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APPLICATION OF ULTRAVIOLET SPECTROSCOPY TO DISCRIMINATE WHEAT α/β- AND γ-GLIADINS SEPARATED WITH HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY – A SHORT REPORT

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Key words: wheat gliadins, aromatic amino acids, reversed-phase high-performance liquid chromatography (RP- HPLC), UV spectroscopy

Studies were carried out to identify and detect potentially toxic proteins of wheat. The gliadin fractions were subjected to chromatographic and spectroscopic analyses to develop the relevant discriminants.

The spectral analysis showed that these proteins differ considerably in their tryptophan-to-tyrosine molar ratios. A standard curve was used. The gliadin fractions were identified by comparing the calculated values of the tryptophan-to-tyrosine molar ratio with the values obtained based on the amino acid sequence of wheat gliadins in the Swiss-Prot/TrEMBL database. Based on the retention times and second derivatives of UV spectra, the particular wheat gliadin fractions were classified as α/β or γ-gliadins.

INTRODUCTION

Reversed-phase high-performance liquid chromatography (RP-HPLC) is currently one of the most common wheat gliadin separation techniques [Bietz *et al*., 1984; Bean *et al*., 1998; Wieser *et al*., 1998; Arangoa *et al*., 2000; Lookhart *et al*. 2003; Ferranti, 2004]. Previous studies have indicated that UV spectra derivatives can be used for the identification of proteins separated by RP-HPLC [Dziuba *et al*., 2001; Dziuba *et al*., 2002; Minkiewicz *et al*., 2006]. A comparison of the amino acid sequences of α-, α/β-, and γ-gliadins, made using the sequence database [Swiss-Prot/TrEMBL], has shown that particular fractions differ in the tyrosine (Tyr)-to-tryptophan (Trp) molar ratio and thus can be distinguished based on UV spectra.

The study involved the discrimination of gliadin proteins by determining their retention times (RP-HPLC) and spectral parameters (UV), as well as a comparison of the experimental results with the wheat protein amino acid sequences.

MATERIALS AND METHODS

Material. The grain of spring wheat cv. Nawra was harvested at the Production and Experimental Station in Bałcyny, in the province of Warmia and Mazury. The grain was ground in a grinder and sieved through a 250 μm-mesh sieve. The material obtained was stored at approx. 5°C in tightly-sealed plastic containers.

Sample preparation. Wheat gliadins were extracted according to Weiss *et al*. [1993] (in 3 replications) and lyophilized. Samples were prepared for HPLC analysis according to Visser *et al*. [1991] with the modification proposed by Minkiewicz *et al*. [2005]. The final protein concentration was 2 mg/mL.

RP-HPLC and UV analysis of wheat gliadins. Wheat gliadins were subjected to analysis using reversed-phase high-performance liquid chromatography (RP-HPLC) online with UV spectroscopy. The RP-HPLC analysis was performed with an adapted technique developed by Dziuba *et al*. [2004]. A Shimadzu HPLC system with a photodiode array detector and Class-Vp 5.03 software were used. Chromatographic separation was carried out using a Jupiter C_{18} column (250 × 2.0 mm) (Phenomenex) in a gradient of two solvents: A and B containing acetonitrile/water/trifluoroacetic acid (100/900/1.0) and (900/100/0.7) (v/v/v), respectively [Visser *et al*., 1991]. A linear gradient from 30% to 60% of solvent B within 50 min was used and the data acquisition time was 70 min. The injection volume was 50 μL. Ultraviolet spectra within 250-300 nm were acquired and analysed for selected peaks (with absorbance at 220nm over 0.02) as recommended by Dziuba *et al*. [2001]. The solvent spectrum at the retention time corresponding to the retention time of a given fraction was used as a background spectrum. All other details of RP-HPLC were as described previously [Dziuba *et al*., 2004].

