JOURNAL OF FOOD AND DRUG ANALYSIS

Vol.11 No.4 December 2003

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Research Article

本刊榮獲九十二年度「行政院國家科學委員會獎助國內學術研究優良期刊」優等獎

Journal of Food and Drug Analysis, Vol. 11, No. 4, 2003, Pages 271-276 藥物食品分析 第十一卷 第四期

Simultaneous Determination of Furostanol and Spirostanol Glycosides in Taiwanese Yam (*Dioscorea* spp.) Cultivars by High Performance Liquid Chromatography

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(Received: April 4, 2003; Accepted: June 3, 2003)

ABSTRACT

A high performance liquid chromatographic (HPLC) method was developed for the simultaneous determination of the steroid saponins, furostanol and spirostanol glycosides in Taiwanese yam cultivars. C18 column kept at 45°C was used as the stationary phase. The mobile phase was a binary solvent system consisting of methanol and deionized water (62/38, v/v) in the first 20 min and 71/29 (v/v) from 21 to 65 min at a flow rate of 1 mL/min. Both evaporative light scattering detector (ELSD) and UV (203 to 215 nm) were employed and compared for the detection of saponins. The detection limits for ELSD and 203 nm UV were 2~3.5 mg and 0.1~0.2 μg, respectively at signal-to-noise ratio of 10. The recoveries of furostanol and spirostanol glycosides in the range of 0.25~3 μg were all above 92%. The presence of steroid saponins in Taiwanese yams was also determined.

Key words: diosgenin, evaporative light scattering detector, furostanol glycoside, high performance liquid chromatography, saponins, spirostanol glycoside, yam (*Dioscorea* spp.)

INTRODUCTION

Yam (*Dioscorea* spp.) is an important tuber plant for edible and medicinal use to promote health and longevity in Chinese tradition[1]. Saponin was reported to be the major physiologically active compound in yam[1-3]. It usually exists as glycoside in nature and many biological activities, such as hemolytic[4,5], hypocholesterolemic[6,7], hypoglycemic[8], anti-thrombotic[5,9], anti-neoplastic[2,3], anti-viral[10] and anti-cancer[11, 12] activities have been observed. Diosgenin, obtained from yam saponins after hydrolysis, is a principal starting material for industrial production of steroidal drugs[13,14].

Seven steroid saponins have been isolated from *Dioscorea colletti* var. hypoglauca[2,3]. They can be divided into two groups: (1) furostanol glycosides including protoneodioscin, protodioscin, protoneogracillin and protogracillin, (2) spirostanol glycosides including prosapogenin A of dioscin, dioscin and gracillin. There is, however, no report on the qualitative and quantitative determination of saponins in yam cultivars for routine work.

In the present investigation, we developed a high performance liquid chromatographic (HPLC) method for the simultaneous determination of the steroid saponins, including furostanol and spirostanol glycosides. Both UV and evaporative light scattering detector (ELSD) were employed and compared for the detection of saponins. Taiwanese yam cultivars were used as reference samples for testing method evaluation and modification.

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MATERIALS AND METHODS

I. *Material*

Tubers of three yam cultivars were collected in 2001 and 2002 from different areas of Taiwan. Two tubers belonged to *Dioscorea alata* L. The common names and the characteristics (shape of tuber/ color of cortex/ flesh) of these tubers were Ja-I yam (cylindrical/ white/ white) and Yang-Ming-Shan yam (cylindrical/ white/ white). One of the tubers belonged to *D. pseudojaponica* Yamamoto, which was commonly called Kee-Lung yam and was thin cylindrical in shape with white cortex and flesh. Ja-I yam was purchased from a farm in Hualien County. Yang-Ming-Shan yam was purchased from a farm near Yang-Ming-Shan National Park (Taipei City) and Kee-Lung yam was purchased from Chidu in Keelung City. Yam tubers were cleaned, peeled and cut into 4 mm slices using Salad Shooter (National Presto Industries, Eau Claire, WI, USA). The slices were freeze-dried by the FreeZone 18L Freeze Dry System (Labconco Co., Kansas City, MO, USA). All of the freezedried yam slices were ground with a grinder (model RT08, Rong-Tsong Co., Taipei, Taiwan). Steroid saponin standards, including three furostanol glycosides and three spirostanol glycosides were prepared in our laboratory from Kee-Lung cultivar of Taiwanese yam. The method was based on that reported by Chludil et al.[15] and DeMarino et al.[16] The furostanol glycosides were 26-*O*-β-D-glucopyranosyl-22α-methoxyl-25-(*R*)-furost-5-en-3β, 26-diol 3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-{[α-L-rhamnopyranosyl-(1→4)]-*O*-[β-L-rhamnopyranosyl-

(1→4)]}-β-D-glucopyranoside (fu1), methyl protodioscin (fu2) and methyl protogracillin (fu3). The spirostanol glycosides were 25(*R*)-spirost-5-en-3β-ol 3-*O*-α-L-rhamnopyranosyl-(1→2)-*O*-{[α-L-rhamnopyranosyl-(1→4)]-*O*-[α-L-rhamnopyranosyl-(1→4)]}-β-D-glucopyranoside (sp1), dioscin (sp2) and gracillin (sp3). Structures of the saponins are shown in Figure 1. The purities of these standards were above 95% as determined by % total peak area on HPLC chromatogram using ELSD detector. Solvents used for the extraction and analysis of steroid saponins, including methanol, n-butanol, chloroform and sulfuric acid, were purchased from Tedia Co. (Fairfield, OH, USA). Deionized water was obtained from Milli-Q water purification system (Millipore Co., Bedford, MA, USA). It was degassed under vacuum and filtered through a 0.2 μm membrane filter prior to use in the HPLC analysis. *p*-Dimethylaminobenzaldehyde (for Ehrlich reagent) was purchased from Sigma Co. (St. Louis, MO, USA).

II. *HPLC Analysis of Six Steroid Saponins Standards*

Analyses were performed with a Hitachi L-7100 HPLC pump (Hitachi Instruments Inc., Tokyo, Japan) using Luna C18 column (4.6 mm i.d. × 250 mm, 5 μm) (Phenomenex, Torrance, CA, USA) as the stationary phase, which was kept at 45°C in a Colbox column oven (Hipoint Scientific Co., Kaohsiung, Taiwan). The mobile phase was a binary solvent system consisting of methanol and deionized water (62/38, v/v) in the first 20 min and 71/29 (v/v) from 21 to 65 min at a flow rate of 1 mL/min. Either Alltech ELSD 2000 evaporative light scattering detector (ELSD) (tube temperature, 95°C; air flow rate, 2.8 L/min) (Alltech Associates Inc., Deerfield, Ireland) or Hitachi L-7420 UV/VIS detector (Hitachi Instruments Inc., Tokyo, Japan) was used to detect these steroidal saponins. The HPLC separation efficiency was evaluated by the separation factor (α) and resolution (Rs). The maximum wavelength

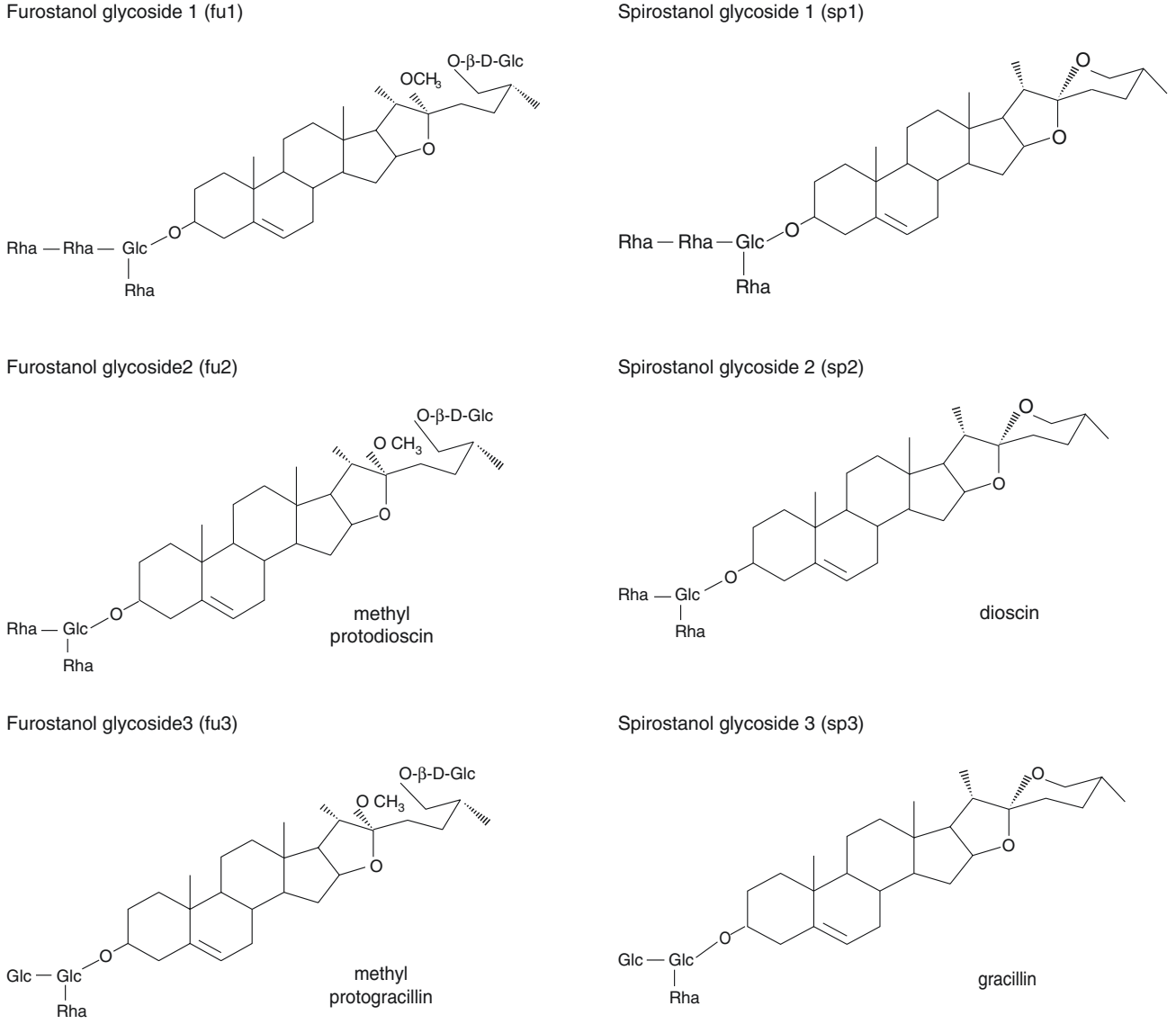

Figure 1. Structures of furostanol glycosides and spirostanol glycosides prepared from *D. pseudojaponica* Yamamoto.

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for the UV/VIS absorption of the six steroid saponins was determined by a Hitachi-U3210 spectrophotometer (Hitachi Instruments Inc., Tokyo, Japan). The detection limit of each steroid saponin was determined as the minimum sample concentration which gave the signal-to-noise ratio (S/N) of 10.

