Jerzy Macewicz

Is quantity and quality of protein in the berley cultivar determined by the proportion of kernels with and witout subaleurone layer?

Studia Ecologiae et Bioethicae 1, 121-126

2003

Studia
Ecologiae et Bioethicae
1/2003

Jerzy MACEWICZ*

Is quantity and quality of protein in the barley cultivar determined by the proportion of kernels with and without subaleurone layer?

Introduction

Protein content in grain yield of barley cultivars may differ up to 50% (e.g. ranging between 9 and 15% of dry mass). The variation is recorded in spite of equal size of aleurone layer, which is a protein tissue. No precise explanation has been created for localization of this surplus of protein, which makes a cultivar is classified as the high- or low-protein one.

In all barley plants, differentiation in protein content in subaleurone tissue among kernels, has been detected by the authors. First, the comparative observation of endosperm structure had been performed in barley cultivars with the highest (Rabel) an the lowest (Rudzik) protein content in grain. The authors used their own histochemical method of staining proteins (Fast green FFC) immediately on a fracture of non-fixed endosperm. This simple procedure (Macewicz 1992) enabled comparative analysis of large numbers of kernels.

Seeds from the same cultivar, could differ with regard to presence or absence of a specific subaleurone layer. In some kernels the subaleurone cells with starchy granules covered with protein are so numerous, that they form a separate subaleurone layer in endosperm. The subaleurone layer proteins are produced independently of those of aleurone layer.

The aim of this work was to find the variability of subaleurone protein presence or absence in seeds of the same barley cultivar and to analyse the structure of protein located in subaleurone layer of endosperm.

* Instytut Ekologii i Bietyki UKSW; Department of Plant Biotechnology and Cytogenetics, Plant Breeding and Acclimatization Institute Radzików.

Material and Methods

Plant Material

Kernels from two cultivars Rabel and Rudzik of spring barley (*Hordeum vulgare* L.), from the collection of the Gene Bank of the Institute of Plant Breeding and Acclimatization Institute in Radzikow, served as experimental material for the analysis of endosperm differentiation and protein analysis.

Grains were taken at random and broken transversely into halves. The remaining kernels' halves (with embryo) were marked with identification numbers and stored. Part of them was used for protein content test. The comparative test of the endosperm structure was performed with the own method trough immersing non fixed grain halves (without embryo) into aqueous solution of Fast green FFC stain, at pH 8,0. The six minutes treatment was followed by drying and glueing the grain halves on microscope slides. The procedure of grain preparation does not require fixing, embedding and microtome cutting. The stained fracture surfaces of endosperm were observed trough a glass slide, using a microscope with the overhead light. The amount of protein presence was expressed, according to the three-step evaluation scale:

0 — no protein structures in the subaleurone region,

1 — scarce, dispersed protein structures,

2 — numerous protein structures filling the subaleurone layer.

Percentages of grains with each score were counted for the investigated cultivars. The kernels halves (with embryo) were grouped conformably to the scores of microscopic test. The groups of score 0 and 2 were subjected to the NIT (Near Infrared Transmittance) investigation of protein content method. The low- and high-protein kernels halves (with embryo) were taken to the protein quality analysis. The following methods and reagents were used in the protein analysis:

Methods of:

Preparation of starch

A sample of 2-10 g of barley grain was homogenized in a blender with 50 ml ice-cold 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5 mM 2-mercaptoethanol, 0,2 mM PMSF. The homogenate was filtered through two layers of Miracloth (Calbiochem) and allowed to sediment for 45 min. The supernatant was discarded and the pellet was washed 4 times with 50 ml of extarctin buffer. The starch was left in the cold room for 45 min to sediment between every wash, finaly starch was air dried overnight at 4°C and stored at – 18°C.

Extraction of granule-bound proteins

Proteins were extracted from starch garnules by SDS-sample buffer [2]. 5 mg of starch was mixed with 300 ml of SDS-sample buffer and stirred 10 min in RT.

Denaturating electrophoresis

One-dimensional SDS-PAGE was performed using methods described by Laemmli (2) on the Bio-Rad Mini-Protean II system (7 cm x 10 cm minigels). 9% acrylamide gels of 1 mm thickness were used in model experiments. Gels were stained with 0,2% Coomassie Brilliant Blue R250 in 40% MeOH in water containing 5% acetic acid for 1 h (which also fixes the proteins) and destained overnight with the same solvent, excluding the dye.

