Presence of Ferulic Acid in Wheat Glutenin Fraction and its Enzymatic Hydrolysates – a Short Report

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Abstract

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Three protein fractions: albumin-globulin, gliadin, and glutenin were extracted from wheat grains. Enzymatic hydrolysates were obtained from each fraction using trypsin, protease from *Bacillus subtilis* and Subtilisin Carlsberg. Ferulic acid was detected neither in albumin-globulin and gliadin fractions nor in their hydrolysates. The RP-HPLC and SE-HPLC chromatograms of the glutenin fraction and its hydrolysates revealed the presence of peaks originated from ferulic acid and ferulic acid derivative/complex. The content of ferulic acid in the glutenin fraction was 1.12 mg/g. During HPLC analysis, the peptide products of glutenin hydrolysis should be recorded at 220 nm because the peaks of the peptides recorded at 280 nm can be overlapped by the peaks of ferulic acid and its derivatives.

Keywords: wheat; albumin; globulin; gliadin; glutenin; ferulic acid; enzymatic hydrolysate; RP-HPLC; SE-HPLC

Enzymatic hydrolysis is one of the methods of food proteins modification. The hydrolysates offer better functional properties such as solubility, emulsifying capacity, foaming or coagulation properties than the native proteins (Mahmoud 1994). They are used for special and clinical nutrition, e.g. for the protein supplementation in the dietary management of phenylketonuria, food allergy and chronic liver disease (Schmidl *et al*. 1994; Clemente 2000). The properties of hydrolysates depend on the size of peptides obtained during proteolysis. High performance liquid chromatography in reversed phase (RP-HPLC) and size-exclusion mode (SE-HPLC) as well as electrophoretic methods are commonly used for the investigation of the composition of protein hydrolysates (Silvestre 1997; Wróblewska & Karamać 2003).

Ferulic acid is the major phenolic acid of cereal grains such as wheat, triticale, and rye (Weidner *et al*. 1999, 2000, 2002). It can exist as an extractable form, as free, esterified, and glycosylated phenolic constituents (Weidner *et al*. 1999) as well as an insoluble-bound occurring in the outer layers of wheat grains (Kim *et al*. 2006). Alkaline hydrolysis is reported to release ferulic acid from the insoluble form (Kim *et al*. 2006; Steinhart & Renger 2000). Also, enzyme preparations are used for the same purpose (Moore *et al*. 2006). There are numerous reports on antiradical, oxidase inhibitory, antiinflammatory, antimicrobial,

anticancer activities of ferulic acid and its derivatives in the literature data (Ou & Kwok 2004; Hirata *et al*. 2005; Wang *et al*. 2007). Ferulic acid and its derivatives are also responsible for the antioxidant properties of cereal grains (Yang *et al*. 2001; Kim *et al*. 2006). Those compounds are proven to play a significant role in the dormancy of cereal caryopses. Some studies have shown that phenolic acids can act as germination inhibitors (Weidner *et al*. 1993, 1999).

The aim of the present study was to investigate the presence of ferulic acid in protein fractions of wheat and their enzymatic hydrolysates, as well as to check up how ferulic acid interferes with the detection of peptides during chromatographic separation using HPLC methods.

MATERIALS AND METHODS

Materials. Winter bread wheat (*Triticum aestivum* L.) cultivar Axis was obtained from cultivation in Dolná Malanta (Slovak Republic) in 2005. Grains were used for analysis after four months storage. Hydrolysis was carried out using commercial enzymes: trypsin (Fluka), protease from *Bacillus subtilis* (Fluka) and Subtilisin Carlsberg (Sigma-Aldrich Co.).

