doi:10.7251/ZSAN2302429G **Original scientific paper**

EXAMINATION OF THE EFFECT OF COLD ATMOSPHERIC PLASMA ON GLUTEN PROTEINS FROM GLUTEN-FREE FLOUR BY THE CGE METHOD

***Vesna Gojković Cvjetković*[1], *Željka Marjanović-Balaban*[2], *Radoslav Grujić*[3]**

[1]University of East Sarajevo, Faculty of Technology Zvornik, Republic of Srpska, Bosnia and Herzegovina,

[2]University of Banja Luka, Faculty of Forestry Banja Luka, Republic of Srpska, Bosnia and Herzegovina,

[3] PI College of Health Science Prijedor, Nikole Pašića 4A, Prijedor, Republic of Srpska, Bosnia and Herzegovina

Abstract: *According to the Codex Alimentarius, a product is declared gluten-free if it contains less than 20 mg/kg of gluten. This paper aimed to examine the effect of cold atmospheric plasma (CAP) on gluten proteins from gluten-free flour (protein content 3.6 g/100 g). The samples were treated with cold atmospheric plasma in a solid state (4 min) and as extracts (1 min) and compared with untreated samples. Gluten proteins, gliadin, were extracted with 70% (v/v) ethanol and glutenin with 50% (v/v) 1-propanol. The absorbance was read at 220 nm. After electrophoretic separation on a CGE apparatus, the total amount of gliadin and glutenin proteins, the amount of protein within the fractions, and their relative concentrations were determined. Based on the obtained results, after treatment with cold atmospheric plasma, the highest amount of gliadin proteins was isolated from untreated samples and amounted to Xav = 8.00 and the lowest from samples that were treated as extracts and amounted to Xav = 5.50. In the case of glutenin proteins, the highest amount of protein was isolated from untreated samples and amounted to Xav = 6.67 and the lowest amount from samples that were treated in the solid state amounted to Xav = 5.67.*

Keywords: *gluten, gluten-free flour, cold atmospheric plasma (CAP), CGE method*

Introduction

In recent years, gluten-free (GF) products have become popular, as they not only cater to individuals with medical needs but also to consumers who seek a GF diet [1]. According to the *Codex Alimentarius,* a product is declared gluten-free if it contains less than 20 mg/kg of gluten [2]. The only effective treatment for people with celiac disease is a gluten-free diet, which, over time, results in clinical and mucosal recovery [3]. Foods not allowed in a gluten-free diet include any bread, cereal, or other food made with wheat, rye, barley, triticale, Dinkel, kamut, or oat flour or ingredients, and by-products made from those grains, processed foods that contain wheat and gluten derivatives as thickeners and fillers, and medications that use gluten as the pill or table binders [4].

Gluten is the main protein in flour. It is responsible for the elastic characteristics of dough. It contributes to the appearance and crumb structure of many baked goods [5-

6]. It consists of two fractions that are present in approximately equal amounts. These are gliadins and glutenins [7]. By electrophoretic separation of gliadins, α+β, γ, ω1,2 and ω5 gliadins appear, and by electrophoretic separation of glutenin, LMW glutenins, HMW glutenins, and ωb gliadins appear [8]. Gliadins are soluble in an aqueous solution of alcohol, and glutenins are soluble in an aqueous solution of alcohol with the addition of a reducing agent [9].

Plasma processing is getting a lot of attention in recent applications as a new, environmentally friendly, and very efficient approach. Cold atmospheric plasma is mainly used to reduce the number of microorganisms in food and biological materials, as well as in different levels of packaging [10]. Recently, the influence of cold atmospheric plasma treatment on the protein structures of food and pharmaceutical systems, as well as in the packaging industry, has been studied. Proteins, as an integral part of food, play an important role in the techno-functional characteristics of processed food. Some proteins are responsible for reduced quality and nutritional value and cause allergic reactions in the human body [11].

Taking into account that the number of people who are allergic to gluten is increasing every day in the world, this work aimed to examine the effect of cold atmospheric plasma on gluten proteins extracted from gluten-free flour samples. After treatment, capillary gel electrophoresis was used for protein separation. The total amount of gliadin and glutenin proteins, the amount of protein within the fractions, and their relative concentrations were determined.

