PAPER

COMPARATIVE 1D- AND 2D-ELECTROPHORETIC PROTEIN PROFILES OF ANCESTRAL AND MODERN BUCKWHEAT SEEDS GROWN IN THE ITALIAN ALPINE REGION

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ABSTRACT

Buckwheat is an old crop whose seeds are under-utilized. The protein composition of these seeds, however, makes them suitable as much needed ingredients for the production of gluten-free products. Several buckwheat species and local cultivars are known worldwide. In this work, 1D and 2D electrophoresis were used to characterize and compare the seed protein profiles of two buckwheat species (*Fagopyrum esculentum* and *Fagopyrum tataricum*). The two analyzed cultivars of *F. esculentum* represent authentic landraces of an Italian Alpine valley, named Valtellina. The protein profiles of *F. tataricum* and the two *F. esculentum* cultivars did not show major differences. However, narrow but significant differences were present between these two landraces, allowing their discrimination at protein level. This work represents a molecular-based approach to the designation of origin and authenticity of local buckwheat varieties and their tracing in flours for human food.

Keywords: buckwheat, electrophoresis, *Fagopyrum* spp., historical landraces, proteome, traditional cultivation

1. INTRODUCTION

Buckwheat is a pseudo-cereal seed of the class Dicotyledoneae, genus Fagopyrum, and smartweed family. It originated from and was domesticated in Eastern Himalaya regions and can be cultivated in flat and mountainous regions, as long as the climate is cold. Buckwheat came to Europe in the late Middle Age (OHNISHI, 1993) and, early traces of its cultivation in Italy were found in property documents of a family in Teglio, Valtellina, dating back to the middle of the sixteenth century (FERRANTI *et al*., 2002).
After a long history of growth and food use and a remarkable decline in the last decades, there has been a renewed interest in the crop. Among the reasons for this recent reappraisal are the growing worldwide need for sustainable nutrient sources (DURANTI and SCARAFONI, 2015) and the claimed health benefits of various buckwheat components (LI *et al*., 2008; IZYDORCZYK *et al*., 2014; ZHOU *et al*., 2015), as it occurs for many other seeds (SCARAFONI *et al*., 2007).
Buckwheat seeds (*Fagopyrum* spp.) display a high fiber content, ranging from 12 to 18% with very low lipid levels (about 3%) and relatively low carbohydrate levels, around 50% (EGGUM *et al*., 1980). The presence of biologically-relevant compounds, such as flavonoids, flavones, phytosterols, thiamin-binding proteins (LI and ZHANG, 2001) and rutin, with antioxidant properties (KREFT *et al*., 2002), all add nutritional value to this seed. The protein content is similar to or greater than that of wheat, from 12% d.w. upward. The peculiar composition of the protein fraction makes these seeds and their proteins suitable as main ingredients for many food applications, including foods for coeliac patients.
Phylogenetic relationships and polymorphisms of buckwheat have been studied at both genetic and protein levels (LI *et al*., 2008; OHNISHI and MATSUOKA, 1996; YASUI and OHNISHI, 1998; DU *et al*., 2004; ZELLER *et al*., 2004; ROUT and CHRUNGOO; 2007).
Wide margins for implementing tailored and finalized applications exist, because not all molecular and compositional features of these seeds have been thoroughly investigated for their optimal exploitation.
Modern analytical approaches allow the use of molecular-based strategies for the comparison, selection and improvement of crops and these activities are becoming crucial for mankind in the near future. Electrophoretic techniques applied to the protein fraction may represent a complementary and effective approach to the genetic/genomic analysis of plants (GORINSTEIN *et al*., 2005; CAPRARO *et al*., 2008). In this work, we applied a fast and reliable methodology based on electrophoretic analyses of seed proteins to identify candidate quality marker to be used to test and guarantee the designation of origin and authenticity of local buckwheat varieties. We focused our attention on two remnant cultivars of *Fagopyrum esculentum*, L.; one which is locally named 'Nustran' and the other called 'Furest' or 'Francese' or else 'Curunin' which has recently been identified as an authentic Valtellina landrace by genetic analyses (BARCACCIA *et al*., 2016). In this work we will refer to this latter cultivar as 'Furest'. It is still cultivated in Teglio (Valtellina), an Italian Central Alpine valley village. The first landrace, 'Nustran', is an original Teglio ecotype, whereas 'Furest' has been cultivated in Teglio only since the beginning of the twentieth century. In appearance, the two cultivar seeds show only slight morphological differences (BARCACCIA *et al*., 2016). In addition, a modern variety of *Fagopyrum tataricum* has also been included in our comparative work.