Six concentrations of each steroid saponin standards were injected at 20 μL into HPLC (the ranges for UV 203 nm and ELSD detection are described in Table 3), and the linear regression equation of the standard curve for each steroid saponin was obtained by plotting the amount of saponin injected against the peak area. The regression equation and the correlation coefficient (r^2) were calculated using CHEM-WIN computer software system (Shuen-Hua Co., Taipei, Taiwan). The standard curves of all steroid saponins were determined in triplicate and the mean values were calculated.

III. *HPLC Analysis of Saponins in Yam*

Steroid saponins were extracted from the three cultivars of yams with method similar to that reported in the literatures[2,3,17]. Freeze-dried yam powder (100 g) was extracted with 1 L of methanol for 24 hr at 25°C followed by filtration and concentration in the Rotavapor RE111 rotary evaporator (Büchi Co., Flawil, Switzerland). The residue was suspended in 25 mL of distilled water and partitioned against 25 mL of n-butanol for three times to yield saponin extract. The extract was washed with 50 mL of distilled water for three times and then concentrated to dryness by evaporating the solvent in the rotary evaporator. The dried crude extract was dissolved in 1 mL of methanol prior to the quantitative determination of steroid saponins by HPLC.

Yam extracts (20 μL) were injected into HPLC and analyzed under the same condition as the saponin standards. The steroid saponins in yam extracts were identified by: (1) comparison of retention time and mass spectrum (fu 1, M.W. = 1208; fu 2, M.W. = 1062; fu 3, M.W. = 1078; sp1, M.W. = 1014; sp 2, M.W. = 868; sp 3, M.W. = 884) obtained on a VG platform II LC-MS (Micromass Co., Cheshire, UK). Conditions of LC-MS: ESI^+ mode, cone voltage = 40 eV, source temperature = 200°C; (2) co-chromatography of furostanol and spirostanol glycoside standards with sample; (3) confirmation by thin layer chromatography (TLC). A TLC method similar to that developed by Konishi et al.[18] and Pierre et al.[19] was used. The yams extracts were spotted on silica gel 60 F_{254} TLC plate (No.1.05715, Merck Co., Darmstadt, Germany) and developed with $CHCl_3/CH_3OH/H_2O$: (8/4/1, v/v/v). Visualization was carried out with two spraying agents: (1) Ehrlich reagent (3.2 g *p*-dimethylaminobenzaldehyde in 60 mL 95% ethanol and 60 mL 12N HCl) and heated at 110°C for 5 min after spraying. Furostanol glycosides would show bright red color, while spirostanol glycosides did not show any color change; (2) 10% sulfuric acid (in methanol) and heated at 110°C for 5 min after spraying. In this case, furostanol and spirostanol glycosides were all yellow-green colored.

The recoveries were determined by adding mixture of these six steroid saponin standards (each weighing 0.25, 0.5, 1 and 3 mg) to 100 g of freeze-dried Kee-Lung yam (*D. pseudojaponica* Yamamoto) powder and extracted as

Table 1. Separation factor (α) and resolution (Rs) of the six steroid saponins standards

Compound	Peak number	α[a]	Rs[b]
Furostanol 1	fu1	–	–
glycoside 2	fu2	1.085 (fu1/fu2)[c]	1.247 (fu1/fu2)
3	fu3	1.141 (fu2/fu3)	1.851 (fu2/fu3)
Spirostanol 1	sp1	–	–
glycoside 2	sp2	1.046 (sp1/sp2)	1.613 (sp1/sp2)
3	sp3	1.042 (sp2/sp3)	1.395 (sp2/sp3)

[a]$\alpha = t_{R2}\text{-}t_0 / t_{R1}\text{-}t_0$, where t_{Rn} = retention time of an analyte, t_0 = retention time of an unretained peak.

[b]$Rs = 2\ (t_{R2}\text{-}t_{R1}) / (w_1+w_2)$, where w_n = band width of an analyte at the baseline.

[c]Values in parentheses represent two neighboring peaks.

Table 2. UV λ_{max} and the detection limits of the six steroid saponins standards using UV and ELSD detections

Steroidal saponins		UV λ_{max} (nm)	Detection limit (μg)[a] UV 203 nm	UV 208 nm	UV 214 nm	UV 215 nm	ELSD
Furostanol	1	202.0	0.1	0.5	1	1	2
glycoside	2	202.4	0.1	0.5	1	1	2
	3	202.4	0.2	0.75	1.5	1.5	2.5
Spirostanol	1	201.6	0.2	0.75	1.5	1.5	3.5
glycoside	2	201.2	0.1	0.5	1	1	2
	3	202.0	0.2	0.75	1.5	1.5	3

[a]Detection limit determined at S/N = 10.

Table 3. The linear regression equations of the six steroid saponins standards using UV 203 nm and ELSD detections

Compound		UV 203 nm Linear range (μg)	UV 203 nm Linear regression equations[a]	UV 203 nm Correlation coefficient (r^2)[a]	ELSD Linear range (μg)	ELSD Linear regression equations[a]	ELSD Correlation coefficient (r^2)[a]
Furostanol	1	0.1~60	Y = (8.35 X- 3.84) E4[b]	0.9994	2~60	Y = (1.76 X - 5.33) E4	0.9981
glycoside	2	0.1~60	Y = (10.91 X- 6.49) E4	0.9993	2~60	Y = (2.03 X - 5.29) E4	0.9984
	3	0.2~60	Y = (7.14 X- 2.71) E4	0.9996	2.5~60	Y = (1.13 X - 2.59) E4	0.9982
Spirostanol	1	0.2~60	Y = (6.07 X- 3.02) E4	0.9997	3.5~60	Y = (0.53 X - 1.83) E4	0.9988
glycoside	2	0.1~60	Y = (18.42 X- 4.65) E4	0.9996	2~60	Y = (2.44 X - 7.89) E4	0.9984
	3	0.2~60	Y = (10.54 X- 4.67) E4	0.9997	3~60	Y = (0.76 X - 2.41) E4	0.9984

[a]All data are the means of triplicate analyses.

[b]Y is the value of the peak area, X is the value of sample quantity (μg).

described above. The extracts were then subjected to HPLC analysis for steroid saponins contents, and then recoveries were calculated. The quantitative analyses and determination of recoveries of all steroid saponins were conducted in triplicate and the mean values were determined.

RESULTS AND DISCUSSION

I. *HPLC Separation of Six Steroid Saponins Standards*

Due to the polar nature of glycosidated saponins, high performance liquid chromatography (HPLC) is usually chosen for quantitative analysis. Steroid saponins lack remarkable chromophore for UV and visible detection, so the refractive index (RI) detection and the low-wavelength ultraviolet detection were used in HPLC analysis. Chen et al.[20] has employed a C18 column, a binary solvent system consisting of methanol and water, and RI detection to separate five spirostanol glycosides (the aglycone part was diosgenin) from *Paris* plants simultaneously. The nine spirostanol glycosides present in *Rhizoma paridis* were separated with a C8 column, which was a binary solvent system consisting of acetonitrile and water, and UV 203 nm detection. Five of them contained diosgenin in their aglycone parts[21]. In order to overcome the limit of the gradient elution using RI detection or low-wavelength ultraviolet detection, evaporative light scattering detector (ELSD) has been used to determine the triterpenoid saponins, ginsenosides[22].

We refered to these methods first. After repeated experiments and modification, we employed a C18 column and kept it at 45°C as the stationary phase. The mobile phase was a binary solvent system consisting of methanol and deionized water (62/38, v/v) in the first 20 min and 71/29 (v/v) from 21 to 65 min at a flow rate of 1 mL/min. The three furostanol and three spirostanol glycosides could be separated simultaneously (Figure 2). The furostanol glycosides were eluted at about 20 min, while spirostanol glycosides were eluted at about 60 min. Table 1 shows the separation factors (α) and resolutions (Rs) of the saponins. The α values between fu1 and fu2, fu2 and fu3 were 1.085, 1.141 and the Rs values between fu1 and fu2, fu2 and fu3 were 1.247, 1.851, respectively. Those for the three spirostanol glycosides were 1.046, 1.042 and 1.613, 1.395, respectively. Therefore, the HPLC condition developed in this study showed good separation efficiency of the three furostanol glycosides and the three spirostanol glycosides on one chromatogram. This has not been reported previously. It can be used to determine the steroid saponins in yams for routine work.

UV spectra of the six yam steroid saponins were obtained by dissolving the standard compounds in methanol. The maximum absorption wavelengths of furostanol glycoside 1, 2 and 3 were 202.0, 202.4 and 202.4 nm, respectively, while those of spirostanol glycoside 1, 2 and 3 were 201.6, 201.2 and 202.0 nm, respectively (Table 2). We employed both UV and ELSD detectors during the HPLC analyses of yam saponins and compared their detection ability. The detection limits of the six steroid saponins were determined by ELSD and UV at 203, 208, 214 and 215 nm at signal-to-noise ratio (S/N) of 10 under the developed elution condition. Table 2 shows that the detection limits for 203 nm were 0.1~0.2 mg, which had the highest sensitivity among all UV detections. On the other hand, the detection limits for ELSD were 2~3.5 μg, which had a lower sensitivity compared to UV detection. However, there were more interferences and apparent baseline shift following solvent gradient elution during the determination of the steroid saponins with low-wavelength ultraviolet detection. ELSD could tolerate the interferences of impurities and the gradient elution of mobile phase, and therefore it would improve the accuracy for saponins determination (Figure 2 and 3).

Solutions containing 0.1~60 μg and 2~60 μg of the six saponin standards for UV 203 nm and ELSD detection respectively, were used to obtain standard calibration curves. They were linear and reproducible. The linear regression equations are shown in Table 3. The correlation coefficient (r^2) for UV 203 nm and ELSD detection were above 0.999 and 0.998.

II. *Determination of Steroid Saponins in Yam*

Extraction of steroid saponins in yams usually used methanol or ethanol. After evaporating alcohol, the residue was suspended in water and partitioned against n-butanol to extract saponins for analysis[2, 3, 17]. This method was adopted in our experiment. The freeze-dried yam powder was extracted with methanol followed by liquid-liquid extraction with water and n-butanol. The abovementioned HPLC method developed for the simultaneous analysis of six steroid saponins was employed. Table 4 shows that all

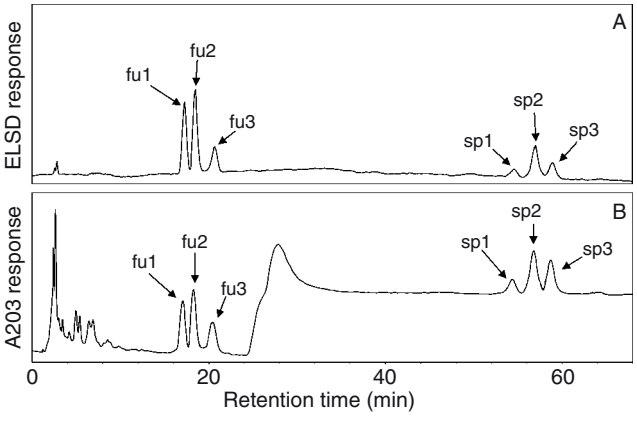

Figure 2. HPLC chromatograms of six steroid saponins. HPLC conditions: column, Luna C18 (4.6 mm i.d. × 250 mm, 5 μm); column temperature, 45°C; mobile phase, CH_3OH/H_2O = 62/38 from 1 to 20 min; 71/29 from 21 to 65 min; flow rate = 1 mL/min; detection, ELSD (tube temperature, 95°C; air flow rate, 2.8 L/min) (A) and 203 nm (B).