Material and method

Sample extraction

Extraction of gliadin and glutenin proteins from gluten-free flour (protein content 3.6 g/100 g) samples was performed according to a modified Osborne method, as described by Lockhart and Bean [12] and Grujić *et al*. [13]. Gliadin extraction was performed with 70% (v/v) (refined REAHEM, 96% v/v ethyl alcohol, Srbobran) and glutenins were extracted with 50% (v/v) 1-propanol solution (Lach-Ner, Czech Republic) and 1% dithioerythritol solution (DTT, erythro-1,4-mercapto-2,3 butanediol, ACROS Organics, Switzerland).

Sample and standard preparation for analysis at CGE

Samples were diluted with sample buffer (SDS-MW sample buffer, Beckman Coulter, USA) before analysis. Then 2 µl of internal standard (10 kDa, Beckman Coulter, USA) and 5 µl of 2-mercaptoethanol (Sigma-Aldrich Chemie GmbH, Germany) were added. Then, the samples were heated on a thermo-shaker (Thermo-Shaker, Biosan) at 100 °C for 3 minutes. After cooling to room temperature, the samples were ready for analysis.

The standard was taken to room temperature for 15 minutes after removal from the refrigerator. After that, 10 µl of standard (SDS-MW standard, USA) was pipetted into the vial. Then 85 µl of buffer (SDS-MW sample buffer, Beckman Coulter, USA) and 2 µl of internal standard (10 kDa, Beckman Coulter, USA) were added. Afterward, 5 µl of 2-mercaptoethanol (Sigma-Aldrich Chemie GmbH, Germany) was added. Then, it was heated on a thermo-shaker (Thermo-Shaker, TS-100, Biosan) at a temperature of 100 °C for 3 min. After heating, the standard vial was cooled to room temperature.

Gliadin and glutenin protein separation on the CE apparatus

Separation of gliadin and glutenin proteins by capillary gel electrophoresis (CGE) was performed on an Agilent CE 7100 apparatus (capillary temperature 25 °C, voltage -16.5 kV, duration analysis 30 min, absorbance measured at 220 nm). The SDS-MW analysis kit (Beckman Coulter, USA) was used to separate gliadin and glutenin.

Measurement procedure

Gluten-free flour samples in their solid state and their extracts were treated with cold atmospheric plasma. During the treatment, the input frequency was 50 Hz and the input voltage was 50 V. Solid samples were treated for 4 minutes and extracts for 1 minute.

Statistical data analysis

Statistical data analysis was done in IBM SPSS, Statistics 26. The average value (Xav), relative concentration (RC), standard deviation (SD), min, and max values were calculated. To assess the influence of treatment on the average amount of total isolated proteins and the number of proteins within the fractions, as well as their relative concentrations, analysis of the variance of different groups was used, and the significance of the differences between average values at the level of significance $p = 0.05$ was assessed by posthoc Tukey's HSD test.

Results and discussion

Table 1 shows the total amount of gliadin protein and the amount of protein within the fractions after electrophoretic separation.

Descriptive analysis showed that the highest amount of gliadin protein was observed in T1 samples (Xav=8.00), and the lowest in T3 samples (Xav = 5.50). One-factor analysis of the variance of different groups revealed a statistically significant difference in the amount of protein, $F(2.15) = 25.73$, $Sig.=0.000<0.05$. A subsequent comparison with the Tukey HSD test of real differences found that the amounts of protein (T1 and T2; T1 and T3) differed statistically significantly. The further comparison revealed that the amounts of protein (T2 and T3) do not differ statistically significantly. Descriptive analysis showed that the highest amount of protein within the α+β gliadin fraction was observed in T1 samples (Xav = 5.00) and the lowest in

T3 samples (Xav = 2.17). Within the γ gliadin fraction, the highest amount of protein was obtained in T3 samples (Xav = 1.33), and no amount of protein was observed in T2 samples. Within the ω1,2 and ω5 gliadin fractions from samples T1, T2, and T3, the same amount of protein was observed.