2. MATERIALS AND METHODS

2.1. Materials

Buckwheat seeds were kindly supplied by Raetia Biodiversità Alpine, Teglio, Valtellina, Italy. *Fagopyrum esculentum*, L. was of the varieties Nustran and Furest; *Fagopyrum tataricum*was of variety n'Zibaria.

2.2. Methods

2.2.1 Protein extraction

Dry seed kernels were manually ground to a meal in a mortar. The protein fractions of the resulting flours were extracted under stirring at room temperature for two hours following two procedures: a non-denaturing one, consisting of a solution containing 50 mMTris-HCl and 0.5 M NaCl at pH 7.5, and a denaturing one (redry solution), consisting of a solution containing 8 M urea, 2 M thiourea, 20 mg/mL3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate(CHAPS) and 65 mM 1,4-dithiothreitol (DTT) at pH 8.5. The ratios, 1/20 (w/v) in non-denaturing conditions and 1/40 (w/v) in the denaturing buffer, were used. The slurries were centrifuged at 12,000*g* at room temperature for 20 min, and the extracted proteins were conserved at -20 °C until used. Two experimental replicas for each condition were carried out.

2.2.2 Electrophoretic techniques

Isoelectric focusing (IEF) was performed on 7 cm pH 3–10 linear IPG strips (Amersham Biosciences) as described by CAPRARO *et al*. (2008).
SDS-PAGE for either 1D and 2D analyses was carried out according to LAEMMLI (1970) on 12% polyacrylamide gel using a mini-Protean II cell (Bio-Rad). The gels were stained with Coomassie blue. Protein extracts were analyzed in triplicate.

3. RESULTS AND DISCUSSION

Buckwheat seed proteins include either albumins and globulins, typical of the legume grains, and prolamins at high and low molecular weight (HMW and LMW) (NAIŁĘCZ *et al*., 2009), typical of cereals seeds. For this reason, the performances of two extraction protocols were assessed. The extraction of proteins under non-denaturing conditions using a saline alkaline buffer (Fig. 1, lanes marked with ND, namely T^{ND}, N^{ND} and F^{ND}) resulted in the prevalent solubilization of buckwheat globulins, as shown by 1D SDS-PAGE analysis. The presence of two main bands at about 40 and 25 kDa (L_A and L_B, respectively in Fig. 1) is typical of the reduced 13S globulin family of most species (CASEY *et al*., 1985) and the greater $M_r$minor band components around 60 kDa likely correspond to the 7S globulin subunits (V in Fig. 1). Under these conditions, a lower number of high M_r bands with respect to the samples extracted under denaturing conditions was visible. According to NAIŁĘCZ *et al*. (2009), these new bands likely correspond to the prolamin family, which are insoluble, unless denatured and reduced.

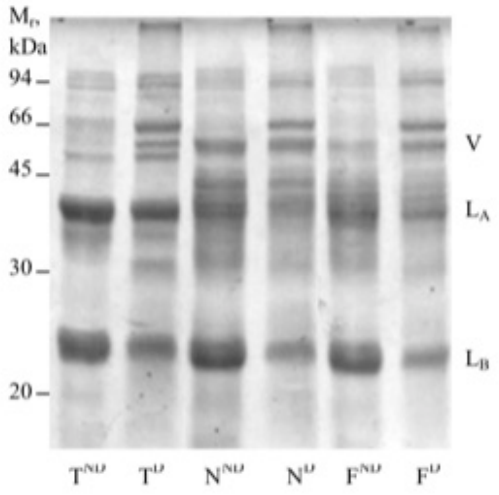

Figure 1. SDS-PAGE under reducing conditions of proteins from seeds of *Fagopyrum* species, namely *tataricum* (T), and *esculentum* landraces, 'Nustran' (N) and 'Furest' (F) extracted under non-denaturing (ND) and denaturing extraction conditions (D). L_A and L_B stand for 13S globulin acidic and basic subunits, respectively; V stands for 7S globulin family. See text for further details.

