CADERNO DE CIÊNCIAS AGRÁRIAS
Agrarian Sciences Journal

ICA INSTITUTO DE CIÊNCIAS AGRÁRIAS

UFMG

Physicochemical characterization of ice structuring proteins in Brazilian wheat and rye varieties

Claudia Regina Vieira[1]*, Mônica Ribeiro Pirozi[2], Flávia Assunção Campelo[3]

Abstract

Wheat and rye leaves from plants grown for seven weeks under natural (NA) and cold acclimated (CA) conditions were used to obtain crude extracts. Chemical composition, protein composition in SDS-PAGE and the morphology of ice crystals of these extracts were analyzed. The total solids content ranged from 0.74 to 0.86 % and 0.92 to 1.70 % for CA and NA extracts, respectively, while the protein content ranged from 51.69 to 60.69% for the extracts CA and from 43.78 to 52.18 % for the NA extracts. The SDS-PAGE revealed that NA extracts have very little and weak protein bands between 14.4 and 97.0 kDa. In contrast, several bands were observed in all CA extracts. Furthermore, CA extracts showed the ability to modify the shape of ice crystals, confirming the ice structuring activity of the proteins present.

Keywords: Antifreeze protein; cereal leaves; cryopreservation.

Caracterização físico-química de proteínas estruturadoras de gelo em variedades brasileiras de trigo e centeio

Resumo

Folhas de plantas de trigo e centeio, cultivadas por sete semanas em condições naturais e ao frio, foram usadas na obtenção de extratos brutos, que foram reportados como não aclimatados (NA) e aclimatados (AF). Os extratos foram analisados quanto à sua composição química, eletroforese em SDS-PAGE e pela morfologia dos cristais de gelo. O teor de sólidos totais variou de 0,74 a 0,86 % e 0,92 a 1,70 % para os extratos AF e NA, respectivamente; o teor de proteína de 51,69 a 60,69 % para os extratos AF e 43,78 a52,18 % para os NA. A eletroforese permitiu constatar que extratos NA praticamente não apresentam bandas na faixa de 14,4 e 97,0 kDa, em contrapartida, várias bandas foram observadas em todos extratos AF. Os extratos AF apresentaram capacidade em modificar o formato dos cristais de gelo, comprovando atividade das proteínas presentes.

Palavras-chave: Criopreservação; Folhas de cereais; Proteínas anticongelantes.

Introduction

Low temperature is the major environmental limitation for agricultural production. Late frosts delay seed germination, early frosts reduce the quality and yields of crop and low temperatures decrease the survival of some winter crops such as cereals, for example. Some plants, however, have the ability to withstand temperatures below freezing for long periods of time. Studies have shown that this is possible due to the synthesis of specific proteins that are involved in the development of freezing tolerance in these plants (Hew *et al*., 1999).

In winter, the temperature of some cold areas can reach below - 40°C. Winter plants found in the Arctic, Antarctic, and Alpine have developed a high tolerance to cold. It is believed that the proteins associated with freezing tolerance are endogenously produced by plant

[1]Professora Adjunto III at the Instituto de Ciências Agrárias at the Universidade Federal de Minas Gerais - *Campus* Montes Claros - MG - crvieira@ica.ufmg.br
[2]Professora Associado II at the Departamento de Tecnologia de Alimentos at the Universidade Federal de Viçosa - *Campus* Viçosa - MG - mrpirozi@gmail.com
[3]Doutoranda em Ciência de Alimentos at the Departamento de Alimentos da Faculdade at the Farmácia da Universidade Federal de Minas Gerais - *Campus* Pampulha - MG - facampelo85@gmail.com
*Correspondingauthor: crvieira@ica.ufmg.br

Received 12 april 2018. Accepted 15 may 2018.
ISSN: 2447-6218 /

cells and are secreted through the plasma membrane into the intercellular spaces, to effect and change in the formation of ice crystals during exposure to freezing temperatures (Atici; Nalbantoğlu, 2003).

Rye is a plant that can withstand temperatures below - 20°C (Chun *et al*., 1998). Winter wheat varieties have survived in temperatures below - 25°C and some grass have withstood temperatures below - 30/C, confirming that certain plants have their special mechanisms to protect against cold stress (Atici; Nalbantoğlu, 2003).