In gel digestion

Stained protein spots were excised and destained for 2-6 h in a mixture of 40% [v/v] acetonitrile and 60% (v/v) 50 mM NH_4HCO_3. Destained gel pieces were dried by vacuum centrifugation. An aliquot of 20 µl of a sequencing grade trypsin solution (30 ng/µl in 50 mM NH_4HCO_3) was added to the dried gel piece and soaked. Subsequently, 30 µl of 50 mM NH_4HCO_3 was added and digestion was allowed to proceed overnight at 37° C. Proteolytic peptides were extracted by incubation with 15 µl of 5% (v/v) formic acid for 10 min followed by the addition of 30 µl of acetonitrile. The supernatant was removed after 30 min and the 5% formic acid/acetonitrile treatment was repeated once. The combined supernatants were lyophilized, dissolved in 3 µl acetonitrile and 7 µl 2% (v/v) TFA and were then used for MALDI-TOF MS.

Mass spectrometry

Mass spectra of peptide mixtures as well as post source decay (PSD) spectra were obtained using a Reflex II MALDI-TOF mass spectrometer (Bruker-Daltonik, Bremen, Germany). a-cyano-4-hydroxy cinnamic acid [saturated solution in 6 vol. acetonitrile and 4 vol. 0.1% (v/v) TFA] served as matrix. Samples were prepared by mixing 0.5 µl of 2% aqueous TFA, 0.5 µl of sample and 0.3 µl of matrix solution directly on the target and letting it air-dry. Spectra were recorded in the reflector mode and calibrated using the monoisotopic peaks from a known autodigestion product of bovine trypsin (residue 50 to 69, M+H+=2163.06 Da) and the matrix trimer ion (3M+H+=568.14 Da). Mass accuracy was better than ±0.1 Da up to a mass of 3000 Da.

Primary ion selection for post source decay (PSD) measurements was performed using a timed ion selector providing a mass resolution of ±20 Da. Therefore, PSD spectra can be recorded for all peptides of sufficient

intensity which show no intense neighboring signals within ±20 Da. The mass accuracy of the fragment ions is better than ±0.5 Da.

Database search

The database search using the proteolytic peptide masses was performed with the PeptideSearch program (http://www.narrador.embl-heidelberg.de/GroupPages/Homepage.html). The search was done with an assumed peptide mass accuracy of ±0.1 Da and the database was restricted to proteins in the mass range below 100 kDa. Database searches using the amino acid sequence stretches derived from PSD spectra were performed with the same program using amino acid sequence as input.

Reagents

Tripsin was purchased from Roche Diagnostics GmbH, Mannheim, Germany. Other reagents were from commercial sources.

List of abbreviations: EDTA ethylenedinitrilotetraacetic acid disodium salt, MALDI matrix-assisted laser-desorption ionisation, PMSF phenylmethylsulfonyl fluoride.

Results

Great variability of protein content was observed among individual kernels belonging to the registered , uniform cultivars. Differences were noted in the volume of subaleurone layer among the morphologically similar grains from the same cultivar. The volume of this layer was positively correlated with the grain protein content. Low-protein grains were found together with the high-protein ones in the both cultivars.

Using microscope test, it was possible to distinguish single grains with low- or high-protein content and use them for chemical analysis.

A database search of the masses of the peaks detected in the spectrum allowed to identify a protein from barley called z-type Serpin (sptrembl:Q40076). Peaks belonging to Serpin are marked with a star in the spectrum (Figure B). However, a number of peaks can not be attributed to serpin and it is suspected that the gel band contains more than one protein (as a matter of fact the band appears to be a doublet when less material is loaded on the gel). Therefore, it was attempted to record post-source decay (PSD) (Kaufmann 1994) fragment spectra from unknown peaks. The peak at m/z=997.60 gave a good quality fragment ion spectrum which could be used to sequence the peptide. The following amino acid sequence was determined: L/I-V-P-L/I-A-L/I-D-T-R. A database search based on this sequence allowed to identify the 30

kD fragment of the protein B3-hordein (swissprot P06471, pir S07975). Some peaks still remain unidentified. Possibly they belong to the part of the B3-hordein protein which is not in the database yet. However, a third, so far unidentified, protein component in the gel band can not be excluded.

Disscusioin

Brej (1973) and Kaczmarek (1977) proved similar differences existing between kernels of a single plant, using the method of combustion and calorymetry on grain halves. Gaines et. al. (1985) reported kernel-to-kernel differences in protein content revealed by staining of microtome cuttings. The authors recommended the separate treatment of every kernel.

Our applied method of kernels' halves staining, enabled manifestation of variation in protein structures among morphologically uniform kernels of a single cultivar. The microscopic observation showed that a part of protein may be stored beyond the aleurone layer — on the starchy granules in subaleurone layer. A number of low-protein kernels was present even within the high-protein cultivar Rabel. In the other cultivar Rudzik, with low-protein content, some high-protein kernels may also occur (Macewicz 2000).

