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REGULAR ARTICLE

THE COMPARISON OF PROLAMINS EXTRACTED FROM DIFFERENT VARIETIES OF WHEAT, BARLEY, RYE AND TRITICALE SPECIES: AMINO ACID COMPOSITION, ELECTROPHORESIS AND IMMUNODETECTION

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ABSTRACT

The aim of this study was to compare the prolamin complex of several varieties of cereals: 16 varieties of wheat (including common, durum and spelt wheat), 8 varieties of barley, 3 varieties of triticale and 1 variety of rye. In amino acids composition the major part represent glutamic acid in all type of prolamins (38 – 43 %) but there were some differences between content of proline (in wheat and triticale it was 17 %, in rye 20 % but in barley 25 %). By ELISA based on monoclonal antibody R5 it was showed positive reaction in relation to coeliac disease active peptides. Immunoblot based on polyclonal gluten antibody detected only proteins with molecular weight higher than 35 kDa.

Keywords: Cereals; Prolamins; Amino Acid; ELISA; Western blot

INTRODUCTION

The storage protein fractions of the cereal grains endosperm are classified into four classes depending on their solubility: the albumins soluble in water, the globulins soluble in salt solution, the prolamins soluble in alcohol solution and the glutenins insoluble in neutral aqueous or saline solution and ethanol **(Osborne, 1924; Ciccocioppo et al., 2005)**. The prolamins are monomeric polypeptide chains with molecular weights between 30 to 80 kDa **(Van Eckert et al., 2010)**. They are rich in proline and glutamine (20 - 55 %). Prolamins in wheat which are known as gliadins, in barley as hordeins, in rye as secalins, and in oats as avenins are main triggering factor in celiac disease **(Weber et al., 2009)**.

The prolamins are assigned to three groups: sulphur-poor (S-poor), S-rich and high molecular weight (HMW) prolamins. The S-poor prolamins consist essentially of ω-gliadins, account for about 11% of total storage proteins and contain little or no cysteine residues. They are predominantly monomeric, with molecular weight ranging from 30,000 to 80,000 Da. The S-rich prolamins, accounting for about 70-80 % of the prolamin fraction, have molecular weight from about 30,000 to 55,000 Da and include both monomeric α/β- and γ-gliadins. They consist of a repetitive N-terminal domain, representing up to half of the molecule, and a non-repetitive cysteine rich C-terminal domain. The HMW prolamins constitute 10% of the prolamin fraction, they can be grouped into x- and y-type subunits, with molecular weight ranging from 83,000 to 88,000 Da and 67,000 to 74,000 Da, respectively **(Vaccino et. al., 2009).**

Immunochemical detection of gluten proteins is based on reactivity of gluten-detecting antibodies with prolamins extracted from cereals. The most popular immunochemical methods of evaluation of prolamins presence and toxicity are ELISA and Western blot **(Battais et al., 2003)**. Immunological tests for determination of gliadin content use monoclonal or polyclonal anti-gliadin antibodies, eg polyclonal antibodies developed against wheat gliadin, or an anti-ω-gliadin monoclonal antibody. There were also other antibodies developed, such as monoclonal antibody PN3 or R5. PN3 antibody is raised against a synthetic peptide equivalent to the amino acids sequence 31 - 49 of α-gliadin, *i.e.* the sequence of toxic peptide of α-gliadin, which has been shown to cause mucosal damage to the small bowel of celiac patients. Monoclonal antibody R5 was developed against a secalin extract and recognizes the epitopes with the amino acid sequences QQPFP, QQQFP, LQPFP and QLPFP **(Kahlenberg et. al., 2006; van Eckert et. al., 2010)**. These epitopes occur repeatedly to a similar level in α-, γ- and ω-gliadins, hordeins and secalins of wheat, barley

and rye **(Konic-Ristic et. al., 2009).**

In our study were analyzed the prolamin complex of cereal grains by comparison of protein fractions, amino acids composition and electrophoresis. The immunoreactivity was tested by methods Western blot (polyclonal antibody was used) and ELISA (with monoclonal antibody).

