SE-HPLC Separation of Myosin Complex with Tannins of Bearberry (*Arctostaphylos uva-ursi* L. Sprengel) Leaves – A Short Report

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Abstract

Amarowicz R., Pegg R.B., Kosińska A. (2009): **SE-HPLC separation of myosin complex with tannins from bearberries** (*Arctostaphylos uva-ursi* L. Sprengel) **leaves – a short communication.** Czech J. Food Sci., **27**: 386–391.

Phenolic compounds were extracted from bearberry (*Arctostaphylos uva-ursi* L. Sprengel) leaves into 95% (v/v) ethanol. The tannin constituents were separated from the crude extract using Sephadex LH-20 column chromatography with 95% (v/v) ethanol and 50% (v/v) acetone as the mobile phases. Myosin was isolated and purified from excised pork knuckle muscles using the standard salt-solution extraction procedure followed by gel filtration chromatography. Myosin was precipitated from the solution with bearberry-leaf tannins at pH 5.0. The recovered complex was washed, lyophilised, and subjected to size-exclusion high performance liquid chromatography (SE-HPLC). Based on the basic conditions of the HPLC analysis, a portion of the tannin constituents was liberated from the complex. The UV spectra of these compounds were characterized by a maximum at ~ 300 nm. A portion of the tannins was present in the complex with myosin, and this was confirmed by UV spectra.

Keywords: myosin; tannins; SE-HPLC; bearberry; protein-tannins complex

Nutritionists define tannins as antinutrients of plant origin because they can precipitate proteins, inhibit the action of digestive enzymes, and decrease the utilisation/bioavailability of vitamins and minerals (Lachman *et al*. 1998). The presence of tannins in foods can also be highly undesirable, as they contribute to astringent or bitter tastes in certain foods and beverages (Amarowicz *et al.* 2004b). On the other hand, tannins have also been considered as "health-promoting" components in plant-derived foods and beverages. For instance, they have been reported to possess anticarcinogenic and antimutagenic potentials as well as antimicrobial properties (Cos *et al*. 2003; Awika *et al*. 2005; Amarowicz *et al*. 2000b, 2008). Several studies have reported on the antioxidant and antiradical activities of tannins (Amarowicz 2007; Amarowicz *et al*. 2000a; 2004a, 2005).

The phenomenon of protein-tannins interaction is attributed to some combinations of hydrogen and/or hydrophobic bonding (Hagerman & Butler 1981; Siebert 1999; Papadopoulou & Frazier 2004). Tannins bind to salivary proteins which leads to the precipitation of insoluble complexes and the perception of astringent flavour (Baxter *et al*. 1997). The findings of some studies

have shown that tannins are potent free-radical scavengers, even when bound to protein, and that the complexes formed may act as a radical sink within the gastrointestinal tract (Riedl & Hagerman 2001).

The antioxidative properties of tannin-rich extracts have been observed in pork model system studies, as shown by the reduction in the formation of 2-thiobarbituric acid reactive substances (TBARS) during the storage of cooked meat. Noteworthy is that the antioxidant activity was also observed with the myosin-tannins complex so prepared; the complex incorporated into a pork model system at 500 and 750 ppm levels exhibited an antioxidant efficacy equivalent to that of free tannins from a bearberry-leaf (*Arctostaphylos uva-ursi*) extract added at 50 ppm (Pegg & Amarowicz 2004).

The objective of the present study was to examine SE-HPLC as a tool to separate and partially characterise myosin-tannins complexes. The leaves of the bearberry (*Arctostaphylos uva-ursi* L. Sprengel) plant were used as the source of tannin constituents, due to the fact that a strong antioxidant activity has been reported for bearberry-leaf polyphenolics (Amarowicz *et al*. 1999; 2004a; Pegg *et al*. 2005).