According to Silva *et al*. (2008), wheat is able to withstand the freezing of the cells by a process called cold acclimation. The phenomenon causes physiological, biochemical and molecular changes that make the plants resistant not only to freezing temperatures but also to dehydration induced by freezing. According to these authors, the acclimation occurs according to the sequential action of cooling temperatures (higher than 0°C) and freezing temperatures (-3 to -5°C). Among the cereals, there is a great difference in the requirement of temperature above 0°C, necessary for initiation of acclimation. Wheat, for example, acclimatizes in temperature below 2/°C.

The decrease of water potential in tissues, due to the decrease in osmotic potential by sugar accumulation in the vacuole is an important aspect. This process is correlated to significant increases in abscisic acid and results in modification of protein synthesis (Silva *et al*., 2008).

Called antifreeze proteins (AFPs) in several studies, they are also known as ice structuring proteins (ISPs), for connection and influence in the growth of ice crystals. Proteins with these characteristics have been identified in fish living in areas susceptible to ice formation and in plant and in insects subject to these environments (Antikainen; Griffith,1997; Cheng, 1998; Feeney; Yeh, 1998; Griffith, 1999; Zhang *et al*.,2007, 2008).

According to Hew *et al*. (1999), the secretion of polypeptides into the apoplast followed by an increase in antifreeze activity is a common response in all plants of the Poaceae family exposed to cold. These authors report that the polypeptides and proteins associated with freezing tolerance are produced in smaller quantities in plants that have not grown at low temperatures.

In a study by Hon *et al*. (1994), the authors were able to isolate six AFPs with molecular weights ranging from 16-35 kDa from the apoplast of winter rye leaves acclimated to cold. Antikainen and Griffith (1997) examined the accumulation of proteins in the apoplast of plants during cold acclimation for seven weeks by electrophoresis (SDS-PAGE). In barley and winter wheat, spring wheat and spring rye, the proteins secreted into the apoplast of leaves were similar to winter rye.

This study aims to investigate the presence of ISPs in Brazilian cultivars of wheat and rye acclimated to cold, and does a physical-chemical characterization in the extracts obtained from leaves of these plants.

Material and methods

Experimental material

The field experiment was conducted following a 6x2 factorial arrangement in a completely randomized design (CRD), where each treatment had three replications, totaling 36 experimental units. For the preparation of the ISPs extracts were used the spring wheat varieties: BRS Guabiju, Ônix and LD 062212 developed by EMBRAPA, OR Sementes and IAPAR, respectively, a rye variety: IPR 89 developed by IAPAR and two winter wheat varieties: IPR 84 and CD 104 developed by IAPAR and COODETEC, respectively.

Seeds were sown in plastic pots of 4 L of soil containing $(NH_4)_2SO_4$ (0.5 g/L), KCl (0.5 g/L), superphosphate (1 g/L) and lime (15 g/L) growing under natural conditions (green house, no temperature, light or humidity control) for 12 days in the greenhouse at the Setor de Agronomia at the Universidade Federal de Viçosa.

After this period, plants were transferred (three replicates of each one) for acclimation in a photoperiod of 10/14 h (day / night) for seven weeks in a cold chamber with cooling system (0-3°C, RH ~ 80%) and light condition (1 400LUX) adapted for the research in the Departamento de Tecnologia de Alimentos atthe Universidade Federal de Viçosa. These samples were reported ascold acclimated (CA). Those that remained under natural conditions (greenhouse, no temperature, light or humidity control, with three replications of each one), also for seven weeks, were reported as non acclimated (NA). The plants were irrigated with water when necessary.

At the end of seven weeks of culture, were obtained extracts of ISPs from the leaves of plants.

Preparation of the extracts of ISPs

The crude extracts of ISPs were prepared according to the method (vacuum infiltrating) described by Zhang *et al*. (2008) with some modifications. The leaves of plants after harvest, were cut with scissors into three sections in the transverse direction, rinsed with deionized water and mashed in a blender (Arno Brand, model Faciliq) at speed 1 for 5 min with Tris-HCl buffer (pH 7.4) at 1:200 (sample: buffer g/mL) for CA plants and in the proportion 1:20 (sample: buffer g/mL) for NA plants. The mixture was centrifuged at 3500 xg at 4°C for 30 min and filtered through a Whatman filter paper nº 40. The filtrates containing the ISPs were placed in plastic pots identified and kept refrigerated until the time of analysis.