MATERIAL AND METHODS

Biological material

The collection of varieties of cereals was from gene bank of seed´s species Slovak Agricultural Research Centre, Research Institute of Plant Production, Piestany (Slovakia).

Analytical methods

The composition of protein fractions from milled grains was performed according to Osborne method with modifications, and extracted prolamins were lyophilized (lyophylizer Christ® Alpha1-2 LD Plus, Martin Christ, Germany). The protein content was determined by Kjeldahl method (nitrogen analyzer, VELP Scientifice, Italy).

Amino acid analysis was done after liquid-phase acid hydrolysis under an argon atmosphere. Amino acids were determined by ion-exchange chromatography with post-column derivatization with ninhydrin (amino acid standard solution SIGMA, USA; automatic amino acid analyzer AAA400, INGOS, Czech Republic).

SDS-PAGE under reducing conditions was performed according to the Tris-Tricine method (**Schägger and Von Jagov, 1987**; molecular weight protein markers FERMENTAS Int. Inc., Canada). Electrotransfer to PVDF membrane was performed using CAPS transfer buffer according to protocol of manufacturer (ImmobilonP^{SQ} PVDF transfer membrane, MILLIPORE, USA, electrophoresis and transfer equipment from BIO-RAD Laboratories Inc., USA). Proteins were visualized by Ponceau S-red. In western blot (primary antibody: anti-gluten wheat antibody, USBiological, USA, secondary antibody: anti-rabbit HRP antibody, BD Pharmingen, USA) the chromogenic detection was proceeded with diaminobenzidine (SIGMAFAST™ 3,3′-Diaminobenzidine tablets, SIGMA, USA).

For immunodetection by ELISA was used a kit RIDASCREEN® Gliadin based on monoclonal antibody R5 (R-BIOPHARM, Germany; ELISA reader BIO TEK, USA).

RESULTS

The composition of protein fractions of cereal grains are presented in Table 1. Prolamins are main protein fraction (approximately 30 – 40 %).

From the amino acid compositions of prolamins (Table 2) follows that all wheat prolamins have similar amino acid compositions: very high content of glutamic acid (40.7 - 42.9 %) and proline (16.8 - 18.9 %), and very low level of lysine (approximately 0.5 %). In group of essencial amino –acids the highest contens was detected in phenylalanine (4.9 – 5.2 %).

Prolamins of barley are more reach in proline (23.8 - 27.6 %) as wheat one but glutamic acid content is similar (38 – 40 %) and also low is level of lysine. All triticale varieties have amino acid composition more similar to wheat than rye (e.g. leucin content).

Table 1 Content of crude protein and individual protein fractions in examined varieties of wheat, barley, rye and triticale

Variety	Crude protein[a] (%)	Alb + Glo[b] (%)	Prolamins (%)	Glutelins (%)	Calculated prolamin content[c] (%)	ELISA gliadin (%)
Common wheat – spring						
Granny	12.31	26.66	36.69	24.67	4.48	6.35
Saxana	11.86	25.67	36.51	27.02	4.32	6.70
Common wheat – winter						
Arida	12.83	20.00	38.75	31.22	4.96	11.90
Balaton	10.37	25.38	35.36	27.69	3.68	10.90
Blava	11.69	22.61	36.33	29.44	4.24	8.30
Brea	11.86	23.65	36.51	29.72	4.32	10.85
Hana	12.83	21.87	38.75	28.11	4.96	11.35
ID Karpatia	11.97	21.34	39.35	32.65	4.72	9.75
Ignis	10.37	24.62	36.90	31.52	3.84	7.30
Markola	10.37	26.92	35.36	30.76	3.68	11.20
Viginta	11.17	22.86	38.59	29.99	4.32	11.05
Vlada	14.08	21.02	39.77	27.26	5.60	12.60
Durum wheat						
Riveldur	12.78	24.99	39.38	28.73	5.04	12.75
Soldur	11.97	26.00	36.69	27.33	4.40	14.00
Spelt wheat						
Ceralio	14.25	24.71	44.93	23.59	6.39	9.05
Rubiota	14.42	22.22	44.44	28.32	6.40	12.15
Barley – spring						
Levan	9.58	27.51	33.33	24.18	3.20	2.80
Ludan	11.17	22.86	35.69	30.70	4.00	2.60
Radegast	12.31	22.73	33.80	29.21	4.16	2.95
Sladar	13.11	23.77	36.59	26.21	4.80	3.00
Barley – winter						
Amsterdam	12.83	21.25	39.38	26.86	5.04	2.55
Babette	11.63	22.61	30.13	34.23	3.52	2.63
Gerlach	8.66	25.02	27.79	32.41	2.40	2.73
Luran	9.58	24.18	30.01	31.67	2.88	2.55
Rye – winter						
Dankowskie Nowe	8.34	39.20	25.51	19.64	2.13	3.45
Triticale						
Wanad - spring	10.89	32.34	33.07	21.33	3.60	12.60
Kendo - winter	8.66	33.33	32.41	23.17	2.80	10.95
Kinerit - winter	9.92	32.24	31.44	24.20	3.12	10.60
average	11.49	25.27	35.84	27.92	4.18	8.06
standard deviation	1.66	4.33	4.41	3.58	1.05	4.08