Higher amount of protein structures in subaleurone layer of endosperm in the Rabel cultivar, is related to higher kernel protein content.

References

Brej S., 1973 – Zawartość białka w ziarniakach mieszańcówF$_2$ pszenicy zwyczajnej formy jarej. Hod.Rośl. Aklimat. 17/6: 419-423.

Gaines R., Bechtel D., Pomeranz Y., 1983 – Endosperm structural and biochemical differences between the high-protein amphiploid and its progenitors. Cereal Chem., 62/1: 25-31.

Kaczmarek J., 1977 – Zawartość białka w ziarnie linii wsobnych żyta. Biul. IHAR.,131: 69-80.

Kaufmann R., Kirsch D., Spengler B., 1994 – Sequencing of peptides in a time-of-flight mass spectrometer - Evaluation of postsource decay following matrix-assisted laser-desorption ionisation (MALDI). Int. J. Mass Spectrom. Ion Proc.; 131: 355-385.

Laemmli U. K., 1970 – Cleavage of structural proteins during the assembly of the haed of bacteriophage T4. Nature 227: 680-685.

Macewicz J., 1992 – Subaleurone tissue and high proteincontent in barley cultivars. Hod. Rośl. Aklim. 36: 47-59

Macewicz J., 2000 – Differential occurenceof protein subaleurone layer in seeds of barley cultivars for brewery industry. Biul. IHAR: 237-242

Czy proporcja udziału ziarniaków z warstwą i bez warstwy subaleuronowej determinuje poziom i jakość białka w plonie jęczmienia

STRESZCZENIE

Wysokobiałkowe odmiany jęczmienia mogą różnić się zawartością białka w plonie nawet o 50%. Stwierdzamy tę między odmianową zmienność, mimo że warstwa aleuronowa, gromadząca białka zapasowe ma jednakową wielkość w ziarniakach wszystkich odmian. Dotąd brak było morfologicznej interpretacji, wskazującej gdzie w ziarniakach gromadzona jest ta dodatkowa ilość białka, którą wyróżniają się odmiany wysokobiałkowe.

Wykonano porównawcze obserwacje struktury endospermu w odmianie najwyżej- i najniżej białkowej. Autorzy barwili białka własną metodą histochemiczną: na powierzchni przełomu nie utrwalonego endospermu. Dzięki zastosowaniu tej prostej metody, stały się możliwe badania porównawcze dużej ilości pojedynczych ziarniaków. Wykazały one, że u części ziarniaków każdej rośliny wysokobiałkowej występuje specyficzna tkanka subaleuronowa. W obrazach mikroskopowych widoczna jest ona między skrobią endospermu a warstwą aleuronową i nie występuje w pozostałych ziarniakach tej samej rośliny. Zatem w niektórych ziarniakach białko gromadzone jest nie tylko w warstwie aleuronowej ale dodatkowo także w tkance subaleuronowej endospermu.

Obecność białka subaleuronowego w części ziarniaków rośliny, zwiększa zawartość białka w plonie z tej rośliny czyniąc ją wysokobiałkową. U pozostałych roślin, ziarniaki wysokobiałkowe występują z niższą częstością lub nie występują wcale – rośliny te są niskobiałkowe. Udowodniliśmy w ten sposób, że wysokobiałkowe odmiany jęczmienia wyróżniają się zwiększonym udziałem ziarniaków wysokobiałkowych, zawierających obie tkanki gromadzące białka zapasowe: aleuronową i subaleuronową. Potwierdzeniem uzyskanego wyniku jest 100%-wy udział ziarniaków z tkanką subaleuronową we wszystkich roślinach etiopskiej formy jęczmienia – Hiproly.

Ponadto celem pracy jest wykazanie, że ziarniaki zbierane z pojedynczej rośliny różnią się nie tylko ilością białek, ale i ich jakością. Podjęto w tym celu próbę identyfikacji białek obecnych w obu typach ziarniaków stosując metody analityki biochemicznej. We frakcji białek wydzielonej z ziarniaków zawierających tkankę subaleuronową (wysokobiałkowych), w porównaniu do analogicznej frakcji białek wyizolowanej z ziarniaków bez tej tkanki (niskobiałkowych), stwierdzono znacznie silniejszą obecność kilku peptydów o masach cząsteczkowych w przedziale od 41 kDa do 34 kDa. Przypuszcza się, że są to izoformy endo-B-1, 4-ksylanazy.