Analysis of total solids, protein, and ash in the extracts of ISPs

In this step, all tests were performed according to methods described by Association of Official Analytical Chemists (AOAC, 1997): total solids (nº 925.10), crude protein (nº 960.52) and ash (nº923.03).

Identification of the ice structures protein (ISPs)

The molecular weight distribution of ISPs was studied by electrophoresis in the gel of polyacrylamide in the presence of sodium dodecyl sulfate (SDS-PAGE). It was used the vertical method with plates of 10x10 cm, the discontinuous SDS-PAGE described by Laemmli (1970) and modified by Fullington *et al.* (1983) employing stacking gel of polyacrylamide 4% (w/v) and runnig gel of polyacrylamide 15% (w/v). The electrophoresis was conducted at a constant voltage of 100 V for 4 h. The standard of low molecular weight used was: phosphorylase b 97.0 kDa, bovine serum albumin 66.0 kDa, egg albumin, 45.0 kDa, carbonic anhydrase 30.0 kDa, trypsin inhibitor 20.1 kDa, lysozyme purified from clear Egg 14.4 kDa. The coloration of the gels with silver was done according to the procedure described by Creste *et al.* (2001).

Statistical analysis

The statistical analysis was performed in SAS System software v 9.1, where the characteristics were subjected to analysis of variance (ANOVA) and the results, for the ones whichwere detected significant differences ($p < 0.05$), were analyzed by t test, with a significance level of 5%.

Results and discussion

Total solids, protein and ash content in the extracts of ISPs

The data presented in Table 1 refer to the chemical composition of the extracts of ISPs obtained from the leaves of different cultivars. It is noted that higher total solids content was found in the NA, which remained for seven weeks at room temperature after germination. Independent of the treatment used, cultivar had no significant effect on total solids, except for IPR 84 that differed from the others in the NA extract.

Table 1 – Total solids and protein content in the extracts of ISPs obtained from wheat and rye

Variety	Total solids (g/100 g)		Protein[1] (g/100 g)	
	CA[2]	NA[3]	CA[2]	NA[3]
Spring Wheat:				
BRS Guabiju	0,74 ± 0,03 a(A)	1,70 ± 0,05 a(B)	60,69 ± 2,61 a(A)	52,18 ± 3,41 a(B)
Ônix	0,75± 0,01 a(A)	1,11± 0,09 a(B)	58,23 ± 3,19 ac(A)	43,78 ± 0,75 b(B)
LD 062212	0,78± 0,09 a(A)	1,07± 0,01 a(B)	56,75 ± 4,39 acd(A)	48,13 ± 1,55 ac(B)
Rye:				
IPR 89	0,78± 0,05 a(A)	1,10± 0,06 a(B)	51,69 ± 2,74 bcd(A)	47,06 ± 1,42 bc(B)
Winter Wheat:				
IPR 84	0,86± 0,17 a(A)	0,92 ± 0,15 b(A)	54,76 ± 2,67 cd(A)	51,24 ± 2,50 ac(A)
CD 104	0,74± 0,02 a(A)	1,06 ± 0,04 a(B)	53,15 ± 1,48 cd(A)	47,18 ± 1,64 bc(B)

[1]The results are expressed as dry matter; [2] CA: cold acclimated; [3] NA: non acclimated. Different lowercase letters on the same column and uppercase letters on the same line between the mean of solids content or protein showed a significant difference ($p < 0.05$) by t test.

The lower solids content found in CA varieties was probably due to blockage of plant growth caused by low temperature. This is due to the presence of plant hormones, including abscisic acid, which is a regulator of plant growth and development that promotes physiological responses to abiotic stresses, as in this case, cold (Leung ; Giraudat, 1998; Finkelstein *et al.*, 2002), making the plant synthesize smaller amounts of nutrients, such as minerals, decreasing the number of total solids in the extracts.

In contrast, the crude protein content was significantly higher for the extracts of plants acclimated to cold, except for cultivar IPR 84, in which were not found a statistical difference at 5% of significance by t test between the two treatments. Chun *et al.* (1998) also found higher levels of protein in the extract of a cultivar of spring wheat acclimated to cold when compared to non acclimated.

According to Modesto *et al.* (2002), as the grasses reach maturity, there is a decrease in crude protein associated with increases in levels of the cell wall. In this study, was observed that plants grown under natural conditions have developed more than the plants acclimated to cold, which explains the difference observed in the protein.