[a] total N substances x protein factor
[b] albumins and globulins
[c] crude protein x prolamin content from fractionation / 100

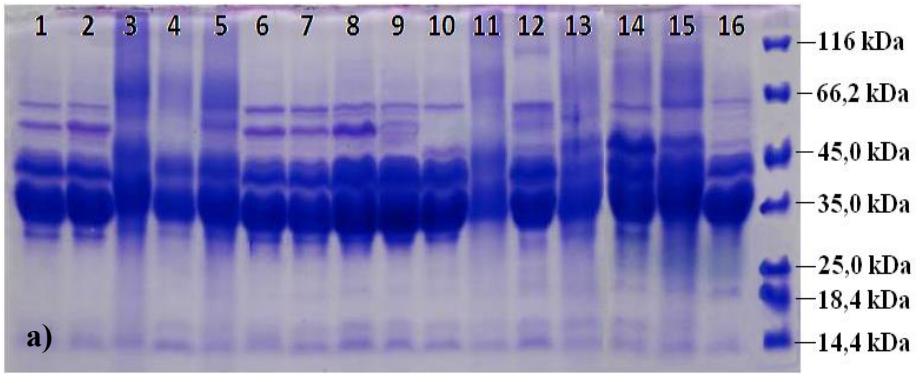
Table 2 Amino acid composition of prolamins