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recoveries of the furostanol and spirostanol glycosides (each weighing 0.25 to 3 mg) were above 92%, regardless of the type and the amount of saponin added.

Figure 3 shows the HPLC chromatograms of the methanol extract of *Dioscorea pseudojaponica* Yamamoto. It was clearly indicated that the extraction and analytical methods established in this study could be successfully applied to the analysis of steroid saponins (furostanol and spirostanol glycosides) in Taiwanese yam cultivars without interference peaks, especially using ELSD. Thin layer chromatography (TLC) was also employed to assist in confirming the presence of steroid saponins. The R_f values for furostanol and spirostanol glycosides were around 0.44 and 0.67, respectively (data not shown). Table 5 shows individual content of the six steroid saponins in the three Taiwanese yam cultivars. Furostanol glycosides were found to be the major saponins in Kee-Lung yam and Yang-Ming-Shan yam. The two cultivars of *D. alata* L., Ja-I yam and Yang-Ming-Shan yam collected from different areas of Taiwan, had quite different steroid saponin composition. Dinan et al.[17] indicated that saponins content depended on many factors, e.g., age, geographic location of the plant and the cultivation conditions. It is assumed that these factors caused the difference in the amounts of furostanol and spirostanol glycosides in Ja-I yam and Yang-Ming-Shan yam. Chen and Wu[13] reported that diosgenin contents of the major *Dioscorea* species (*D. colletti*, *D. althaeoides*, *D. colletti* var. hypoglauca, *D. gracillima*, *D. nipponica*, *D. panthaica*, *D. parviflora* and *D. zingiberensis*) in China were 1.5~3.6% of fresh weight. In Indian yams, the contents of diosgenin were 1.42, 3.48 and 0.62% of dry weight in *D. floribunda*, *D. deltoidea* and *D. prazeri*, respectively[23]. However, contents of saponins in the glycoside forms, e.g., furostanol, spirostanol glycosides in yams, have not been reported yet.

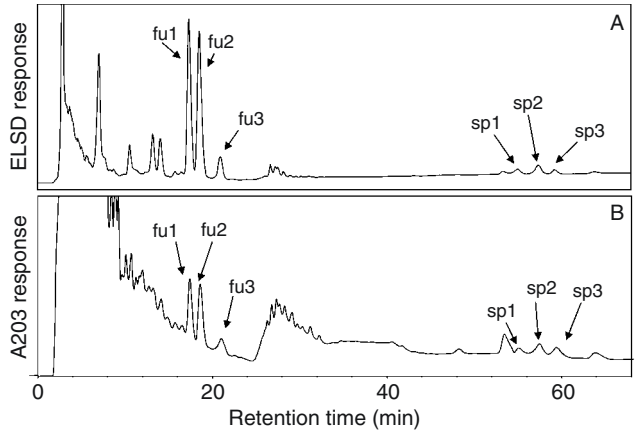

Figure 3. HPLC chromatograms of the methanol extract of *Dioscorea pseudojaponica* Yamamoto. HPLC conditions: same as in Figure 2.

In this study, the total saponin contents in the three Taiwanese yams were found to be in the range of 114.48 to 216.66 μg/g dw. Assuming saponins could be completely converted to diosgenin in acid hydrolysis, the diosgenin content in the three Taiwanese yams would be around 49.06, 57.55 and 84.41 μg/g dw. It is therefore evident that these Taiwanese yams had much lower diosgenin content than Chinese and Indian yams.

CONCLUSION

The three furostanol and three spirostanol glycosides

Table 4. The recovery (%) of added steroid saponins

Compound		Recovery (%)[a]			
		Amount of saponin added (mg)			
		0.25	0.5	1	3
Furostanol glycoside	1	92.57 (7.38)[b]	93.32 (3.74)	94.11 (3.24)	93.89 (1.96)
	2	93.62 (6.54)	94.06 (7.18)	94.52 (1.68)	94.58 (2.52)
	3	92.84 (4.12)	94.13 (8.06)	93.43 (3.06)	93.96 (1.73)
Spirostanol glycoside	1	93.25 (5.17)	93.16 (5.38)	94.16 (3.38)	94.35 (2.06)
	2	92.75 (2.48)	94.21 (3.66)	94.74 (2.66)	94.71 (3.17)
	3	93.28 (6.92)	93.66 (2.34)	93.66 (2.68)	94.25 (1.27)

[a]All data are the means of triplicate analyses.
[b]Values in parentheses are the coefficient of variation (%).

Table 5. Steroid saponins contents in yam fleshes (*Dioscorea* spp.)

Curtivars		Steroidal saponins contents (μg / g dw)[a]								
		Furostanol glycoside				Spirostanol glycoside				Total
		1	2	3	Total	1	2	3	Total	saponins
D. alata L.	Ja-I	10.44 ± 3.22	13.43 ± 2.58	10.94 ± 4.04	34.81	24.37 ± 3.12	34.23 ± 1.69	21.07 ± 3.53	79.67	114.48
	Yang-Ming-Shan	30.49 ± 3.57	36.57 ± 2.19	30.52 ± 1.85	97.58	8.03 ± 3.14	12.09 ± 4.03	25.88 ± 2.33	46.00	143.58
D. pseudojaponica Yamamoto.	Kee-Lung	49.57 ± 2.48	50.74 ± 2.78	47.42 ± 4.96	147.73	20.72 ± 2.87	27.37 ± 2.12	20.84 ± 2.95	68.93	216.66

[a]All values are mean ± S. D. obtained by triplicate analyses.

present in yam could be separated simultaneously by reversed-phase high performance liquid chromatography. C18 column kept at 45°C was used as the stationary phase, the mobile phase was a binary solvent system consisting of methanol and deionized water (62/38, v/v) in the first 20 min and 71/29 (v/v) from 21 to 65 min at a flow rate of 1 mL/min. The recoveries of furostanol and spirostanol glycosides were above 92%. In the three Taiwanese yam cultivars, the contents of furostanol glycosides were found to range from 34.81 to 147.73 μg/g dw, while the contents of spirostanol glycosides ranged from 46 to 79.67 μg/g dw.

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Journal of Food and Drug Analysis, Vol. 11, No. 4, 2003, Pages 277-282 藥物食品分析 第十一卷 第四期

Antibacterial and DPPH Free Radical-scavenging Activities of the Ethanol Extract of Propolis Collected in Taiwan

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(Received: March 19, 2003; Accepted: May 22, 2003)

ABSTRACT

In the present study, ethanolic extracts of propolis (EEP) collected from various regions (Taipei, Mingchen and Fangliao) in Taiwan during different time periods (June, August and October-November, 2000) were tested for their antibacterial and antioxidative activities. In addition, the thermal stabilities of these activities exerted by EEP were also investigated.

It was found that the EEP samples, depending on collecting location and time period, exerted various antioxidative activities in terms of scavenging α,α-diphenyl-2-piorylhydrasyl (DPPH) free radicals and showed various extents of antibacterial activity against *Staphylococcus aureus* and *Listeria monocytogenes*, but not against *Escherichia coli* and *Salmonella typhimurium*. In general, the Taiwanese propolis extract collected in June showed the most profound antibacterial and free radical-scavenging activities than those collected during other time periods. Among all of the samples tested, EEP collected from the Mingchien area in June exhibited the highest antibacterial activity, while that collected from the Taipei area during the same time period showed the highest free radical-scavenging activity. Further tests of EEP collected from Taipei in June revealed that its DPPH free radical-scavenging effects reduced significantly after heating at 50, 80 or 100°C for 1 hr, while its antibacterial activity remained unchanged.

Key words: ethanolic extract of propolis, antibacterial and DPPH free radical-scavenging activity, thermal stability

INTRODUCTION

Propolis is a natural product derived from plant resins collected by honey bees. It is used by bees as glue, a general-purpose sealer, and as draught-extruder for beehives. Propolis has been used in folk medicine for centuries[(1)]. It is known that propolis possesses antimicrobial, antioxidative, anti-ulcer and anti-tumor activities[(2,3)]. Therefore, propolis has attracted much attention in recent years as a useful or potential substance used in medicine and cosmetics products[(4)]. Furthermore, it is now extensively used in foods and beverages with the claim that it can maintain or improve human health[(5,6)].

The chemical composition of propolis is quite complicated. More than 150 compounds such as polyphenols, phenolic aldehydes, sequiterpene quinines, coumarins, amino acids, steroids and inorganic compounds have been identified in propolis samples[(2,7)]. The contents depend on the collecting location, time and plant source[(7-9)]. As a consequence, biological activities of propolis gathered from different phytogeographical areas and time periods vary greatly. In Taiwan, Chang et al.[(10)] reported that the three Taiwanese propolis samples examined contained various amounts of flavonoids, which are generally considered to be the key active biological compounds in propolis.

Although numerous reports concerning the biological activities of propolis collected in Europe and South America have been documented, information concerning the characteristics of Taiwanese propolis is still quite limited. Therefore, antibacterial and antioxidative activities of the ethanolic extract of propolis (EEP) collected from different regions of Taiwan at different time periods in 2000 were determined.

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MATERIALS AND METHODS

I. *Propolis Origins*

Taiwanese propolis samples tested in the present study were all obtained from Professor K. K. Ho, Dept. of Entomology, National Taiwan University, Taipei, Taiwan. These propolis samples were originally collected from beehives located at different regions in Taiwan: Taipei (northern part), Mingchien (middle part) and Fangliao (southern part) in June, August and October-November, 2000.

Brazilian propolis and Chinese propolis samples, originally obtained from Research & Development Division, Institute of Agricultural Research, Chinese Academy of Agricultural Science, Beijing, China, were also provided by Prof. Ho. These propolis samples were stored at -20°C.

II. *Preparation of Ethanolic Extracts of Propolis (EEP) Solution*

Propolis samples were cut into small pieces, ground and extracted with 80% ethanol (1:10, w/v) by shaking (150

rpm) at 25°C for 48 hr. The ethanolic extract solution was then filtered through a Whatman # 1 filter paper and restored to the original volume with 80% ethanol. Based on the individual dry weight determined in the solution, the EEP solution was further adjusted with appropriate amounts of 80% ethanol to obtain solutions containing various amounts of EEP.

