher relative viscosities after gelation of rye pentosan were probably due to the higher MW, as evidenced by gel chromatography.

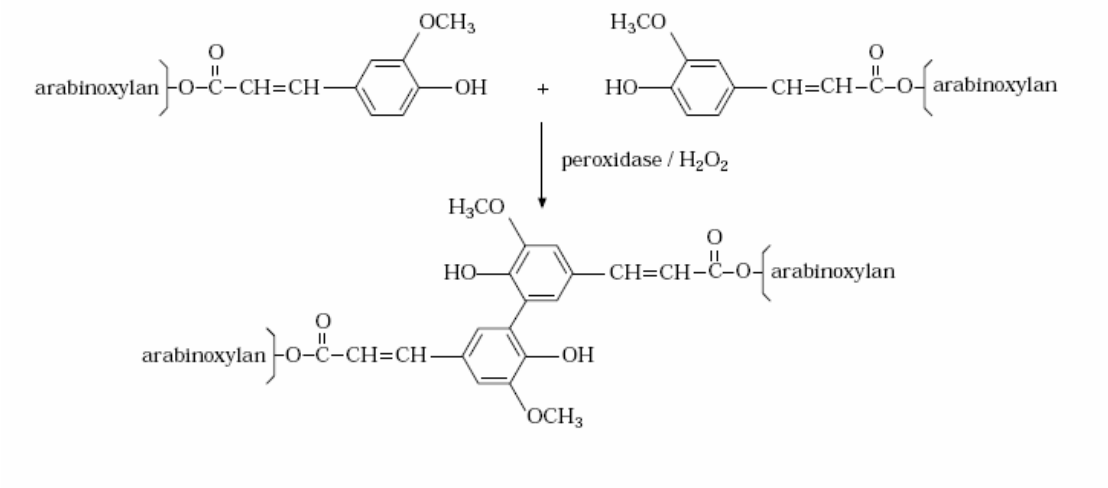


Figure 2.3 Cross-linking of arabinoxylans by formation of diferulic acid during oxidative gelation

Source: Vinkx and Delcour (1996)

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CHAPTER III

WATER-UNEXTRACTABLE ARABINOXYLANS FROM JOB'S TEARS (*COIX LACHRYMA-JOBI* L.) : COMPOSITION AND MOLECULAR CHARACTERIZATION

Abstract

Two types, dark and white husk, of polished Job's tears (*Coix lachryma-jobi* L.) were ground and refluxed with 70% ethanol. The residues were sequentially extracted with water (75 ˚C) and 0.5 M NaOH solution. Non-starch polysaccharides were not found in water-extractable (WE) fraction but present in alkali extractable fraction. The major component of alkali extracts or water-unextractable (WU) fraction from dark and white husk Job's tears was protein (47.8% and 38.4%) with similar amino acid profiles which were high in glutamic acid and methionine sulfone. In addition, both WU fractions were rich in ash contents with large amounts of phosphorus and calcium. The results revealed that WU fraction of dark husk Job's tears consisted of 6.6% arabinose, 5.3% xylose, 4.9% glucose, and 2.1% uronic acid, whereas that of white husk Job's tears contained 5.1% arabinose, 4.1% xylose, 5.6% glucose, and 1.7% uronic acid. Minor amounts of galactose and mannose were observed in both extracts. The high ratio of arabinose/xylose (Ara/Xyl), 1.25 and 1.24 for dark and white husk Job's tears, implied the presence of highly branched arabinoxylans. The arabinoxylans from dark husk Job's tears had much lower an

average molecular weight (MW) of 741,000 Da (Pd 1.5) when compared to that of 1,449,000 Da (Pd 2.6) from white husk Job's tears according with calculations from triple detectors of size exclusion chromatography. The average MW of arabinoxylans reduced after treatment with protease to 369,000 Da (Pd 2.7) and 244,000 Da (Pd 1.6), respectively. The surface activity of WU fraction decreased as the average MW and protein concentration decreased due to enzymatic hydrolysis. The WU fraction at high concentration (20% w/v) exhibited Newtonian flow behavior.

3.1 Introduction

Coix lachryma-jobi L., commonly named Job's tears, is a grain crop in the family of Gramineae (Chang, Huang, and Hung, 2003; W. Chiang, Cheng, M-T. Chiang, and Chung, 2000; Li and Corke, 1999). It has been widely cultivated in many Asian countries such as Philippine, Burma, China, Sri Lanka, and Thailand (Duke, 1983; D.A. Bender and A.E. Bender, 1999). Job's tears is variable in size, shape, color and hardness; however the edible seed is pear-shaped, around 5 mm in diameter covered with a hard, shiny dark brown to gray-black hull (Li and Corke, 1999) which is dehulled during milling process. The dehulled seed is usually polished to obtain kernel before use. This plant can also be used for forage and fodder and the bran can be used as a substitute for wheat bran in poultry feed (Purseglove, 1972).

Recently, animal and human clinical trials showed that consumption of Job's tears in various forms may potentially improve lipid metabolism, thus decreasing the risk of heart diseases. However, the active components and associated mechanism are not clear (Tsai, Yang, Su, and Chen, 1998; Yu, Chang, Liu, and Tsai, 2004). Yu, Lu, M-T. Chiang, and W. Chiang (2005) reported that water soluble polysaccharide

enriched fraction of Job's tears lowered the level of serum total cholesterol, LDL cholesterol and triglyceride in hamsters. Kim et al. (2004) also reported that the levels of triglyceride, total-cholesterol, and leptin in blood serum of rats were significantly decreased when they were fed by crude water extract of Job's tears. Water soluble β-glucans from cereals such as oats and barleys have been well known to have such hypolipidemic effects (Wood, 2002). Similarly, arabinoxylans from corn bran were also reported to reduce cholesterol level (Lopez et al., 1999). Limited information on non-starch polysaccharides (NSP) from Job's tears was reported. Therefore, NSP of Job's tears prepared by a consecutive extraction with hot water and alkali solution were studied. The chemical and physical properties of extracted NSP were then investigated.

3.2 Materials and methods

3.2.1 Materials

Two types of polished Job's tears seed, dark and white husk, grown in Thailand, were obtained from CCP Northern Co., Ltd, Thailand. Thermostable α-amylase 3,000 U/ml (45 U/mg) from *Bacillus licheniformis* was obtained from Megazyme International (Bray, Co. Wicklow, Ireland) and protease from *Streptomyces griseus* (4 U/mg) was obtained from Sigma-Aldrich. All chemicals used were of reagent grade.

3.2.2 Composition analysis of Job's tears

Moisture and ash was determined according to AOAC (2000). Protein was analyzed by an NA2100 Nitrogen and Protein Analyzer/ThermoQuest Itallia S.P.A.

EA/NA 1110 Automatic Elemental Analyzer (Strada Rivoltana, Milan, Italy), using the factor of 6.25 to convert nitrogen to protein content. Lipid content was analyzed by a 2050 Soxtec Avanti autoextraction unit connecting with a 2050 Soxtec autocontrol unit and drive unit (Foss Tecator, Sweden). Total dietary fiber content was determined using the total dietary fiber assay kit from Sigma-Aldrich based on the method of AOAC (1997).

The content of starch was determined with slight modification as described by Wood, Weisz, and Blackwell (1991). The samples were weighed with addition of thermostable α-amylase, α-amyloglucosidase, and buffer. The samples were then incubated with constant stirring and centrifuged at 8,500 rpm for 10 min. The supernatant was diluted to fit the range of glucose standard solutions and automatically analyzed as a result of the reaction of glucose with glucose oxidase from Megazyme International (Bray, Co. Wicklow, Ireland). The intensity of the reaction was read at 505 nm by colorimeter. Corn starch was used as a check of enzymatic action in each analysis.

Total sugar content was determined according to the method of Dubois, Gilles, Hamilton, Rebers, and Smith (1956). The aliquot 72% H_2SO_4 was added in the samples and hydrolyzed for 30 min at room temperature. The concentration of samples was diluted to fit the curve of glucose standard solution. The aliquot of standard and samples was added with 80% phenol, followed by the addition of concentrated H_2SO_4. The mixed solution allowed to stand for 30 min before the reading was taken by a UV-Visible spectrophotometer (Varian Cary 3C) at the absorbance of 490 nm.

3.2.3 Sample preparation

Samples were cleaned to remove dust and other defects (such as small fractured husk) before grinding and passing through a 35-mesh sieve. The ground samples were refluxed with 70% EtOH at the ratio of 1 : 10. The ethanol extract was filtered and the residue was dried at 70 °C, overnight, then reground and passed a 35-mesh sieve. This obtained sample designated as residue A.

3.2.4 Extraction of non-starch polysaccharides (NSP)

Residue A was hydrolyzed by α-amylase at 90 ºC for 30 min and then decreased temperature to 75 ºC for 2.30 hr (Figure 3.1). The sample was filtered and air-dried overnight after washing with deionized water (75 °C, 15 min), followed by once with 100% EtOH. This sample was noted as residue B. The filtrate was heated to 100 ºC for 15 min and adjusted pH to 4.5 with 2 M HCl for protein precipitation. The mixture was centrifuged at 10,000 rpm for 15 min to obtain supernatant. After dialysis against deionized water for 2 days, the supernatant was concentrated to about one-forth of its volume by vacuum rotary evaporation and then freeze-dried; the sample was assigned as water-extractable NSP. Residue B was extracted with 0.5 M NaOH for 3 hr at room temperature (25 ºC) as described by Cui, Wood, Weisz, and Beer (1999) and then centrifuged at 10,000 rpm for 15 min to obtain supernatant. After adjusted pH to 4.5, the supernatant was centrifuged and dialyzed against water for 2 days. The supernatant was concentrated to about one-forth by vacuum evaporation and then freeze-dried. The dried sample was redissolved in water and adjusted pH to 7. After centrifugation, the supernatant was freeze-dried and designated as water-unextractable NSP.

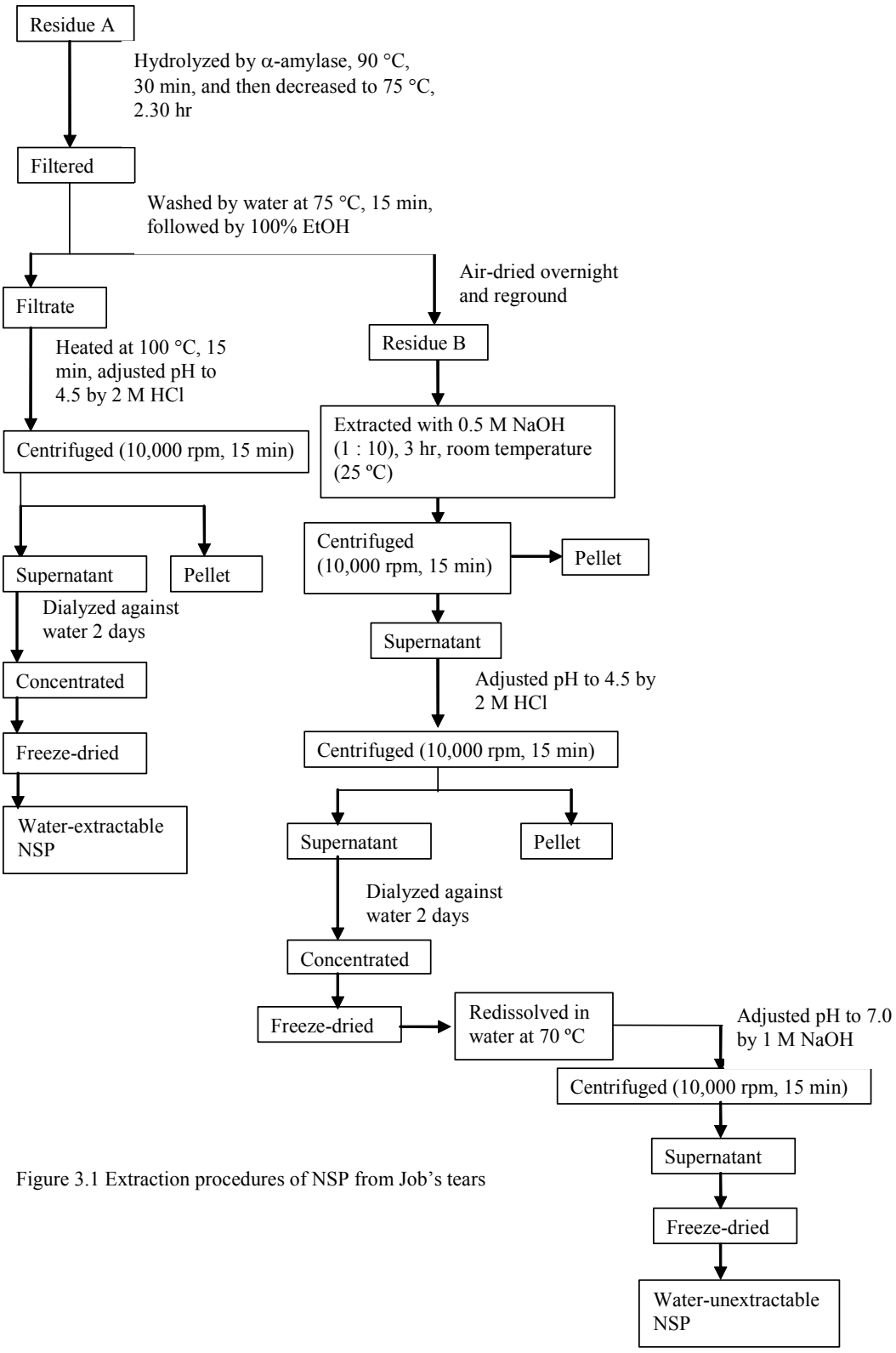

Figure 3.1 Extraction procedures of NSP from Job's tears

3.2.5 Determination of uronic acid, mineral and amino acid composition

The uronic acid content was determined according to Blumenkrantz and Asboe-Hansen (1973). The samples were dissolved in water at 50 ºC for 3 hr and diluted to fit the range of standard curve of galacturonic acid. The diluted samples, as well as standard solution was pipetted and mixed with 12.5 mM tetraborate in concentrated H_2SO_4, and then placed in a crushed ice bath. The samples were heated in a water bath at 100 ºC for 5 min and placed in the ice bath to cool down. The 0.15% m-hydroxydiphenyl reagent was added and left for 20 min at room temperature. The developed color was read by a UV-Visible spectrophotometer (Varian Cary 3C) at the absorbance of 520 nm. Mineral content was determined by Atomic Absorption Spectroscopy (AAS) and the amino acid composition was determined according to the AOAC 994.12 AA in Feeds and AOAC 985.28 Sulphur AA in Food and Feed Ingredient by Laboratory Service, University of Guelph, Canada.

3.2.6 Monosaccharide analysis

The samples were hydrolyzed in 1 M H_2SO_4 at 100 ºC for 2 hr, and diluted. The monosaccharide hydrolysates were filtered (0.45μm) and the volume of 50 μl was injected onto the High Performance Anion Exchange Chromatography (HPAEC) system (DIONEX) and differentiated by a CaboPac PA1 column connected with pulsed amperometric detection (PAD) as described by Wood, Weisz, and Blackwell (1994). Gradient elution was carried out using 100 mM NaOH and deionized water at the flow rate of 1.0 ml/min, with 8/92 to 0/100 of a linear gradient. The initial condition with 300 mM NaOH for 15 min was performed to wash column before each

sample was run. The monosaccharide content was quantitatively analyzed by comparison with known individual standard curves (rhamnose, arabinose, galactose, glucose, xylose, and mannose).

3.2.7 Molecular size distribution

According to Wang, Wood, Huang, and Cui (2003) method, molecular weight analysis was determined using High Performance Size Exclusion Chromatography (HPSEC) coupled with refractive index, right angle laser light scattering (RALLS) and viscosity detectors (Model Dual 250, Viscotek, Houston, USA). The Shimadzu SCL-10Avp pump unit (Shimadzu Scientific Instruments Inc., Columbia, MD, USA) was operated and two serially connected columns (Shodex OHpak SB-806M HQ, Showa Denko K.K., Tokyo, Japan and Ultrahydrogel linear, Waters, Milford, USA) were maintained at 40 ºC. The eluent was 100 mM sodium nitrate ($NaNO_3$) composed of 0.03% sodium azide (NaN_3), running at the flow rate of 0.6 ml/min. A set of different known molecular weight and intrinsic viscosity standard of Pullulans 100, 400, 800 were used for calibrating the detectors. A specific refractive index increment (d*n*/d*c*) of 0.146 and 0.190 ml/g was used for the MW calculation of NSP. The sample was dissolved in deionized water (5 mg/ml) at 50 ºC for 3 hr before the solution was filtered through a 0.45 μm filter and the volume of 100 μl was then injected onto the column system. In addition, the sample was monitored by HPSEC coupled with multi angle laser light scattering (MALLS), refractive index and ultraviolet detectors in order to determine the fractions of polysaccharides and proteins. The aliquot of samples was filtered (0.45 μm) and injected onto the biosep 4,000 column (phenomenex), running by 100 mM $NaNO_3$ (pH 7) at the flow rate of 0.5 ml/min.

3.2.8 d*n*/d*c* measurement

The refractive index increment (d*n*/d*c*) was determined with the BI-DNDC differential refractometer (Brookhaven) at 30 ºC and λ_0 = 535 nm. The NSP were dissolved in 100 mM $NaNO_3$. The d*n*/d*c* value was determined from the plot of the refractive index against various concentrations, ranging from 2.0 to 0.2 mg/ml to obtain the slope of the increment.

3.2.9 Intrinsic viscosity measurement

The NSP of Job's tears were dissolved in water, and then measured at 23 ± 0.05 ºC using a glass capillary viscometer (Ubbelohde viscometer, size 50 ml, USA). The relative viscosity and specific viscosity against concentration were plotted and extrapolated to zero concentration. The intrinsic viscosities were then calculated according to the Huggins (1942) and Kraemer (1938) equations. The effect of ionic on the charge of the polysaccharide solution was determined by dissolving samples in 0.05 M NaCl.

3.2.10 Surface tension of NSP

The surface tension of NSP of Job's tears was determined by a semi-automatic surface tensionmeter (Surface Tensionmat 21 model, Fisher Scientific, Toronto, Canada) at 23–24 ºC. This tensionmeter is based on the Du Nouy ring method to measure static surface tension. The various concentration of solution was placed in the dish with diameter 7.5 cm.

3.2.11 Rheological measurement

The steady shear flow measurement of NSP of Job' s tears solution with concentration at 5%, 10%, and 20% (w/v) was carried out using a Bohlin VOR rheometer. A cone and plate geometry with diameter 50 mm and cone angle 4º was used. The temperature was maintained at 23 ºC during all measurements.

3.2.12 Enzymatic treatment of NSP

The NSP were dispersed in 0.1 M phosphate buffer pH 7.5 and subjected to protease from *Streptococcus griseus* (Sigma-Aldrich) to hydrolyze protein. The mixer was placed at 37 ºC and incubated for 16 hrs. The enzyme was inactivated by heating at 100 ºC for 15 min and precipitated by centrifugation at 10,000 rpm. The supernatant was dialysed against deionized water for 2 days and then freeze-dried.

3.3 Results and discussion

3.3.1 Composition of polished Job's tears seed

The main composition of polished Job's tears seed was starch, dietary fiber, and protein with minor differences between the two types (Table 3.1). The dark husk type contained higher amount of protein and dietary fiber compared to the white husk type. The content of total dietary fiber of polished Job's tears was similar to other sources of cereal; barley, wheat, oats, corn, and sorghum (17.7, 14.9, 11.5, 13.1, and 11.5%, respectively) (Silva and Ciocca, 2005). Pornkitprasarn (1987) found 6% fat, and 1.6% ash in Job's tears flour, similar to 5.9% and 5.5% fat; 1.6 and 1.7% ash, which were obtained in the dark and white husk types, respectively. However, the

starch content of 67.5% and 69.7% present in dark and white husk types was significantly higher than it was reported by Pornkitprasarn (1987).

Table 3.1 Chemical composition of polished Job's tears seed (dry weight basis)

Components	Dark husk type	White husk type
Total sugar (%)	70.4 ± 0.35	74.4 ± 0.18
Starch (%)	67.5 ± 0.69	69.7 ± 1.63
Total dietary fiber (%)	17.4 ± 1.96	14.7 ± 1.45
Protein (%)	15.4 ± 0.05	13.6 ± 0.10
Moisture (%)	9.6 ± 0.06	9.3 ± 0.06
Fat (%)	5.9 ± 0.12	5.5 ± 0.45
Ash (%)	1.6 ± 0.00	1.7 ± 0.00

3.3.2 Extraction and composition of NSP extracts

There was no evidence of water soluble polysaccharides in both types of polished Job's tears. The major component in the water soluble extracts was determined as starch (83% dry weight), which remained after hydrolysis by amylase, and it was consequently confirmed as only glucose detected by HPAEC. This might be as a result of the physical entanglement and the degree and substitution pattern of arabinoxylans in the endosperm (Girhammar, Nakamura, and Nair, 1986), which were not able to be extracted by hot water. The covalent ester bonds between ferulic acids and other components such as lignin in the cell wall matrix is also a possible explanation (Scalbert, Monties, Lallemand, Guittet, and Rolando, 1985). The residue B of the dark husk type contained 5.56% total sugar, 1.73% arabinose, 0.36% galactose, 2.13% glucose, 1.24% xylose, and 0.10% mannose and the residue B of the

white husk type contained 5.54% total sugar, 1.63% arabinose, 0.35% galactose, 1.85% glucose, 1.25% xylose and 0.07% mannose.