	Amino acids (mol %)														
Variety	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	His	Lys	Arg
Common wheat – spring															
Granny	2.26	1.96	4.95	40.99	17.81	3.36	2.74	4.13	3.99	7.07	1.76	4.96	1.80	0.57	1.64
Saxana	2.41	1.88	4.88	40.69	17.99	2.87	2.83	4.28	4.14	7.19	1.84	5.06	1.77	0.54	1.65
Common wheat – winter															
Arida	2.33	1.73	4.89	42.36	16.94	2.94	2.58	4.00	4.02	7.13	2.11	5.09	1.79	0.49	1.60
Balaton	2.40	1.86	5.00	40.67	16.84	3.74	2.77	4.28	4.05	7.41	2.00	4.86	1.94	0.54	1.65
Blava	2.30	1.77	4.83	41.81	17.59	2.50	2.60	4.14	4.15	7.18	1.97	5.21	1.82	0.53	1.59
Brea	2.35	1.77	4.86	41.92	17.44	2.51	2.66	4.05	4.13	7.23	2.00	5.15	1.82	0.54	1.58
Hana	2.38	1.79	4.91	41.47	17.83	2.43	2.72	4.08	4.08	7.36	2.07	5.02	1.75	0.50	1.61
ID Karpatia	2.39	1.84	5.07	41.23	17.18	2.65	2.74	4.18	4.18	7.32	2.00	5.19	1.80	0.52	1.71
Ignis	2.36	1.81	4.81	41.47	17.79	2.72	2.67	4.06	4.15	7.23	1.98	5.05	1.78	0.49	1.61
Markola	2.33	1.67	4.63	42.94	17.58	2.28	2.81	4.02	3.98	6.98	1.97	4.98	1.80	0.49	1.54
Viginta	2.34	1.78	4.83	41.71	17.76	2.53	2.68	4.09	4.07	7.19	2.01	5.11	1.81	0.52	1.58
Vlada	2.46	1.77	4.97	40.97	17.54	2.76	2.69	4.23	4.09	7.46	2.21	4.90	1.80	0.50	1.66
Durum wheat															
Riveldur	2.43	1.89	5.09	41.32	17.65	2.41	2.68	4.01	4.06	7.36	1.99	5.25	1.79	0.46	1.61
Soldur	2.38	2.02	5.34	41.06	16.72	2.68	2.82	4.34	4.08	7.68	1.88	4.84	1.89	0.50	1.77
Spelt wheat															
Ceralio	2.29	1.85	5.07	40.76	18.86	2.78	2.53	4.07	4.07	7.04	1.82	5.14	1.54	0.57	1.62
Rubiota	2.19	1.97	5.09	41.14	17.97	2.91	2.53	4.21	4.01	7.13	1.97	5.00	1.72	0.57	1.61
Barley – spring															
Levan	1.37	1.66	3.80	38.75	25.68	1.46	1.81	3.47	3.47	5.85	2.33	6.88	1.42	0.52	1.54
Ludan	1.35	1.87	4.04	37.83	24.63	1.77	2.20	4.17	3.92	6.44	2.39	5.91	1.23	0.42	1.83
Radegast	1.31	1.75	3.97	38.48	23.80	1.75	2.04	4.23	3.72	6.53	2.39	6.20	1.36	0.54	1.93
Sladar	1.37	1.89	3.78	38.05	25.52	1.61	1.99	3.84	3.73	6.15	2.40	6.40	1.16	0.35	1.79
Barley – winter															
Amsterdam	1.25	1.78	3.69	39.40	25.40	1.59	1.60	3.22	3.31	6.13	2.30	7.00	1.25	0.47	1.60
Babette	1.14	1.53	3.38	40.03	27.58	1.42	1.44	2.87	3.08	5.53	2.25	6.91	0.96	0.43	1.45
Gerlach	1.39	1.75	3.57	39.68	25.74	1.72	1.70	3.31	3.24	5.99	2.14	6.51	1.23	0.49	1.56
Luran	1.40	1.91	3.93	38.59	23.96	1.81	1.91	3.80	3.52	6.64	2.31	6.49	1.34	0.57	1.81
Rye – winter															
Dankowskie Nowe	1.98	2.18	5.53	39.28	20.58	4.68	2.52	4.66	3.19	5.63	1.28	4.88	1.73	0.69	1.20
Triticale															
Wanad	2.56	2.10	5.07	39.94	18.36	2.50	2.83	4.50	4.16	7.22	1.49	5.24	1.72	0.62	1.69
Kendo	2.55	2.30	5.49	39.94	17.70	3.08	3.00	4.45	3.97	6.95	1.49	4.96	1.81	0.65	1.66
Kinerit	2.51	2.06	5.02	40.15	17.90	2.97	2.83	4.35	4.23	7.08	1.46	5.25	1.85	0.62	1.71
average	2.06	1.86	4.66	40.45	19.94	2.52	2.46	4.04	3.89	6.86	1.99	5.48	1.63	0.53	1.64
standard deviation	0.49	0.16	0.61	1.32	3.57	0.73	0.43	0.40	0.34	0.59	0.30	0.72	0.27	0.07	0.13

SDS-PAGE protein pattern was similar for all varieties of wheat and on the basis of molecular weight it is possible to identify presence of gliadins subfractions, as they have molecular masses about 32 kDa (α-gliadins), 38 - 42 kDa (γ-gliadins) and 55 - 79 kDa (ω-

gliadins) **(Belitz et al., 2009)**. There are some differences in quantity of these fractions and it depends on variety of wheat (Fig 1a). The main protein fractions of barley had molecular weight between 30 - 45 kDa. Prolamins of rye consisted from two main protein bands approximately > 35 kDa and > 66 kDa, additional weak bands of proteins of molecular weight about 100 kDa and higher were observed. Results obtained for triticale showed protein bands characteristic for both species (wheat and rye) – main protein fraction about 35 - 45 kDa (resembling wheat) and additional protein bands about 66 kDa (similar to rye) (Fig 2a).