III. *Heat Treatment of EEP Solution*

Ethanol solution containing EEP (Taipei-6) was heated at 50, 80 or 100°C for 1 hr by a refluxing system. The heating sample was restored to its original volume with the addition of 80% ethanol and served as the heating sample.

IV. *Microorganisms and Preparation of Inoculum*

To determine the antibacterial activity of EEP, *Staphylococcus aureus* CCRC 12657 and *Listeria monocytogenes* CCRC 19730 were obtained from the Culture Collection and Research Center, Food Research and Development Institute, HsinChu, Taiwan.

After two successive transfers of the test organism in tryptic soy broth (TSB, Difco, Detroit, MI, USA) at 37°C for 12 hr, the activated culture was inoculated into TSB and incubated at the above temperature 37°C for 12 hr. When the population was about 3~7 $\times 10^8$ CFU/mL, it was appropriate to serve as the inoculum.

V. *Determination of Balsam Content*

The EEP solution was evaporated under 105°C until dry. Weight was determined and expressed as weight percentage of balsam in the ethanolic extract solution.

VI. *Measurement of Antibacterial Activity*

Saline solution (0.85% NaCl) in a quantity of 8.9 mL was first added with 0.1 mL of the prepared EEP solution (750 μg/mL) or 0.1 mL 80% ethanol, which served as the control. The mixture was then inoculated with 1.0 mL of the test organism at an initial concentration of 10^7 CFU/mL. Viable population of the test organism was determined after 6 hr of incubation at 37°C.

To enumerate the viable population of the test organism, cultures were first serially diluted with saline solution. One mL of the serially diluted sample was pour-plated onto tryptic soy agar (TSA, Difco, Detroit, MI, USA). Colonies appearing on the plates after 48 hr of incubation at 37°C were counted. In addition, the population reduction (log CFU/mL) was obtained by subtracting the final population (log CFU/mL) in test sample from that in the control.

VII. *Measurement of α,α-Diphenyl-2-piorylhydrasyl (DPPH) Free Radical Scavenging Activity*

The DPPH radical scavenging ability of EEP was determined basically according to the method of Shimada et al.[11]. The EEP solution (20-640 μg/mL) was mixed with 400 μM DPPH (Sigma Chemical Co., St Louis MI.) methanol solution at a ratio of 1:3. The mixture was left in the dark at room temperature for 90 min. The absorbance of the resulting solution was measured by a spectrophotometer at 517 nm. The capability of scavenging DPPH radicals was then calculated by the following equation:

$$\text{Scavenging effect \%} = [1-(A_{517} \text{ of sample}/A_{517} \text{ of control})]$$

VIII. *Statistical Analysis*

The mean values and the standard deviations were calculated from data obtaining triplicate trials. These data were then compared with the least significant difference test[12].

RESULTS AND DISCUSSION

I. *Color and Balsam Content of Propolis*

Differences in the color of Taiwanese propolis harvested at different time periods were noted. In general, the Taiwanese propolis collected in June, regardless of collecting location, appeared yellowish-green, while those collected in August and October-November appeared yellowish-brown and brown, respectively. On the other hand, the color of the Brazilian propolis, similar to that of Taiwanese propolis collected in August, appeared yellowish-brown in color; while the color of the Chinese propolis was dark brown.

In the present study, propolis was extracted with 80% ethanol. The fraction of the propolis soluble in alcohol was usually called "propolis balsam" and it leaves the alcohol-insoluble or wax fraction separate[1]. Balsam contents have been reported to vary with the source of propolis[13,14]. As shown in Table 1, the balsam contents in Taiwanese propolis, ranging between 24.3% and 70.9%, varied not only with collecting regions but also with the collecting time of propolis. In general, a relatively higher content of

Table 1. Balsam contents of various propolis extracted with 80% (v/v) ethanol

Source of propolis	Balsam contents (%, w/w)
Taipei-6	53.7
Taipei-8	31.4
Taipei-11	24.3
Mingchien-6	70.9
Mingchien-8	56.7
Mingchien-10	49.6
Fangliao-6	56.8
Fangliao-8	42.7
Fangliao-10	37.3
Brazil	45.2
China	49.5

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Table 2. Antibacterial activities of the ethanolic extracts of Taiwanese propolis obtained from different regions during different time periods

	Staphylococcus aureus		*Listeria monocytogenes*		*Salmonella typhimurium*		*Escherichia coli* O157:H7	
EEP[a]	Final population[b] (log CFU/mL)	Population reduction[c] (log CFU/mL)	Final population (log CFU/mL)	Population reduction (log CFU/mL)	Final population (log CFU/mL)	Population reduction (log CFU/mL)	Final population (log CFU/mL)	Population reduction (log CFU/mL)
Control[d]	6.95 ± 0.05A[e]		7.02 ± 0.05A		7.41 ± 0.05AB		7.10 ± 0.10A	
Taipei-6	0.84 ± 0.33FG	6.11	0.78 ± 0.28I	6.24	7.38 ± 0.01AB	0.03	7.08 ± 0.10A	0.02
Taipei-8	2.74 ± 0.12C	4.21	2.55 ± 0.25EF	4.47	7.39 ± 0.04AB	0.02	7.11 ± 0.03A	-0.01
Taipei-11	4.54 ± 0.22B	2.41	3.20 ± 0.12D	3.82	7.45 ± 0.03A	-0.04	7.07 ± 0.01AB	0.03
Mingchien 6	0.48 ± 0.41G	6.47	0.10 ± 0.17J	6.92	7.40 ± 0.03AB	0.01	6.95 ± 0.15B	0.15
Mingchien 8	2.06 ± 0.09E	4.89	2.02 ± 0.21G	5.00	7.37 ± 0.01B	0.04	7.07 ± 0.06AB	0.03
Mingchien 10	2.24 ± 0.22DE	4.71	2.25 ± 0.44FG	4.77	7.41 ± 0.04AB	0.00	7.08 ± 0.03A	0.02
Fangliao 6	0.98 ± 0.60F	5.97	0.44 ± 0.47IJ	6.58	7.35 ± 0.03B	0.06	7.06 ± 0.04AB	0.04
Fangliao 8	2.61 ± 0.42CD	4.34	1.54 ± 0.19H	5.48	7.41 ± 0.08AB	0.00	7.09 ± 0.10A	0.01
Fangliao 10	2.76 ± 0.31C	4.19	2.72 ± 0.42E	4.30	7.40 ± 0.05AB	0.01	7.08 ± 0.05A	0.02
Brazil	6.89 ± 0.09A	0.06	4.88 ± 0.02C	2.14	7.39 ± 0.04AB	0.02	7.12 ± 0.08A	-0.02
China	6.95 ± 0.06A	0.00	6.51 ± 0.10B	0.51	7.41 ± 0.04AB	0.00	7.16 ± 0.06A	-0.06

[a]Saline solution (0.85% NaCl) containing 7.5 μg/mL EEP was inoculated with test organism at initial concentration of 10^7 CFU/mL.
[b]Determined after 6 hr of incubation at 37°C.
[c]Population reduction = log (final population in control)- log (final population in test sample).
[d]EEP was substituted with ethanol solution.
[e]Values with the same organism with different capital letters are significantly different ($p < 0.05$) by least significant difference (LSD) test.

balsam was noted in propolis collected in June, regardless of collecting area, than those collected in other months. Among the propolis samples tested, that collected from Mingchien in June showed the highest balsam content. Besides, the Brazilian and Chinese propolis tested showed a balsam content of 45.2% and 49.5%, respectively.

II. *Antibacterial Activity of the Ethanol Extract of Propolis*

It is reported that the antimicrobial activity of propolis reflected its constituent, which may differ from area to area, and season to season depending on its chemical composition[15-18]. Flavonoids and esters of phenolic acids are regarded to be responsible for the anti-microbial activity of propolis[18,19]. Kujumgiev et al.[13] found that tropical propolis did not contain such substances but still showed similar antibacterial activity and indicated that different substance combinations in the propolis are essential for its biological activity. On the other hand, Kedzia et al.[20] reported that the mechanism of anti-microbial activity is complicated and could be attributed to the synergy between flavonoid hydroxyacids and sesquiterpenes. Krol et al.[21] also observed this effect.

Antibacterial activities of the ethanolic extract of propolis gathered from different regions at different time intervals against test organisms are summarized in Table 2. In agreement with the reports of Kujumgiev et al.[13], Nieva Moreno et al.[14] and Dobrowalski et al.[22], the ethanolic extracts of propolis tested did not show antibacterial activity against *Sal. typhimurium* and *E. coli* O157:H7 which were gram (–) bacteria. On the other hand, the Taiwanese propolis extracts exhibited various extents of antibacterial activity against *Sta. aureus*, which showed a marked population reduction, ranging from 2.41 to 6.11 log CFU/mL, under the present test conditions. A population reduction ranging between 6.92 and 3.82 log CFU/mL was also observed with *L. monocytogenes* after 6 hr of exposure to the Taiwanese propolis extracts tested. In general, *L. monocytogenes* was more susceptible to the EEP than *Sta. aureus*. The ethanolic extracts of Chinese and Brazilian propolis tested also showed antibacterial activity against *Sta. aureus* and *L. monocytogenes*, while not against *Sal. typhimurium* and *E. coli* O157:H7.

Sforcin et al.[18] reported that there are no significant difference between the antibacterial activity of Brazilian propolis collected during different seasons. However, we do find a significant difference in the antibacterial activity of Taiwanese propolis due to differences in the collecting time as shown in Table 2. This is in agreement with the report of Santos et al.[23] who found that the propolis extract collected in summer exhibited higher antibacterial activity against *Actinobacillus actinomycetemcomitans* than those collected during other seasons. Among the Taiwanese propolis extracts tested, in general, propolis collected in June exhibited the most profound antibacterial activity than those collected during other time periods against *Sta. aureus* or *L. monocytogenes*. For example, Taipei-6 EEP reduced the viable *Sta. aureus* population by 6.11 log CFU/mL, while Taipei-11 EEP caused a smaller population reduction of only 2.41 log CFU/mL under similar test conditions. Variations in the antibacterial activity of propolis collected in the same time period, while from different locations were also observed. Among the samples collected at the same period, in general, EEP from the Mingchien area exhibited a higher antibacterial activity than those from other areas.

III. *Antioxidative Activity of Propolis Extracts*

Various investigators have reported that propolis

possesses antioxidative activities[24-28]. Among them, Nagai et al.[28] demonstrated the antioxidative activity in commercially available propolis. They postulated that flavonoids, such as quercetin, flavones, isoflavones, flavonones, anthocyanins, catechin and isocatechin may contribute to the antioxidative activity they observed. Nieva Moreno et al.[14] also found that the ethanolic extracts of the Argentina propolis showed free radical-scavenging activity. However, they indicated that different flavonoid compositions and other factors might be involved in the free radical-scavenging activity.

In the present study, the antioxidative activity, in terms of the scavenging of the radical DPPH, of the ethanolic extracts of various propolis was determined and compared. The proton-radical scavenging action has been known as an important mechanism of antioxidation. DPPH was used to determine the proton-radical scavenging action of the propolis extract, since it possesses a proton free radical and shows a characteristic absorption at 517 nm. The purple color of the DPPH solution would fade rapidly when it encounters proton-radical scavengers[29].