Discrimination of wheat gliadins. The apparent Trp/Tyr molar ratio in particular peaks was calculated on the basis of the measured values of parameter Y, defined according to equation (1) [Dziuba *et al*., 2001]:

$$Y=[(d^2A/d\lambda^2)_{295}-(d^2A/d\lambda^2)_{290}]/[(d^2A/d\lambda^2)_{288}-(d^2A/d\lambda^2)_{283}] \quad (1)$$

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The data concerning the following polypeptides and proteins from bovine milk: β-casein fragments 1-105/107, β-casein fragments 29-105/107, κ-casein, α_{s2}-casein, α_{s1}-casein, β-casein, β-casein fragments of 106/108/114-209 and β-lactoglobulin, published by Dziuba *et al*. [2001, 2002], were used for standard curve calculation. The following equation was formulated:

$$Trp/Tyr = -0.0687Y^3 + 0.1538Y^2 + 0.2033Y + 0.0482 \qquad (2)$$

and the correlation coefficient was 0.995. Following that, the wheat gliadins were classified as α- or α/β- and γ-fractions, based on a comparison of the observed Trp/Tyr molar ratios with the values of this ratio calculated based on the wheat gliadin amino acid sequences available in the Swiss-Prot/TrEMBL database. The statistical significance of the differences between the values of the apparent (measured *via* UV spectroscopy) and calculated Trp/Tyr molar ratios was determined using Student's t-test.

RESULTS AND DISCUSSION

Figure 1 presents an example of a chromatogram of wheat gliadins obtained using RP-HPLC.

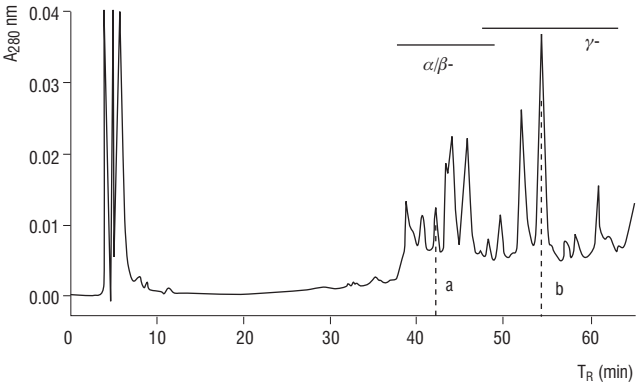

FIGURE 1. Chromatogram of wheat gliadins. The second derivatives of UV spectra of fractions indicated as (a) and (b) are presented in Figure 2.

Examples of the UV spectra of a typical α- or α/β-gliadin fraction (a) and a typical γ-gliadin fraction (b) are presented in Figure 2. For spectrum (a), the deepest minimum occurred

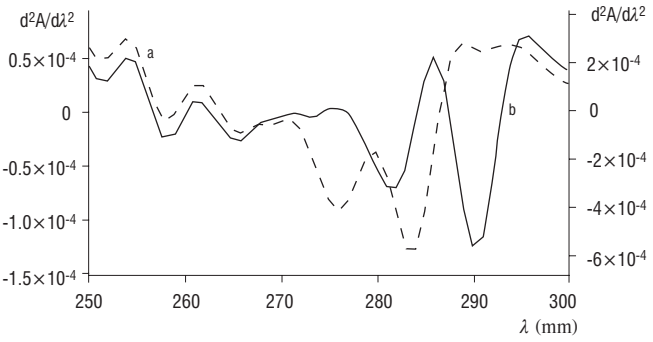

FIGURE 2. The second derivatives of UV spectra from the chromatogram presented in Figure 1: (a) spectrum of the fraction with a retention time of 42.3 min (dashed line and left scale); (b) spectrum of the fraction with a retention time of 54.4 min (solid line and right scale). 1 Unit on the absorbance derivative axis corresponds to absorbance = 10^{-4}.

at 283±2 nm, characteristic of tyrosine absorbance. The minimum at 290±2 nm, characteristic of tryptophan absorbance, was very shallow, which indicates that this fraction contains a low amount of this amino acid. The deepest minimum in the spectrum (b) occurred at 290 nm, indicating that this fraction contained tryptophan-rich proteins. Other, similar examples of the second derivatives of spectra typical of tryptophan-rich proteins as well as a review of the methods of interpretation of chemical compounds UV spectra acquired using diode-array detector have been presented by Minkiewicz *et al*. [2006].

The apparent values of the Trp/Tyr molar ratio presented in Table 1 were compared with the values calculated based on the wheat gliadin amino acid sequences available in the Swiss-Prot/TrEMBL database. The data (based on a comparison of the experimental value of the Trp/Tyr molar ratio and calculated from the amino acid sequences) showed that the fractions with retention times from 39 to 46 min contained α- and/or α/β-gliadins. The fractions with retention times ranging within 47–49.5 min could contain both α- and α/β-gliadins and γ-gliadins. The fractions with retention times longer than 49.5 min contained mainly γ-gliadins.