In the 1D separation of Fig. 1, a clearly distinct pattern of *F. tataricum* protein profile from those of the two *F. esculentum* cultivars was visible in the range 40-70 kDa. In particular, in samples T^D, three distinct bands were visible in the range 50 - 66 kDa while only two polypetides were detectable in the other two samples.
Overall, the 1D protein profiles of *F. esculentum* 'Nustran' and 'Forest' were more similar to each other than that of *F. tataricum*.
The greater resolution of 2D electrophoretic analysis was used to get a more detailed comparison of the three protein patterns (Fig. 2). Based on the results described above, the 2D IEF/SDS-PAGE analyses were carried out under denaturing and reducing extraction conditions to get the most complete picture of the respective proteomes. Indeed, the 2D maps of the analyzed *Fagopyrum* spp. allowed the identification of some of the seed's main protein components. The spot groups marked with L_A and L_B display intensities and positions which definitely identifies these spots as acidic and basic subunits of the 13S globulin, respectively. The 'train spot' group is typical of the high M_r 7S globulin chains with their peculiar pI isoforms (RADOVIĆ *et al*., 1996; MAGNI *et al*., 2007). In this latter case, however, closely migrating HMW prolamins with variable pI and M_r around 50 kDa were likely present too, as detailed by NAIŁĘCZ *et al*. (2009). Prolamins of intermediate and low M_r, though barely recognizable, seemingly spread in the map at more acidic pIs and with greater migration coefficients, according to NAIŁĘCZ *et al*. (2009).
The 2D electrophoretic maps confirm and detail the 1D profiles by showing a greater similarity between the two *F. esculentum* cultivars and their difference with *F. tataricum* spot pattern. Indeed, the region of the 13S globulin acidic subunits appears quite different and some major spots, which are circled in the panel T of Fig. 2, contributed to significantly diversify the map of *F. tataricum* with respect to those of *F. esculentum*. However, differences between the two *F. esculentum* landraces were also detectable. Quantitative differences among common spots, could be noted too. Most of these differences were found in the low M_r, neutral pH region of the 2D maps. The observed intervarietal differences cannot be attributed to climatic, pedological or edaphological causes, since the seeds arise from same cultivation area and grower. Likely, these

differences represent authentic biodiversity source. In an extension of this work, it would be interesting to identify those differing spots, which are unlikely related to classical storage proteins because of their low M_r and clear-cut shape of the spots, and to associate them with peculiar phenotypic or nutritional features of the two cultivars.