Immunological features were investigated by reaction of prolamins with polyclonal anti-wheat gluten antibody by Western blot and R5 monoclonal antibody by ELISA method. In immunoblotting of wheat samples, polyclonal antibody recognized all protein fractions of molecular weight higher than 35 kDa. Antibody did not react with low molecular weight proteins present in all wheat extracts (about 15 - 30 kDa) (Fig. 1b). These small molecules were not recognized neither in any variety of barley, rye, and triticale. In case of barley some additional bands were visualized on immunoblot, mainly about 60 kDa. In case of rye, two main protein bands visualized on the gel were not as good visualized by immunoreaction, on the other hand, the third band with molecular weight higher than 100 kDa reacted strongly with antibody (Fig. 2b). ELISA assay is based on monoclonal antibody R5 which recognizes allergenic epitopes in wheat gliadins and corresponding proteins from barley (hordeins) and rye (secalins). Quantitative data obtained from ELISA analysis are compared with prolamin content calculated from protein fractionation in Table 1. Generally, all ELISA results obtained for wheat, rye and triticale varieties are much higher than calculated prolamin content.



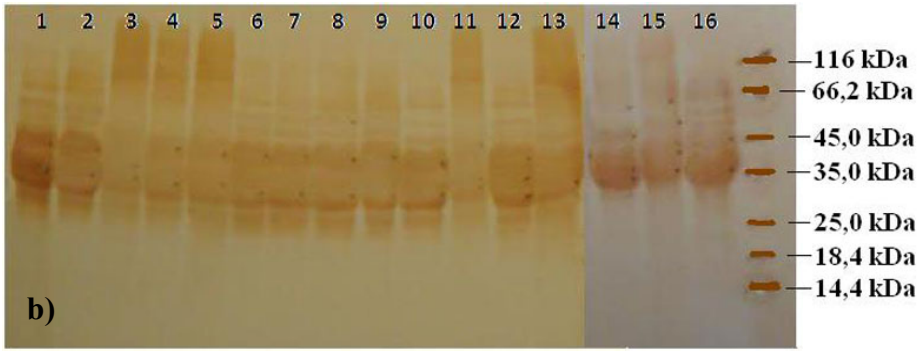
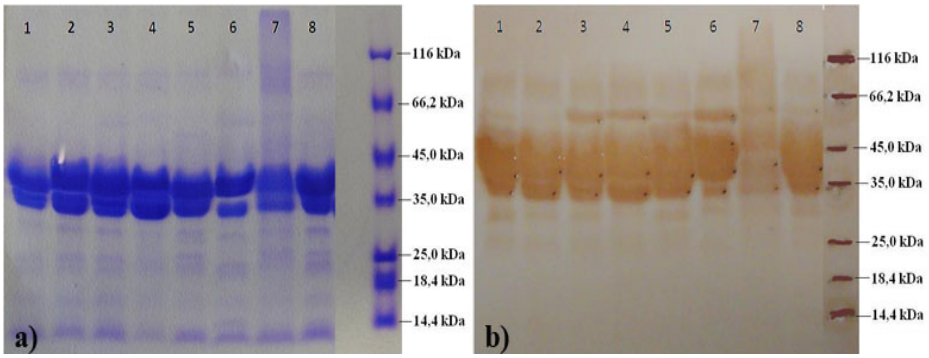


Figure 1 a) SDS-PAGE and b) Western blot of common wheat, durum wheat and spelt wheat prolamins: 1. Ignis, 2. Markola, 3. Arida, 4. Balaton, 5. Blava, 6. Viginta, 7. Brea, 8. ID Karpatia, 9. Hana, 10. Vlada, 11. Granny, 12. Saxana, 13. Riveldur, 14. Soldur, 15. spelt wheat Rubiota, 16. spelt wheat Ceralio, standard.