The total yields of the alkali extracts or water-unextractable (WU) fractions obtained from the dark and white husk types were 0.10 and 0.09% (w/w), respectively. Protein was found to be the major component in WU fractions from both dark (47.8%) and white husk (38.4%) Job's tears (Table 3.2). Both types of Job's tears have similar amino acid profiles, being high in glutamic acid and methionine sulfone, but low in cysteic acid, phenlyalanine and isoleucine. Interestingly, it was found that the WU fraction was very rich in ash content, 24.4% in dark husk type and 30.1% in white husk type. As shown in Table 3.2, the WU fraction was high in phosphorus (P) and calcium (Ca). The majority of phosphorous in cereals was reported being associated with phytic acid (Lolas, Palamidis, and Markakis, 1976), which may occur in some regions of the seeds as a mixed calcium-magnesium-potassium salt (Lott and Ockenden, 1986). The uronic acid content expressed as galacturonic acid equivalent was 2.1% and 1.7% in dark and white husk types, respectively. Both WU fractions from dark and white husk Job's tears were composed of low content of total sugar, 18.5% and 15.2% respectively, as determined by the phenol-sulfuric acid method.

The composition of monosaccharide was analyzed by HPAEC, which peptides and amino acids did not interfere. The WU fraction of dark husk type was composed mainly of 6.6% arabinose, 5.3% xylose, and 4.9% glucose, followed by 1.0% galactose and 0.2% mannose. Similarly, the WU fraction of white husk type was high in 5.1% arabinose, 4.1% xylose, and 5.6% glucose, followed by 0.8% galactose and 0.2% mannose. The arabinose and xylose are considered to be parts of arabinoxylans

Table 3.2 Chemical analysis of WU fraction from Job's tears (dry weight basis)

Chemical Composition	Dark husk type	White husk type
Protein (%)	47.8 ± 0.19	38.4 ± 0.98
Ash (%)	24.4 ± 0.03	30.1 ± 0.13
Total sugar (%)	18.5 ± 0.55	15.2 ± 0.54
Uronic acid (%)	2.1 ± 0.14	1.7 ± 0.01
Monosaccharide (%)		
Ara	6.6 ± 0.02	5.1 ± 0.04
Xyl	5.3 ± 0.02	4.1 ± 0.06
Glu	4.9 ± 0.17	5.6 ± 0.18
Gal	1.0 ± 0.14	0.8 ± 0.04
Man	0.2 ± 0.00	0.2 ± 0.02
Ara/Xyl	1.25	1.24
Minerals (%)		
P	5.9	8.4
Ca	3.0	3.8
Na	1.5	2.2
S	0.6	0.4
Mg	0.09	0.09
K	0.02	Tr[1]
Cu	Tr	0.02
Zn	Tr	0.02
Fe	Tr	< 0.01
Cd, Cr, Ni, Pb, Mo, Co	Tr	Tr
Amino acids (%)		
Cysteic acid	1.8	2.3
Aspartic acid	5.6	5.9
Serine	4.8	5.1
Glutamic acid	19.3	18.5
Glycine	5.0	5.2
Histidine	4.5	4.5
Arginine	8.1	9.0
Threonine	2.8	2.8
Methionine sulfone	12.0	11.7
Alanine	6.3	6.0
Proline	8.7	8.4
Tyrosine	3.1	3.1
Valine	4.3	4.2
Lysine	3.8	4.4
Isoleucine	2.3	2.2
Leucine	5.4	4.9
Phenlyalanine	2.1	2.0

WU = Water-unextractable, Ara = Arabinose, Xyl = Xylose, Glu = Glucose, Gal = Galactose, Man = Mannose, [1]Tr = Trace

in cereals, possibly esterified with the ferulic acids in the cell wall of endosperm (Gubler, Ashford, Bacic, Blakeney, and Stone, 1985) and released by hydrolysis with alkali (Gruppen, Hamer, and Voragen, 1992; Gubler et al., 1985; Cui, 2001). The ratio of arabinose/xylose (Ara/Xyl) was 1.25 in dark and 1.24 in white husk types, indicating a much higher degree of branching than that was reported in arabinoxylans of wheat (0.6-0.8) (Annison, Choct, and Cheetham, 1992), barley (0.3-0.6) (Izydorczyk, Macri, and MacGregor, 1998) and ryes (0.2-0.6) (Vinkx, Stevens, Gruppen, Grobet, and Delcour, 1995). The galactose found is possibly from arabinogalactan which is also present in some cereals at low levels (Fincher and Stone, 1986; Neukom and Markwalder, 1975). Mannose might be derived from glucomannans which have been found in various cereal cell walls (Fincher and stone, 1986). The level of glucose was high although starch was removed and less than 1% was left in the residue B.

3.3.3 Molecular size distribution and intrinsic viscosity ([η])

The size exclusion chromatograph profiles of WU fraction detected by triple detectors clearly showed that there were two distinctive fractions (Figure 3.2 and 3.3) of different size. Both refractive index (RI) and viscometer detectors represented two fractions, while the light scattering (LS) detector showed only the high molecular weight fraction. This is due to the reason that LS signal is less sensitive to small molecules. After protein was hydrolyzed by protease, both peaks shifted to the longer retention volume, indicating a reduction of average molecular weight (MW) of both fractions. In fraction 1, the enzymatic-treated sample of white husk type showed much lower in average MW. Similar results were found in an arabinoxylan-protein

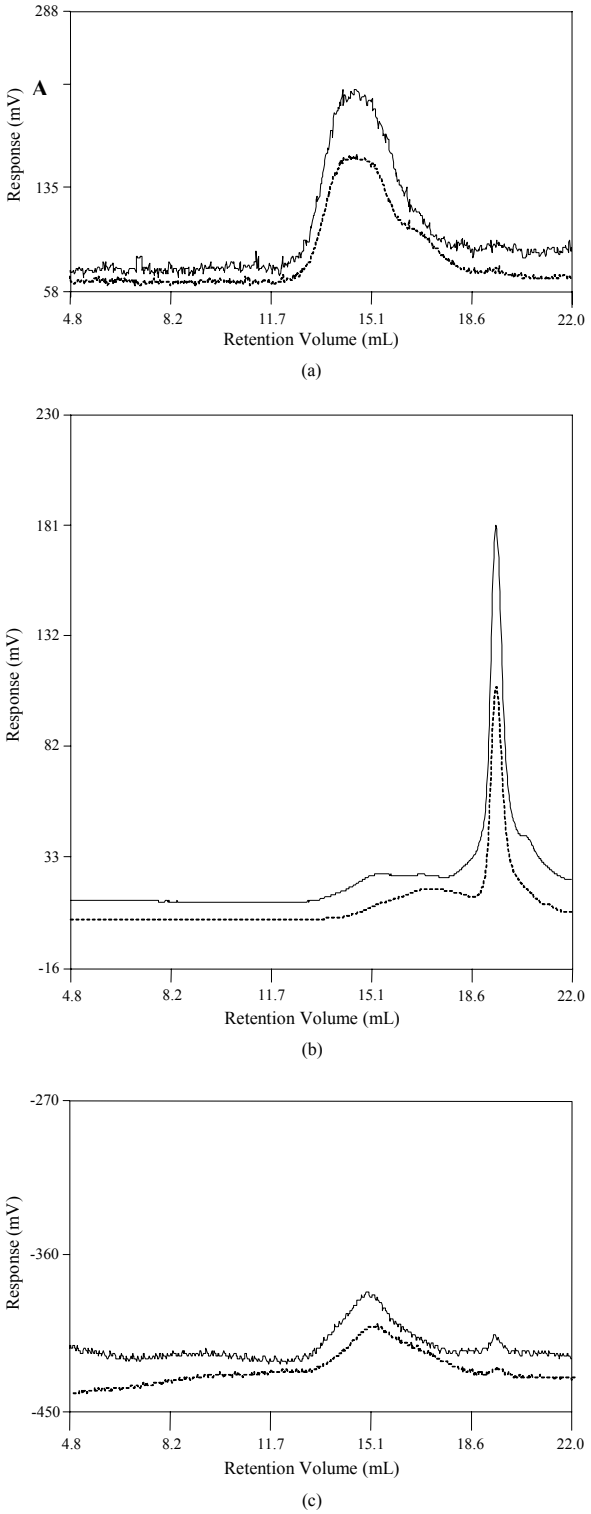

Figure 3.2 Elution profile of WU fraction from dark husk Job's tears on two serially connected columns coupled with triple detectors (a) LS = Light scattering detector, (b) RI = Reflective index detector, (c) viscometer detector
Solid line was WU fraction before enzymatic treatment.
Dashed line was WU fraction after enzymatic treatment.

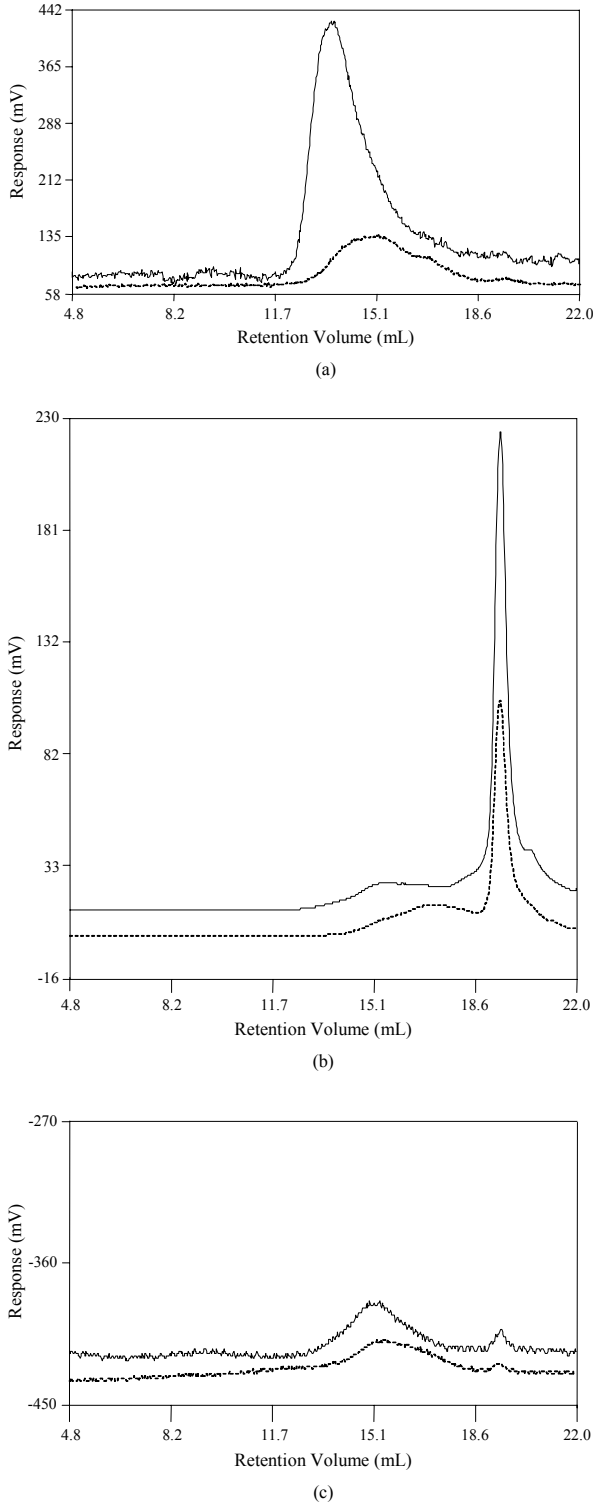

Figure 3.3 Elution profile of WU fraction from white husk Job's tears on two serially connected columns coupled with triple detectors (a) LS = Light scattering detector, (b) RI = Reflective index detector, (c) viscometer detector
Solid line was WU fraction before enzymatic treatment.
Dashed line was fraction after enzymatic treatment.

complex isolated from rye bran, indicating that MW decreased after it was treated by pronase (Ebringerová, Hromádková, and Berth, 1994). The average MW of the fraction 1 with high MW was estimated to be 741,000 (Pd 1.5) and 1,449,000 Da (Pd 2.6) in WU fraction of dark and white husk Job's tears respectively and reduced to 369,000 (Pd 2.7) and 244,000 Da (Pd 1.6) after protease treatment (Table 3.3). The strong LS signal from the high MW fraction with relatively low from RI detector was likely explained by the highly branched arabinoxylans rather than protein polymers. Fraction 2 probably was present as protein polymers with a low average MW of 3,355 and 4,372 Da because of considerable protein contents in the WU fraction. The higher value of radius of gyration (Rg) and intrinsic viscosity ([η]) was in accord with the higher average MW of polymer. The values of average MW of Job's tears arabinoxylans were within the range of hull-less barley arabinoxylans which were reported between 640,000 to 2,220,000 Da measured by laser LS (Storsley, Izydorczyk, You, Biliaderis, and Rossnagel, 2003).

To obtain more details on protein and carbohydrate fractions, the samples were run onto the biosep 4,000 column coupled with LS, RI and ultraviolet (UV) detectors. The elution profiles supported that WU fraction of dark and white husk types were composed of two fractions (Figure 3.4 and 3.5). Both fractions showed UV absorbance, suggesting the presence of protein component which might be chemically associated to carbohydrate polymers. The results were consistent with Storsley et al. (2003) who reported that arabinoxylans were associated with some extent protein through covalent interaction, as evidenced by UV detector. The glucose and protein content of WU fraction obviously reduced after enzymatic treatment while arabinose and xylose increased and the ratio remained likely the same level (Table 3.4). The results

Table 3.3 Average molecular weight (MW), radius of gyration (Rg), polydispersity (Pd), and intrinsic viscosity [η] of WU fraction by HPSEC

	MW (Da)		Rg (nm)		Pd		[η] (dl/g)	
WU fraction	F1[1]	F2[2]	F1	F2	F1	F2	F1	F2
Dark husk Job's tears								
A	741,000	3,355	44.5	1.8	1.5	1.0	3.1	0.05
B	369,000	3,032	27.2	1.6	2.7	1.8	2.2	0.04
White husk Job's tears								
A	1,449,000	4,372	52.3	1.7	2.6	2.1	3.5	0.04
B	244,000	2,382	22.9	1.4	1.6	1.0	1.7	0.03

A = Before enzymatic treatment, B = After enzymatic treatment, F1 = Fraction 1, F2 = Fraction 2

[1] d*n*/d*c* = 0.146 ml/g was used to MW calculation; [2] d*n*/d*c* = 0.190 ml/g was used to MW calculation; the averages of duplicate were expressed in data.

Table 3.4 Comparison of monosaccharide and chemical composition of WU fraction before and after enzymatic treatment

	Dark husk Job's tears		White husk Job's tears	
WU fraction	A	B	A	B
Ara (%)	6.6 ± 0.02	18.3 ± 0.05	5.1 ± 0.04	15.0 ± 0.09
Xyl (%)	5.3 ± 0.02	14.6 ± 0.00	4.1 ± 0.06	12.4 ± 0.13
Glu (%)	4.9 ± 0.17	0.60 ± 0.02	5.6 ± 0.18	0.8 ± 0.05
Gal (%)	1.0 ± 0.14	2.6 ± 0.07	0.8 ± 0.04	2.1 ± 0.10
Man (%)	0.2 ± 0.00	0.3 ± 0.10	0.2 ± 0.02	0.4 ± 0.01
Ara/Xyl	1.25	1.25	1.24	1.21
Protein (%)	47.8 ± 0.19	21.4 ± 0.30	38.4 ± 0.98	22.4 ± 0.37
Ash (%)	24.4 ± 0.03	20.8 ± 0.03	30.1 ± 0.13	25.5 ± 0.22
Uronic acid (%)	2.1 ± 0.14	4.6 ± 0.19	1.7 ± 0.01	3.5 ± 0.02

A = Before enzymatic treatment, B = After enzymatic treatment

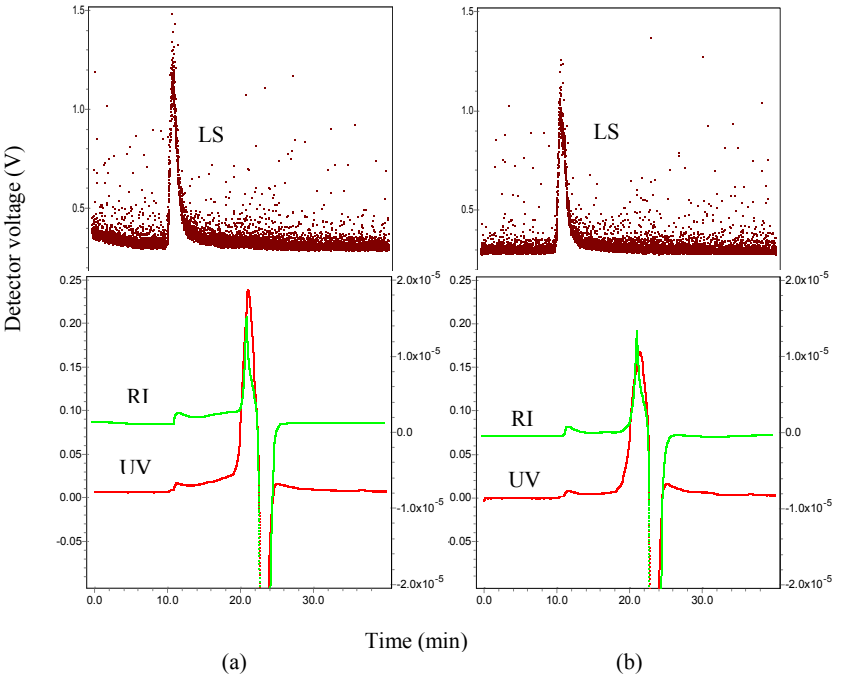

Figure 3.4 Elution profile of WU fraction from dark husk Job's tears on column coupled with triple detectors; LS = Light scattering detector, RI = Reflective index detector, UV = Ultraviolet detector
(a) = Before enzymatic treatment, (b) = After enzymatic treatment

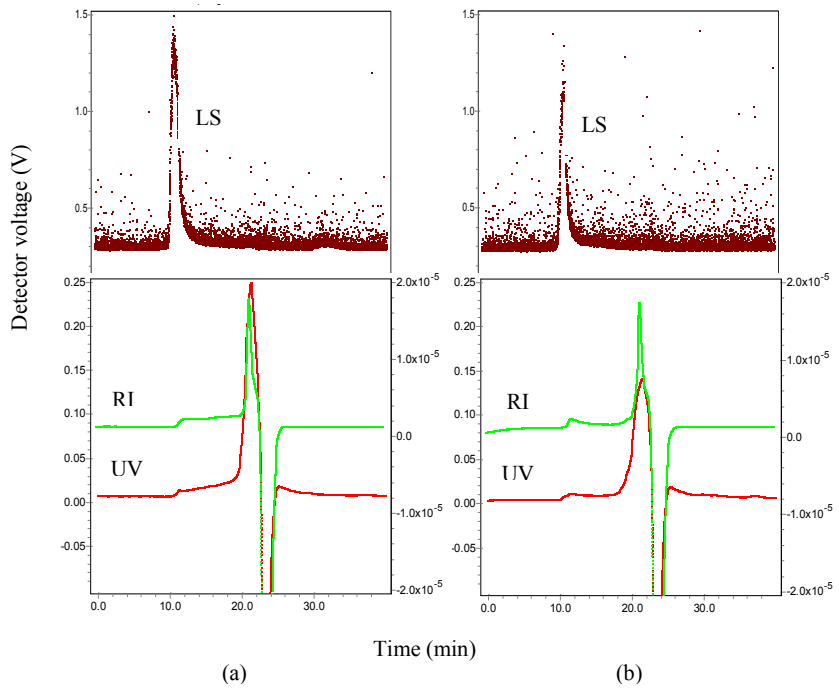

Figure 3.5 Elution profile of WU fraction from white husk Job's tears on column coupled with triple detectors; LS = Light scattering detector, RI = Reflective index detector, UV = Ultraviolet detector
(a) = Before enzymatic treatment, (b) = After enzymatic treatment

suggested that glucose may be involved in polysaccharide-protein complex which was released by protease. The values of average MW of WU arabinoxylans from polished Job's tears were higher than that was reported in dehulled Job's tears (70,000-160,000 Da) by Yamada, Yanahira, Kiyohara, Cyong, and Otsuka (1987), possibly due to variation in seed components. The values of d*n*/d*c* obtained by differential refractometer were 0.16 and 0.14 ml/g for WU fraction of dark husk Job's tears, and 0.15 and 0.14 ml/g for WU fraction of white husk Job's tears before and after enzymatic treatment, respectively. It could be explained that the reduction of protein and increment in polysaccharide content affected the value of d*n*/d*c* although the average MW significantly decreased.