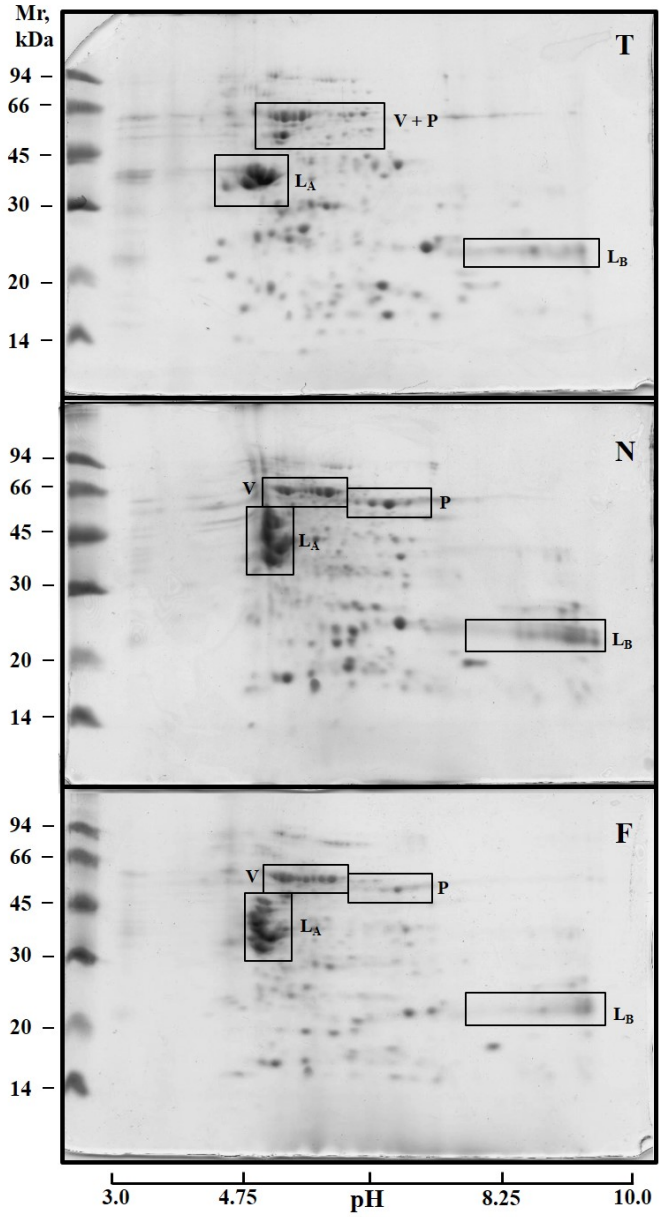

Figure 2. Two-dimension electrophoretic maps of proteins extracted from seeds of *Fagopyrum* species, namely *tataricum* (T) and *esculentum* landraces: 'Nustran' (N) and 'Furest' (F), all extracted under denaturing conditions (see details under Methods). V: 7S globulin chains; P: prolamins chains; L_A: 13S globulin acidic subunits; L_B: 13S globulin basic subunits.

4. CONCLUSIONS

In the perspective of future uses of landraces and their derivatives in foods suitable for people with coeliac disease or in nutraceutical formulations, the development of an agile

methodology to identify landraces components is needed. This work represents a first step in this direction, making available specific 2D electrophoretic maps for given landraces and thus helping in their identification and tracing in flours for human food. It is worthy of note that the two cultivar seeds are very similar in appearance, with 'Furest' being smaller in size and of lighter grey color (BARCACCIA *et al*., 2016). These minimal differences make the need for molecular fingerprinting more compelling.
This work, by revealing even minor interspecific and intervarietal differences in the protein expression patterns of buckwheat seeds, may open the gateway to the identification of hidden useful properties, such as resistance to adverse environmental conditions and seed quality characteristics, and to the valorization of these crop populations.

REFERENCES

Barcaccia G., Volpato M., Gentili R., Abeli T., Galla G., Orsenigo S., Citterio S., Sgorbati S. and Rossi G. 2016. Genetic identity of common buckwheat (*Fagopyrum esculentum* Moench) landraces locally cultivated in the Alps. Genet. Resour. Crop Evol. 63: 639.

Capraro J., Magni C., Fontanesi M., Budelli A. and Duranti M. 2008. Application of two-dimensional electrophoresis to industrial process analysis of proteins in lupin-based pasta. LWT - Food Sci. Technol. 41: 1011.

Casey R., Domoney C. and Ellis N. 1985. Legume storage proteins and their genes. Oxford Surv. Plant Mol. Cell Biol. 3: 1.

Du S.K., Li Z.X. and Yu X.Z. 2004. Research progress on buckwheat protein. Food Sci. 10: 409.

Duranti M. and Scarafoni A. 2015. Can legume seed proteins be a valid alternative to animal sources to fulfill the growing need of food proteins? Agro Food Ind. Hi. Tech. 26: 8.

Eggum B.O., Kreft I., Javornik B.1980. Chemical composition and protein quality of buckwheat (Fagopyrum esculentum Moench). Plant Foods Hum. Nutr. 30: 175.