Figure 1 shows the dose-response curve for the radical-scavenging activity of the EEP. While Table 3 summarizes the calculated half-inhibition concentration (IC_{50}), the efficient concentration required for decreasing initial DPPH concentration by 50%. IC_{50} was obtained by interpolation from linear regression analysis of data shown in Figure 1. It was found that the propolis extracts tested showed various potencies for free radical-scavenging activity with an EEP where IC_{50} ranged between 17.90 and 108.05 μg/mL (Table 3). The antioxidative activity of all the EEP tested, except Taipei-6 propolis extract, increased with the concentration of propolis extract to 80 μg/mL (Figure 1). From the same region, the Taiwanese propolis extract collected in June exhibited a higher activity than those collected at other time periods. Variations in the free radical-scavenging activity were also noted on the extracts of propolis collected from different regions. Among the propolis extracts tested in the present study, propolis from Taipei collected in June (Taipei-6) exhibited the highest free radical-scavenging activity, while extract of Taipei-11 propolis showed the lowest activity. On the other hand, the IC_{50} for the propolis extract from Brazil and China was found to be 41.41 and 24.53 μg/mL, respectively.

Table 3. Half-inhibition (IC_{50})[a] of EEP in scavenging DPPH radicals

EEP	IC_{50} (μg/mL)
Taipei-6	17.90 ± 0.22I[b]
Taipei-8	42.33 ± 0.12C
Taipei-11	108.05 ± 1.75A
Mingchien-6	28.17 ± 0.32G
Mingchien-8	37.40 ± 1.09E
Mingchien-10	40.12 ± 2.00D
Fangliao-6	31.16 ± 1.48F
Fangliao-8	37.14 ± 0.42E
Fangliao-10	51.48 ± 2.26B
Brazil	41.41 ± 0.46CD
China	24.53 ± 0.57H

[a]IC_{50}, the efficient concentration decreasing initial DPPH concentration by 50%, was obtained by the interpolation from linear regression analysis.

[b]Each value is given as means ± standard deviation (n = 3). Values with capital letters are significantly different ($p < 0.05$) by least significant difference (LSD) test.

IV. *Thermal Stability of the Antibacterial and Antioxidative Activities of Taiwanese Propolis Extract*

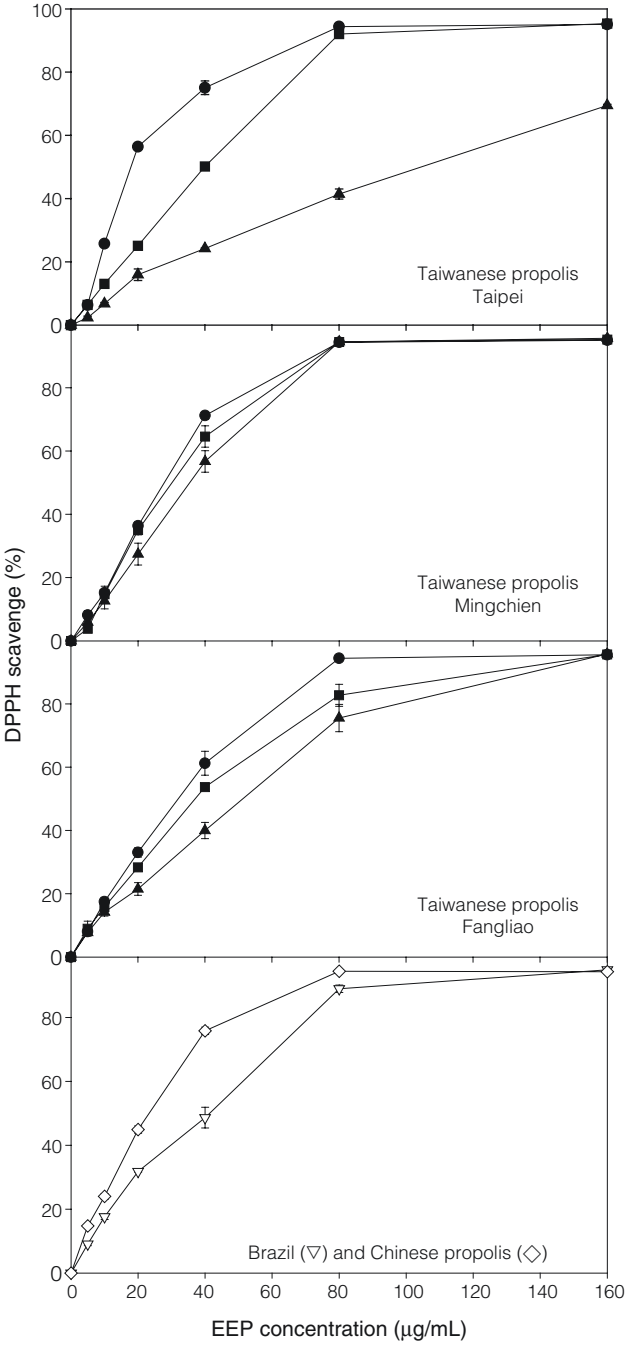

Figure 1. DPPH free radical scavenging effects of EEP obtained from different regions during different time periods. Correcting time: June, ●; August, ■; October (Mingchien and Fangliao) and November (Taipei), ▲.

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Table 4. Effects of heating on antibacterial activities of EEP[a]

Heating temperature (˚C)	*Staphylococcus aureus*		*Listeria monocytogenes*	
	Final population[b] (log CFU/mL)	Population reduction[c] (log CFU/mL)	Final population (log CFU/mL)	Population reduction (log CFU/mL)
Control[d] without heating	6.95 ± 0.05A[e]		7.02 ± 0.05A	
50	0.84 ± 0.33B	6.11 ± 0.33A	0.78 ± 0.28B	6.24 ± 0.28A
80	0.60 ± 0.39B	6.35 ± 0.39A	0.69 ± 0.09B	6.33 ± 0.09A
100	0.97 ± 0.23B	5.98 ± 0.23A	0.33 ± 0.56B	6.69 ± 0.56A
	0.72 ± 0.47B	6.23 ± 0.47A	0.29 ± 0.11B	6.73 ± 0.11A

[a]Saline solution (0.85% NaCl) containing 7.5 μg/mL Taipei-6 EEP which has been heated at the specified temperature for 1 hr, was inoculated with test organism at an initial concentration of 10^7 CFU/mL.
[b]Determined after 6 hr of incubation at 37˚C.
[c]Population reduction = log (final population in control)- log (final population in test sample).
[d]EEP was substituted with ethanol solution.
[e]Values with the same organism with different capital letters are significantly different ($p < 0.05$) by least significant difference (LSD) test.

The effect of heat treatment on the antibacterial and antioxidative activities was investigated on Taipei-6 propolis extract. After each sample was heated at 50, 80 and 100˚C for 1 hr, the antibacterial and antioxidative activities were measured.

As shown in Table 4, the finial populations of either *Sta. aureus* or *L. monocytogenes* in saline solution containing EEP were all less than that found in saline solution containing no EEP (control). However, the final population or population reduction of each test organism noted in the saline solution containing EEP with or without heat treatments did not show any significant difference ($p > 0.05$). This indicated that antibacterial activity of the propolis extract was quite stable under the heat treatments tested.

Table 5 shows the IC_{50} of the Taipei-6 propolis extract after subjecting to various heat treatments. IC_{50} of propolis extracts subjected to 50, 80 or 100˚C heat treatment showed no significant difference ($p > 0.05$) among them. While they are all higher than that of propolis extract without heat treatment, this demonstrated that heat treatments, contrary to that observed on antibacterial activity, reduced the antioxidative activity of propolis extract. The antioxidative activity of propolis was not thermally stable. Porpolis components associated with the antibacterial and antioxidative activities may be different or not entirely the same and thus led to the observed difference in the thermal stability. However, the exact reason remained to be further examined.

Table 5. Half-inhibition (IC_{50}) of heated EEP[a] in scavenging DPPH radicals

Treatment temperature (˚C)	IC_{50} (μg/mL)
50	28.48 ± 0.28A[b]
80	28.25 ± 0.46A
100	28.42 ± 0.98A
Without heating	17.90 ± 0.22B

[a]Taipei-6 EEP was subjected to heating for 1 hr.
[b]Each value is given as means ± standard deviation (n = 3). Values with capital letters are significantly different ($p < 0.05$) by least significant difference (LSD) test.

CONCLUSION

The results of this study demonstrated that ethanolic extracts of Taiwanese propolis possess antibacterial activities and DPPH radicals-scavenging effects which varied with the source and collecting time. Regardless of collecting locations, the extracts of propolis harvested in June, in general, exhibited higher antibacterial and DPPH radical-scavenging activity than those harvested during other time periods. Among the samples tested, extract of propolis collected from Taipei in June (Taipei-6) exhibited the highest DPPH radicals scavenging activity, while Mingchien-6 propolis extract showed the highest antibacterial activity. In addition, although the DPPH radicals scavenging effects of Taipei-6 propolis extract significantly reduced after heating at 50, 80 or 100˚C for 1 hr, its antibacterial activity remained unchanged.

Difference in the plant source available to honey bees at different locations and time periods might lead to differences in biologically active components present in the propolis[7-9]. This may in turn result in the variations of the antibacterial and antioxidative activities of propolis observed in the present study. Therefore, both the identity of the plant source available in Taiwan and the biological active components in Taiwanese propolis merit further investigation. However, results obtained from the present study, along with reports of other investigators[7-9], further stress the importance of quality control when commercial propolis products for these biological activities are being prepared from raw materials.

ACKNOWLEDGMENTS

Appreciation was extended to professor Ho, K. K., Dept. of Entermology, National Taiwan University for providing the propolis samples. This research was financially supported by the National Science Council, ROC (Taiwan). (NSC90-2313-B002-353).

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Journal of Food and Drug Analysis, Vol. 11, No. 4, 2003, Pages 283-289 藥物食品分析 第十一卷 第四期

Physicochemical and Morphological Analyses on Damaged Rice Starches

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(Received: December 31, 2002; Accepted: May 5, 2003)

ABSTRACT

Two types of rice cultivars, indica type (Tainung Sen 19, TNuS19) and waxy (Taichung Waxy 70, TCW70) were used as samples to investigate the changes in the physicochemical properties of damaged rice starch by ball-milling treatment. Rapid Visco Analyzer (RVA) analysis indicated that onset temperature ($T_{o,\eta}$), peak viscosity (P), setback (C-H), and cold-paste viscosity (C) decreases were more pronounced in TNuS19 than in TCW70 starch during the ball-milling treatment. Similar phenomena were also found in peak temperature (T_p) and enthalpy changes (ΔH) by differential scanning calorimetry (DSC), and the peak intensities measured by X-ray diffraction. However, both types of starch showed that extended treatment time resulted in higher median particle size (PS 50). Polarized light micrographs showed that damaged starch granules lost their birefringence, suggesting that the order structure of starch granule was disrupted. The morphological changes in the microstructure of rice starch during ball-milling treatment was also examined by scanning electron microscopy (SEM). The results indicated that TNuS19 starch granules may be more susceptible to mechanical action than TCW70 starch.

