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Rapid methods for selecting single kernels of waxy barley

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Summary

The rapid estimation of the amylose content in waxy barley is of particular importance in breeding new varieties with a low amylose content and in controlling the amylose content in the industrial production process, e.g. milling companies. Therefore, an amylose test measuring the quotient of the absorbance of the starch-iodine complex in the absorption maximum for amylose and for amylopectin was modified and adapted for the determination in single kernels of commercial and waxy barley. To achieve this, single kernel were crushed, dissolved with 1N sodium hydroxide solution and stained with iodine-potassium iodide solution. Next, the amylose iodine and the amylopectin iodine complex were measured colorimetrically in wells of microtitre plates (96 samples). The modified two-wavelength iodine binding procedure was used as a standard chemical analysis for developing a non destructive Near Infrared (NIR) method to distinguish single kernel of commercial barley and waxy barley. The calibration was calculated using the transmission spectra and the Modified Partial Least Squares (MPLS)-method. Spectral transmission in a wavelength range between 900 nm and 1100 nm were measured in a cuvette holding 23 single kernels. The quality of the calibration was controlled by cross validation (coefficient of cross validation 1-VR = 0.936; standard error of cross validation SECV = 0.020) and a prediction (coefficient of prediction RSQ = 0.883; standard error of prediction SEP [C] = 0.028) in the range of R-Values between 0.63 and 0.86. Applying this calibration facilitates an unequivocal differentiation between commercial and waxy barley genotypes on the single kernel level.

Introduction

Starch is one of the major polysaccharides in cereal grains. Barley starch, like other cereal grains, comprises the components amylose and amylopectin. Out of these, amylose is the minor component consisting of linear long chains of α-(1→4) linked D-glucose residues. In contrast, amylopectin is a highly branched polymer consisting of many α-(1→6) linked D-glucose side chains attached to the α-(1→4) polymer (Mac Gregor and Bhathy, 1993).

Grains of barley normally contain about 20-30 % amylose (Mac Gregor and Morgan, 1984; Morrison et al., 1984). Waxy barley, consisting nearly exclusively of amylopectin, contains according to Banks (1970) only 0.4-13 % and according to Morrison et al. (1984) 2-8 % amylose.

Many different procedures have been published for the determination of the amylose in starch using differential scanning calometry (Mestres et al., 1996; Moorthy et al., 2006; Creek et al., 2007), capillary electrophoresis (Herrero-Hartinez et al., 2004), thermogravimetric analysis (Stawki, 2008), high performance size exclusion chromatography (Cheu and Bergmann, 2007), potentiometric titration (Schwank et al., 1990), and amperometric titration (Larsen et al., 1953). A lot of these procedures use colorimetric methods (Mahmood et al., 2007), such as "Blue-value" (Sowbhagya and Mysore, 1971; Knutson and Grove, 1994; Anaro et al., 2009), and "R-value" (Hovenkamp-Hermelink et al., 1988; Haase, 1993). The blue iodine-amylose-complex is measured at an absorption maximum at approximately 620 nm (Stark and Yin, 1986). The amylopectin reacts weakly with iodine at about 530-550 nm and gives a red coloured complex (Mac Gregor and Bhathy, 1993).

Most of the described methods are very time consuming and it is necessary to isolate the starch or to mill the grains for the determination of amylose. With the release of the waxy barley "Waxyma" in Europe in 2008 the determination of amylose content in barley became an important issue for breeders and industries.

The aim of this work is therefore to develop a new simple and rapid method for the determination of amylose and amylopectin in single barley grains based on a non destructive NIR-technique.

Material and methods

Plant material

Analyses were carried out using the waxy barley cultivar "Waxyma", new lines of waxy barley as well as the varieties 'Lomerit', 'Action' and 'Malwinta' supplied by Dieckmann GmbH & Co. KG Nienstädt, Germany. Additional barley cultivars (nearly twenty varieties) were used to develop the NIR calibration. These cultivars were grown under field conditions in Groß Lüsewitz, Germany, in 2008 and 2009 applying cultural management practices common for the region.

Whole meal sample preparation

Barley awns were removed and the grains were ground in a falling number mill (Laboratory Mill 3100, PERTEN) to pass a 0.8 mm sieve (Flamme et al., 1999).

Starch sample preparation in laboratory scale

The starch was prepared using a Glutomatic System (PERTEN instruments) combined with a flow stream channel, described previously (Flamme et al., 1999). Isolated starches had a high purity with lower than 0.3 % raw protein.

Amylose measurement using amperometric titration

Amperometric titration method was used as the standard method to determine the iodine binding capacity of isolated barley starches mainly according to Larson et al. (1953) and Richter et al. (1968). The procedure was modified as follows:

40 mg of isolated starch was put into a volume of 1.0 ml of distilled water to swell the starch granules over night. Next 1.0 ml of 2N sodium hydroxide solution was added while stirring constantly. The starch was extracted for 2h in a refrigerator and then the solution was neutralized with 1N hydrochloric acid and filled to a volume of 25 ml with distilled water.