Ferranti R., Pirola A. and Penati F. 2002. Il paesaggio vegetale della Provincia di Sondrio, Suppl. Il Naturalista Valtellinese, Atti del Museo Civico di Storia Naturale di Morbegno 13: 38.

Gorinstein S.,Drzewiecki J.,Delgado Licon E.,Pawelzik E., Martinez Ayala A.L., Medina O.J., Haruenkit R. and Trakhtenberg S. 2005. Relationship between dicotyledone-amaranth, quinoa, fagopyrum, soybean and monocots-sorghum and rice based on protein analyses and their use as substitution of each other. Eur. Food Res. Technol. 221(1): 69.

Izydorczyk M.S., McMillan T., Bazin S., Kletke J., Dushnicky L. and Dexter J. 2014 .Canadian buckwheat: A unique, useful and under-utilized crop. Can. J. Plant Sci. 94: 509.

Kreft S., Strukelj B., Gaberscik A. and Kreft I. 2002. Rutin in buckwheat herbs grown at different UV-B radiation levels: comparison of two UV spectrophotometric and an HPLC method. J. Exp. Bot. 53: 1801.

Laemmli U.K.1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 660.

Li J.H., Chen Q.F. and Zeller F.J.2008. Variation in seed protein subunits among species of the genus Fagopyrum Mill. Plant Syst. Evol. 274: 193.

Li S.Q. and Zhang Q.H. 2001.Advances in the Development of Functional Foods from Buckwheat. Crit. Rev. Food Sci.Nutr. 41: 451.

Magni C., Scarafoni A., Herndl A., Sessa F., Prinsi B., Espen L. and Duranti M. 2007. Combined 2D electrophoretic approaches for the study of white lupin mature seed storage proteome. Phytochem. 68(7): 997.

Naiłęcz D., Dziuba J., Minkiewicz P., Dziuba M. and Szerszunowicz I. 2009 .Identification of oat (Avena sativa) and buckwheat (*Fagopyrum esculentum*) proteins and their prolamin fractions using two-dimensional polyacrylamide gel electrophoresis. Eur. Food Res. Technol. 230: 71.

Ohnishi O. and Matsuoka Y. 1996. Search for the wild progenitor of buckwheat II. Taxonomy of Fagopyrum (Polygonaceae) species based on morphology, isozymes and cpDNA variability. Genes Syst. 71: 383.

Ohnishi O. 1993. Population genetics of cultivated common buckwheat, Fagopyrum esculentum Moench. VIII. Local differentiation of land races in Europe and the silk road. Jap. J. Genet. 68: 303.

Radović S.R., Maksimović V.R. and Varkonji-Gašić E.I. 1996.Characterization of buckwheat seed storage proteins. J. Agric. Food Chem. 44(4): 972.

Rout A. and Chrungoo N.K. 2007.Genetic variation and species relationships in Himalayan buckwheats as revealed by SDS PAGE of endosperm proteins extracted from single seeds and RAPD based DNA fingerprints. Genet. Res. Crop. Evol. 54: 767.

Scarafoni A., Magni C. and Duranti M. 2007.Molecular nutraceutics as a mean to investigate the positive effects of legume seed proteins on human health. Trends Food Sci. Tech. 18(9): 454.

Yasui Y. and Ohnishi O. 1998. Phylogenetic relationships among Fagopyrum species revealed by the nucleotide sequences of the ITS region of the nuclear rRNA gene. Genes Genet. Syst. 73: 201.

Zeller F.J., Weishaeupl H. and Hsam S.L.K. 2004. Identification and genetics of buckwheat (*Fagopyrum*) seed storage protein. In: Seung S.H., Choi Y.S., Kim N.S., Proceedings of the VIII International Symposium on buckwheat, Chunchon, Korea. Advances in buckwheat research: 195.

Zhou X., Wen L., Li Z., Zhou Y., Chen Y. and Lu Y. 2015.Advances on the benefits of bioactive peptides from buckwheat. Phytochem. Rev. 14: 381.

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