MATERIAL AND METHODS

Materials. All solvents used were of HPLC or analytical grade, unless otherwise specified. Bearberry (*Arctostaphylos uva ursi* L. Sprengel) leaves were obtained as a gift from Dr. Branka Barl, formerly of the Department of Plant Sciences, University of Saskatchewan, Saskatoon, Canada. Excised pork knuckle muscles were purchased from Mitchell's Gourmet Foods (Saskatoon, Canada).

Extraction of phenolic compounds from bearberry leaves. The leaves were dried at 35°C, cut-up, ground in a Moulinex coffee mill, transferred to dark-coloured flasks, mixed with 95% (v/v) ethanol at the material to solvent ratio of 15:100 (w/v), and then extracted in a shaking Magni Whirl constant temperature bath (Blue M Electric Company, Model MSG-1122A-1, Blue Island, IL) at 50°C for 30 min (Amarowicz *et al*. 1999). The extraction was repeated two more times, the supernates were combined and ethanol was evaporated under vacuum at 40°C using a Büchi Rotavapor/Water bath (Models EL 131 and 461, respectively, Brinkmann Instruments Ltd., Toronto, Canada). The extracts obtained were stored at 4°C until further analysed.

Column chromatography. A 2-g portion of the crude bearberry-leaf extract was suspended in 20 ml of 95% (v/v) ethanol and applied onto a chromatographic column (5 cm × 40 cm) packed with Sephadex LH-20 and equilibrated with 95% (v/v) ethanol. Low-molecular-weight phenolic compounds were eluted from the column using 1000 ml of 95% (v/v) ethanol. To obtain the tannin constituents, the column was washed with ~ 600 ml of 50% (v/v) acetone (Strumeyer & Malin 1975). Then, the organic portion was evaporated using the Rotavapor and water was removed from the aqueous solution by lyophilisation.

Isolation of myosin. Myosin was isolated from the pork knuckle muscles according to Dudziak & Foegeding (1988) with slight modifications. All steps involved are shown in Figure 1. For purification, gel filtration chromatography was employed. The separation conditions were as follows: column was 3 cm × 60 cm packed with Toyopearl WH-55F gel; the mobile phase was 20mM sodium phosphate (pH 7.0) in 0.5M NaCl; 4-ml fractions were collected using a fraction collector (Model SC100, Beckman Coulter, Inc., Fullerton, Canada) in the cold room at 4°C; and absorbance readings at 280 nm were taken to identify the myosin elution. The collected fractions containing myosin were lyophilised and subsequently used for the preparation of myosin-tannins complex.

Preparation of myosin-tannins complex. A 50-mg portion of tannins separated via Sephadex LH-20 column chromatography was dissolved in 50 ml of deionised water. An equal mass of myosin was dissolved in 50 ml of 0.20M acetate buffer (pH 5.0) containing 0.17M sodium chloride (Hagerman & Butler 1978). The two solutions were mixed, followed by a 30-min quiescent period at room temperature. The reaction mixture was transferred to a 250-ml polypropylene centrifugal tube. The tube was centrifuged for 10 min at 10 800 × g in a Sorvall RC-6 Plus Superspeed Centrifuge (Thermo Fisher Scientific Inc., Waltham, USA). The supernate was carefully decanted. The precipitate adhering to the wall of the centrifuge tube was scraped of and the product was mixed with *ca.* 20 ml of deionised water and transferred into a 50-ml beaker. The beaker and its contents were frozen at –30°C and then placed on a shelf in a FreeZone 2.5 Liter Benchtop Freeze Dry System