Table 1. The total amount of gliadin protein and the amount of gliadin within fractions (α+β, γ, ω1,2, and ω5 gliadins)

Treatments		N	Xav	SD	Std. error	Min	Max
The total amount of proteins (TAP)	T1	6	8.00	0.00	0.00	8	8
	T2	6	5.67	0.82	0.33	5	7
	T3	6	5.50	0.84	0.34	5	7
α+β gliadins	T1	6	5.00	0.00	0.00	5	5
	T2	6	3.67	0.82	0.33	3	5
	T3	6	2.17	0.41	0.17	2	3
γ gliadins	T1	6	1.00	0.00	0.00	1	1
	T2	6	0.00	0.00	0.00	0	0
	T3	6	1.33	0.52	0.21	1	2
ω1,2 gliadins	T1	6	1.00	0.00	0.00	1	1
	T2	6	1.00	0.00	0.00	1	1
	T3	6	1.00	0.00	0.00	1	1
ω5 gliadins	T1	6	1.00	0.00	0.00	1	1
	T2	6	1.00	0.00	0.00	1	1
	T3	6	1.00	0.00	0.00	1	1
ANOVA (TNP)	F(2.15)=25.73, Sig.=0.000<0.05, eta square=23.44/30.28=0.77						
ANOVA (α+β)	F(2.15)=43.40, Sig.=0.000<0.05, eta square=24.11/28.28=0.85						
ANOVA (γ)	F(2.15)=32.50, Sig.=0.000<0.05, eta square=5.78/7.11=0.81						
ANOVA (ω1,2)	F(2.15)=inf.						
ANOVA (ω5)	F(2.15)=inf.						

Table 2 shows the total relative concentration of gliadin protein, as well as the relative concentration of gliadin protein within the fractions after electrophoretic separation.

Based on the obtained results, it can be seen that the highest relative concentration within the α+β gliadin fraction was obtained from sample T3 (RC = 28.29%), and the lowest from sample T2 (RC = 18.30%). Within the γ gliadin fraction, the highest relative concentration was obtained from sample T3 (RC = 4.11%), and the lowest from sample T2 (RC = 0.00%). The highest relative concentration within the fraction ω1,2 gliadin was obtained from samples T1 (RC = 40.34%) and the weakest from samples T3 (RC = 35.48%), and within the ω5 gliadin, the highest relative concentration was obtained from samples T2 (RC = 43.35%) and the lowest from samples T3 (RC = 32.12%).

Table 2. The total relative concentration of gliadin protein and relative concentration within fractions (α+β, γ, ω1,2, and ω5 gliadins)

	Treatments	N	RC (%)	SD	Std. error	Min	Max
The total relative concentration (TRC)	T1	6	100	0.00	0.00	100	100
	T2	6	100	0.00	0.00	100	100
	T3	6	100	0.00	0.00	100	100
α+β gliadins	T1	6	25.76	0.84	0.34	24.76	26.81
	T2	6	18.30	1.02	0.41	17.76	19.83
	T3	6	28.29	1.82	0.74	25.58	30.35
γ gliadins	T1	6	1.46	0.21	0.08	1.25	1.81
	T2	6	0.00	0.00	0.00	0.00	0.00
	T3	6	4.11	0.58	0.24	3.64	5.23
ω1.2 gliadins	T1	6	40.34	1.22	0.50	38.17	41.65
	T2	6	38.35	0.90	0.37	36.86	39.20
	T3	6	35.48	1.44	0.59	33.27	37.53
ω5 gliadins	T1	6	32.43	1.69	0.69	29.96	35.26
	T2	6	43.35	1.29	0.53	40.97	44.54
	T3	6	32.12	2.83	1.16	28.00	35.92
ANOVA (α+β)	F(2.15)=96.37, Sig.=0.000<0.05, eta square=323.75/348.95=0.93						
ANOVA (γ)	F(2.15)=204.50, Sig.=0.000<0.05, eta square=52.00/53.91=0.96						
ANOVA (ω1.2)	F(2.15)=24.59, Sig.=0.000, eta square=71.56/93.39=0.77						
ANOVA (ω5)	F(2.15)=58.63, Sig.=0.000, eta square=491.06/553.87=0.89						

Table 3 shows the total amount of glutenin protein and the amount of protein within the fractions after electrophoretic separation.