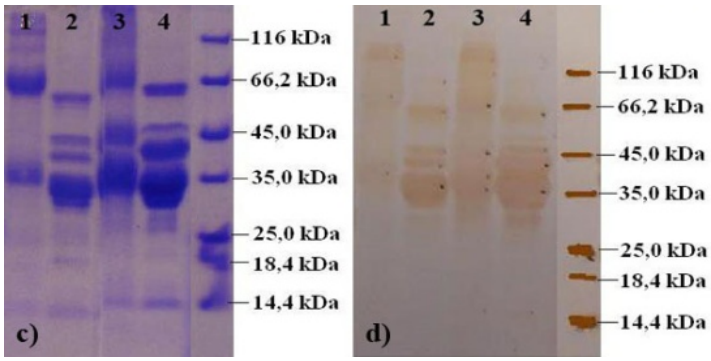


Figure 2 a) SDS-PAGE and b) Western blot of barley prolamins: 1. Levan, 2. Ludan, 3. Radegast, 4. Sladar, 5. Amsterdam, 6. Babette, 7. Gerlach, 8. Luran, standard; and c) SDS-PAGE and d) Western blot of rye and triticale prolamins: 1. rye Dankowskie Nowe, 2. triticale Kendo, 3. triticale Kinerit, 4. triticale Wanad, standard.

DISCUSSION

The biochemical features (molecular weight and amino acid composition) of the prolamin proteins reflect very well the taxonomic relationships of the cereals. Prolamins of wheat, barley and rye have common features – very high glutamic acid and proline content,

and very low lysine level. The results are similar to previous studies **(Wieser, 1995; Shewry, 2004; Belitz et al., 2009)**. There are no significant differences between varieties of species of examined cereals.

In our study the immunoreactivity of cereals and pseudocereals were analyzed by two immunodetection methods, Western blot and ELISA, based on polyclonal and monoclonal antibodies respectively. Generally, anti-wheat antibodies (polyclonal and monoclonal as well) recognizes not only wheat gluten and also barley, rye and triticale prolamins. Polyclonal antibodies can recognize many epitopes of gluten molecule, so cross-reactivity is more possible because of probability of presence chemical or structural similarities in other proteins and these informations are obtained by Western blot.

The use of monoclonal antibodies eliminates cross-reactivity due to very narrow specificity, only against epitopes responsible for celiac disease. Monoclonal antibody R5 used for ELISA tests reacts with the epitopes of amino acid sequences QQPFP, QQQFP, LQPFP, and QLPFP.From our results of ELISA analysis followed that all varieties of wheat (including common, durum and spelt), barley, rye and triticale contained very high level of toxic prolamins. Level of recognized sequences of prolamins depended on variety but generally, gliadin content calculated for all varieties of wheat, triticale and rye exceeded results obtained from protein fractionation. There are some factors which can influence on the uncertainty in determination of gliadin content by ELISA method. Reactivity of monoclonal antibodies can vary significantly against different prolamin preparations due to different prolamin composition, location and concentration of sequences recognized by antibody, its specificity, binding intensity and eventually cross-reactivity **(Denery-Papini et al., 1999; van Eckert et al., 2010)**. Gliadin content results determined by ELISA method can also vary markedly dependent on reference material used **(van Eckert et al., 2006)**. Additionally, using the different method for extraction of prolamins can affect analytical data as there is possibility of obtaining different composition of hydrophobic proteins and peptides in extracts.

CONCLUSION

In the present study the wide scale of varieties of cereals important for human diet was investigated to characterize their prolamins, important from point of view of celiac disease as they are the external trigger factor. Prolamin proteins are found not only in common cereal products (bread, pasta) but also in a wide range of other foodstuffs and additives (wheat starch, dressings, candies, beer, meat products etc.).

Immunodetection methods, ELISA and Western blot, confirms that all varieties of wheat (including common, durum and spelt wheat) have very high level of prolamin proteins, as well as barley, rye and triticale.

Moreover, there are no significant differences in prolamins amino acid profiles and electrophoretic properties between varieties of examined species.

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