Key words: rice starch, ball-milling, damaged starch, rice cultivar changes

INTRODUCTION

When polished rice kernels are ground into rice flour, some starch granules are damaged by the mechanical action of the milling process[1-7]. Recently, damaged starch has been a concern for rice flour production in Taiwan. Chen et al.[1] indicated that the damaged starch and particle size distribution are two key factors affecting the physicochemical properties and the applications of rice flour. The damaged fraction of rice flour may be distinguished microscopically by staining a sample with dyes or from the detection of a loss of birefringence[8,9]. Damaged starch swells extensively in cold water, and the swelling is limited to the damaged portion of the starch granules[10]. The rice flour with a high level of damaged starch generally had high water absorption capacity and was more susceptible to attack by amylase[9-11].

During rice flour milling process, mill type and milling method can profoundly affect the physicochemical characteristics of rice flours[1,2]. Generally, wet-milled rice flour is better than dry-milled flour for making traditional rice-based products baked or steamed[2,7,12]. Chen et al.[2] reported that the wet-milled rice flour gave the lowest damaged starch level and the finest particle size among dry- or semi-dry milled flours. Additionally, the degree of damage is also affected by rice kernel hardness, mill types, milling methods and the soaking process[1,13,14].

Although many milling parameters (i.e. rice cultivar, mill type, milling method and soaking process) on the physicochemical characteristic of rice flour have been investigated, the mechanism of mechanical action on rice flour and starch remains unclear. A better understanding of the mechanical effects would be helpful for the manufacturing of rice flour. It was therefore appropriate to study the effects of physical damages on rice starch before considering the more complex effects of the industrial milling process. In a previous study[8], we have described the effects of the ball-milling treatment on various degrees of starch damage and morphology changes of waxy and non-waxy rice starches. The objectives of this study were to investigate the effects of the ball-milling treatment on the physicochemical properties and the structural integrity of rice starches. The mechanical effects on starch pasting, gelatinization, particle size distribution, microscopic appearance and crystallinity properties were examined.

MATERIALS AND METHODS

I. *Rice Starch and Ball-mill Treatment*

Rice starch from two cultivars, Tainung Sen 19 (TNuS19) and Taichung Waxy 70 (TCW70) were used as samples. Starch isolation and the determination of amylose content were described in a previous work[8]. Ball-milling procedures were also the same: 125 g of rice starch was placed with 10 stainless steel balls (16-mm diameter) in the mill (Fritsch, Type: 06.101, Germany). The operation was

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done at 420 rpm and room temperature. The milled samples were collected at 10, 30 and 60 min, respectively. All tests were run in triplicate.

II. *Analytical Methods*

(I) *Rapid visco analyzer (RVA)*

The pasting behavior of the starch sample was determined with a Rapid Visco Analyzer (RVA 3D, Newport Scientific Pty., Ltd., Narrabeen, Australia). A 28 g of suspension containing 3 g of starch was used. The determination was carried out by heating at 50˚C for 1.5 min, raising to 95˚C in 3.7 min, holding at 95˚C for 2 min, followed by cooling to 50˚C in 3.7 min and keeping at 50˚C for 6.1 min[2].

(II) *Differential scanning calorimetry (DSC)*

The sample of starch: water = 1:3 (w/w) was prepared and kept in a refrigerator overnight for reaching equilibrium. 110 ± 10 mg of the above starch suspension was put into a stainless steel crucible, and sealed with a stainless steel cover with an aluminum O-ring. The sealed sample crucible was scanned by a differential scanning calorimeter (Setaram DSC121; Caluire Cedex, France) from 20˚C to 150˚C at 5˚C /min[2].

(III) *Particle size distribution*

The particle size distribution of 0.02% sample suspended in ethanol was measured by a laser particle size analyzer (Fritsch Analysette 22, Germany). The median particle diameter (PS 50 in μm) was calculated using the software provided by Fritsch Co.

(IV) *Microscopy*

Light microscopy and scanning electron microscopy (SEM) were used to examine the starch granules. Samples were observed using a polarized light microscope (Nikon AFT-IIA, Japan)[1]. For SEM, all samples were mounted on aluminum stubs using double-sided tape, sputter-coated with gold and investigated using an ABT-150S SEM (Topon Corp., Japan) at an accelerated voltage of 15 kV.

(V) *Powder X-ray diffraction*

An X-ray diffractometer (D/MAX-IIIA, Rigaku Denki Co. Ltd., Japan) with the method of Zobel[15] was used.

(VI) *Statistical analysis*

Data were analyzed by Statistical Analysis System (SAS)[16]. Analysis of variance (ANOVA), correlation and Duncan's multiple range test were performed when appropriate.

RESULTS AND DISCUSSION

I. *Pasting Behavior*

The rapid viscosity changes of native and ball-milled starch suspensions during RVA measurements are shown in Figure 1. Rice cultivars and ball-milling treatment time affected all pasting parameters of the starch suspensions, including the onset and peak temperatures ($T_{o,\eta}$ and $T_{p,\eta}$ respectively), peak viscosity (P), hot-paste viscosity (H), breakdown (P-H), cold-paste viscosity (C) as well as setback (C-H). The results indicated that both TNuS19 and TCW70 ball-milled starches tend to exhibit lower $T_{o,\eta}$, P, C-H, and C viscosity than native starches, and were more pronounced in TNuS19 starch than in TCW70 starch. TNuS19 starch showed significant decreases in $T_{p,\eta}$, P, C-H and C as treatment time prolonged. However, TCW70 starch showed a slight decrease for 10 min, but significantly decreased for 30 and 60 min of treatment. After 30 min of treatment, both TNuS19 and TCW70 ball-milled samples showed cold water viscosity (Figure 1). The high proportion of damaged starch probably contributed to their high water retention capacity[8]. The statistical analysis showed

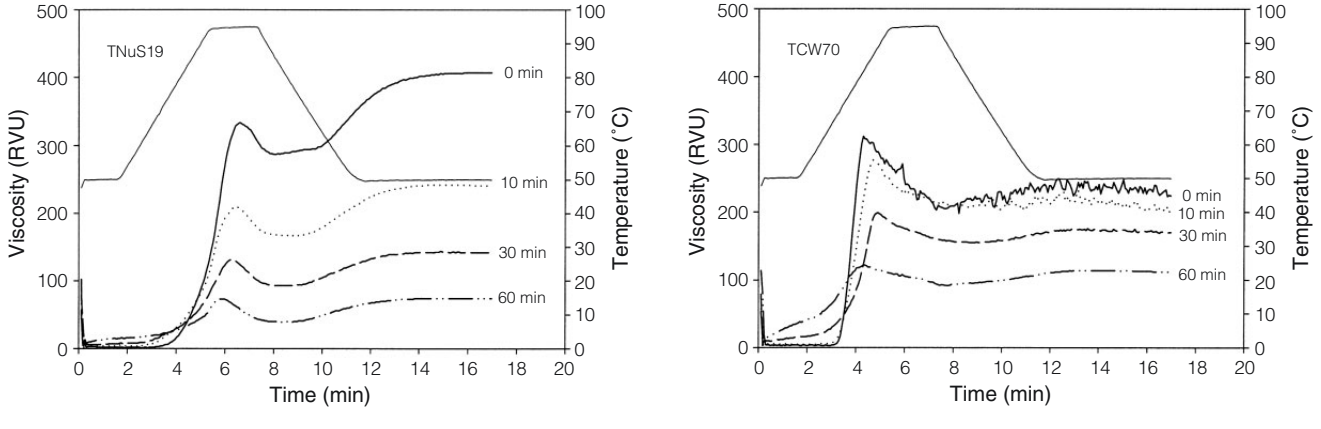

Figure 1. The effect of ball-milling treatment on pasting characteristics of TNuS19 and TCW70 rice starches.

that ball-milling treatment time was negatively correlated with $T_{o,\eta}$ ($r = -0.91$, $p < 0.01$), P ($r = -0.92$, $p < 0.01$), H ($r = -0.89$, $p < 0.01$) and C ($r = -0.83$, $p < 0.01$) (Table 1). Many researchers have reported that ball-milling caused starch molecules (i.e. amylopectin and amylose) to break down into low molecular weight fragments, which could have caused decreases in the paste viscosity of starches[11, 17, 18, 19]. Morrison and Tester[19] suggested that low molecular weight fragments (LMWAP) of amylopectin were formed by breaking glycosidic bonds in B_2, B_3, and B_4 internal chain segments of amylopectin.

II. *DSC*

Figure 2 and 3 show the effect of ball-milling treatment on the DSC thermograms of TNuS19 and TCW70 starches, respectively. The results indicated that the TNuS19 sample significantly ($p < 0.05$) decreased the onset (T_o), peak (T_p), and conclusion (T_c) temperatures as well as enthalpy changes (ΔH) after the ball-milling treatment. However, TCW70 showed slight change for 10 min, and significantly decreased ΔH for 30 and 60 min treatment, which indicated the order structure of the starch granule was disrupted. The statistical analysis showed that ΔH was negatively correlated with ball-milling treatment time ($r = -0.84$, $p < 0.01$), and positively correlated with pasting parameters $T_{o,\eta}$ ($r = 0.73$, $p < 0.05$), P ($r = 0.95$, $p < 0.01$), P-H ($r = 0.87$, $p < 0.01$), H ($r = 0.85$, $p < 0.01$) (Table 1). The results indicated that the effects of ball-milling may be attributed partly to the conversion of starch into a more amorphous form as treatment time increased. Morrison et al.[20] suggested the decreases in T_o, T_p, T_c and ΔH with increasing level of damaged starch for wheat and maize starch. Several studies have reported that amylose in normal starch was much less affected by physical impact compared with amylopectin[17,18,19]. Tester et al.[21] observed that small B-granules of wheat starch were more susceptible to damage than large A-granules. Mok and Dick[22] indicated that isolated starch from hard wheat is more susceptible to physical action than that from soft wheat. From our previous study[8], we have shown that increasing ball-milling periods caused a higher degree of starch damage ($p < 0.05$), and TNuS19 starch were more susceptible to damage than TCW70. Lu et al.[23] reported that the amylopectin of indica rice had lower molecular weight, lower average degree of polymerization (DP), and lower average chain number when compare to japonica and

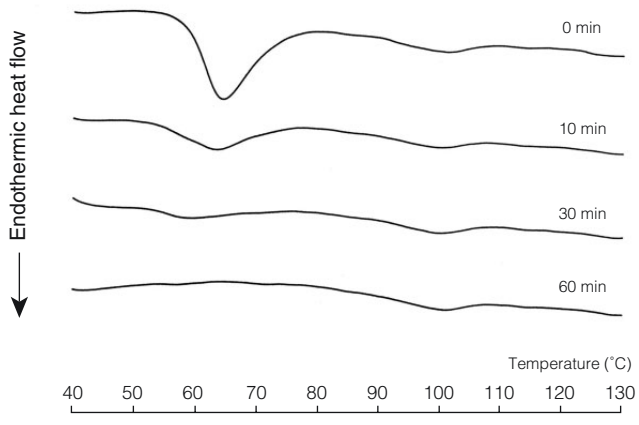

Figure 2. The effect of ball-milling treatment on DSC therograms of TNuS19 rice starch.