Note also that the extract of spring wheat cultivar BRS Guabiju presented a higher protein content when compared with the set of winter varieties (wheat and rye), in the samples treated under cold acclimation. Although this behavior was not observed in all spring wheat varieties studied, in similar works was also observed this difference. Hew *et al*. (1999), in a study involving different varieties of cereals, noted that extracts of spring varieties showed higher protein content when compared to the cultivars of winter. However, this behavior has not been elucidated.

There was not significant interaction for the type of treatment (CA or NA) and type of cultivar on the ash content. The extract of BRS Guabiju showed an ash content (1.91%) significantly lower than the cultivars Ônix (2.21%), LD062212 (2.24%), IPR89 (2.52%), IPR84 (2, 40%) and CD104 (2.34%). The rye extract (IPR89) showed a significantly higher ash content than the spring wheat varieties (BRS Guabiju, Ônix, and LD062212).

The cold acclimation significantly reduced the ash content of the extracts (0.98%) compared to non acclimated varieties (3.56%), confirming that the conditions for plant growth, observed during the experiment, affected the synthesis of nutrients.

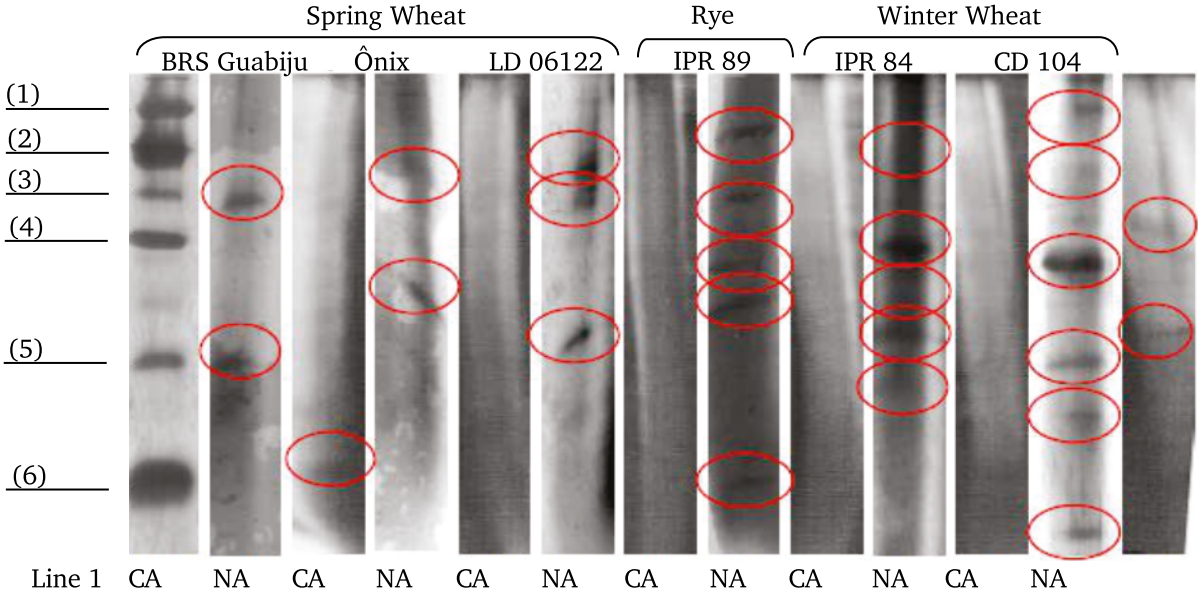
Identification of the ice structures protein (ISPs)

Figure 1, are the images of the gels obtained by SDS-PAGE to identify the ISPs of extracts from the varieties, after seven weeks of cold acclimation (CA) and non acclimated (NA).

In the NA extracts were not observed bands on gels, and only the varieties BRS Guabiju and CD104 contained some proteins of 14.4 and 97.0 kDa under the conditions of analysis.

In Figure 1, it can be observed in the extracts of rye leaves (IPR 89) the following proteins: one of 14.4 kDa, two of 20.1 to 30.0 kDa, one of 30.0 to 45.0 kDa and one of 66.0 kDa. Similarly, winter wheat varieties had a greater number of proteins in the range 14.4 to 66.0 kDa. In the extract of IPR84, it was identified a protein of molecular weight between 14.4 to 20.1 kDa, three from 20.1 to 30.0 kDa and 45.0 to 66.0 kDa, while in the extract of CD104 were found proteins of 14.4, 14.4 to 20.1, 20.1, 20.1 to 30.0, from 45.0 to 66.0 and 97.0 kDa, being this last, identified only in that cultivar.