Extraction of proteins. Ground wheat grains (10 g) were suspended in 100 ml 10% NaCl, stirred for 45 min and centrifuged at 5000 g for 10 min (Maruyama *et al*. 1998). The extraction was repeated twice more. Combined supernatants were adjusted with ammonium sulphate to 75% saturation and the precipitate was collected by centrifugation at 5000 g for 15 minutes. Albumins and globulins present in the pellet were dialysed and lyophilised. The pellet remaining after the NaCl treatment was extracted with 100 ml 70% (v/v) ethanol to separate gliadins. The extraction was carried out three times for 45 min and the mixture obtained was centrifuged at 5000 g for 10 minutes. The supernatants were combined, ethanol was evaporated under vacuum at 45°C and the aqueous residue was lyophilised. The pellet remaining after the extraction of gliadins was treated with 100 ml 0.2% NaOH, stirred for 45 min and centrifuged. Alkaline extraction was repeated three times. The combined supernatants were lyophilised in order to obtain the glutenin fraction. The protein content of every fraction was determined by Kjeldahl method (AOAC 1990).

Enzymatic hydrolysis of wheat proteins. Hydrolysis of albumin-globulin, gliadin, and glutenin fractions was carried out at 50°C, pH 8.0, at the initial protein concentration of 5% and enzyme/substrate ratio of 15 mAU/g of protein (Karamać *et al*. 2002). Each protein fraction was hydrolysed with trypsin, protease from *Bacillus subtilis*, and Subtilisin Carlsberg in separate treatments. Constant pH value of 8.0 during hydrolysis was maintained through titration with 0.2M NaOH and the process was controlled by pH-stat method (Adler-Nissen 1984). After 120 min reaction was terminated by heating at 100°C for 5 minutes. Then the samples were lyophilised.

RP-HPLC method. The protein fractions and their hydrolysates were dissolved in 80% acetonitrile (2 mg/ml) and analysed using a Shimadzu HPLC system consisting of LC-10AD$_{Vp}$ pumps, UV-VIS SPD-M10A$_{Vp}$ photo-diode array detector, SCL-10A$_{Vp}$ system controller. The samples were injected into the Bakerbond BDC (5 µm, 4.6 × 250 mm, Hypersil) column. The elution was carried out with the gradient of 0% to 80% acetonitrile (with 0.1% trifluoroacetic acid, TFA) for 50 min. The flow rate was 1 ml/min, the injection volume was 20 µl.

SE-HPLC method. The same HPLC system was used to conduct size exclusion chromatography. The samples were dissolved in 45% acetonitrile (2 mg/ml). 20 µl of the sample dilution was injected into TSK Gel G2000SW$_{XL}$ (5 µm, 7.86 × 300 mm, TosoHaas) column and eluted with 45% acetonitrile containing 0.1% TFA, with flow rate of 1 ml/min. Additionally, the ferulic acid standard (Fluka) was separated. The contents of ferulic acid in the glutenin fraction and its hydrolysates were calculated on the basis of the calibration curve determined for the concentrations in the range of 0.028–0.14 µg of the standard injected on the column. The limit of detection of the method was 0.2 µg of ferulic acid per ml and the coefficient of variation (*CV*) for six determinations was less than 3%.

RESUTS AND DISCUSSION

The results of RP-HPLC analysis show the presence of two compounds (1) and (2) in glutenins and in the hydrolysate obtained from this fraction using protease from *Bacillus subtilis*. Both compounds gave sharp and well separated peaks with the retention times of 20.5 min (1) and 21.8 min (2), respectively (Figure 1). The UV spectrum of