This polysaccharide solution was used for the titration with iodine-potassium iodate solution (1.78 g KIO_3 + 19.36 g KI + 0.84 g $NaHCO_3$ were dissolved in 1 l of distilled water, titration solution: tenfold dilution of the above solution).

For amperometric titration an automated titrator (ORION 960) equipped with a special beaker and a double platinum electrode for Karl-Fischer-Titration was used. Ten ml of the starch solution were taken into the beaker and dissolved with 30 ml distilled water. Adding of the titration solution was carried out in steps of 0.2 ml. After finishing the titration, an automatic evaluation was performed using the first derivation of the titration curve. Then the amylose content was calculated with the iodine binding capacity of amylose of 19.5 g of iodine per 100 g polysaccharide (BANKS et al., 1974).

Amylose measurement in single barley grains using a two-wavelength colorimetric method

1. Modified method with perchloric acid as solvent:
Barley grains were sliced in two halfes with a razor blade. The half without the embryo was milled in a swing mill (RETSCH). Then a spatula tip of meal was extracted in perchloric acid according to HOVENKAMP-HERMELINK (1988). After staining with I_2-KI solution the absorbance at 620 and 550 nm was measured. The ratios of the absorbencies (R-value) were used to differentiate between waxy and non waxy barley grains.

2. Modified method with sodium hydroxide as solvent:
According to HOVENKAMP-HERMELINK (1988) and HAASE (1993) the ratio of absorption at two wavelengths was used to estimate the amylose content. The method was modified as follows: Barley grains were divided into two halves with a razor blade. The half without the embryo was crushed and extracted over night at room temperature with 2 ml of 1N sodium hydroxid solution. Samples were permanently stirred in glass tubes using a magnetic stirrer. After dissolving with distilled water and neutralisation with 1N hydrochloric acid the starch containing solution was filled to a volume of 100 ml. Then 0.1 ml of the starch solution was stained with 0.1 ml of iodine solution (5.08 g KI and 2.54 g I dissolved in 1l distilled water, fourfold dilution of the above solution). Staining was carried out in microtitre plates in a 96-well formate (HU et al., 2010) and the measuring of the absorbance at 620 and 550 nm was conducted in a microtitre plate photometer (ANTHOS HT III). The knowledge of the R-value enables us to differentiate between waxy (R-value ~ 0.7) and non waxy barley grains (R-value ~ 0.9).

Amylose measurement using NIR

For NIR measurement awns were removed and transmission spectra were measured with an Infratec 1255 (Fa. FOSS) using a wavelength range between 900 nm and 1100 nm. The device was equipped with a special cuvette. In this cuvette it was possible to analyse 23 single kernels in one rotation of the cuvette. Near infrared transmittance spectra were collected from 1104 single barley kernels of different genotypes with normal and low amylose content. As standard chemical method, the modified two-wavelength colorimetric method for single kernels in microtitre plates was used as described above. The spectra and analytical data were separated into 108 samples (calibration set) and into 996 samples (prediction set). Every tenth sample was selected for the calibration set. Using the MPLS-method the calibration was calculated between spectra data and data of chemical analysis.

Statistical analysis

All tests were repeated twice. The error between the duplicates was lower than three percent. All statistical computations were made using SAS version 9.2 (SAS Institute, Inc., Cary, NCI, USA). Correlation analysis was conducted using Pearson's correlation test. Differences between methods were assessed using the Tukey-test.

Results

The amperometric method was performed to measure the amylose content in conventional and in waxy barley. Titration curves of different barley varieties are shown in Fig. 1.

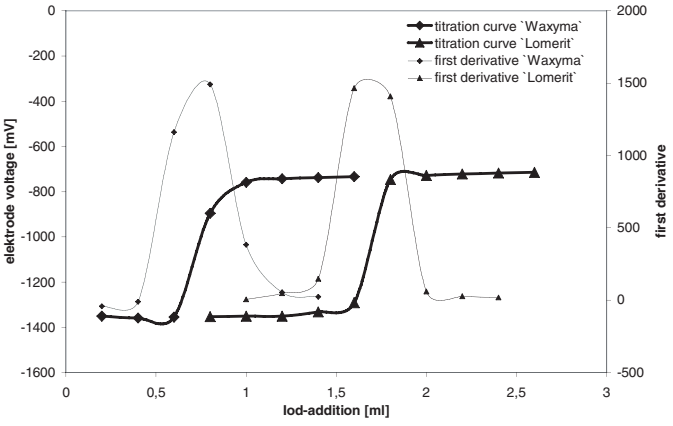

Fig. 1: Amperometric titration curves and first derivative of the waxy barley 'Waxyma' and the standard barley 'Lomerit'.