Table 3.5 Comparison of intrinsic viscosity ([η]) of WU fraction determined by HPSEC and capillary viscometer

WU fraction	[η] (overall)[1] (dl/g)	[η] (water)[2] (dl/g)	[η] (0.05 M NaCl)[2] (dl/g)
Dark husk Job's tears			
A	0.29	0.26	0.25
B	0.38	0.36	0.37
White husk Job's tears			
A	0.20	0.19	0.21
B	0.29	0.27	0.26

A = Before enzymatic treatment, B = After enzymatic treatment, [1] = determination by HPSEC,

[2] = determination by capillary viscometer; the averages of duplicate were expressed in data.

Table 3.5 displayed the overall [η] values of a combination of two fractions. The [η] values determined by HPSEC were slightly higher than those by capillary viscometer. The non-enzymatic and enzymatic-treated samples of dark husk Job's

tears were higher in [η] than those of white husk Job's tears. Likewise, the [η] of the both enzymatic-treated samples increased from 0.29 to 0.38 dl/g and 0.20 to 0.29 dl/g probably because of higher concentration of polysaccharides (Table 3.4). In comparison, the [η] values were not significantly different when WU fraction dissolved in either deionized water or 0.05 M NaCl. The results implied that the polysaccharides have low degree of charge in agreement with the existence of low uronic acid.

3.3.4 Surface tension and rheological flow behavior

The relationship between surface tension and concentration of two WU fractions of Job's tears, before and after enzymatic treatment, was shown in Figure 3.6 and 3.7. The results indicated that the reduction of an average MW and protein concentration affected the surface activity of WU fraction. The surface activity reduced when the average MW and protein concentration reduced according to enzymatic treatment, although the concentration of polysaccharides increased. It appeared that the surface tension of water decreased constantly with increasing concentration of non-enzymatic treated WU fraction.

The steady shear flow curves of WU fraction of Job's tears were present in Figure 3.8. Similar to that of white husk Job's tears, WU fractions of dark husk Job's tears before and after enzymatic treatment exhibited Newtonian flow behavior, which the apparent viscosity is independent on shear rate, even at high concentration (20% w/v). The weak viscosity of WU fraction might be contributed to the great distribution of substituted arabinose to xylose residues (Bengtsson, Andersson, Westerlund, and Åman, 1992), and its combination with high protein and minerals.

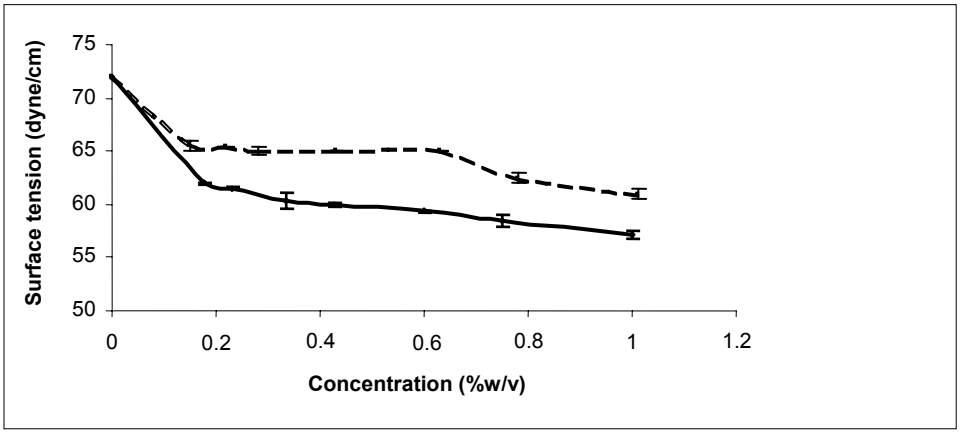

Figure 3.6 Surface tension of WU fraction from dark husk Job's tears at various concentrations at 23-24 °C; Solid line was WU fraction before enzymatic treatment. Dashed line was WU fraction after enzymatic treatment.

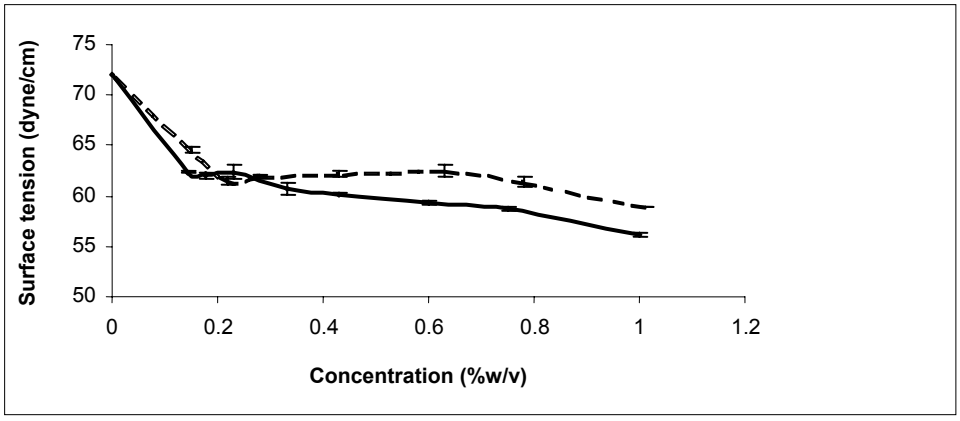

Figure 3.7 Surface tension of WU fraction from white husk Job's tears at various concentrations at 23-24 °C; Solid line was WU fraction before enzymatic treatment.

Dashed line was WU fraction after enzymatic treatment.

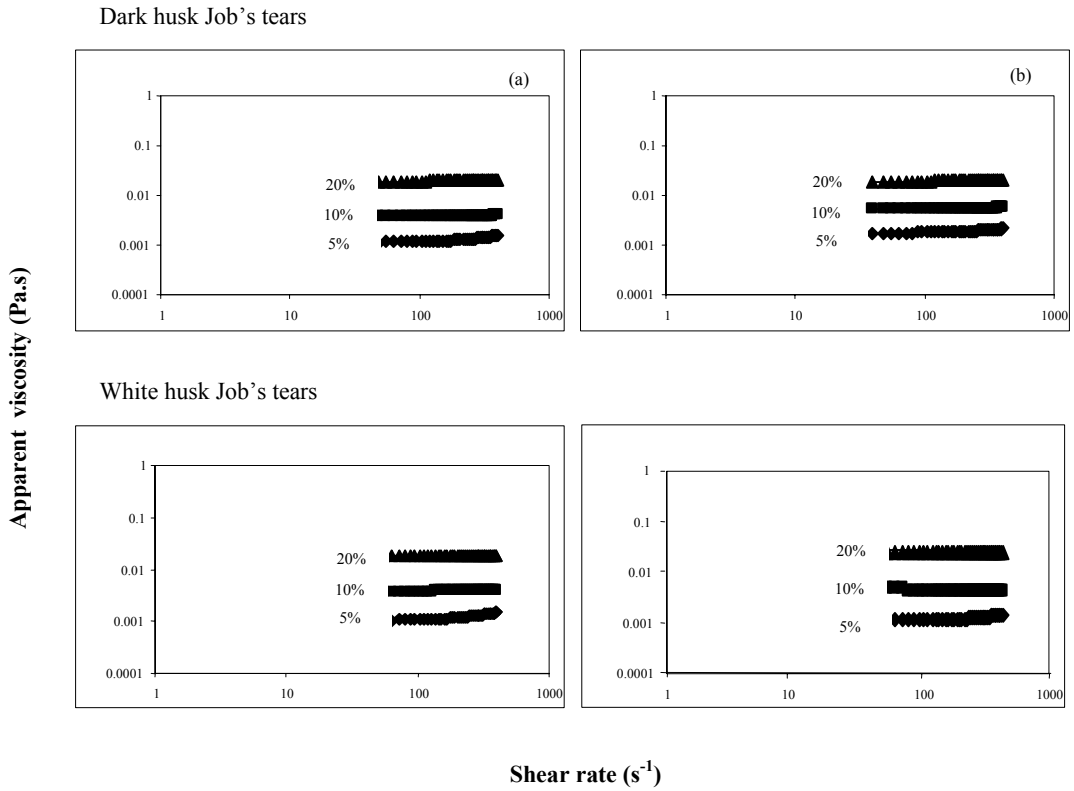

Figure 3.8 Steady shear rheological flow behavior of WU fraction from dark and white husk Job's tears; (a) = Before enzymatic treatment, (b) = After enzymatic treatment

3.4 Conclusion

The WU fraction from dark and white husk Job's tears similarly consisted of water-unextractable arabinoxylans with a high content of protein and minerals. The Ara/Xyl ratio of arabinoxylans was higher than those from other cereals, indicating its high degree of branching with arabinose. The arabinoxylans from dark husk Job's tears had a lower average MW than from white husk type. The average MW reduced after treatment with protease which probably could represent covalent cross linkages between arabinoxylans and protein. WU fraction could reduce surface tension of water which was influenced by its average MW and protein content. The Newtonian

flow behavior at high concentration (20% w/v) of WU fraction was possibly affected by highly substituted arabinoxylans with high protein content and minerals.

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CHAPTER IV

STRUCTURAL CHARACTERIZATION OF WATER-UNEXTRACTABLE ARABINOXYLANS FROM JOB'S TEARS (*COIX LACHRYMA-JOBI* L.)

Abstract

Structural features of water-unextractable (WU) arabinoxylans from polished Job's tears were investigated by methylation analysis, 1D and 2D NMR spectroscopy. The high arabinose/xylose (Ara/Xyl) ratio of 1.27 and 1.25 indicated highly branched WU arabinoxylans extracted from dark and white husk Job's tears, respectively. The WU arabinoxylans were elucidated to be (1, 4)-linked β-D-xylan main chain with highly branched side chains, containing a single arabinose. The results showed α-L arabinofuranosyl residues (Ara*f*) were attached as branched sides mostly at O-3, followed by at both O-2 and O-3 of xylopyranosyl residues (Xyl*p*).

4.1 Introduction

Job's tears (*Coix lachryma-jobi* L.) is a cereal grain crop in the family of Gramineae, mostly cultivating in Asia. Previously, the study was carried out with the extraction of non-starch polysaccharides in polished Job's tears. The water-unextractable non-starch polysaccharides in Job's tears have been shown to compose of arabinoxylans. The results revealed that the neutral sugar composition contained

arabinose, xylose, and glucose, including small amounts of galactose and mannose. Although the extracted polysaccharides contained a high degree of protein and minerals, it appeared that they have high weight average molecular weight (MW) of arabinoxylans, representing 741,064 and 1,449,488 Da with the arabinose/xylose (Ara/Xyl) ratio of 1.25 and 1.24 in dark and white husk Job's tears respectively. It is assumed that some amounts of protein are an integral part of the arabinoxylan structure. The water-unextractable arabinoxylans in wheat and barley flour have been reported to contain a backbone of (1, 4)-linked xylopyranosyl residues which are substituted with arabinofuranosyl residues, mainly on O-3, both O-2 and O-3, as well as to some extent to O-2 position (Viëtor, Angelino, and Voragen, 1992; Gruppen, Hamer, and Voragen, 1992b). The degree of branching and polymerization varies, depending on the species and varieties. It is important to study the structural characterization of polysaccharides which are known to be related to their physical and functional properties. In this Chapter, the water-unextractable (WU) arabinoxylans were isolated from polished Job's tears. Glycosidic linkage distribution and structural details were elucidated with the combination of methylation analysis, 1D and 2D NMR.

4.2 Materials and methods

4.2.1 Extraction of WU arabinoxylans

Dark and white husk Job's tears seeds, cultivated in Thailand, were obtained from CCP Northern Company. The seeds were ground and passed a 35 mesh sieve. The ground sample was refluxed with 70% EtOH at the ratio of 1:10, 70 °C for 3 hr. The residue was dried at 70 °C, overnight and then reground to pass a 35 mesh sieve,

designated as residue A. Residue A was hydrolyzed by α-amylase to remove starch. The destarched residue was then extracted with 0.5 M NaOH to obtain WU arabinoxylans according to the method described previously in Chapter III.

4.2.2 Purification of WU arabinoxylans

The WU arabinoxylans were dispersed in phosphate buffer pH 7.5 (0.1 M) and subjected to protease from *Streptococcus griseus*, obtained from Sigma-Aldrich for protein removal and then they were placed at 37 ºC 24 hr. The enzyme was inactivated by heating at 100 ºC for 15 min and precipitated by centrifugation (10,000 rpm). The supernatant was dialyzed against water for 2 days, concentrated and precipitated by 3 volumes of 100% EtOH, and then the residue was air-dried.

4.2.3 Monosaccharide analysis

The sample of 10 mg was hydrolyzed in 1 ml of 1 M H_2SO_4 at 100ºC for 2 hr and diluted (10X). The monosaccharide hydrolysates were filtered (0.45μm) and the volume of 50 μl was injected onto the CaboPac PA1 column of DIONEX High Performance Anion Exchange Chromatography (HPAEC) system, connected with pulsed amperometric detector (PAD) as described earlier by Wood, Weisz, and Blackwell (1994). Gradient elution was run using 100 mM NaOH and deionised water at the flow rate of 1.0 ml/min at room temperature. The linear gradient was with 8/92 to 0/100 in 17 min, and maintained for 28 min. The initial condition with 300 mM NaOH for 15 min was performed to wash column before each sample was run. The monosaccharide content was quantitatively analyzed by comparison with known standards (rhamnose, arabinose, galactose, glucose, xylose, and mannose).

4.2.4 Methylation analysis and GC-MS

Methylation analysis was carried out using a modified method of the Ciucanu and Kerek (1984). The purified sample (2-3mg) was dried in a vacuum oven at 80 ºC for 4-5 hr and kept in a desiccator under vacuum with phosphorous pentoxide. The volume of 0.5 ml anhydrous dimethylsulfoxide (DMSO) was added under the nitrogen in order to solubilize the sample by stirring in water bath at 60 ºC for 2 hr, followed by sonicating for 2 hr. The approximate 20 mg of dry powdered NaOH was added and the mixture was stirred at room temperature for 2 hr. After the addition of methyl iodide (0.3ml), the mixture was stirred for an additional 2 hr. The partially methylated polysaccharides were extracted by the addition of methylene chloride (3-5ml) and the excess of NaOH was removed by mixing with deionized water. After phase separation, the water layer was removed and discarded. The solvent layer was passed through an anhydrous sodium sulphate column, and filtrate mixture was then collected and dried under a stream of nitrogen. The partially dried methylated polysaccharides were hydrolyzed in 4 M trifluoroacetic acid (TFA) (0.5 ml) at 100ºC for 6 hr and the TFA was evaporated by a stream of nitrogen. The residue was dissolved in deionized water (0.3 ml) and 1% NH_4OH (1 drop). The acid hydrolysates were then reduced with the addition of sodium borodeuteride (1-5 mg) and kept at room temperature for at least 12 hr. The glacial acetic acid was added drop by drop to remove the excess reagent. The mixture was treated with addition and evaporation of 0.5 ml of 5% acetic acid-methanol, followed by of 100% methanol as many times as was necessary to remove the excess borate. The residue was subsequently acetylated with acetic anhydride (0.5 ml) at 100 ºC for 2 hr and evaporated to dryness. The volume of 0.5 ml methylene chloride was added to dissolve the partially methylated

alditol acetates (PMAA) and passed through an anhydrous sodium sulphate column. Aliquots of solution (1 μL) were injected onto the GC-MS system (ThermoQuest Finnigan, San Diego, CA). The SP-2330 column (Supelco, Bellefonte, PA) coated with fused silica capillary (30 m X 0.25 mm, 0.2 μm film thickness) and equipped with an ion trap MS detector. The flow rate of 1 ml/min of helium was used as the gas carrier combined with the condition controlled in the range of 160-210 ºC at a rate of 2 ºC/min, and held at 210 ºC for 8 min. The temperature was then increased to 240 ºC at a rate of 5 ºC/min and held for 6 min.

4.2.5 NMR spectroscopy

Purified arabinoxylans was dissolved in D_2O and freeze-dried three times and finally dissolved in D_2O (4% w/v) before NMR analysis. The ^{1}H NMR and ^{13}C NMR spectra were recorded at 30 ºC on Bruker AMX-500 FT spectrometer. Frequency pulse angle 45º with presaturation was recorded for each sample. Tetramethylsilane (TMS) was used as an internal reference for both ^{1}H and ^{13}C NMR. Two-dimensional NMR (HMQC, COSY, TOCSY, and HMBC) were performed using the standard Bruker pulse sequence.

4.3 Results and discussion

4.3.1 Extraction and purification of WU arabinoxylans from Job's tears

Preliminary study indicated that WU arabinoxylans extracted by 0.5 M NaOH was composed of significantly high protein content (47.8% and 38.4% in dark and white husk types, respectively). The WU arabinoxylans was then subjected to protease in order to hydrolyze the excessive protein. The monosaccharide

composition of purified WU arabinoxylans of dark and white husk types was shown in Table 4.1.

Table 4.1 Monosaccharide composition of purified WU arabinoxylans of Job's tears (dry weight basis)

Monosaccharide (%)	Job's tears	
	Dark husk type	White husk type
Ara	32.3 ± 0.03 (6.6 ± 0.02)*	29.2 ± 0.41 (5.1 ± 0.04)
Xyl	25.4 ± 0.03 (5.3 ± 0.02)	23.3 ± 0.05 (4.1 ± 0.06)
Gal	2.4 ± 0.06 (1.0 ± 0.14)	4.3 ± 0.11 (0.8 ± 0.04)
Glu	- (4.9 ± 0.17)	0.9 ± 0.09 (5.6 ± 0.18)
Man	- (0.2 ± 0.00)	0.2 ± 0.02 (0.2 ± 0.02)
Ara/Xyl	1.27 (1.25)	1.25 (1.24)

*Monosaccharide content before deproteinization (protease treatment)

Ara = Arabinose, Xyl = Xylose, Gal = Galactose, Glu = Glucose, Man = Mannose

The results indicated that the monosaccharide content increased after parts of protein were hydrolyzed. The purified WU arabinoxylans of dark husk seed was relatively high composed of 32.3% arabinose and 25.4% xylose, as well as small amounts of 2.4% galactose. In addition to 29.2% arabinose, 23.3% xylose, and 4.3% galactose, minor component of glucose (0.9%) and mannose (0.2%) was found in WU arabinoxylans of white husk seed. After protease treatment, the Ara/Xyl ratio of WU arabinoxylans of dark husk seed analyzed by HPAEC exhibited 1.27 and 1.25 for WU arabinoxylans of white husk seed. These results suggested that enzymatic protein removal did not affect the ratio of Ara/Xyl of WU arabinoxylans. The reduction of

glucose and mannose was assumed that they might be lost as a result of treatment with enzyme.

4.3.2 Methylation analysis

Methylation analysis was carried out to determine the presence of the glycosidic linkages of polysaccharides. After methylation, the individual peak of PMAA and fragmentation patterns was identified by their retention time in GC and comparison with mass spectra patterns in the literature (Carpita and Shea, 1989) as well as on the basis that the configurations of arabinose and xylose in arabinoxylans of cereals are L-furanose and D-pyranose, respectively. The analysis of PMAA and linkage-patterns of WU arabinoxylans was shown in Table 4.2.

The results revealed that WU arabinoxylans of dark and white husk types had similar linkages. The arabinofuranosyl residues (Ara*f*) and xylopyranosyl residues (Xyl*p*) were assigned to arabinoxylans which were mainly composed of Ara*f* present as non-reducing terminal units (T-Ara*f*) with respect to a high level product of 2, 3, 5-tri-O-methyl arabinitol. The T-Ara*f* reflected that one unit of Ara*f* as side chain attached to (1, 4)-linked xylan. The present Ara*f* were highly substituted at the xylan backbone through position O-3 of the individual Xyl*p*, observed from the high presence of 2-O-methyl pentitol and 2, 4-di-O-methyl pentitol, followed by O-2 and O-3 on the same Xlyp, observed from 1-deuterio pentitol pentaacetate and 4-O-methyl pentitol, respectively. The occurrence of unsubstituted Xyl*p* residues was evidenced as 2, 3-di-O-methyl pentitol. The dark husk WU arabinoxylans ratios of branched to unbranched-Xyl*p* and single-branched to double-branched Xyl*p* were of 4.6 and 1.2, while those of white husk WU arabinoxylans were of 5.4 and 2.4, respectively. There

was no O-2 linked Ara*f* observed in this study. In both dark and white husk Job's tears arabinoxylans, terminal Ara*f* were predominantly linked at O-3 of Xyl*p* similar to water-extractable arabinoxylans reported in wheat flour (Gruppen, Hamer, and Voragen, 1992a) and rye grain (Bengtsson and Åman, 1990). The Ara*f*/Xyl*p* ratio of WU arabinoxylans of dark husk type was 0.6 similar to 0.5 of white husk type. However, it was noticed that the Ara*f*/Xyl*p* ratio was lower than that was determined by HPAEC, possibly because conversion to methylated sugar was not completed or Ara*f* were lost regarding rapid evaporation. The galactopyranosyl residues (Gal*p*) were proposed to be (1, 4)- and (1, 6)-linked backbone of arabinogalactans (Fincher and Stone, 1986) with Ara*f* attached at O-2 as seen by 3, 6-di-O-methyl hexitol.