Based on the obtained results (Table 3), it can be seen that the highest total amount of glutenin protein was obtained from sample T1 (Xav = 6.67), and the lowest from sample T2 (Xav = 5.67). One-factor analysis of the variance of different groups revealed no statistically significant difference, F(2.15) = 1.96, Sig.=0.17>0.05. The highest amount of protein within the LMW glutenin fraction was obtained from sample T1 (Xav = 3.67) and the lowest from sample T2 (Xav = 1.67). Within the HMW glutenin fraction, the highest amount of protein was obtained from sample T2 (Xav = 1.83) and the lowest from sample T1 (Xav = 1.17). The last fraction that appears is ωb gliadin. Within this fraction, the highest amount of protein was obtained from samples T2 and T3 and is Xav = 2.17, and the lowest from samples T1 and is Xav = 1.83.

Table 3. The total amount of glutenin protein and the amount of glutenin within fractions (LMW glutenins, HMW glutenins, and ωb gliadins)

	Treatments	N	Xav	SD	Std. error	Min	Max
The total amount of proteins (TAP)	T1	6	6.67	0.82	0.33	5	7
	T2	6	5.67	0.82	0.33	4	6
	T3	6	6.17	0.98	0.40	5	7
LMW glutenins	T1	6	3.67	0.52	0.21	3	4
	T2	6	1.67	0.52	0.21	1	2
	T3	6	2.33	0.52	0.21	2	3
HMW glutenins	T1	6	1.17	0.41	0.17	1	2
	T2	6	1.83	0.41	0.17	1	2
	T3	6	1.67	0.52	0.21	1	2
ωb gliadins	T1	6	1.83	0.41	0.17	1	2
	T2	6	2.17	0.41	0.17	2	3
	T3	6	2.17	0.41	0.17	2	3
ANOVA (TAP)	F(2.15)=1.96, Sig.=0.17>0.05						
ANOVA (LMW)	F(2.15)=23.33, Sig.=0.000<0.05, eta square=12.44/16.44=0.76						
ANOVA (HMW)	F(2.15)=3.61, Sig.=0.05, eta square=1.44/4.44=0.32						
ANOVA (ωb)	F(2.15)=1.33, Sig.=0.29>0.05						

Table 4 shows the total relative concentration of glutenin protein and the relative concentration of glutenin protein within the fractions after electrophoretic separation.

Descriptive analysis showed that the highest relative concentration within the LMW glutenin fraction was obtained from sample T3 (RC = 25.59%) and the lowest from sample T2 (RC = 6.78%). Within the HMW glutenin fraction, the highest relative concentration was obtained from sample T2 (RC = 42.52%) and the lowest from sample T3 (RC = 20.92%). The highest relative concentration within the ωb gliadin fraction was obtained from sample T1 (RC = 63.14%) and the lowest from sample T2 (RC = 50.70%).

Nooji [14] investigated how pulsed ultraviolet light reduces immunoglobulin E binding to Atlantic white shrimp (*Litopenaeus setiferus*) extract. Results obtained in this research showed that PUV treatment reduced the reactivity of the major shrimp allergen, tropomyosin, and decreased the IgE binding capacity of the shrimp extract.

Sun *et al*. [15] investigated the effects of cold jet atmospheric pressure plasma on the structural characteristics and immunoreactivity of celiac-toxic peptides and wheat storage proteins. Based on the obtained results, the immunoreactivity of gliadin extract declined over time, demonstrating a significant decrease of 51.95% after 60 min of CJAP plasma treatment *in vitro*. CJAP plasma could be employed as a

potential technique for the modification and reduction of celiac-toxic peptides and wheat storage proteins.