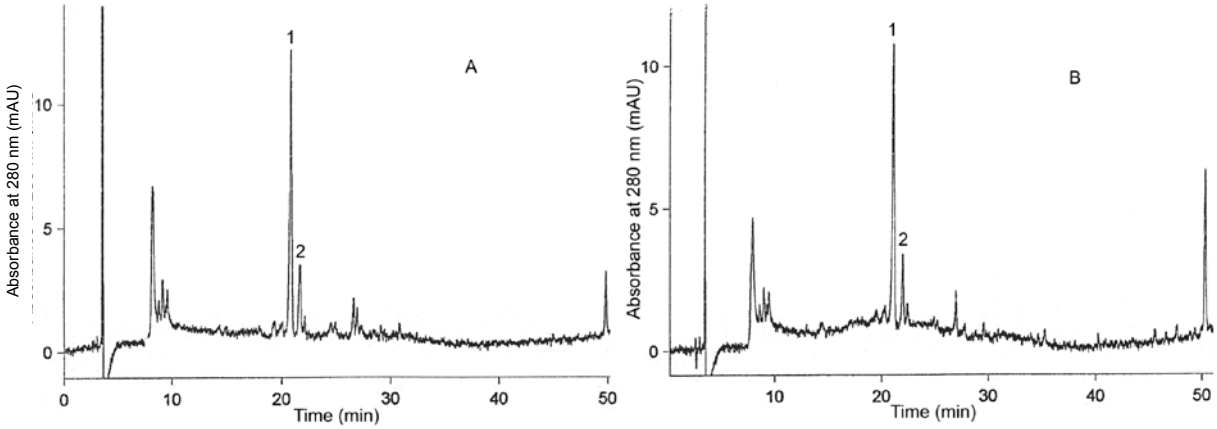

Figure 1. RP-HPLC chromatograms of glutenin fraction (A) and its hydrolysate obtained using *Bacillus subtilis* protease (B)

compound (1) was characterised by a maximum at 320 nm and a shoulder at 279 nm. Maxima at 284 nm and 323 nm were recorded for the spectrum of compound (2) (Figure 2). The peaks originated from constituents (1) and (2) were observed on the chromatograms of trypsin and microbial proteases hydrolysates of glutenins, while they did not appear in albumin-globulin and gliadin fractions and in their hydrolysates (data not shown).

Figure 3 depicts a separation of wheat glutenins (A, C, E) and their *Bacillus* protease hydrolysates (B, D, F) using SE-HPLC method. The chromatograms were recorded at 220 nm (A, B), 280 nm (C, D), and 320 nm (E, F). Those wavelengths are typical for the peptide bonds, aromatic amino acids, and phenolic acids, respectively. The results obtained confirmed the presence of two low molecular weight compounds (3) and (4) in glutenin fraction and its hydrolysates. The UV spectra of compounds (3) and (4) separated using SE-HPLC were the same as those of (2) and (1) separated by RP-HPLC presented in Figure 2. Ferulic acid injected into TSK Gel G2000SW$_{XL}$ column gave a peak with the retention time of 11.6 min (Figure 4). The above mentioned retention time and UV spectrum enable to recognise compound (4) as ferulic acid.

The presence of ferulic acid in glutenin fraction could be explicated when the procedure of wheat proteins separation is considered. Due to the use of 70% ethanol for the gliadin separation, which is an excellent solvent also for the extraction of phenolic compounds, the soluble form of phenolic acids would be present in the gliadin fraction. The alkaline conditions during glutenins extraction give rise to the liberation of insoluble-bound ferulic acid (Steinhart & Renger 2000; Kim *et al*. 2006). The absence of peaks originated from ferulic acid in gliadins can be explained by its low content, below the detection level. Weidner *et al.* (1999) determined the soluble forms of phenolic acids in wheat caryopses and noted that the amounts of free, esterified, and glycosylated ferulic acid were 2.0, 25.0 and 3.5 μg/g dry matter, respectively. In our study, the content of ferulic acid in the glutenin fraction was 1.12 mg/g, i.e. 106.6 μg/g of wheat grains. This concentration is considerably higher than those results reported by Weidner *et al.* (1999), which suggests that insoluble-bound ferulic acid liberated under alkaline conditions was determined in the present work. Most of insoluble-bound phenolic acids occur in the outer layers of cereal grains, which constitute