Table 4.2 Methylation analysis and mode of linkage of WU arabinoxylans from Job's tears

Methylated sugar	Deduced linkage	Molar ratio*	
		Dark husk type	White husk type
2, 3, 5-tri-O-methyl arabinitol	T-Ara*f*	3.6	3.1
2, 3-di-O-methyl pentitol	1, 4-Xyl*p*	1.0	1.0
2-O-methyl pentitol	1, 3, 4-Xyl*p*	2.4	3.7
1-deuterio pentitol pentaacetate	1, 2, 3, 4-Xyl*p*	1.4	1.4
4-O-methyl pentitol	1, 2, 3-Xyl*p* (end)	0.7	0.2
2, 4-di-O-methyl pentitol	1, 3-Xyl*p* (end)	0.1	0.1
2, 3, 6-tri-O-methyl hexitol	1, 4-Gal*p*	1.1	0.5
2, 3, 4-tri-O-methyl hexitol	1, 6-Gal*p*	0.4	0.5
3, 6-di-O-methyl hexitol	1, 2, 4-Gal*p*	0.5	0.3
2, 3, 4, 6-tetra-O-methyl hexitol	1-Gal*p* (end)	0.4	0.1

* Relative molar ratio calculated from the ratio of the peak height and normalized to 1, 4-Xyl*p*

4.3.3 ^{1}H NMR, ^{13}C NMR and 2D NMR analysis

Since there were no major differences between the linkages of WU arabinoxylans from dark and white husk types, the former was given to discuss on NMR analysis. The structural features of arabinoxylans were further elucidated by analysis of NMR spectra. On the basis of the literature for cereal non-starch polysaccharides, the signals in the region of 5.2-5.4 ppm by ^{1}H NMR analysis were assigned to anomeric protons of α-L-linked-Ara*f*, whereas, the signals at 4.4-4.8 ppm were assigned to anomeric protons of β-D-linked-Xyl*p* (Izydorczyk and Biliaderis, 1992; Annison, Choct, and Cheetham, 1992; Bengtsson and Åman, 1990; Westerlund, Andersson, and Åman, 1993; Ebringerová, Hromádková, and Berth, 1994; Ebringerová, Hromádková, Petráková, and Hricovíni, 1990; Hoffmann, Kamerling, and Vliegenthart, 1992). By comparing the chemical shift with known spectra, the region of the observed values at 5.10-5.38 ppm given by ^{1}H NMR spectra (Figure 4.1) was assigned to the typical signals of anomeric protons of Ara*f*. The high signal at 5.38 ppm was attributed to anomeric proton of α-L terminal Ara*f* linked to position O-3 of Xyl*p*. The two signals of similar relative intensity at 5.20 and 5.25 ppm were responsible for anomeric proton of α-L-Ara*f* linked to O-2 and O-3 of the double-branched Xyl*p*, respectively. The results suggested that Xyl*p* were highly substituted at O-3 by α-L-Ara*f* in accordance with the methylation data. There was an additional resonance for anomeric proton at 5.10 ppm which might be associated to β-L-Ara*f* (Ebringerová et al., 1990). However, this regional signal did not appear in ^{13}C NMR spectra. The unsubstitution of Xyl*p* was reflected by the broad resonance at 4.46 ppm, and the two small peaks at 4.58 and 4.61 ppm were assigned to mono- and disubstituted Xyl*p*, respectively. The spectra did not display resonances in the region

of phenolic moieties between 6-8 ppm (Saulnier et al., 1999) in ^{1}H NMR, explaining that there was no phenolic proportion in WU arabinoxylans fraction.

The ^{13}C NMR analysis was used for more accurate identification of the complexity of the structural features of arabinoxylans. The spectra exhibited the well resolved-signal characteristics of Ara*f* and Xyl*p* as shown in Figure 4.2. With respect to anomeric carbons, α-L-linked Ara*f* observed at 111.335, 110.779, and 110.338 ppm were identified as O-2 and O-3, and O-3 linked to individual Xyl*p*, compared with those values reported in the literatures (Hoffmann, Roza, Maat, Kamerling, and Vliegenthart, 1991; Hoffmann et al., 1992; Annison et al., 1992; Bengtsson and Åman, 1990; Ebringerová et al., 1994; Nandini and Salimath, 2002; Izydorczyk and Biliaderis, 1992; Izydorczyk and Biliaderis, 1993). The resonance due to anomeric carbons of β-D-linked Xyl*p* at 103.927, 102.528, and 100.362 ppm were assigned to unsubstituted, monosubstituted at O-3, and disubstituted at O-2 and O-3 Xyl*p*, respectively. ^{1}H and ^{13}C NMR spectra showed the lower signal intensity of substituted than unsubstituted xylan possibly because of shielded electrons. The minority of galactose was not assigned in the spectra, probably because of low degree of intensity. Signals of the carboxyl carbons at lower field between 170-180 ppm (Cui, 2005) were not observed, verifying the absence of uronic acid.

Figure 4.3 was given to show the proposed structure of arabinoxylans so that the discussion of 2D NMR was facilitated. The two-dimensional Heteronuclear Multiple Quantum Coherence (HMQC) applied to show correlation of ^{1}H and ^{13}C peaks (Figure 4.4). The spectrum of distinct three cross peaks in its anomeric region of Ara*f* were observed. The C-1 signal at 111.335 and 110.779 ppm, assigned to α-L-Ara*f* linked to O-2 and O-3 of Xyl*p* (residue C and F, Figure 4.3), showed cross peak with

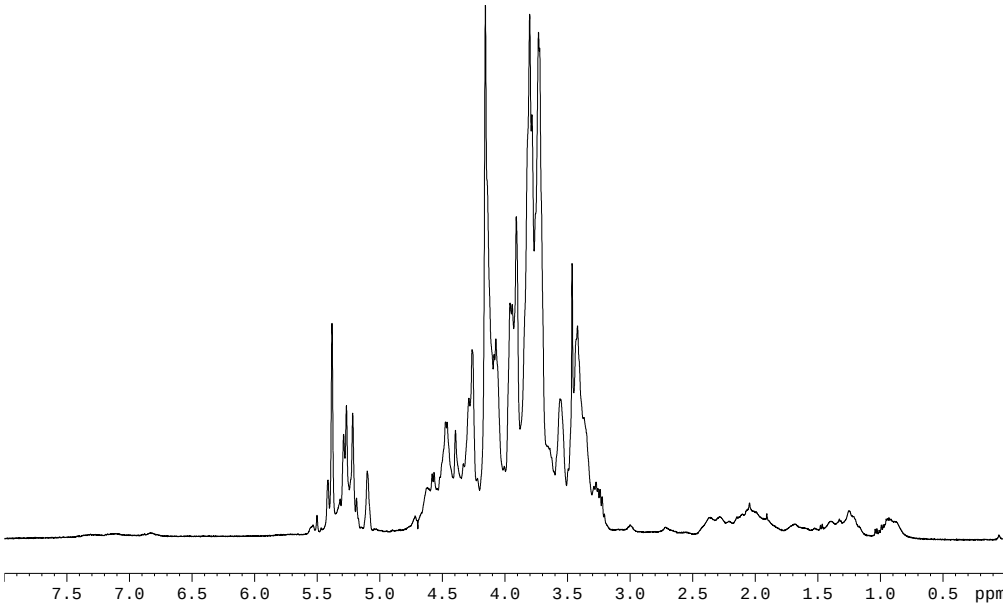

Figure 4.1 ^{1}H NMR spectra of Job's tears WU arabinoxylans relative to internal TMS

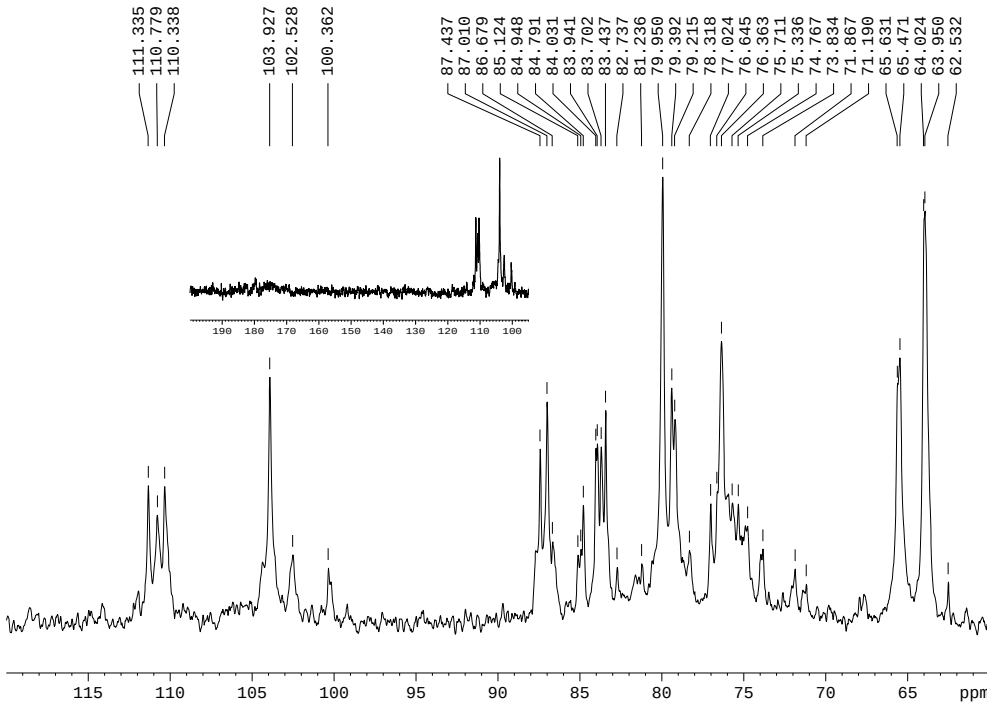

Figure 4.2 ^{13}C NMR spectra of Job's tears WU arabinoxylans, relative to internal TMS

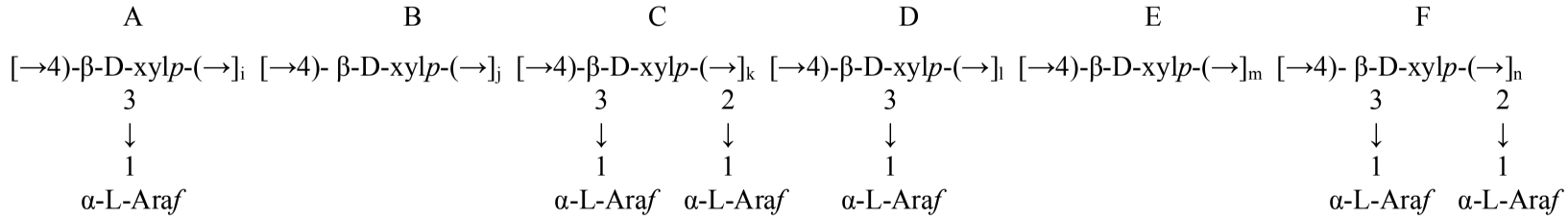

Figure 4.3 Proposed structures of WU arabinoxylans from Job's tears

H-1 resonance at 5.20 and 5.25 ppm, respectively. The C-1 at 110.338 ppm was assigned to α-L-Ara*f* linked to O-3 of Xyl*p* (residue A and D, Figure 4.3) since it was connected to H-1 resonance at 5.38 ppm. It was noted that H-1 at 5.10 ppm assigned to β-L-Ara*f* was not correlated with C-1 region of Ara*f* but C-1 region of Xyl*p*. The H-1 at 4.46 ppm was assigned to unsubstituted β-D-xylopyranosyl core (residue B and E, Figure 4.3), showing correlation to C-1 at 103.927 ppm. The cross peak between C-1 and H-1 resonances of mono- and disubstituted Xyl*p* was not observed in this spectrum.

The proton-proton connectivity of sugar ring was determined by the $^{1}H/^{1}H$ correlation spectrosopy (COSY) (Figure 4.5). The spectrum assigned the chemical shifts of anomeric protons from O-2 and O-3 linked-Ara*f* coupling with respective H-2s but the corresponding of H-1 of O-3 linked-Ara*f* with H-2 was not assigned in this spectrum. In addition, The H-1s of mono- and disubstituted Xyl*p* and respective H-2s were assigned. Due to limited resolution in COSY spectrum as a result of very crowded H-3, H-4, and H-5 protons, it was not clear to assign correlation between these cross peaks.

The total $^{1}H/^{1}H$ correlation spectroscopy (TOCSY) showed the resonances of protons on the same sugar. The TOCSY spectrum provided all intra residue assignments of all protons on each O-3, and O-2 and O-3 linked-Ara*f* as shown in Figure 4.6. Three resonances of mono-, di-, and unsubstituted Xyl*p* were also present in the spectrum.

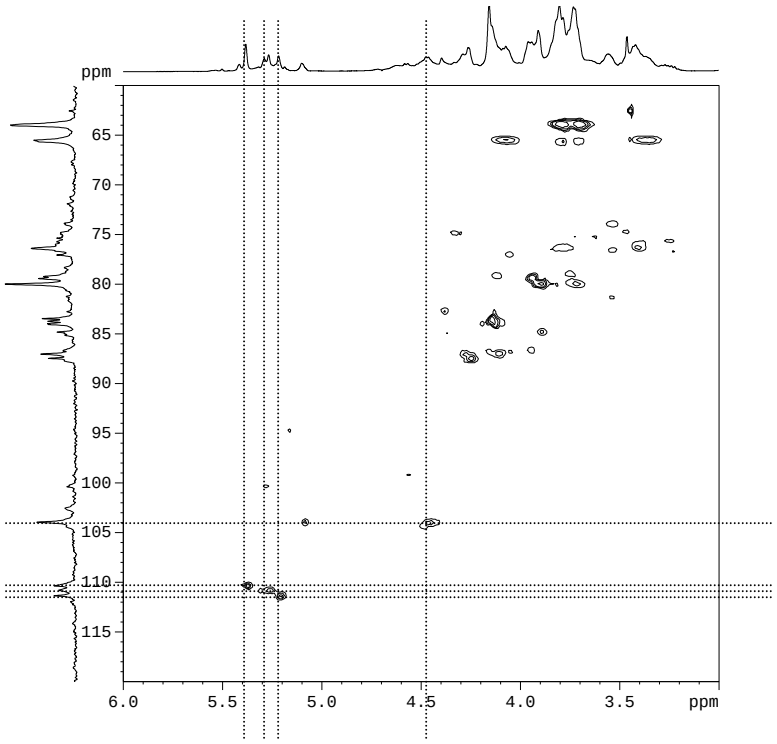

Figure 4.4 $^{1}H/^{13}C$ HMQC correlation of Job's tears WU arabinoxylans

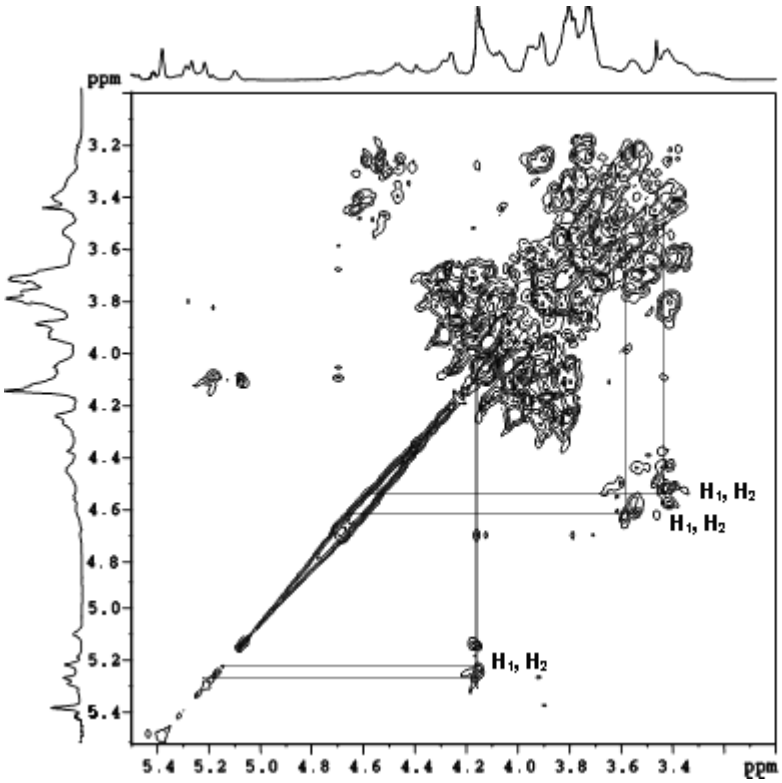

Figure 4.5 $^{1}H/^{1}H$ NMR COSY of Job's tears WU arabinoxylans

To establish the linkage and sequence of the xylan backbone and linked-Ara*f*, the long-range heteronuclear correlation spectrum (HMBC) was investigated (Figure 4.7). Figure 4.7 showed that the cross peaks at H-1/C-3 of O-3 linked-Ara*f* represented the residue A and D, and those at H-1/C-2 of O-3 and O-2 linked-Ara*f* represented the residue C and F in the proposed structures (Figure 4.3). All peak assignments according with ^{1}H and ^{13}C NMR, COSY, TOCSY, HMQC, and HMBC along with the literature were summarized in Table 4.3.