Table 4. The total relative concentration of glutenin protein and relative concentration within fractions (LMW glutenins, HMW glutenins, and ωb gliadins)

	Treatments	N	RC (%)	SD	Std. error	Min	Max
The total relative concentration (TRC)	T1	6	100	0.00	0.00	100	100
	T2	6	100	0.00	0.00	100	100
	T3	6	100	0.00	0.00	100	100
LMW glutenins	T1	6	11.50	1.23	0.50	10.19	13.81
	T2	6	6.78	0.74	0.30	5.91	7.56
	T3	6	25.59	1.31	0.53	23.51	27.25
HMW glutenins	T1	6	25.36	0.64	0.26	24.66	26.31
	T2	6	42.52	1.75	0.71	40.34	44.54
	T3	6	20.92	0.91	0.37	19.55	22.30
ωb gliadins	T1	6	63.14	1.80	0.73	59.88	64.90
	T2	6	50.70	1.93	0.79	47.99	52.97
	T3	6	53.48	1.30	0.53	51.40	54.83
ANOVA (LMW)	F(2.15)=457.98, Sig.=0.000<0.05, eta square=1149.62/1168.45=0.98						
ANOVA (HMW)	F(2.15)=545.45, Sig.=0.000<0.05, eta square=1560.61/1582.07=0.99						
ANOVA (ωb)	F(2.15)=88.62, Sig.=0.000<0.05, eta square=511.15/554.41=0.92						

In the available References examination of the effect of cold atmospheric plasma on gluten proteins from gluten-free flour by the CGE, the method has not been investigated. But, when the results obtained in this paper are compared with those of Nooji [14] and Sun *et al*. [15] who investigated the effect of cold atmospheric plasma on gluten proteins from wheat flour, it can be seen that they agree.

Conclusion

By examination of the effect of cold atmospheric plasma on gluten proteins from gluten-free flour samples by capillary gel electrophoresis, the following results were reached: Cold atmospheric plasma leads to a decrease in the total amount of gliadin and glutenin proteins when compared to the untreated sample. Based on the obtained results, the highest amount of gliadin proteins was isolated from untreated samples and the lowest from samples that were treated as extracts. In the protein glutenin, the highest amount was isolated from untreated samples and the lowest from samples that were treated in the solid state. Therefore, cold atmospheric plasma was

shown to be a promising treatmeant for reducing the number of gluten proteins in gluten-free flours.

References

[1] Pellegrini N, Agostoni C. Nutritional aspects of gluten-free products. J Sci Food Agric. 2015;95(12):2380-2385. https://doi.org/10.1002/jsfa.7101.

[2] Codex Alimentarius. The standard for foods for special dietary use for persons intolerant to gluten. 2008.

[3] Mandile R, Maglio M, Mosca C, Marano A, Discepolo V, Troncone R, Auricchio R, et al.Mucosal healing in celiac disease: villous architecture and immunohistochemical features in children on a long-term gluten-free diet. Nutrients. 2022;14(18): 3696. https://doi.org/10.3390/nu14183696.

[4] Silvester JA, Weiten D, Graff LA, Walker JR, Duerksen DR. Is it gluten-free? Relationship between self-reported gluten-free diet adherence and knowledge of gluten content of foods. Nutrition. 2016;32(7-8): 777-783. https://doi.org/10.1016/j.nut.2016.01.021.

[5] Barak S, Mudgil D, Khatkar BS. Relationship of gliadin and glutenin proteins with dough rheology, flour pasting, and bread making a performance of wheat varieties. LWTFood Sci. Technol. 2013;51(1): 211-217. https://doi.org/10.1016/j.lwt.2012.09.011.

[6] Cappelli A, Oliva N, Cini E. A systematic review of gluten-free dough and bread: doughrheology, bread characteristics, and improvement strategies. Appl Sci. 2020;10(8):6559. https://doi.org/10.3390/app10186559.

[7] Wieser H, Koehler P, Scherf KA. Chemistry of wheat gluten proteins: Qualitative composition. Cereal Chem. 2023;100: 23-35. https://doi.org/10.1002/cche.10572.

[8] Zang P, Gao Y, Chen P, Lv C, Zhao G. Recent advances in the study of wheat protein and other food components affecting the gluten network and the properties of noodles. Foods. 2022;11: 3824. https://doi.org/10.3390/foods11233824.