The presented chromatogram is typical of wheat gliadin proteins. Although protein reduction is usually not included as a step of sample preparation, the order of α/β- and γ-gliadins was the same as in previous studies [Bietz *et al*., 1984; Bean *et al*., 1998; Wieser *et al*., 1998; Arangoa *et al*., 2000;

TABLE 1. Results of wheat gliadin discrimination based on the comparison of the Trp/Tyr ratio value obtained from a spectral analysis with the Swiss-Prot/TrEMBL database.

T_R range (min)	Y ± SEM	Y range	Apparent Trp/Tyr ± SEM	Apparent Trp/Tyr range	Gliadin fraction
39–46	$0.062 \pm 4.08\times10^{-4}$ (n = 21)	0.017–0.104	$0.062 \pm 4.83\times10^{-4}$ [a] (n = 21)	0.052–0.071	α/β
47–49.5	$0.592 \pm 4.45\times10^{-4}$ (n = 6)	0.432–0.752	$0.209 \pm 4.66\times10^{-3}$ [b] (n = 6)	0.159–0.259	α/β + γ
> 49.5	$1.535 \pm 5.65\times10^{-3}$ (n = 24)	1.237–1.751	$0.469 \pm 4.75\times10^{-3}$ [c] (n = 24)	0.405–0.504	γ

n – number of measurements; SEM – standard error of the mean; [a] – non-significant differences between the data from the Table and the Trp/Tyr ratio mean values calculated based on the Swiss-Prot-TrEMBL database for α or α/β-gliadins (n=18), significant differences at a level of 0.001 for γ-gliadins (n=28), significant differences at a level of 0.05 for ω-gliadins (n=3); [b] – significant differences at a level of 0.02 for α or α/β-gliadins (n=18) and ω-gliadins (n=3), significant differences at a level of 0.05 for γ-gliadins (n=28); [c] – non-significant differences between the data from the Table and the Trp/Tyr ratio mean values calculated based on the Swiss-Prot-TrEMBL database for γ-gliadins (n=28), significant differences at a level of 0.001 for α or α/β-gliadins (n=18) and ω-gliadins (n=3)

Ferranti, 2004]. The reduction was used in our previous studies [Dziuba *et al*., 2001, 2002; Minkiewicz *et al*., 2006] to enable the unfolding of protein chains and to eliminate the influence of disulphide bonds on solvent accessibility and, hence, on the spectral properties of aromatic amino acids. The gliadin proteins were eluted according to their superficial hydrophobicity in the following order: α/β- and γ-gliadins. ω-Gliadins have retention times shorter than the other fractions. Fractions with such retention times occurred in our chromatograms (Figure 1), but their abundance was too low to enable their recognition *via* derivatives of UV spectra. The low abundance of ω-gliadins has also been reported by Arangoa *et al*. [2000].

CONCLUSION

The α/β- and γ-gliadins can be discriminated on the basis of the retention times and the second derivatives of the UV spectra.

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ZASTOSOWANIE SPEKTROSKOPII W NADFIOLECIE DO ROZRÓŻNIANIA α/β- I γ-GLIADYN PSZENICY ROZDZIELONYCH METODĄ WYSOKOSPRAWNEJ CHROMATOGRAFII CIECZOWEJ – KRÓTKI KOMUNIKAT

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Celem badań była identyfikacja i wykrywanie potencjalnie toksycznych białek pszenicy. Badania obejmowały chromatograficzno-spektroskopową analizę frakcji prolaminowej białek pszenicy, w tym rozdział chromatograficzny (RP-HPLC) i opracowanie wyróżników chromatograficzno-spektralnych.

Analiza spektralna chromatograficznie rozdzielonych frakcji gliadyn pszenicy wykazała, że są to białka różniące się znacznie stosunkiem molowym aminokwasów aromatycznych tryptofanu (Trp) do tyrozyny (Tyr). Do obliczania stosunku molowego Trp/Tyr wykorzystano drugie pochodne widm UV poszczególnych frakcji. Identyfikacji frakcji gliadynowych dokonano porównując obliczone wartości stosunku molowego tryptofanu do tyrozyny z wartościami otrzymanymi na podstawie sekwencji aminokwasowych gliadyn pszenicy dostępnych w bazie danych Swiss-Prot/TrEMBL. Na podstawie czasów retencji oraz drugich pochodnych widm UV sklasyfikowano poszczególne frakcje gliadyn pszenicy jako α/β- lub γ-gliadyny.