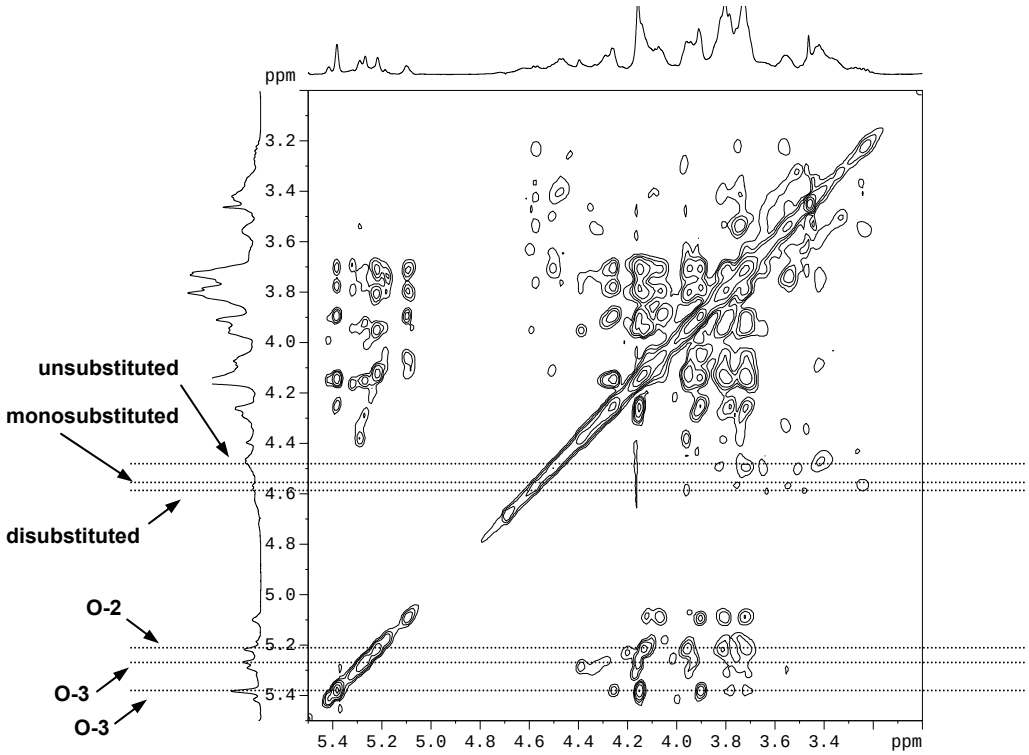

Figure 4.6 $^{1}H/^{1}H$ NMR TOCSY of Job's tears WU arabinoxylans

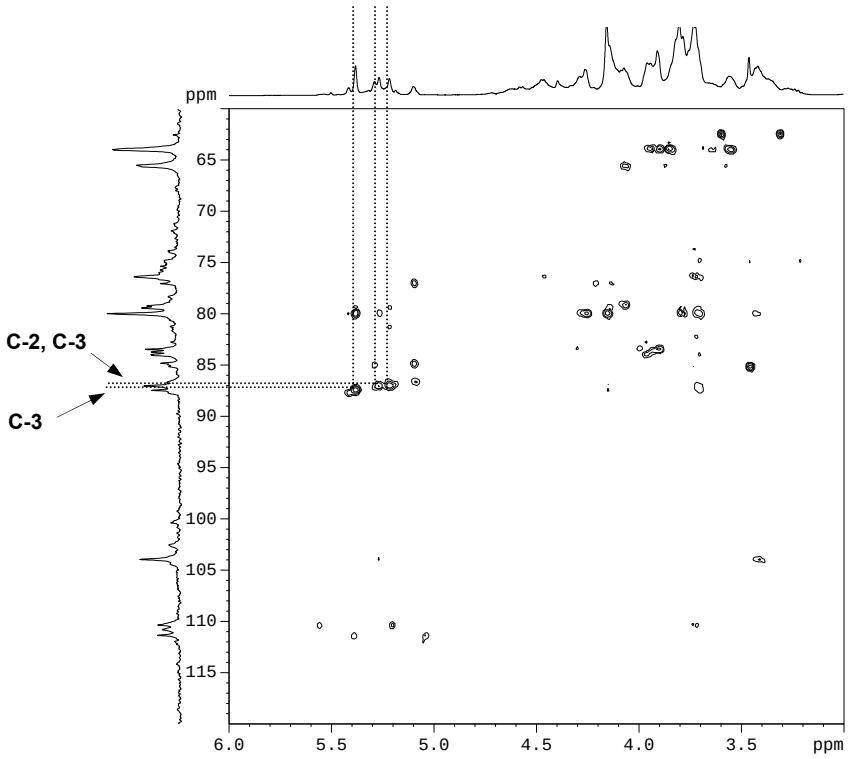

Figure 4.7 $^{1}H/^{13}C$ HMBC correlation of Job's tears WU arabinoxylans

4.4 Conclusion

WU arabinoxylans isolated from dark and white husk Job's tears had a high Ara/Xyl ratio of 1.27 and 1.25, indicating a highly branched structure. There were no significant differences in linkage structure between two types, as determined by methylation analysis. The terminal α-L-Ara*f* were predominantly linked to O-3 of the backbone of (1, 4)-linked β-D-Xyl*p* and some were also substituted at both O-2 and O-3. The highly branched structure of WU arabinoxylans can be expected to affect its physical properties.

Table 4.3 Chemical shift (δ) assignments of ^{1}H NMR and ^{13}C NMR spectra of WU arabinoxylans from Job's tears based on TOCSY, HMQC, COSY, and HMBC

Glucosyl residues	Assigned H, C position[1]	^{1}H (ppm)[2]	^{13}C (ppm)[3]
α-L-Ara*f* O-3 linked	1	5.38	110.338
	2	3.90	79.950
	3	4.14	83.437
	4	4.25	87.437
	5-eq	3.77	65.471
	5-ax	3.72	
α-L-Ara*f* O-3 linked (also at O-2)	1	5.25	110.779
	2	4.15	84.791
	3	3.94	79.392
	4	4.38	83.437
	5-eq	3.79	64.024
	5-ax	3.71	
α-L-Ara*f* O-2 linked (also at O-3)	1	5.20	111.335
	2	4.13	87.010
	3	3.95	79.392
	4	4.20	-
	5-eq	3.81	64.024
	5-ax	3.72	
β-L-Ara*f*	1	5.10	103.927 region of Xyl*p* anomeric carbon
	2 (from COSY)	4.07	65.471
	-	4.12	-
	-	3.91	-
	-	3.81	65.471
	-	3.72	
β-D-Xyl*p* disubstituted	1	4.61	100.362
	2	-	87.010 (from HMBC)
	3	- (not present in TOCSY)	87.010 (from HMBC)
	-	- (not present in TOCSY)	-
	-	- (not present in TOCSY)	-
	-	- (not present in TOCSY)	
β-D-Xyl*p* monosubstituted at O-3	1	4.58	102.528
	2	3.55	74.767
	3	3.64	87.437 (from HMBC)
	4	3.75	79.950
	5-eq	3.95	-
	5-ax	3.48	
β-D-Xyl*p* unsubstituted	1	4.46	103.927
	2	3.43	62.532
	3	3.65	-
	4	3.72	-
	5-eq	3.83	76.645
	5-ax	3.52	

[1] Compared to literatures, [2] assigned from ^{1}H NMR and TOSCY, [3] assigned from ^{13}C NMR and HMQC if not indicated

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CHAPTER V

WATER-EXTRACTABLE AND WATER-UNEXTRACTABLE ARABINOXYLANS FROM BRAN OF JOB'S TEARS (*COIX LACHRYMA-JOBI* L.) : COMPOSITION AND MOLECULAR CHARACTERIZATION

Abstract

Water-extractable (WE) and water-unextractable (WU) fractions were isolated from bran of two types of Job's tears. Both fractions were composed of high protein and ash with some uronic acid. Dark husk Job's tears bran (DHB) had significantly higher WE and WU polysaccharides (22.2% and 34.1%) than those from white husk Job's tears bran (WHB) (17.0% and 24.0%, respectively). The degree of branching (arabinose/xylose ratio) of arabinoxylans from Job's tears bran ranged between 0.93-1.19. The elution profile of WE fraction showed two splitting high molecular weight (MW) peaks, considered as arabinoxylans and arabinogalactans in accord with its monosaccharide composition whereas the WU fraction showed only one high MW peak of arabinoxylans with average MW of 382,000 Da (Pd 1.3) and 488,000 Da (Pd

1.1) for DHB and WHB, respectively. WE and WU fractions of Job's tears bran exhibited Newtonian flow behavior (20% w/v) and a liquid-like character (30%).

5.1 Introduction

Bran of Job's tears (*Coix lachryma-jobi* L.) is a by-product obtained during the milling. Bran is usually a valuable source of dietary fiber (DF) and has been added to breakfast cereals and other baking food products to increase DF content. Source of ferulic acids in bran was reported (Yadav, Moreau, and Hicks, 2007; Kim, Tsao, Yang, and Cui, 2006) and found to have a good antioxidant activity (Andreasen, Kroon, Williamson, and Garcia-Conesa, 2001; Emmons, Peterson, and Paul, 1999). Wheat bran increased concentration of short chain fatty acids (SCFA) in cecum (Treem, et al., 1995) and it was investigated as a significant source of arabinoxylans (Cui, Wood, Weisz, and Beer, 1999). Recently, it was found that arabinoxylans extracted from rice bran may be relevant for anti-cancer activities (Ghoneum and Gollapudi, 2003). Arabinoxylans, a soluble DF extracted from corn bran, could reduce the level of serum cholesterol in rats fed a cholesterol diet. Moreover, water-soluble arabinoxylans from corn bran increased the quantities of SCFA in the cecum (Miyasaka, Sanada, and Ayano, 1992; Lopez et al., 1999).

Many investigations on arabinoxylans have been concerned with the isolation and structural analysis. However, arabinoxylans from Job's tears bran have not been reported yet. To understanding non-starch polysaccharide components from Job's tears bran might lead to an increased use in this by-product. In previous chapter of this thesis, the alkali extract of Job's tears kernel contained a relatively low level of arabinoxylans (~9-12%) with high protein and minerals. This chapter reports the

results on isolation and characterization of water-extractable and water-unextractable fractions of Job's tears brans.

5.2 Materials and methods

5.2.1 Materials

Bran of two types of Job's tears, dark and white husk, grown in Thailand, were obtained from CCP Northern Co., Ltd, Thailand. Thermostable α-amylase 3,000 U/ml (45 U/mg) from *Bacillus licheniformis* and α-amyloglucosidase (36 U/mg) from *Aspergillus niger* were obtained from Megazyme International (Bray, Co. Wicklow, Ireland). Protease from *Streptomyces griseus* (4 U/mg) was obtained from Sigma-Aldrich. All chemicals using were of reagent grade.

5.2.2 Composition analysis of Job's tears bran

Moisture and ash was determined according to the methods described by AOAC (2000). Protein was analyzed by an NA2100 Nitrogen and Protein Analyzer/ThermoQuest Itallia S.P.A. EA/NA 1110 Automatic Elemental Analyzer (Strada Rivoltana, Milan, Italy), using the factor of 6.25 to convert nitrogen to protein content. Lipid content was analyzed by a 2050 Soxtec Avanti autoextraction unit connecting with a 2050 Soxtex autocontrol unit and drive unit (Foss Tecator, Sweden). Total dietary fiber content was determined by using the total dietary fiber assay kit from Sigma-Aldrich based on the method of AOAC (1997).

The content of starch was determined with slight modification as described by Wood, Weisz, and Blackwell (1991). The sample was weighed with addition of

thermostable α-amylase, α-amyloglucosidase, and buffer. The sample was then incubated with constant stirring and centrifuged at 8,500 rpm for 10 min. The supernatant was diluted to fit the range of glucose standard solutions and automatically analyzed as a result of the reaction of glucose with glucose oxidase from Megazyme International (Bray, Co. Wicklow, Ireland). The intensity of the reaction was read at 505 nm by colorimeter. Corn starch was used as a check of enzymatic action in each analysis.

Total sugar content was determined according to the method of Dubois, Gilles, Hamilton, Rebers, and Smith (1956). The addition of aliquot 72% H_2SO_4 was added in the sample and hydrolyzed for 30 min at room temperature. The concentration of sample was diluted to fit the curve of glucose standard solution. The aliquot of standard and sample was added with 80% phenol, followed by the addition of concentrated H_2SO_4. The mixed solution was left for 30 min before the reading was taken by a UV-Visible spectrophotometer (Varian Cary 3C) at the absorbance of 490 nm.

5.2.3 Sample Preparation

Bran of two types of Job's tears, dark and white husk, was cleaned by removing other defects (such as small fractured husk) before grinding and passing through a 30-mesh sieve. The ground bran was refluxed with 70% EtOH at the ratio of 1 : 10. The ethanol extract was separated by filtration. The residue was dried at 70 °C, overnight, and reground, designated as Residue A.

5.2.4 Extraction of non-starch polysaccharides (NSP)

Residue A was hydrolyzed by α-amylase at 90 ºC for 30 min and then decreased temperature to 75 ºC for 2.30 hr (Figure 5.1). The sample was filtered and air-dried overnight after washing with water (75 °C, 15 min), then the sample was noted as Residue B. The filtrate was incubated with α-amyloglucosidase at 50 °C overnight. The solution was heated to 100 ºC for 15 min, cooled down and adjusted pH to 4.5 by 2 M HCl and centrifuged at 10,000 rpm for 15 min to remove some proteins from the supernatant. After dialysis against deionized water for 2 days, the supernatant was concentrated to about one-forth of its volume by vacuum rotary evaporation and freeze-dried. The freeze-dried sample was redissolved in water at 70 °C and adjusted pH to 7.0. The sample was then centrifuged to obtain supernatant. The supernatant was freeze-dried and noted as water-extractable (WE) NSP.

The Residue B was extracted with 0.5 M NaOH for 3 hr at room temperature (25 ºC) and then centrifuged at 10,000 rpm for 15 min to obtain supernatant. After adjusted pH to 4.5 by 2 M HCl, the supernatant was centrifuged and dialyzed against water for 2 days. The supernatant was concentrated to about one-forth of its volume by vacuum evaporation and freeze-dried. The dried sample was then redissolved in water and adjusted pH to 7. After centrifugation, the supernatant was freeze-dried and designated as water-unextractable (WU) NSP.

5.2.5 Determination of uronic acid and mineral contents

The uronic acid content was determined according to Blumenkrantz and Asboe-Hansen (1973). The sample was dissolved in water at 50 ºC for 3 hr and diluted to fit the range of standard curve of galacturonic acid. The diluted sample, as

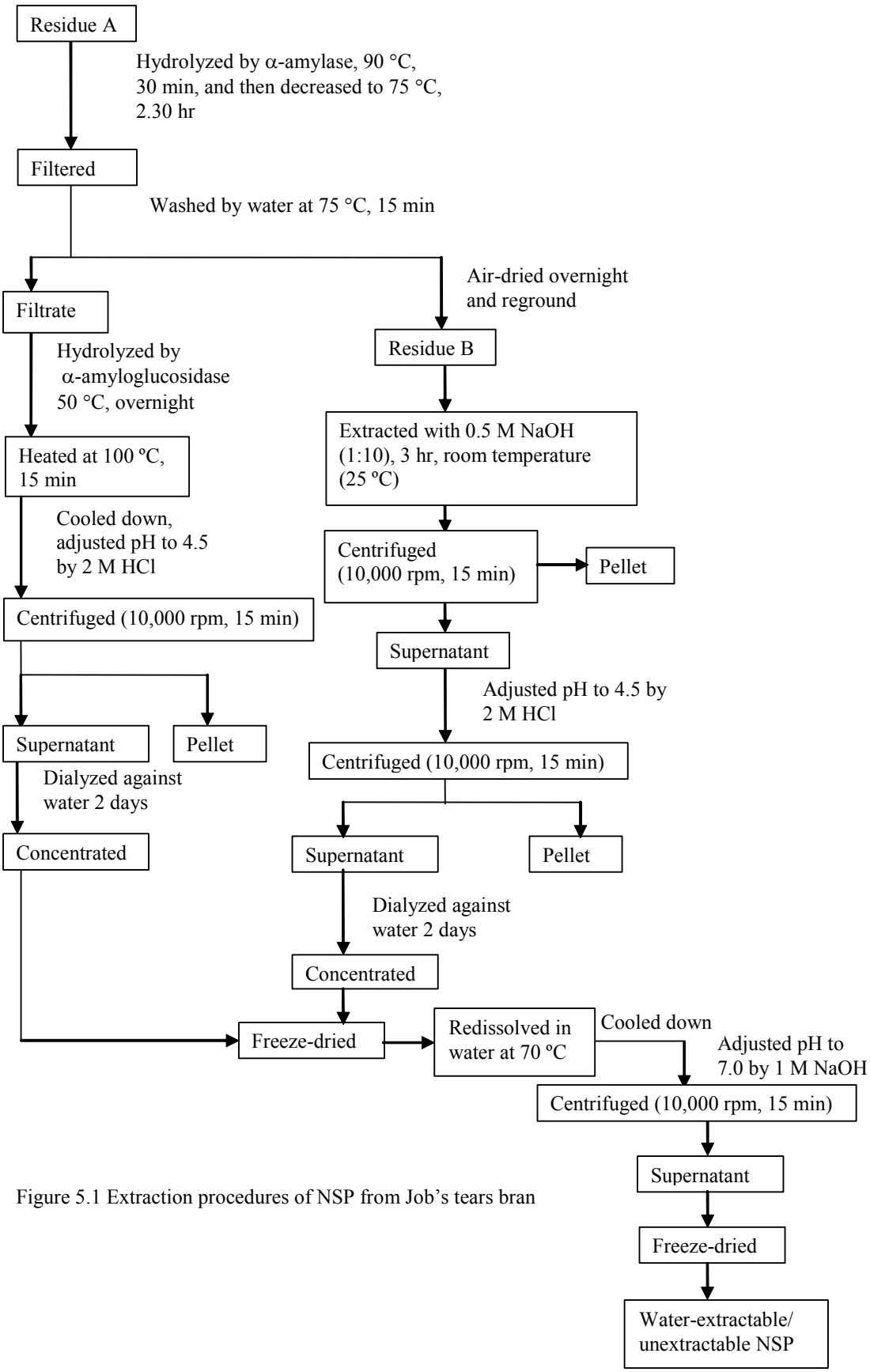


Figure 5.1 Extraction procedures of NSP from Job's tears bran

well as standard solution was pipetted and mixed with 12.5 mM tetraborate in concentrated H_2SO_4, and then placed in a crushed ice bath. The sample was heated in a water bath at 100 ºC for 5 min and placed in the ice bath to cool down. The 0.15% m-hydroxydiphenyl reagent was added and left for 20 min at room temperature. The developed color was read by a UV-Visible spectrophotometer (Varian Cary 3C) at the absorbance of 520 nm.

5.2.6 Monosaccharide analysis

The sample was hydrolyzed in 1 M H_2SO_4 at 100 ºC for 2 h, and diluted. The monosaccharide hydrolysates were filtered (0.45µm) and the volume of 50 µl was injected onto the High Performance Anion Exchange Chromatography (HPAEC) system (DIONEX) and differentiated by a CaboPac PA1 column connected with pulsed amperometric detection (PAD) as described earlier by Wood, Weisz and Blackwell (1994). Gradient elution was carried using 100 mM NaOH and deionized water at the flow rate of 1.0 ml/min, with 8/92 to 0/100 of a linear gradient. The initial condition with 300 mM NaOH for 15 min was performed to wash column before each sample was run. The monosaccharide content was quantitatively analyzed by comparison with known individual standard curves (rhamnose, arabinose, galactose, glucose, xylose and mannose). The ratio of arabinose to xylose in each sample was calculated. Corrections were made for arabinose in samples contained with galactose in the form of arabinogalactans with the average arabinose to galactose ratio of 0.7 according to Loosveld et al. (1998).

5.2.7 Molecular size distribution

According to Wang, Wood, Huang and Cui (2003), molecular weight analysis was determined using High Performance Size Exclusion Chromatography (HPSEC) coupled with refractive index, right angle laser light scattering (RALLS) and viscosity detectors (Model Dual 250, Viscotek, Houston, USA). The Shimadzu SCL-10Avp pump unit (Shimadzu Scientific Instruments Inc., Columbia, MD, USA) was operated and two serially connected columns (Shodex OHpak SB-806M HQ, Showa Denko K.K., Tokyo, Japan and Ultrahydrogel linear, Waters, Milford, USA) were maintained at 40 ºC. The eluent was 100 mM sodium nitrate ($NaNO_3$) composed of 0.03% sodium azide (NaN_3), running by the flow rate of 0.6 ml/min. A set of different known molecular weight and intrinsic viscosity standard of Pullulans 100, 400, 800 were used to calibrate the columns. A specific refractive index increment (d*n*/d*c*) of 0.146 ml/g was used for the molecular weight calculation of NSP. The samples were dissolved in deionized water (5mg/ml) at 50 ºC for 3 hr before the solution was filtered through a 0.45 μm filter and the volume of 100 μl was then injected onto the column system.

5.2.8 Rheological measurement

The steady shear flow measurement of NSP of Job' s tears solution with concentration at 5%, 10% and 20% (w/v) was carried out using a Bohlin VOR rheometer. A cone and plate geometry with diameter 50 mm and cone angle between cone and plate 4º was used. The temperature was maintained at 23 ºC during all measurements. Samples at 30% (w/v) were subjected to frequency sweeps between 0.01 and 20 Hz at 23 ºC with strain of 2.0%.

5.3 Results and discussion

5.3.1 Composition of Job's tears bran

The chemical composition of two types of Job's tears bran was listed in Table 5.1. The bran had significantly higher contents of protein, fat, ash, and dietary fiber and lower content of starch as compared with those from the kernel (see Chapter III). Total dietary fiber (DF) was 22.3% in the dark husk Job's tears bran (DHB) slightly higher than 20.2% of white husk Job's tears bran (WHB), while protein was 21.0% much higher than that of 16.9%, respectively. There were small differences of DF content when compared with that of 18.7% for oat bran and 22.9% for rice bran. However, Job's tears bran displayed a good source of protein with higher content than rice bran (13.7%) and oat bran (0.5%) (Kahlon, 2001). The fat and ash content were 19.9% and 6.1% in DHB, whereas there were 21.5% and 6.8% in WHB, which were much higher than in kernel.