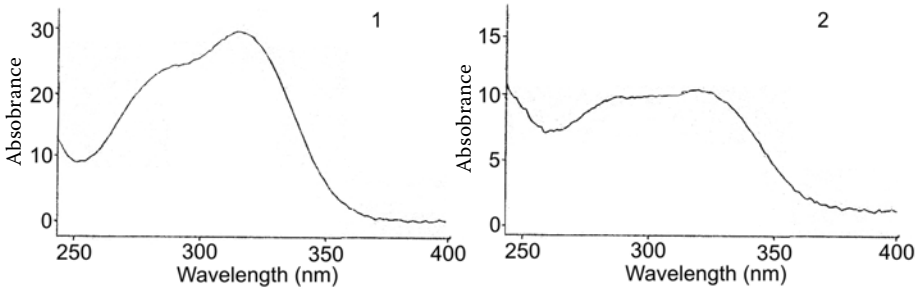

Figure 2. UV spectra of compounds (1) and (2) separated by RP-HPLC (see Figure 1)

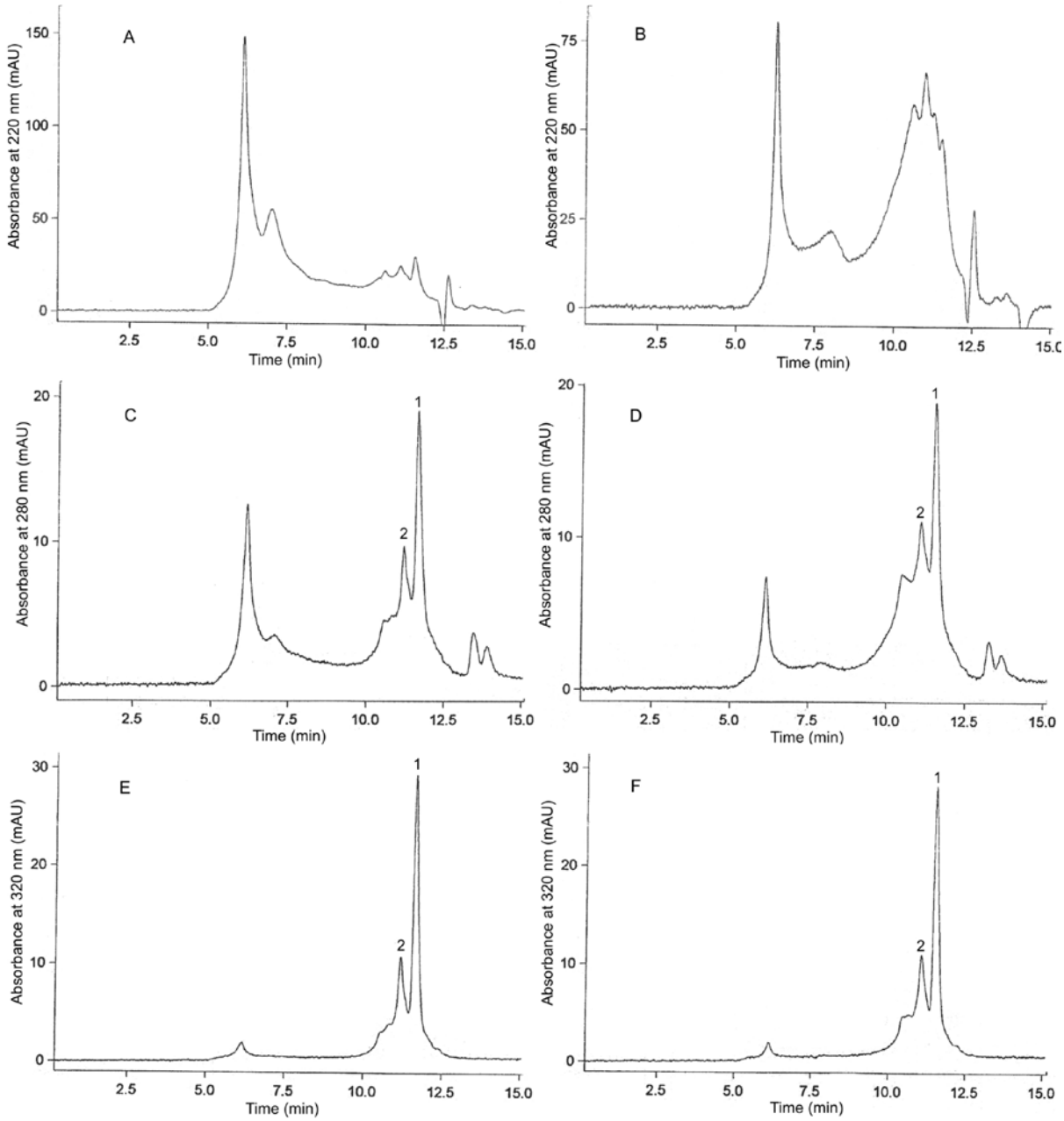

Figure 3. SE-HPLC chromatograms of glutenin fractions (A, C, E) and their hydrolysates (B, D, F) obtained using *Bacillus subtilis* protease

the bran. Kim *et al.* (2006) reported that alkaline hydrolysis of wheat bran liberated 1.36–1.93 mg of ferulic acid per gramme. Furthermore, the authors revealed that ferulic acid is the dominant phenolic acid liberated after alkaline hydrolysis and its content is at least 50 times higher than those of other acids identified.

The similarity between the spectra of ferulic acid and compound (3) in Figure 3 supported the explanation that this compound is a derivative of ferulic acid. On the other hand, due to the strong absorption band at 280 nm we can suggest that compound (3) is a complex of ferulic acid and peptides. Rawel and co-workers (Rawel *et al.* 2001, 2002; Kroll *et al.