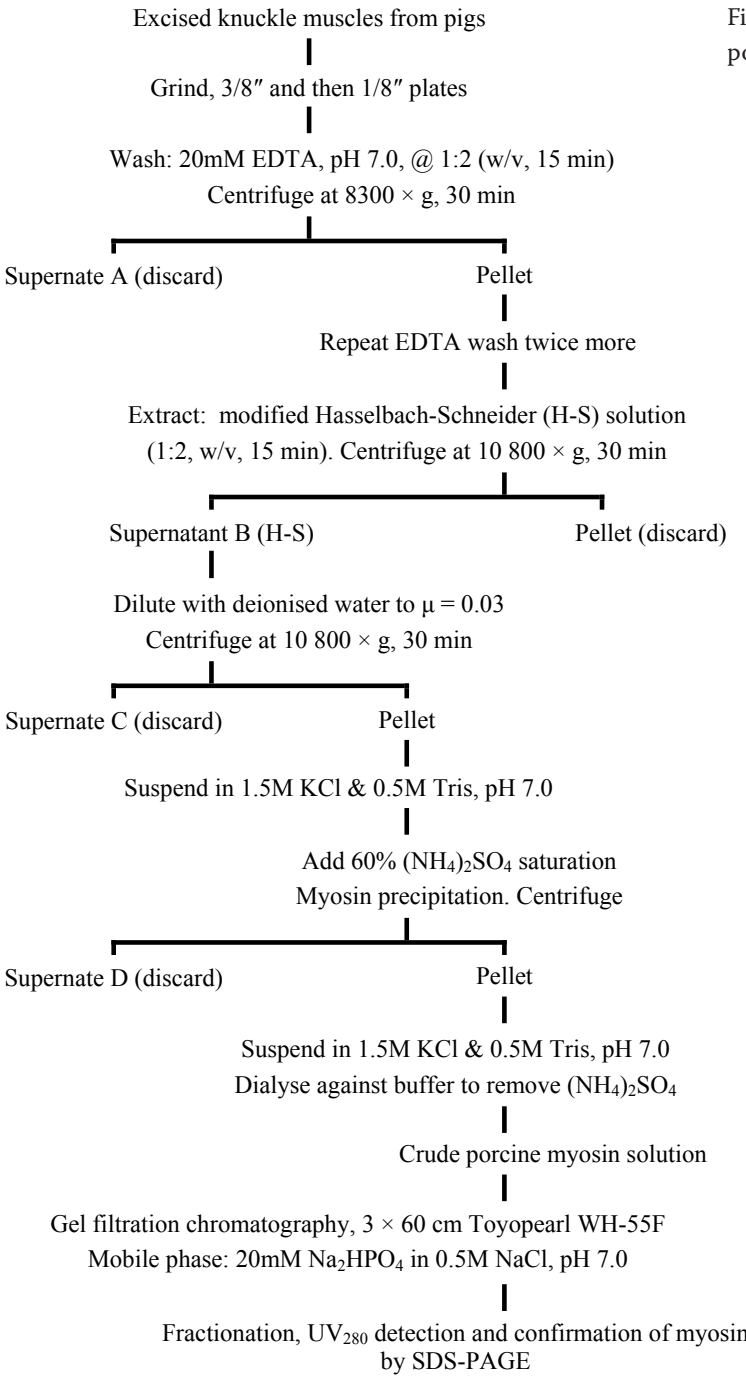

Figure 1. Isolation and purification of myosin from porcine knuckle muscles

(Labconco Corporation, Kansas City, USA) until all water was removed.

SE-HPLC analysis. The tannins fraction and myosin-tannins complex were analysed using a Shimadzu HPLC system (Shimadzu Corp., Kyoto, Japan) consisting of a LC-10AD pump, SCTL 10A system controller, and SPD-M 10A photodiode array detector. The separation was achieved with a TSK G2000SW$_{XL}$ column (7.8 mm × 300 mm, 5 μm; Tosoh Bioscence LLC, Montgomeryvile, USA); the mobile phase of borate buffer (0.2M, pH 8.3) was delivered at a flow rate of 0.8 ml/min; the detection was monitored at 220 nm; and the samples were dissolved in the mobile phase (i.e., 2 mg of complex/ml and 0.25 mg of tannins/ml) before their injection (20 μl) onto the column (PEGG *et al.* 2008).