Table 5.1 Chemical composition of Job's tears bran (dry weight basis)

Components	DHB	WHB
Total sugar (%)	35.6 ± 0.73	37.7 ± 0.24
Starch (%)	14.4 ± 0.69	17.1 ± 0.42
Total dietary fiber (%)	22.3 ± 1.64	20.2 ± 0.68
Protein (%)	21.0 ± 0.31	16.9 ± 0.05
Moisture (%)	6.60 ± 0.14	6.9 ± 0.02
Fat (%)	19.9 ± 0.11	21.5 ± 0.29
Ash (%)	6.1 ± 0.03	6.8 ± 0.00

DHB = Dark husk Job's tears bran, WHB = White husk Job's tears bran

5.3.2 Extraction and composition of NSP from Job's tears bran

The total yield of WE and WU fractions isolated from DHB and WHB contained 0.5 and 1.1%, and 0.5 and 1.0%, respectively. They contained relatively high protein and ash contents (Table 5.2) similar to the WU fraction obtained from kernel. Protein content in the WE fraction of DHB was higher but that in the WU fraction was lower, when compared to WHB. Both WE and WU fractions of WHB consisted of significantly higher in ash than those of DHB. In contrast, a content of WE and WU polysaccharides from DHB (22.2% and 34.1%) was much higher than that from WHB (17.0% and 24.0%). The higher level of uronic acid in the WE fraction than the WU fraction was observed. Distinctively, the WE fraction from DHB and WHB represented minor level of rhamnose and higher galactose, compared with the WU fraction. Other sugars detected were arabinose, glucose, xylose, and mannose. It was noted that arabinose was at a high level except for the WE fraction from WHB. The great amount of galactose and arabinose in WE fraction suggested the presence of arabinogalactans and arabinoxylans in this fraction. The high ratios of arabinose/xylose (Ara/Xyl), an indicative of a highly branched structure of arabinoxylans, were observed for all the fractions (1.17-1.19), except for the WE fraction from WHB (0.93), which was less branched than the others. However, these values were slightly lower than that was isolated from Job's tears kernel (1.24 and 1.25). It was observed that the ratios of Ara/Xyl (~1.19 and 0.93) of WE arabinoxylans from two Job's tears brans were higher than that was reported in WE arabinoxylans from wheat bran (0.59) (Annison, Choct, Cheetham, 1992) and rye bran (0.56-0.58). Furthermore, 5.8% and 5.5% galactose of the WE fraction was higher than ~1.6% galactose which was found in rye bran (Cyran and Saulnier, 2005).

For the WU arabinoxylans of both Job's tears, the Ara/Xyl ratio (1.17) were higher than those of the alkali extractable arabinoxylans from wheat bran (0.33) (Cui et al., 1999) and rye bran (0.98) (Ebringerová, Hromádková, and Berth, 1994).

Table 5.2 Chemical analysis of water-extractable (WE) and water-unextractable (WU) fractions from Job's tears bran (dry weight basis)

Components	DHB		WHB	
	WE fraction	WU fraction	WE fraction	WU fraction
Protein (%)	35.3 ± 1.40	23.6 ± 1.83	31.5 ± 0.86	26.1 ± 0.57
Ash (%)	20.5 ± 0.58	21.7 ± 0.03	29.9 ± 0.09	30.2 ± 0.48
Uronic acid (%)	6.9 ± 0.17	3.6 ± 0.15	4.6 ± 0.05	2.4 ± 0.09
Monosaccharide (%)				
Rham	0.4 ± 0.03	-	0.3 ± 0.12	-
Ara	8.7 ± 0.08	15.6 ± 0.21	5.7 ± 0.04	10.5 ± 0.01
Gal	5.8 ± 0.05	2.2 ± 0.09	5.5 ± 0.15	2.2 ± 0.11
Xyl	3.9 ± 0.37	13.3 ± 0.24	2.0 ± 0.02	9.0 ± 0.17
Glu	2.7 ± 0.10	2.7 ± 0.06	1.4 ± 0.16	1.8 ± 0.06
Man	0.7 ± 0.30	0.3 ± 0.01	1.1 ± 0.09	0.5 ± 0.06
Total	22.2	34.1	17.0	24.0
Ara/Xyl	1.19[1]	1.17	0.93[1]	1.17

Rham = Rhamnose, Ara = Arabinose, Gal = Galactose, Xyl = Xylose, Glu = Glucose, Man = Mannose

[1] Corrected for WE arabinogalactans with the average Ara/Gal ratio of 0.7

5.3.3 Molecular size distribution

The size exclusion chromatography profiles of WE fractions from DHB and WHB differed considerably from those of the WU fraction (Figures 5.2 and 5.3). The two carbohydrate polymers were detected in the WE fractions, as shown by the presence of two splitting peaks by light scattering (LS) detector; the first peak at

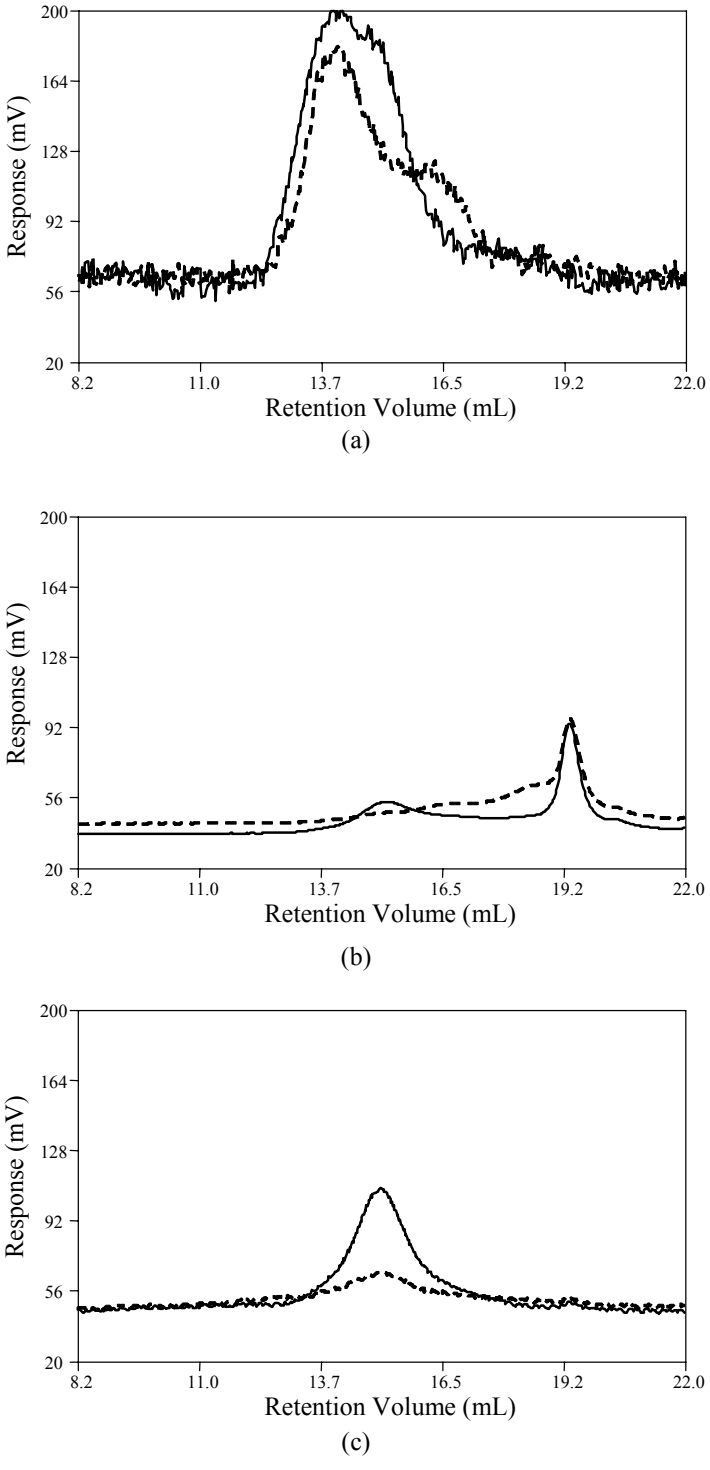
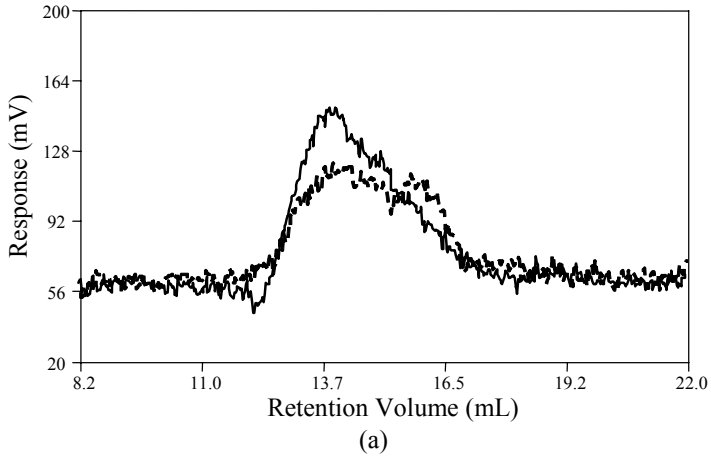
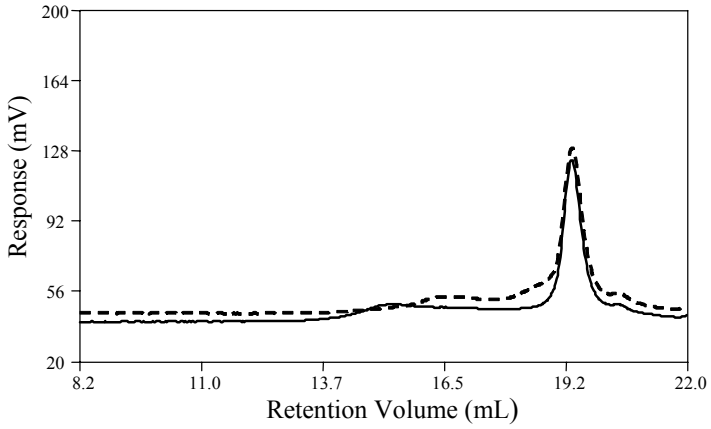


Figure 5.2 Elution profile of WE and WU fractions from DHB on two serially connected columns coupled with triple detectors (a) LS = Light scattering detector, (b) RI = Reflective index detector (c) viscometer detector.
Solid line was WU fraction
Dashed line was WE fraction



(a)



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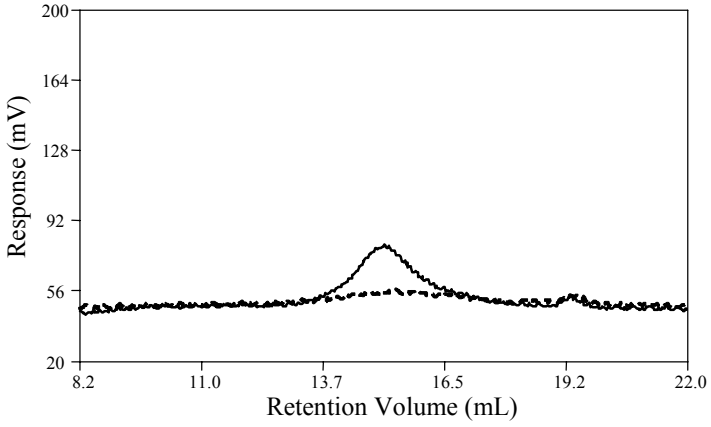


(c)

Figure 5.3 Elution profile of WE and WU fractions from WHB on two serially connected columns coupled with triple detectors (a) LS = Light scattering detector, (b) RI = Reflective index detector (c) viscometer detector.
Solid line was WU fraction
Dashed line was WE fraction

retention volume ~14.0 ml for both DHB and WHB and the second peak at ~16.5 and 15.8 ml for DHB and WHB, respectively. Correlation to its monosaccharide composition, the first polymer, eluting in the higher molecular weight (MW) region (~14.0 ml) and the splitting of second eluting polymer (~16.5 and 15.8 ml), very close to the first peak, were probably ascribed to arabinoxylans and arabinogalactans respectively (Trogh, Courtin, and Delcour, 2004; Izydorczyk, Biliaderis, and Bushuk, 1991; Michniewicz, Biliaderis, and Bushuk, 1990). The elution polymer detected by reflective index (RI) detector at retention volume of ~19.5 ml was deduced to likely protein since high proteins were determined and the elution profiles were similar to those from the previous study (see Chapter III). Regrettably, the MW distribution of the WE fractions was not able to be calculated. The single peak determined by LS detector of the WU fraction of DHB and WHB was assigned to arabinoxylans with average MW of 382,000 Da (Pd 1.3) and radius of gyration (Rg) of 35.9 nm, and 488,000 Da (Pd 1.1) and Rg of 40.4 nm, respectively (Table 5.3).

Table 5.3 Weight average molecular weight (MW), radius of gyration (Rg), polydispersity index (Pd), and Intrinsic viscosity [η] of WU arabinoxylans from Job's tears bran

WU arabinoxylans	MW (Da)	Rg (nm)	Pd	[η] (dl/g)
DHB				
	382,000	35.9	1.3	3.7
WHB				
	488,000	40.4	1.1	4.0

d*n*/d*c* = 0.146 ml/g was used to MW calculation; the averages of duplicate were expressed in data.

The higher average MW of WU arabinoxylans of WHB was in accord with the higher values of Rg and intrinsic viscosity ([η]). In comparison, the values of average MW (741,000 and 1,449,000 Da) and Rg (44.5 and 52.3 nm) of WU arabinoxylans from two Job's tears kernel were higher but [η] values were lower (3.1 and 3.5 dl/g). This is likely because the arabinoxylans from the kernel has a greater degree of branching than that from bran.

5.3.4 Rheological properties

The solution of the WE and WU fractions exhibited Newtonian flow behavior at a concentration as high as 20% (w/v) over the entire shear rate range (Figures 5.4 and 5.5). The high degree of branching of arabinoxylans might explain for this phenomenon whereas arabinoxylans of wheat, which had a lower degree of branching, exhibited shear thinning behavior at low concentration of 1% (w/v) (Izydorczyk, et al., 1991).

Figure 5.6 showed the frequency sweeps of the dynamic moduli (G′ and G″) of the WU fraction of two Job's tears bran at 30% (w/v). G″ values were over those of G′ at all frequencies, indicating a liquid-like character and both moduli were highly dependent on frequency (as seen by a high slope).

5.4 Conclusion

WE and WU fractions from two Job's tears bran were abundant in protein and ash with some differences in content. The level of WE and WU polysaccharides (22.2% and 34.1%) from DHB was significantly higher than that from WHB (17.0% and 24%). The degree of substitution (Ara/Xyl) of arabinoxylans from Job's tears

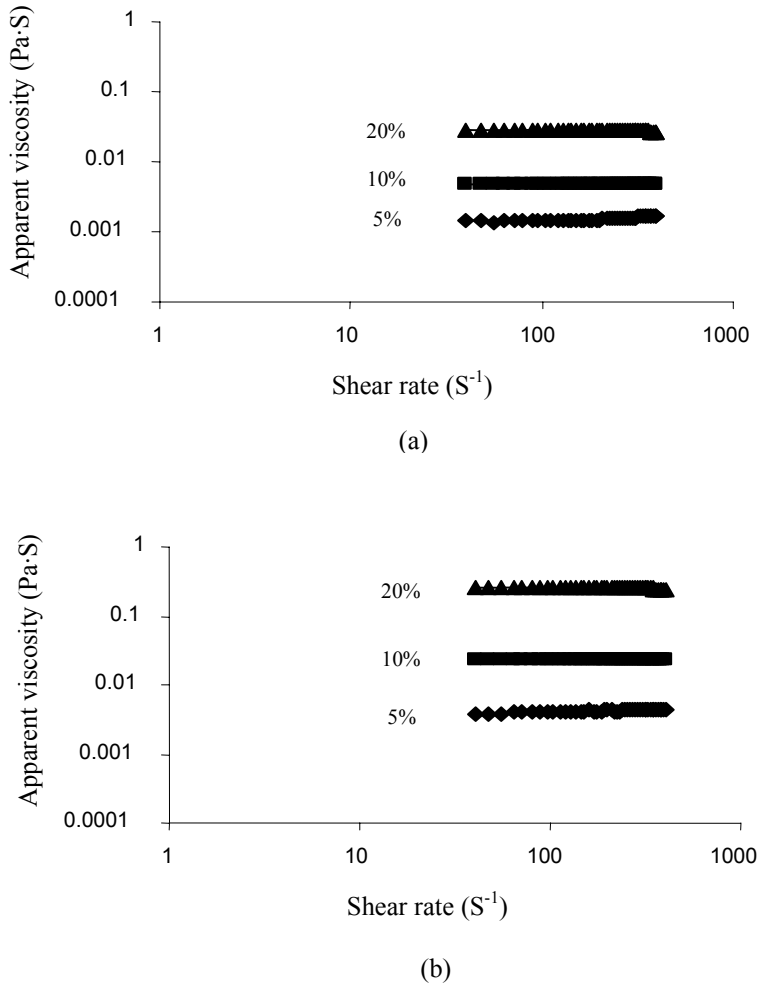
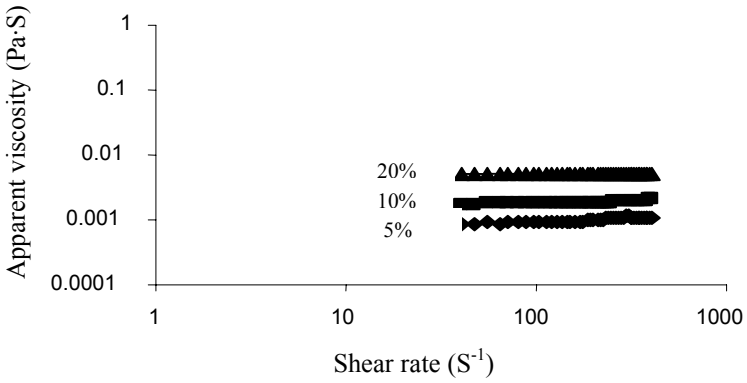
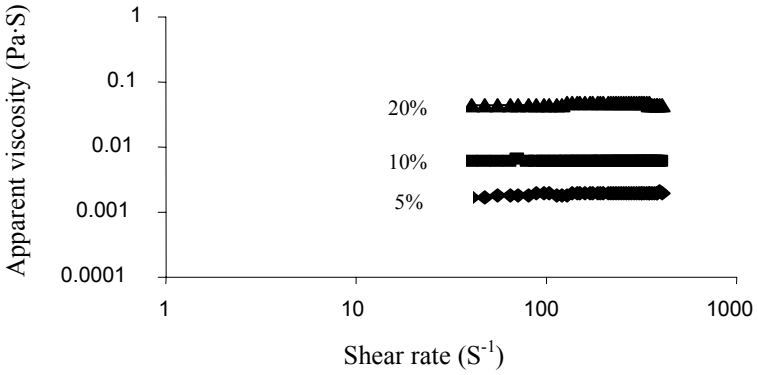


Figure 5.4 Steady shear rheological flow behavior of WE fraction (a) and WU fraction (b) from DHB at various concentrations (% w/v)

bran varied between 0.93 and 1.19, lower than that from its kernel. Two major types of polysaccharides, arabinoxylans and arabinogalactans were found in the WE fraction while WU fraction contained only arabinoxylans. The rheological properties of the extracts showed Newtonian flow behavior and a liquid-like character at high concentration which may not be useful to increase viscosity of food but they may be added as nutritious and soluble dietary fiber sources in a relatively higher amount.



(a)



(b)

Figure 5.5 Steady shear rheological flow behavior of WE fraction (a) and WU fraction (b) from WHB at various concentrations (% w/v)

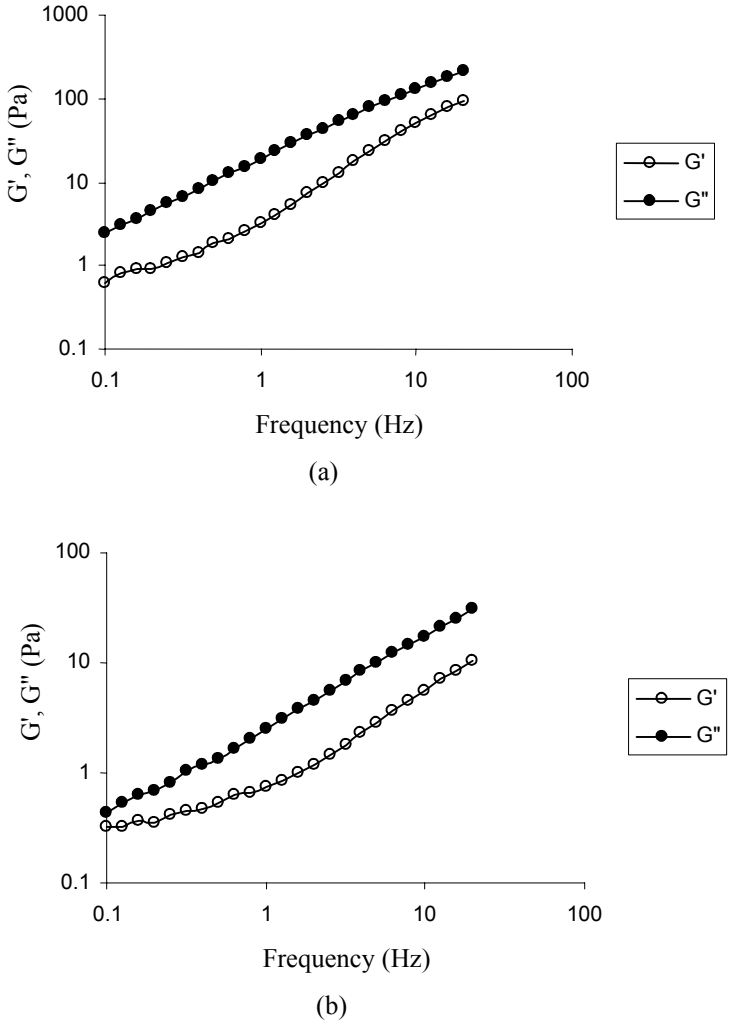


Figure 5.6 Frequency dependence of storage (G?) and loss (G?) moduli of WU fraction; (a) DHB (b) WHB, determined at 30% (w/v)

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CHAPTER VI

SUMMARY

The alkali extract /or water-unextractable (WU) fraction from polished seed of dark and white husk Job's tears similarly consisted of water-unextractable arabinoxylans with a high content of protein and minerals. The arabinose/xylose (Ara/Xyl) ratio of arabinoxylans was higher than those from other cereals, indicating its high degree of branching with arabinose. The arabinoxylans from dark husk Job's tears had a lower average molecular weight (MW) than from white husk type. The average MW reduced after treatment with protease which probably could represent covalent cross linkages between arabinoxylans and protein. WU fraction could reduce surface tension of water which was influenced by its average MW and protein content. As determined by methylation analysis, there were no significant differences in linkage of arabinoxylan structure between two types. Methylation and NMR analysis revealed that the terminal α-L-arabinofuranosyl residues (Ara*f*) were predominantly linked to O-3 of the backbone of (1, 4)-linked β-D-xylopyranosyl residues (Xyl*p*) and some were also substituted at both O-2 and O-3. The Newtonian flow behavior at high concentration (20% w/v) of WU fraction from Job's tears kernel was possibly affected by highly substituted arabinoxylans with high protein content and minerals.