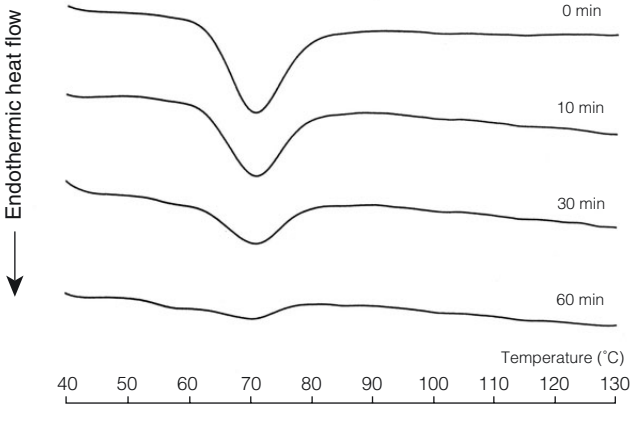

Figure 3. The effect of ball-milling treatment on DSC therograms of TCW70 rice starch.

Table 1. Correlation matrix between ball-milling time, pasting behaviors[a] and thermal characteristics[b] of ball-milled rice starches

	Treatment time	$T_{o,\eta}$	P	P-H	C-H	H	C	T_o	T_p	T_c
$T_{o,\eta}$	-0.91**[c]									
P	-0.92**	0.81*								
P-H	-0.66	0.57	0.70							
C-H	-0.54	0.67	0.48	-0.07						
H	-0.89**	0.79*	0.97**	0.51	0.59					
C	-0.83**	0.81*	0.87**	0.31	0.82*	0.94**				
T_o	-0.55	0.41	0.61	0.35	0.06	0.61	0.46			
T_p	-0.55	0.40	0.60	0.35	0.04	0.60	0.45	1.00**		
T_c	-0.57	0.43	0.60	0.35	0.07	0.61	0.47	1.00**	1.00**	
ΔH (mJ/mg)	-0.84**	0.73*	0.95**	0.87**	0.25	0.85**	0.69	0.55	0.54	0.53

[a]All pasting parameters of the starch suspensions, including the onset temperatures ($T_{o,\eta}$), peak viscosity (P), hot-paste viscosity (H), breakdown (P-H), cold-paste viscosity (C) as well as setback (C-H).

[b]Thermal parameters: T_o: onset temperature; T_p: peak temperature; T_c: conclusion temperature; ΔH: enthalpy.

[c]One asterisk indicates signifcance at the 0.05 level; two asterisks indicate significance at the 0.01 level.

waxy for 14 Taiwan rice cultivars. It was suggested that the mechanical effects on pasting and gelatinization properties were more pronounced in TNuS19 starch than TCW70 starch granules and may differ with respect to compositional or granule structural differences between these two starches.

III. *Particle Size Distribution*

The particle size distribution of native and ball-milled starches were determined by laser particle size analyzer (Figure 4). The data showed that the particle size in TNuS19 starch significantly increased after 10 min of treatment, but those in TCW70 did not. After 30 min of treatment, the median particle diameter (PS 50 in μm) of TNuS19 increased from 6.2 μm to 12.1 μm, but those of TCW70 (6.2 μm) did not change. Both rice varieties showed a significant increase in PS 50 after 30 and 60 min of treatment. PS 50 increased with increasing ball-milling time and was probably due to some stacked starch granules. Meuser et al.[17] reported that ball-milling increased the number of free hydroxy groups to form hydrogen bonding of starch molecules.

IV. *Microscopic Examination*

Polarized light micrographs of native and damaged TNuS19 starch are shown in Figure 5. The result indicated that TNuS19 starch granules significantly lost their birefringence as treatment time prolonged, suggesting that the ordered structure of the starch granule was disrupted. Similar phenomena were also found in TCW70 starch (data not shown). From our previously study[8], iodine and congo red staining analysis showed that the morphology of

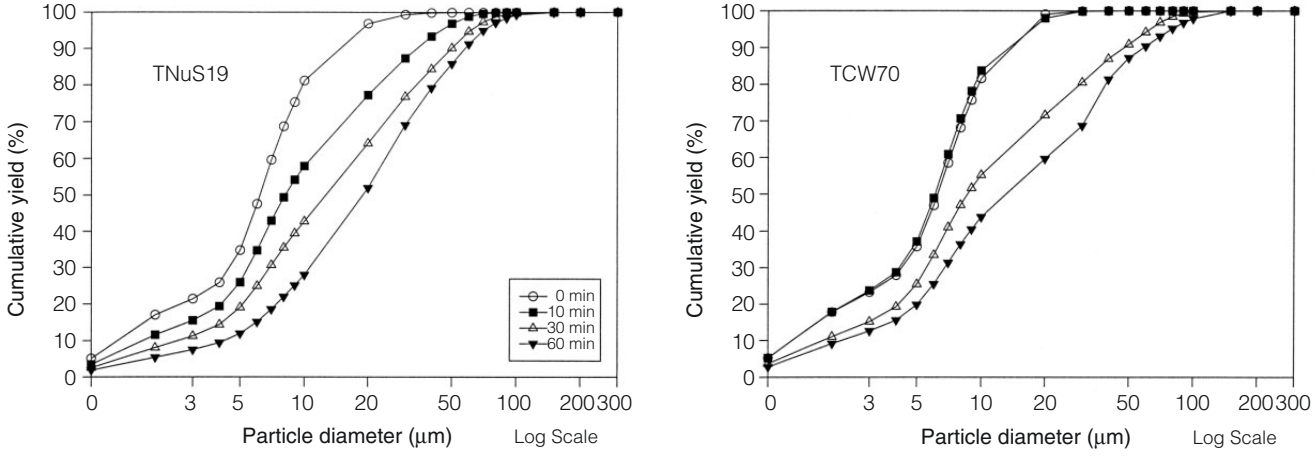

Figure 4. The effect of ball-milling treatment on particle size distributions of TNuS19 and TCW70 rice starches.

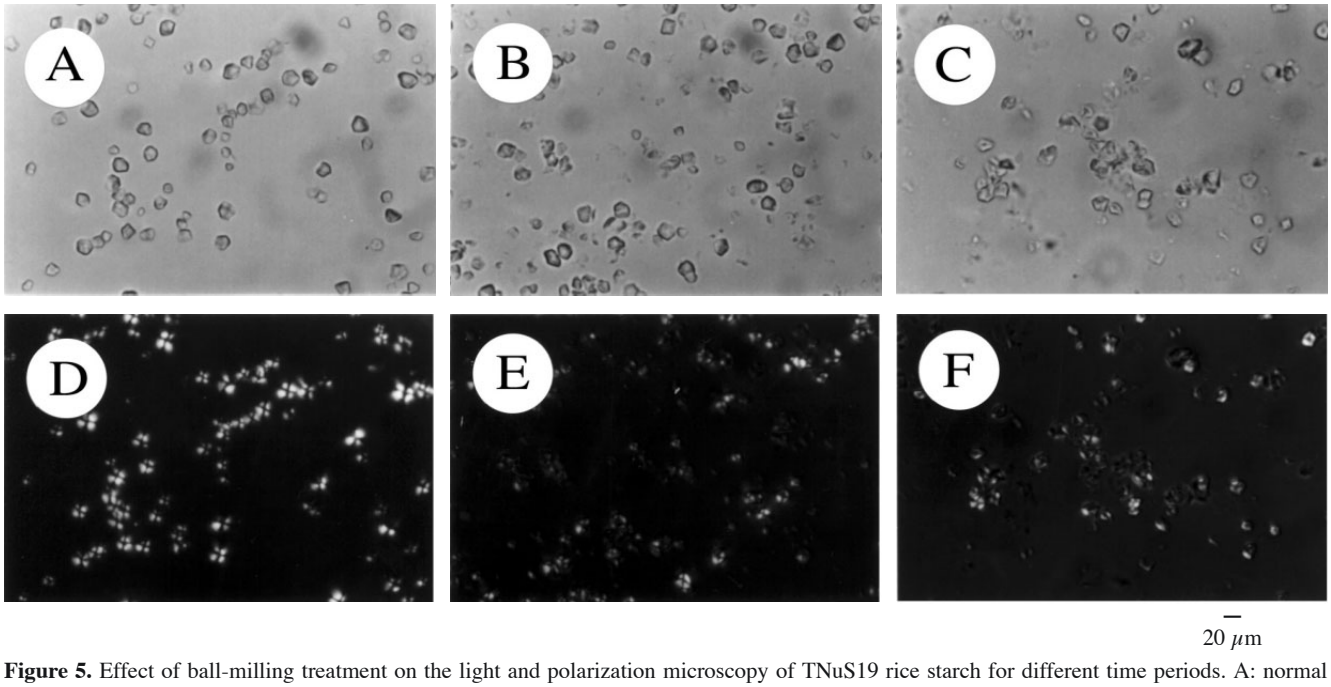

Figure 5. Effect of ball-milling treatment on the light and polarization microscopy of TNuS19 rice starch for different time periods. A: normal light, 0 min; B: normal light, 10 min; C: normal light, 30 min; D: polarized light, 0 min; E: polarized light, 10 min; F: polarized light, 30 min.

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damaged TNuS19 starch was very different to TCW70 starch. Damaged TNuS19 starch gave a more granular form, while TCW70 starch gave more random disrupted shapes after the absorption of water. This result may be due to amylose molecules that can hold the starch molecules together[8].