RESULTS AND DISCUSSION

Figure 2 depicts a chromatogram of the myosin-tannins complex (A) and the tannins frac-

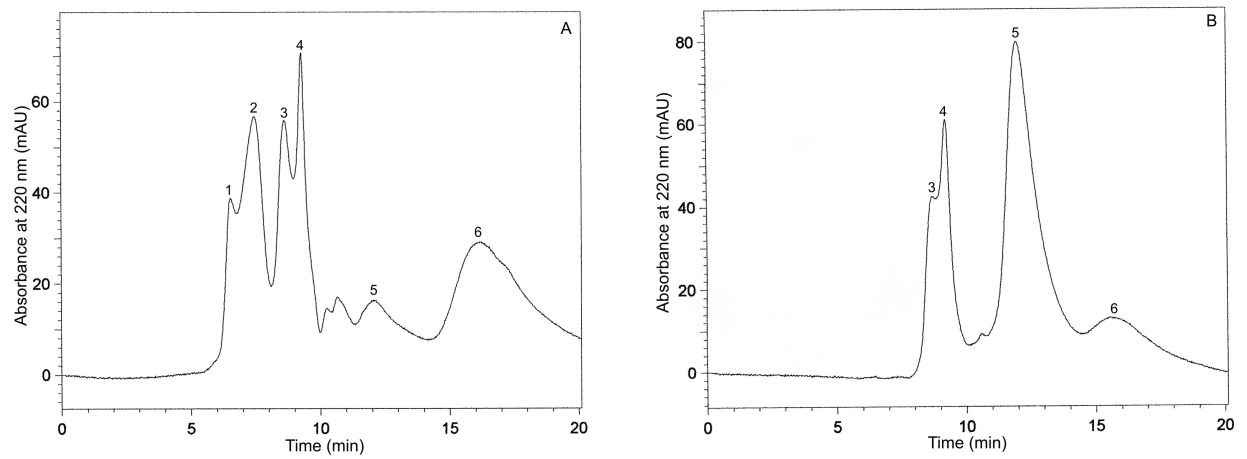

Figure 2. SE-HPLC separation of myosin-tannins complex (A) and tannin fraction from bearberry (*Arctostaphylos uva ursi* L. Sprengel) leaves

tion isolated from the ethanolic bearberry-leaf extract (B). Size-exclusion chromatography of the tannins-myosin complex (Figure 2A) was characterised by the presence of six peaks with the retention times of 6.57, 7.37, 8.65, 9.25, 11.99, and 15.59 minutes. Peaks 1 and 2 originated from the tannins complex of myosin, as confirmed by UV spectra. Figures 3: 1 and 3: 2 show an absorption band at 284 nm which is typical for proteins, and a shoulder at 304 nm that originated from the tannin constituents.

The SE-HPLC chromatogram of isolated tannins (Figure 2B) was characterised by four peaks (numbers 3 to 6) with the retention times same as those in chromatogram 2A. UV spectra of the separated compounds attributed to peaks 2–6 showed maxima at 300, 292, 301, and 302 nm (Figures 3: 2, 3, 6), respectively. Compounds 3, 5,