Water-extractable (WE) and WU fractions from two Job's tears bran were abundant in protein and ash with some differences in content, similar to the WU

fraction obtained from its kernel. The level of WE and WU polysaccharides (22.2% and 34.1%) from dark husk Job's tears bran (DHB) was significantly higher than that from white husk Job's tears bran (WHB) (17.0% and 24.0%). The degree of substitution (Ara/Xyl) of arabinoxylans from Job's tears bran varied between 0.93-1.19, lower than that from its kernel (1.27 and 1.25). Two populations of arabinoxylans and arabinogalactans were found in WE fraction while WU fraction contained only arabinoxylans. The rheological properties of the extracts showed Newtonian flow behavior and a liquid-like character at high concentration which may not be useful to increase viscosity of food but they may be added as nutritional and soluble dietary fiber sources.

Future work, more detailed studies on the nature of association between protein and arabinoxylans are required to elucidate. It is also obviously to investigate the nutritional and biological activities of these mixed non-starch polysaccharides to fully understand their functional role.

APPENDIX

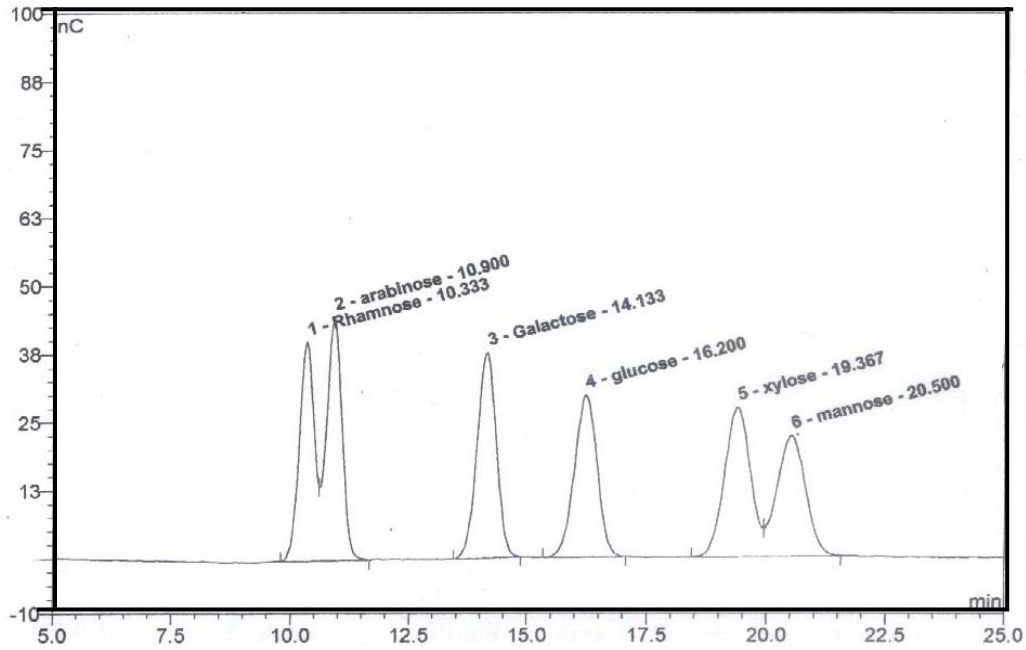

Figure 7.1 Chromatogram of HPAEC of standard monosaccharides

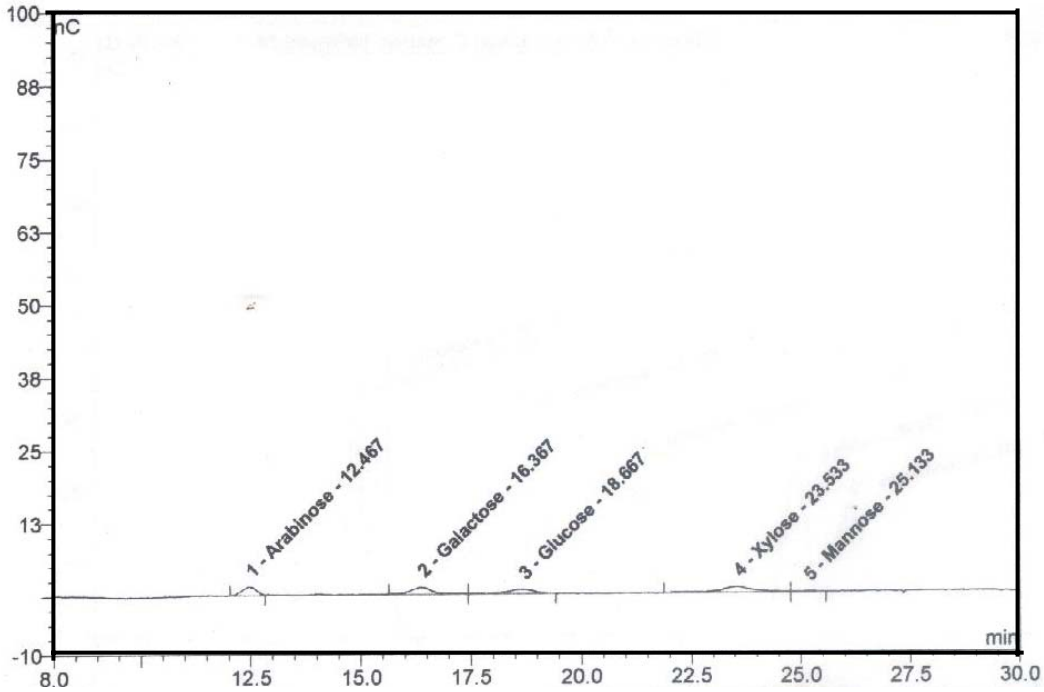

Figure 7.2 Chromatogram of HPAEC of monosaccharides of WE fraction of dark husk Job's tears bran

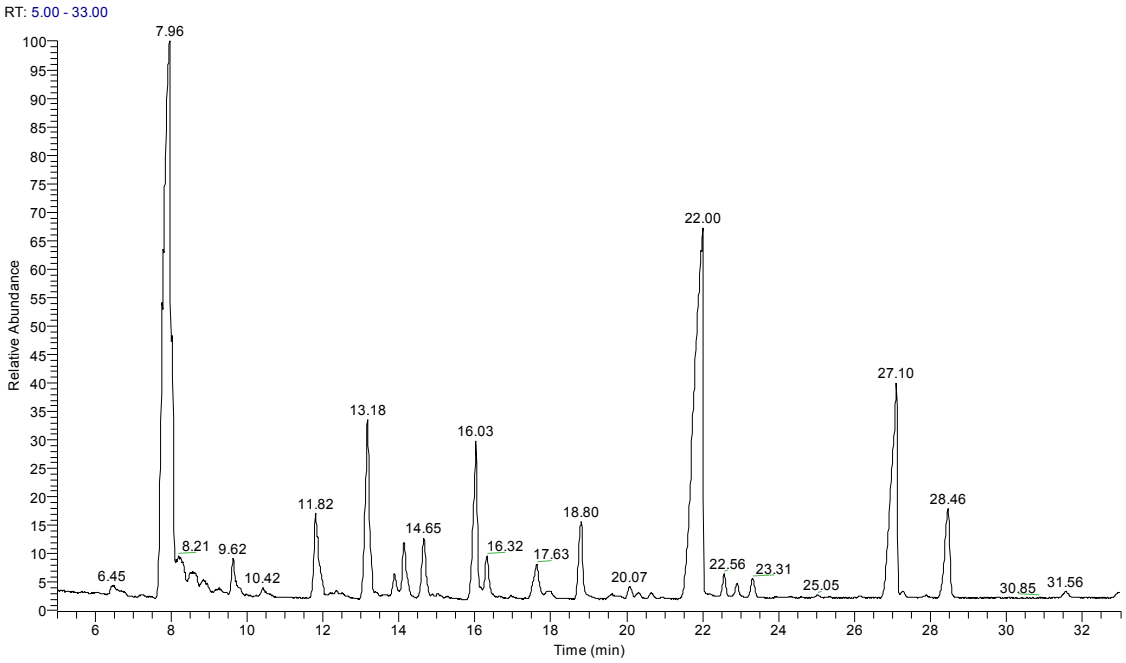

Figure 7.3 Chromatogram by GC of PMAA of WU fraction isolated from waxy Job's tears

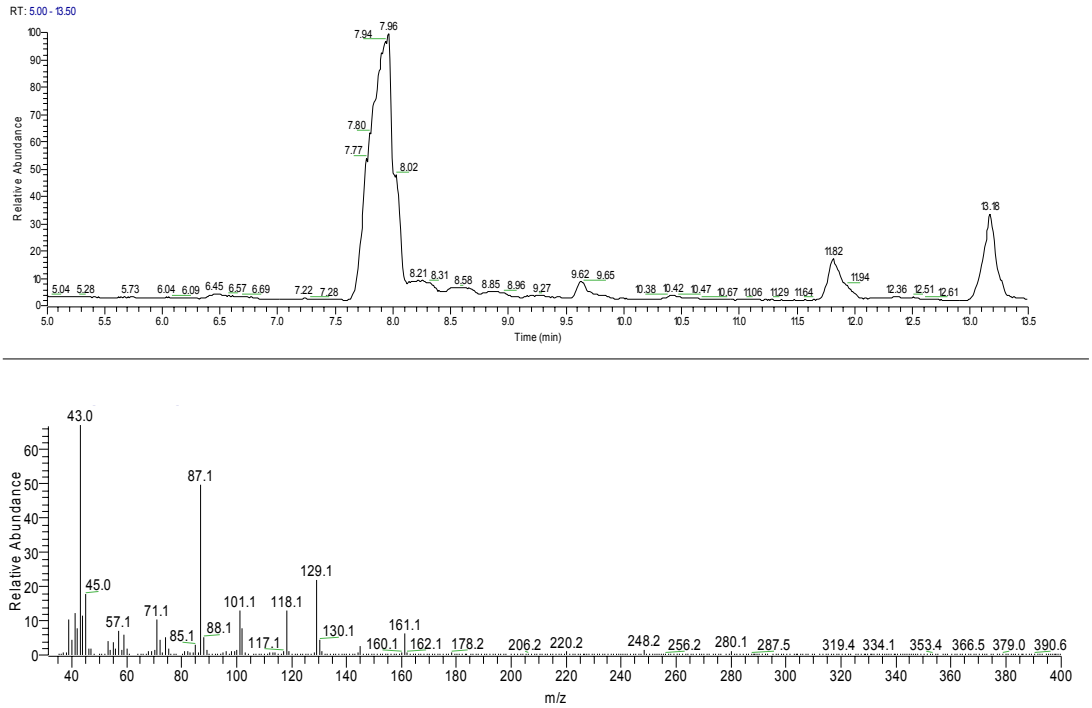

Figure 7.4 Mass spectrum by GC-MS of PMAA of WU fraction isolated from waxy Job's tears at RT 7.96 which matched 2, 3, 5-tri-O-methyl arabinitol

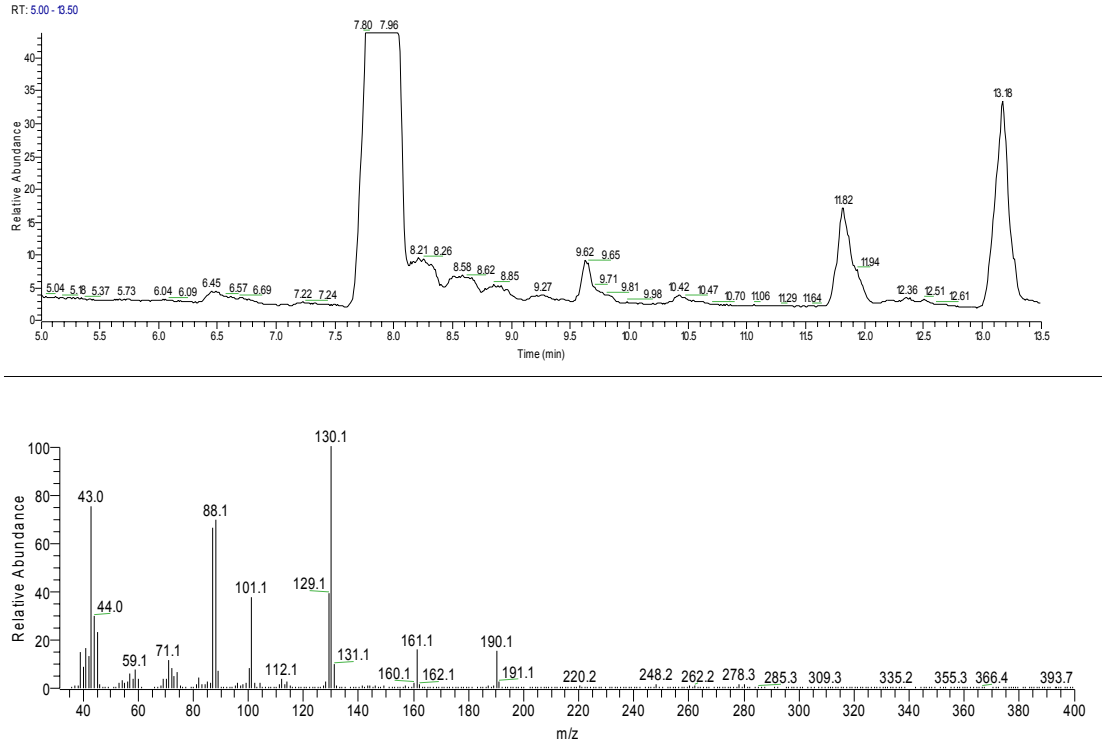

Figure 7.5 Mass spectrum by GC-MS of PMAA of WU fraction isolated from waxy Job's tears at RT 11.82 which matched 3, 6-di-O-methyl hexitol

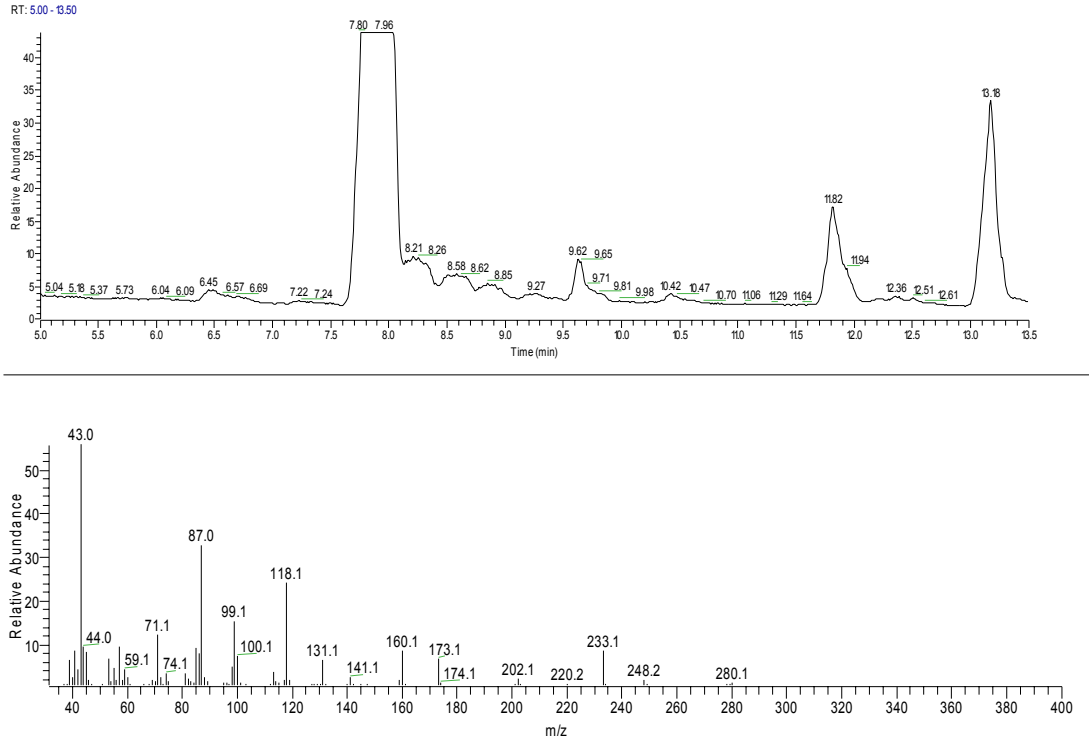

Figure 7.6 Mass spectrum by GC-MS of PMAA of WU fraction isolated from waxy Job's tears at RT 13.18 which matched 2, 3, 6-tri-O-methyl hexitol

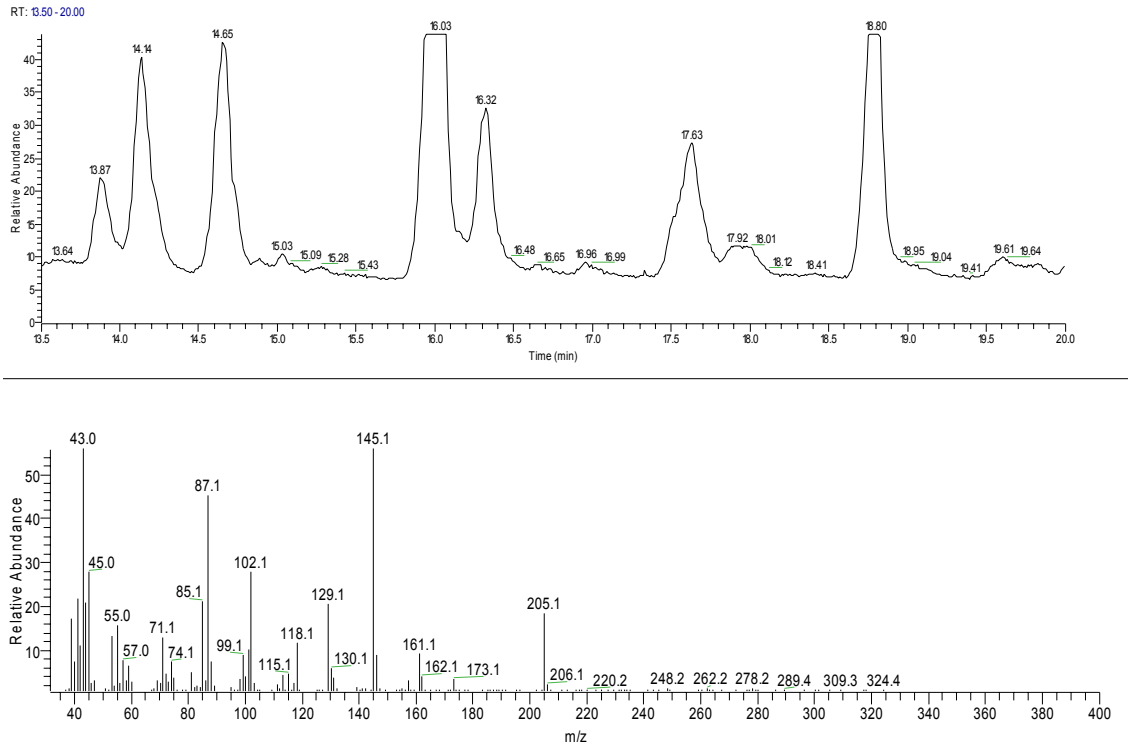

Figure 7.7 Mass spectrum by GC-MS of PMAA of WU fraction isolated from waxy Job's tears at RT 14.14 which matched 2, 3, 4, 6-tetra-O-methyl hexitol