SEM clearly indicated that the surfaces of the starch granules lost flatness and smoothness, and became rough as milling time continued (Figure 6). Both rice cultivars significantly showed that some granules had attached to each other after 60 min of treatment (Figure 7). These results suggest that glycosidic bonds were broken during ball-

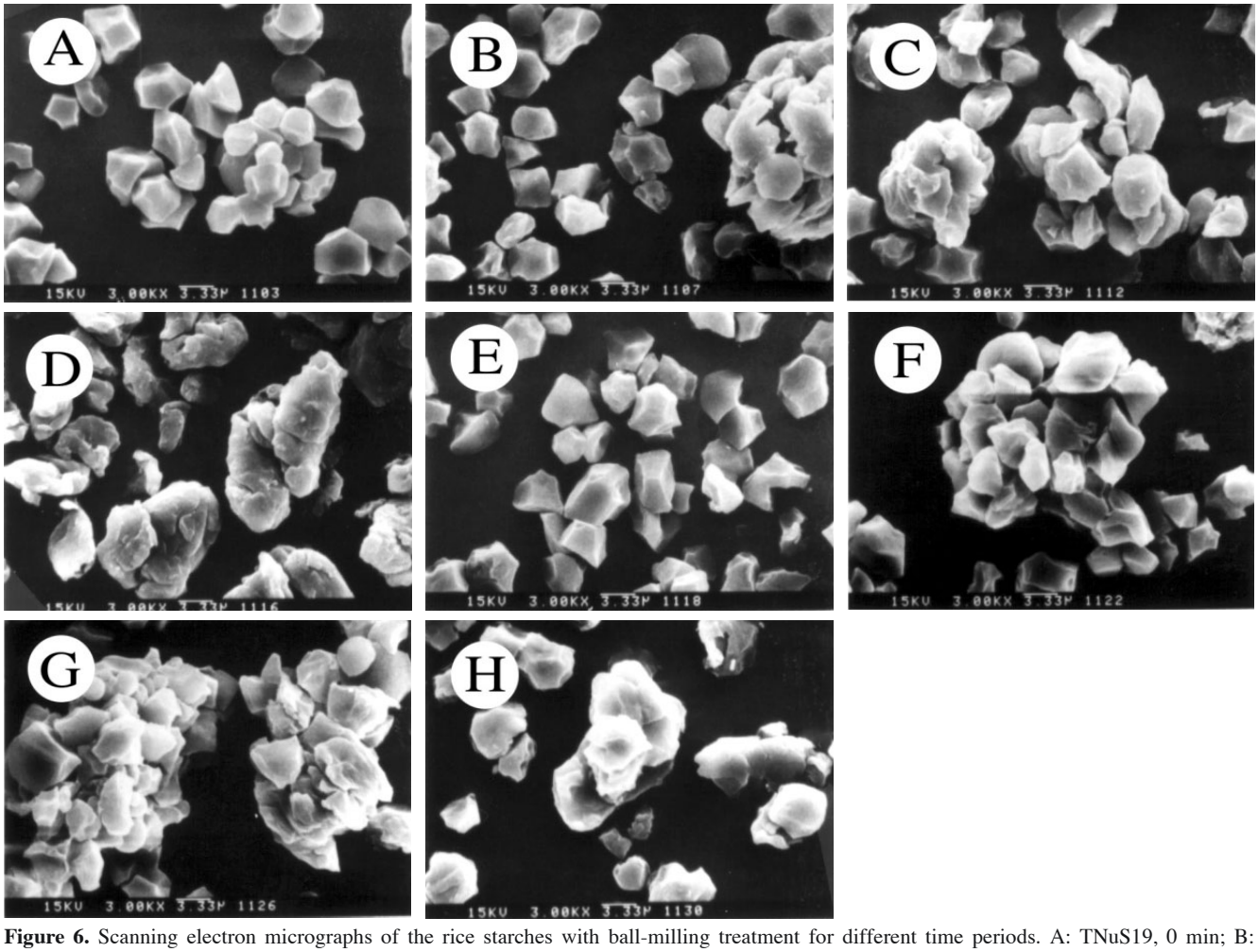

Figure 6. Scanning electron micrographs of the rice starches with ball-milling treatment for different time periods. A: TNuS19, 0 min; B: TNuS19, 10 min; C: TNuS19, 30 min; D: TNuS19, 60 min; E: TCW70, 0 min; F: TCW70, 10 min; G: TCW70, 30 min; H: TCW70, 60 min.

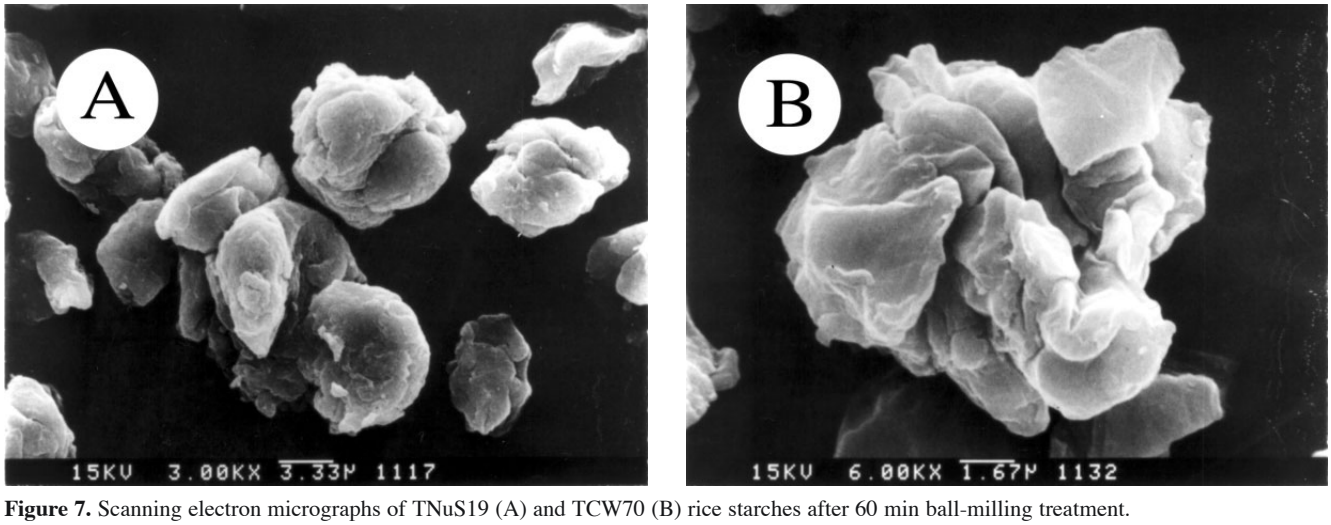

Figure 7. Scanning electron micrographs of TNuS19 (A) and TCW70 (B) rice starches after 60 min ball-milling treatment.

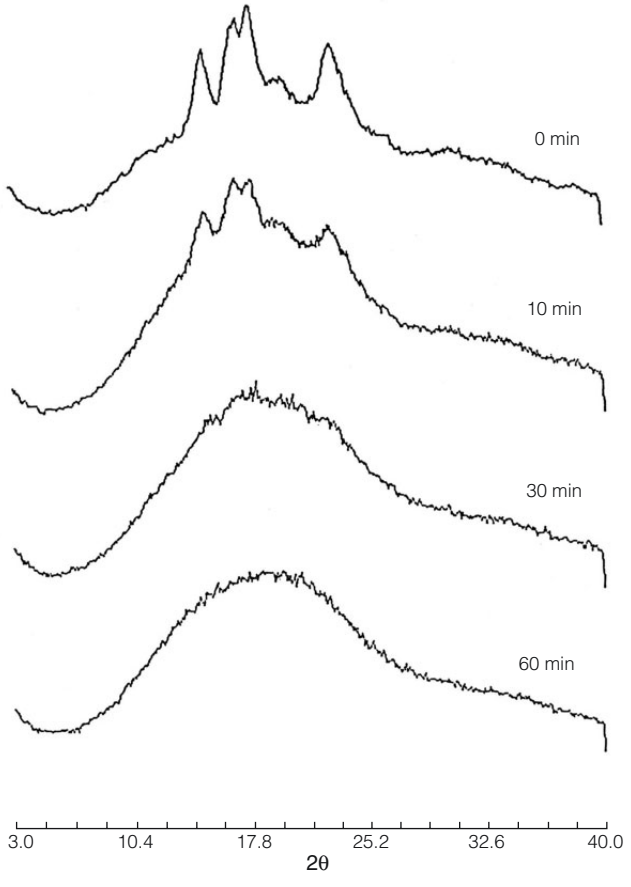

Figure 8. Effect of ball-milling treatment on X-ray diffraction patterns of TNuS19 rice starch.

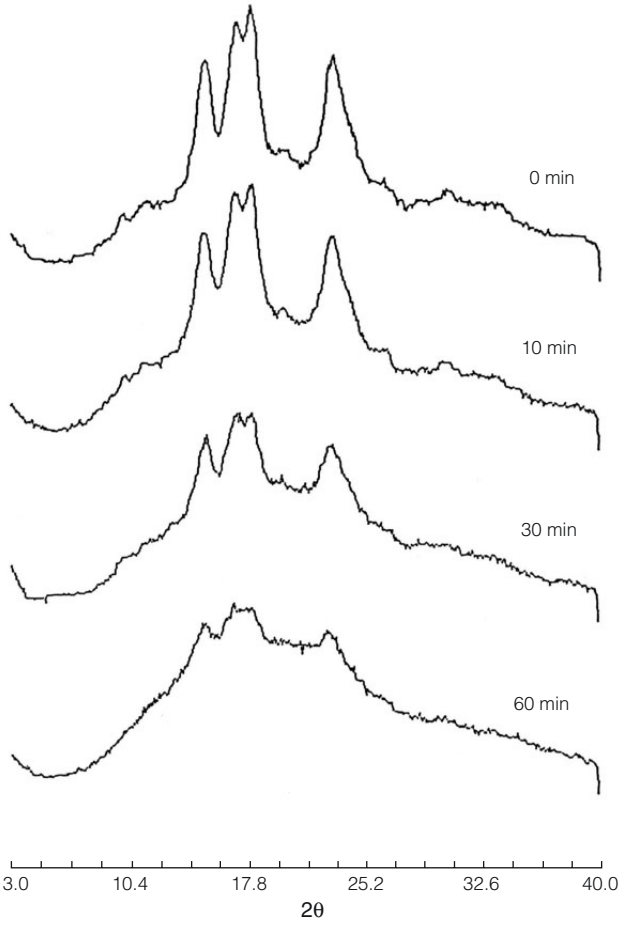

Figure 9. Effect of ball-milling treatment on X-ray diffraction patterns of TCW70 rice starch.

milling and the result increased free hydroxy groups to form hydrogen bonding between starch molecules. These results confirmed the findings of a previous researcher[17]. The surface of the granules was rough and cracks were also found in corn and barley starch after ball-milling[11, 21].

V. *X-ray Diffraction*

The effects of ball-milling treatment on X-ray diffraction properties of TNuS19 and TCW70 starches are shown in Figure 8 and 9. The results of X-ray diffraction indicated that both starches showed A type pattern, and ball-milled samples showed significant decrease in X-ray peak intensities. TNuS19 showed significant decreases in peak intensities after 10 min of treatment, but TCW70 did not. TNuS19 starch almost lost peak intensity after 30 min and completely lost it after 60 min. However, TCW70 starch retained the pattern through 60 min of ball-milling treatment. The results indicated that the effects of milling may be attributed partly to the conversion of starch into a more amorphous form as treatment time increased. Morrison and Tester[19] reported that amylose is barely affected by physical damage to the granule, but amylopectin is progressively degraded into low-molecular-weight fragments. A ^{13}C solid-state-NMR spectra of wheat starches suggests that disruption occurs at the glycosidic linkage during ball-milling and resulted in increases in free hydroxyl groups and the decomposition of polymers[24].

ACKNOWLEDGMENTS

We wish to thank the Taichung District Agricultural Improvement Station, Taiwan, for all material support.

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