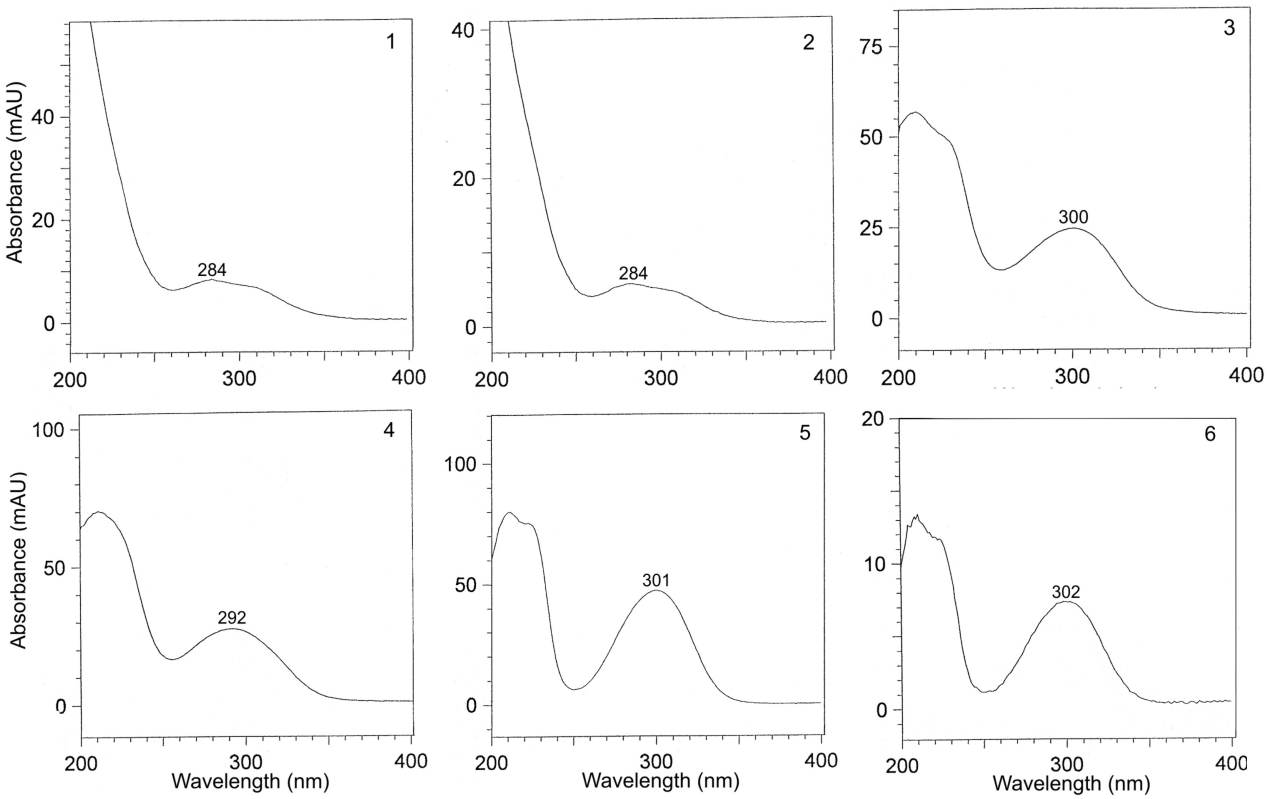

Figure 3. UV spectra of compounds separated by SE-HPLC; the numbers are related to the peaks from Figure 2

and 6 are most likely hydrolysable tannins, whereas compound 4 was clearly a condensed tannin. This conclusion is in accordance with our previous results showing the presence of both condensed and hydrolysable tannins in the bearberry-leaf extract (Pegg *et al*. 2005).

The presence of the peaks coming from tannin constituents in chromatogram 2A indicates that the myosin-tannins complex is not stable. Based on the conditions of the SE-HPLC (i.e., the basic pH of the mobile phase, 8.3), a majority of tannins are liberated from the complex. An interesting observation is that compound 5, which is the main constituent in the tannins fraction, shows a weak activity in the formation of a complex with myosin.

The application of SE-HPLC reported in this study is a new method used for the detection of complexes of phenolic compounds with proteins. Before us, Bartolomé *et al*. (2000) used gel filtration on Sephadex G-50 to investigate the interaction of procyanidins with bovine serum albumin (BSA). The main methods used by scientists are RP-HPLC and capillary electrophoresis. Hagerman *et al*. (1998), for example, employed a C_{18} (ODS) column to study the mechanism of protein precipitation with pentagalloyl glucose and procyanidin. To estimate the binding constants between the protein and phenolic ligand, capillary electrophoresis was applied. The presence of complexes of rapeseed proteins and phenolic constituents was monitored by capillary electrophoresis and RP-HPLC methods (Amarowicz *et al*. 2003; Kosińska *et al*. 2006). Preferential binding of sorghum tannins with γ-kafirin was investigated using RP-HPLC and free zone capillary electrophoresis (FZCE) (Taylor *et al*. 2007).

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