Figure 1 – SDS-PAGE photograph of extracts of spring wheat varieties (BRS Guabiju, Ônix and LD 062212 AF), rye (IPR 89) and winter wheat (IPR 84 and CD 104) cold acclimated (CA) and non acclimated (NA) for seven weeks of culture



Line 1 is the low molecular weight marker including (1) phosphorylase b 97.0 kDa, (2) bovine serum albumin 66.0 kDa, (3) egg albumin 45.0 kDa, (4) carbonic anhydrase 30.0 kDa, (5) trypsin inhibitor 20.1 kDa, (6) lysozyme purified from egg white 14.4 kDa.

For spring wheat varieties, the extract of BRS Guabiju CA, showed only two proteins in the range of molecular weights investigated, one with a 20.1 kDa and other in the range 20.1 to 30.0 kDa, whereas in the extract ofLD062212 and Ônix, were found proteins from 20.1 to 30.0 kDa and 45.0 to 66.0 kDa, one of each, and another of 45.0 kDa only for LD062212.

It is noted that spring varieties (BRS Guabiju, Ônix and LD 062212) had less protein with molecular weights between 14.4 and 97.0 kDa when compared with winter varieties (IPR 84 and CD 104). This is not, however, an indication that these plants have an activity of ISPs lower or less efficient than the winter varieties, since, according to Hon *et al*. (1994), the activity of ISPs depends not only the kind but also the concentration of ISP in this extract.

Similar results were reported by Hon *et al*. (1994), to identify and characterize ISPs, individually, in extracts obtained from leaves of cold acclimated rye. The authors separated the proteins by SDS-PAGE in non reducing conditions, and a total of seven bands were observed. The bands were cut and five of the proteins were eluted, with molecular weights of 19, 26, 32, 34 and 36 kDa, showed a high degree of activity of ISPs, since they changed themorphology of normal growth of ice crystals, forming bipyramidal hexagonal when the solutions were frozen. However, the proteins of 11 and 13 kDa showed low activity.

In a study involving the accumulation of ISPs in cold tolerant cereals, Antikainen and Griffith (1997) also identified different proteins in extracts of leaves acclimated to low temperatures that have been shown to modify the shape of ice crystals, which was not observed in extracts not acclimated. In the extract of winter wheat varieties, were identified proteins of molecular weight of 31.0 to 45.0, 21.5, 14.4 to 21.5, 14.4 and littler than 14.4 kDa, is one of each, and two from 21.5 to 31.0 kDa. Spring wheat varieties produced an extract with similar composition, except that the protein with a molecular weight in the range of 31.0 to 45.0 kDa was not present.

Kontogiorgos *et al*. (2007) isolated and characterized ISPs from the extract of winter wheat leaves acclimated to cold. The extracts were concentrated by ultrafiltration and purified before the identification of proteins. The authors had the objective to identify the presence of ISPs able to inhibit recrystallization of ice. The molecular weight determination by mass spectrometry confirmed the presence of proteins of 21.3 and 12.9 kDa, identified by SDS-PAGE. It was also detected the presence of a protein with molecular weight of 40 kDa. The authors also verified that the non acclimated extracts did not produced ISPs activity, in other words, they did not inhibit recrystallization of ice.

From the observations reported by these investigators and according to the results, it is believed that the cultivars in this study have the potential to produce extracts capable of exhibit ISPs activity. Therefore, further studies were conducted to test this assumption.

Conclusion

The cold acclimation caused a reduction in total solids and ash content and an increase in protein concentration in extracts from all cultivars.

InCA extracts were identified different proteins with molecular weights ranging from 14.4 to 66.0 kDa for rye and winter wheat varieties, while the spring wheat extracts exhibited a lower number of bands between 20.1 to 66.0 kDa, values which are within the range of molecular weight of ISPs in plants acclimated to cold.

The NA extracts exhibited almost no protein bands, compared to CA extracts, indicating that cold acclimation induces the formation of ISPs in Brazilian cultivars of wheat and rye.

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