* 2003) observed in model studies that phenolic acids can react with proteins and peptides at alkaline pH. Their studies were conducted on standards as well as soy and whey proteins. The presence of phenolic compounds in 2S and 12S fractions of rapeseed proteins was also observed (Amarowicz *et al.* 2003; Karamać *et al.* 2004; Kosińska *et al.* 2006).

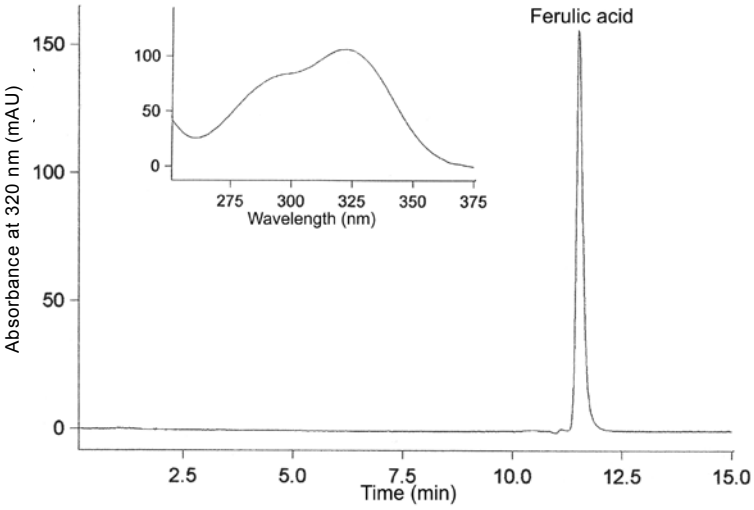

Figure 4. SE-HPLC chromatogram of ferulic acid and its UV spectrum

The presence of ferulic acid and its derivatives in the glutenin fraction and hydrolysates causes an analytical problem with the interpretation of HPLC chromatograms. Some peaks recorded at 280 nm resulted from phenolic compounds, not from aromatic peptides. The products of enzymatic hydrolysis can be determined only when the short wavelength of 220 nm is applied for the detection.

CONCLUSIONS

Ferulic acid and ferulic acid derivative/complex were detected in the glutenin fraction of wheat and its enzymatic hydrolysates. The peptide products of glutenins hydrolysis should be recorded at a shorter wavelength, i.e. 220 nm, if HPLC methods are applied, because the peaks of peptides recorded at 280 nm can be overlapped by the peaks of ferulic acid and its derivatives.

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