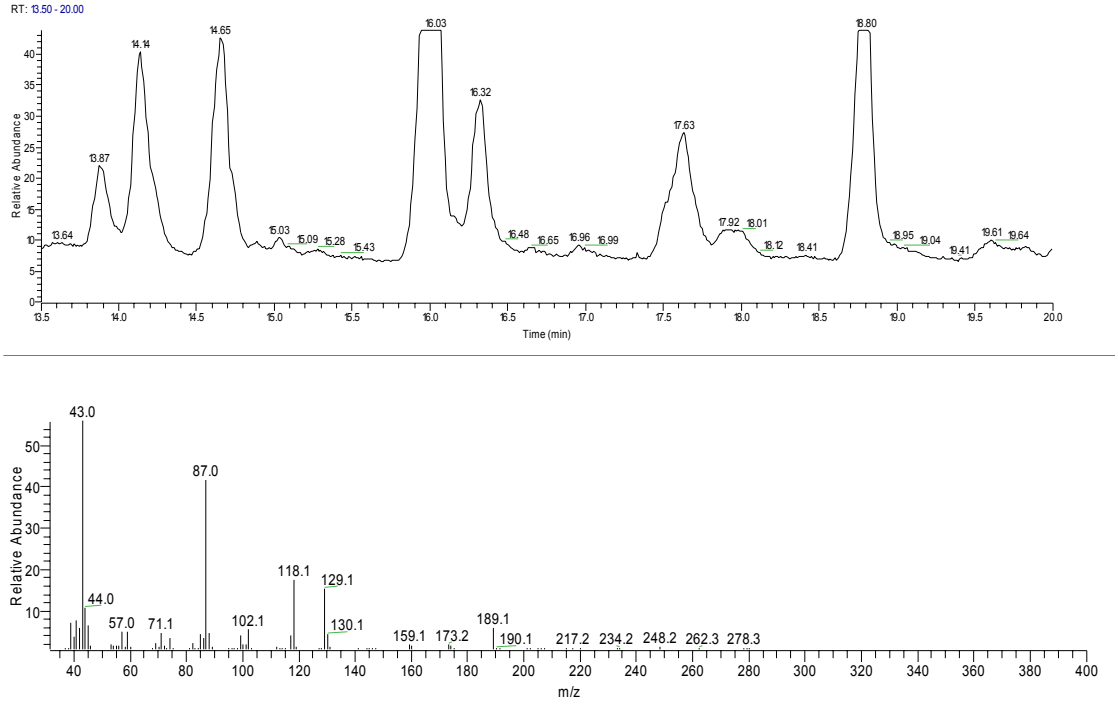

Figure 7.8 Mass spectrum by GC-MS of PMAA of WU fraction isolated from waxy Job's tears at RT 14.65 which matched 2, 3, 4-tri-O-methyl hexitol

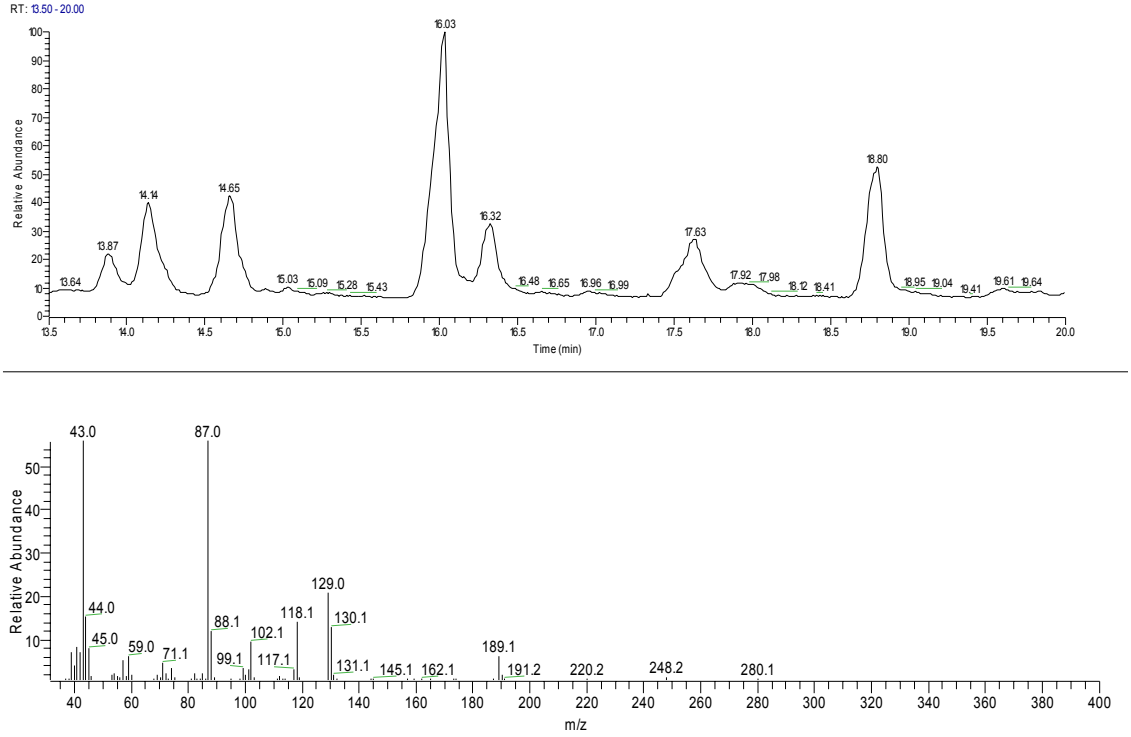

Figure 7.9 Mass spectrum by GC-MS of PMAA of WU fraction isolated from waxy Job's tears at RT 16.03 which matched 2, 3-di-O-methyl pentitol

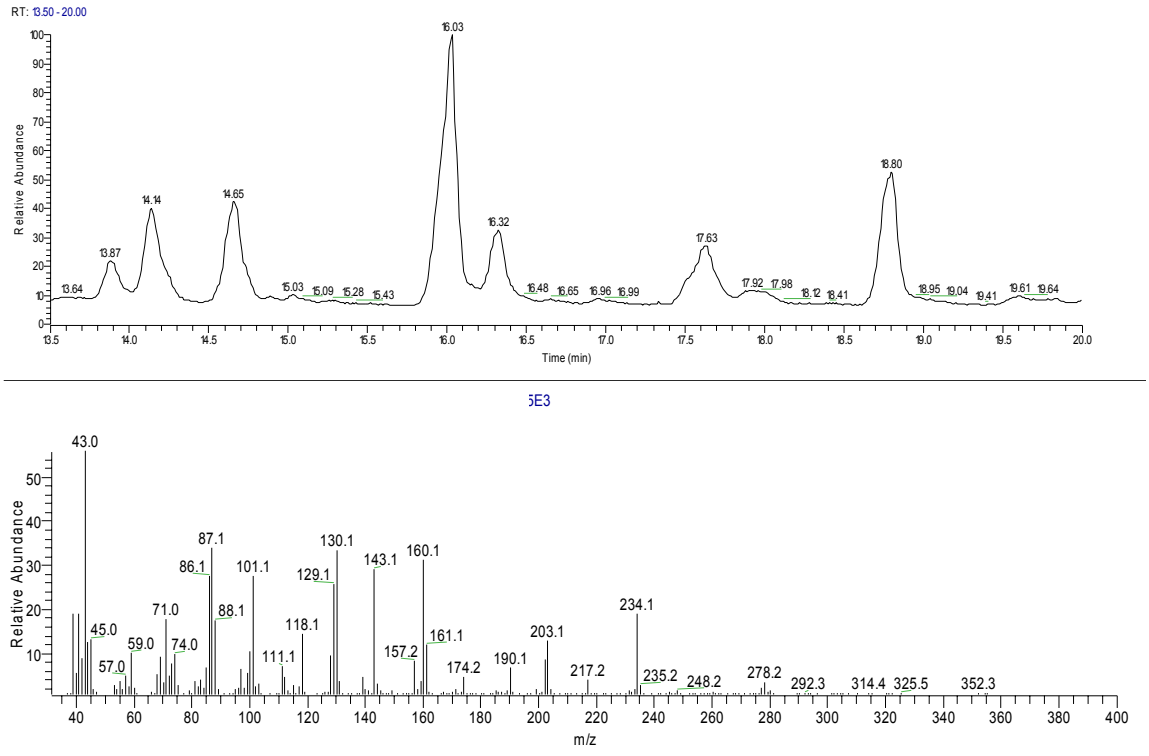

Figure 7.10 Mass spectrum by GC-MS of PMAA of WU fraction isolated from waxy Job's tears at RT 17.63 which matched 2-O-methyl pentitol

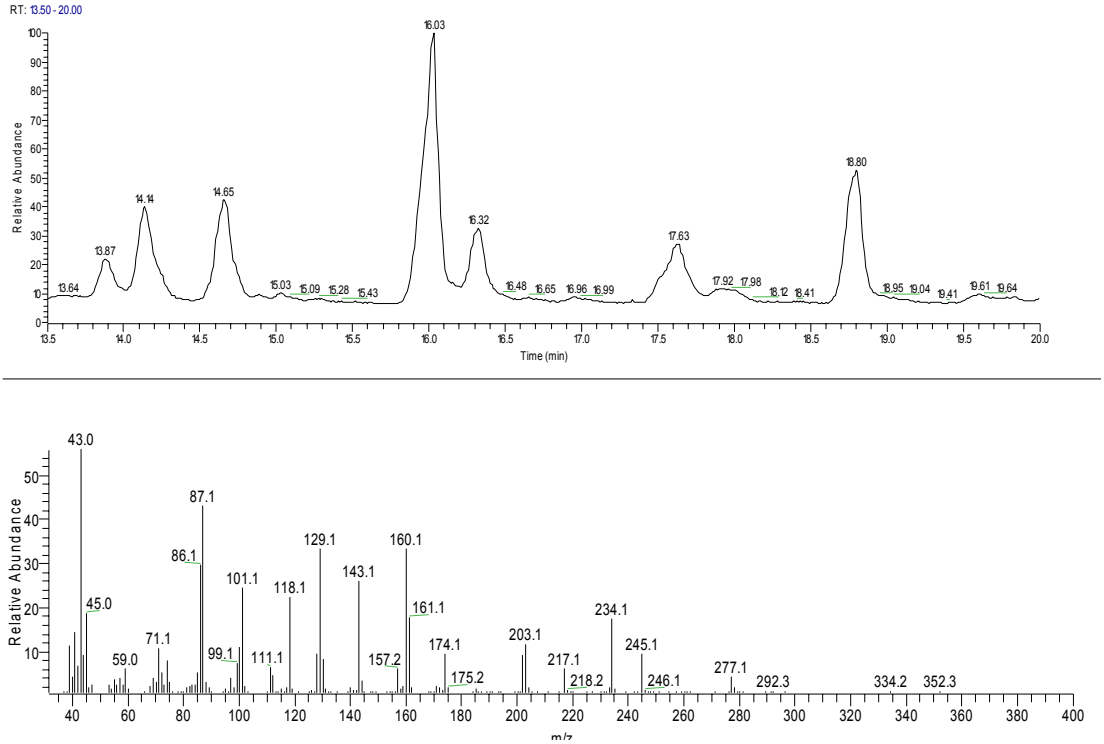

Figure 7.11 Mass spectrum by GC-MS of PMAA of WU fraction isolated from waxy Job's tears at RT 18.80 which matched 2-O-methyl pentitol and 4-O-methyl pentitol

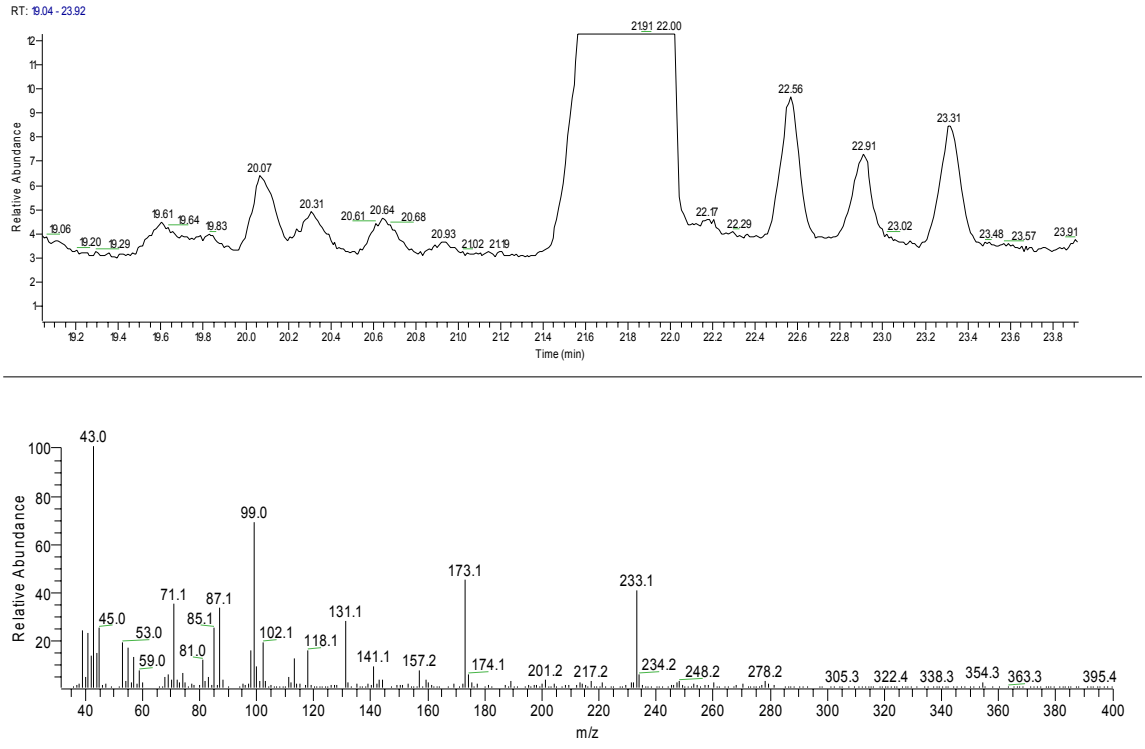

Figure 7.12 Mass spectrum by GC-MS of PMAA of WU fraction isolated from waxy Job's tears at RT 20.07 which matched 2, 4-di-O-methyl pentitol

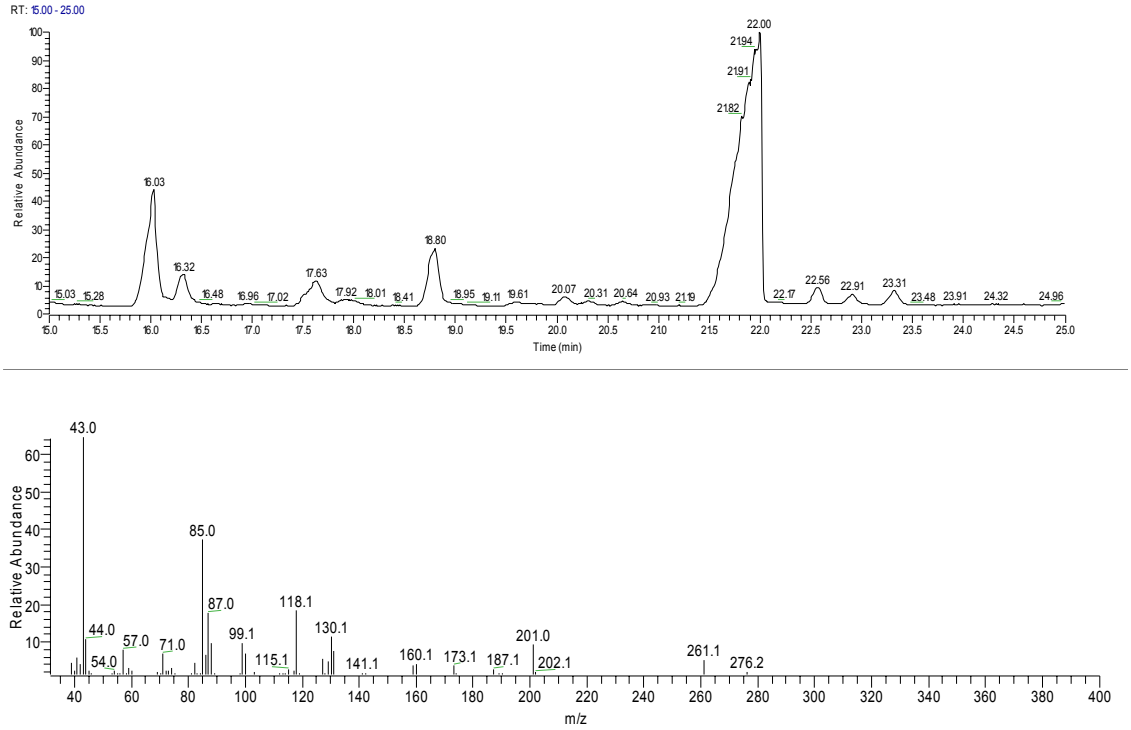

Figure 7.13 Mass spectrum by GC-MS of PMAA of WU fraction isolated from waxy Job's tears at RT 22.00 which matched 2-O-methyl pentitol

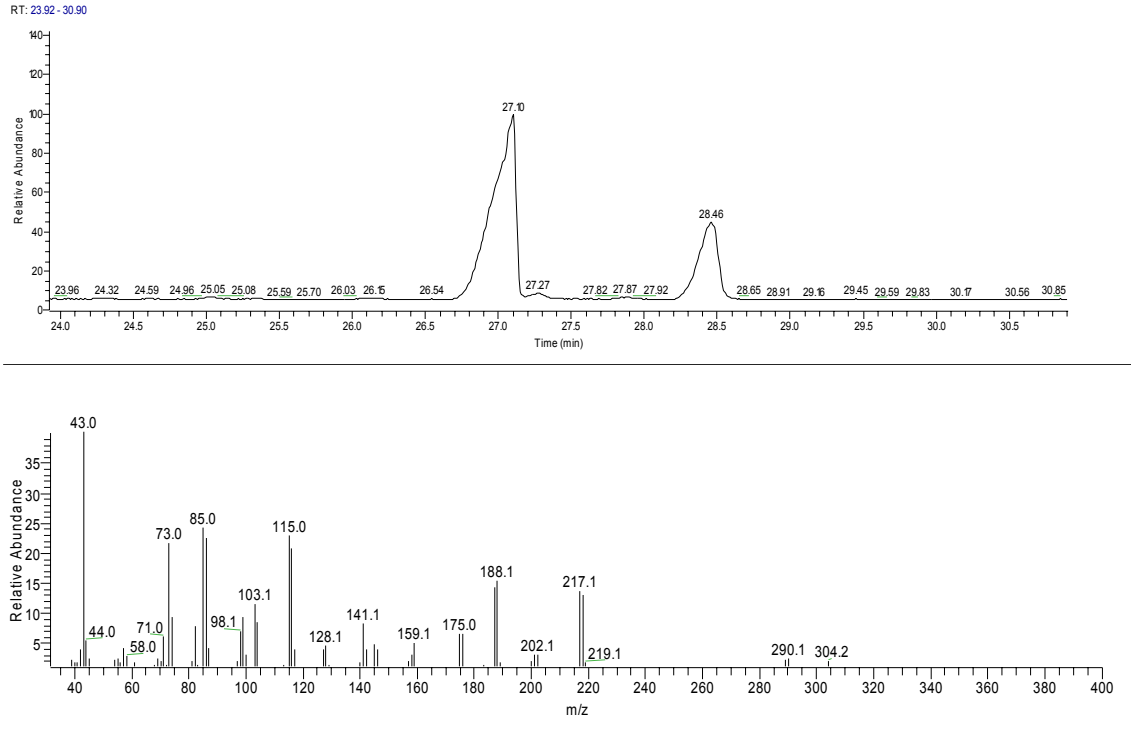

Figure 7.14 Mass spectrum by GC-MS of PMAA of WU fraction isolated from waxy Job's tears at RT 27.10 which matched 1-deuterio pentitol pentaacetate

BIOGRAPHY

Supaporn Apirattananusorn was born and brought up in Pattani, Thailand. She attended Prince of Songkla University, Thailand and received her Bachelor's degree in Agro-Industry (1994). She worked at Chotiwat Manufacturing, Songkla in Quality Assurance for 1 year. In 1996, she got a scholarship from Office of the Civil Service Commission (OCSC), Royal Thai Government and received a Master's degree in Food Technology at University of New South Wales, Australia. She worked as a lecturer in Food Science and Technology Program, Science and Technology Faculty, at Suratthani Rajabhat University. In 2002, she was granted by her University to attend Ph.D. Program at School of Food Technology, Suranaree University of Technology, Thailand.