[9] Wujun MA, Zitong YU, Maoyun SH, Yun Z, Shahidul I. Wheat gluten protein and its impacts on wheat processing quality. Front Agr Sci Eng. 2019;6(3): 279-287.

[10] Ucar Y, Ceylan Z, Durmus M, Tomar O, Cetinkaya T. Application of cold plasma technology in the food industry and its combination with other emerging technologies. Trends Food Sci Technol. 2021;114: 355-371. https://doi.org/10.1016/j.tifs.2021.06.004.

[11] Domonkos M, Ticha P, Trejbal J, Demo P. Applications of cold atmospheric pressure plasma technology in medicine, agriculture, and food industry. Appl Sci. 2021;11(11): 4809. https://doi.org/10.3390/app11114809.

[12] Lockhart G, Bean S. Separation and characterization of wheat protein fractions by high-performance capillary electrophoresis. Cereal Chem. 1995;72(6): 527-532.

[13] Grujić R, Gojković Cvjetković V, Marjanović-Balaban Ž. Separation of gliadins from wheat flour by capillary gel electrophoresis: optimal conditions. Foods Raw Mater. 2020;8(2): 411-421. http://doi.org/10.21603/2308-4057-2020-2-411-421.

[14] Nooji J. Reduction of wheat allergen potency by pulsed ultraviolet light, high hydrostatic pressure, and nonthermal plasma. Gainesville, Florida: University of Florida; 2011.

[15] Sun F, Xie X, Zhang Y, Duan J, Ma M, Wang Y, et al. Effects of cold jet atmospheric pressure plasma on the structural characteristics and immunoreactivity of celiac-toxic peptides and wheat storage proteins. Int J Mol Sci. 2020;21(3): 1012. https://dx.doi.org/10.3390/ijms21031012.

ISPITIVANJE UTICAJA HLADNE ATMOSFERSKE PLAZME NA PROTEINE GLUTENA IZ BEZGLUTENSKOG BRAŠNA CGE METODOM

Vesna Gojković Cvjetković[1], *Željka Marjanović-Balaban*[2], *Radoslav Grujić*[3]
[1]Univerzitet u Istočnom Sarajevu, Tehnološki fakultet Zvornik, Republika Srpska, Bosna i Hercegovina,
[2]Univerzitet u Banja Luci, Šumarski fakultet Banja Luka, Republika Srpska, Bosna i Hercegovina,
[3] JU Visoka Medicinska škola Prijedor, Republika Srpska, Bosna i Hercegovina

Sažetak: *Prema Codex Alimentariusu proizvod se deklariše kao bezglutenski, ukoliko je sadržaj glutena manji od 20 mg/kg. Cilj ovog rada je bio da se ispita uticaj hladne atmosferske plazme (eng. Cold atmospheric plasma, CAP) na proteine glutena iz bezglutenskog brašna (sadržaj proteina 3,6 g/100 g). Uzorci su tretirani hladnom atmosferskom plazmom u čvrstom stanju (4 min) i kao ekstrakti (1 min) i upoređivani sa netretiranim uzorcima. Proteini glutena, glijadini su ekstrahovani 70% (v/v) etanolom, a glutenini 50% (v/v) 1-propanolom. Apsorbancija je izmjerena na 220 nm. Nakon elektroforetskog razdvajanja na CGE aparatu, ukupna količina proteina glijadina i glutenina, količina proteina unutar frakcija, kao i njihova relativna koncentracija je određena. Na osnovu dobijenih rezultata, nakon tretmana hladnom atmosferskom plazmom, najveća količina proteina glijadina je izdvojena iz netretiranih uzoraka i iznosi Xav=8,00, a najmanja iz uzoraka koji su tretirani kao ekstrakti i iznosi Xav = 5,50. Kod glutenina, najveća količina proteina je izdvojena iz netretiranih uzoraka i iznosi Xav = 6,67, a najmanja iz uzoraka koji su tretirani u čvrstom stanju i iznosi Xav = 5,67.*

Ključne riječi: *gluten, bezglutensko brašno, hladna atmosferska plazma (CAP), CGE metoda*