The consumption of KI-KIO3-solution was measured by using the first derivative of titration curve. Amylose contents were calculated on the basis of the iodine binding capacity of amylose.
Different amylose contents and starch contents of barley varieties are given in Tab. 1.
Whereas 'Waxyma' showed an amylose content of approximately 5 %, the amylose content of conventional barley varieties varied between 20.3 % and 24.1 % amylose. In tendency, the starch content of cultivar 'Waxyma' is a little bit lower in comparison to the starch content in the conventional variety 'Lomerit'.

Tab. 1: Starch content and amylose content of different barley varieties.

Variety	Starch content [% in dm]	Amylose content [%]
'Waxyma' (harvest 2007)	59.17	4.6
'Waxyma' (harvest 2008, Altfeld)	67.42	5.5
'Waxyma' (harvest 2008, Bördegrün)	65.39	5.3
'Lomerit' (harvest 2008)	70.22	23.3
'Malwinta' (harvest 2008)	65.42	20.3

Several methods have been tested to extract and dissolve the starch of whole barley grains using the two-wavelength iodine binding procedure. Out of these the extraction medium perchloric acid was not suited, because whole grains can not be dissolved (Fig. 2) resulting in very low extinctions at 550 nm (amylopectin) and 620 nm (amylose).

Also the use of the 1N sodium hydroxide was not suited without stirring the solution. The best result was achieved, when whole grains were stirred over night (16 h) in 1N sodium hydroxide at room temperature, although the highest R-value was achieved, when using perchloric acid as extraction medium to dissolve the starch (Tab. 2).

When using perchloric acid as a solvent the barley grains have to be milled. Nevertheless, both established two-wavelength colorimetric methods, as described above, had the ability to clearly separate waxy from non waxy grains. There was a significant correlation (Pearson correlation coefficient, R^2= 0.79, n = 12) between R-values

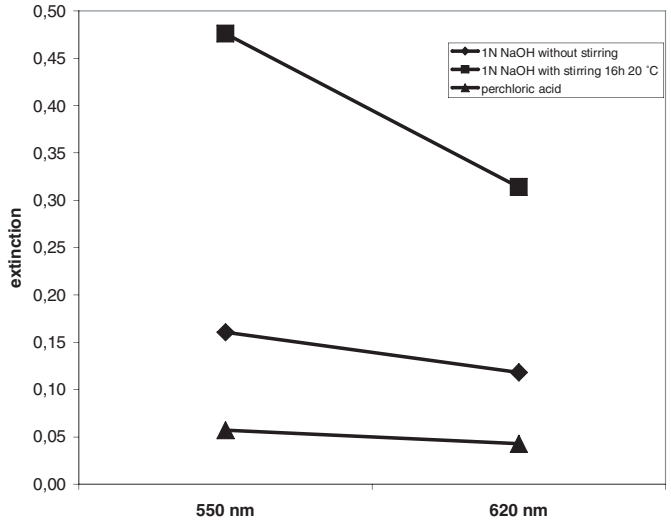

Fig. 2: Extinction of the amylose iodine and the amylopectin iodine complex at 620 and 550 nm using different solvents for whole grains.

Tab. 2: R-values of barley lines (harvest 2009), determined with perchloric acid and sodium hydroxide as grain solvent.

line	R-value (solvent perchloric acid)	R-value (solvent sodium hydroxide)
1	1.213	0.837
2	1.226	0.844
3	0.697	0.647
4	0.771	0.644
5	0.766	0.649
6	1.084	0.660
7	0.736	0.654
8	0.778	0.648
9	0.755	0.665
10	0.790	0.662
11	0.715	0.642
12	0.739	0.644

determined with perchloric acid as solvent and the procedure with sodium hydroxide as solvent.
R-values, determined with the solvent perchloric acid were significantly higher (P = 0.0011) than R-values determined with the solvent sodium hydroxide (Tab. 2).

In breeding it is very useful to have a rapid non-destructive test to characterize waxy and non waxy barley. Near infrared spectroscopy provides an alternative non-destructive technique for measuring the amylose content. Results of calibration and prediction are given in Fig. 3.

MPLS-method was conducted by correlating the spectra of the calibration set and the R-value determined by the two-wavelength colorimetic method. Different mathematical pre-treatments of the raw spectra were tested and the calibration with the highest correlation coefficient (R^2) and the lowest standard error of calibration (SEC) was selected. An R^2 of 0.945 with a (SEC) of 0.019 was found, when using non pre-treated grains. The quality of the calibration was then controlled by a cross validation resulting in an R^2 of 0.936 and the SECV of 0.020. The calibration provided acceptable results for predicting the R-Value in barley, showing a R^2 of 0.883 and a SEP [C] of 0.028 in the prediction set. The R-Value ranged between 0.63 and 0.86.
The developed method can be used for the identification of waxy and non waxy barley types by analysing single barley grain. However, for a further classification within different waxy genotypes and non waxy genotypes this method is not well suited.

Discussion

There are many different procedures to measure the amylose content in starch derived from plants resulting in problems of standardizing these methods for quantifying the amylose content. FITZGERARD et al. (2009) highlighted the need to standardize the way amylose is measured in rice, because five different versions of the iodine binding method are in use. The repeatability was high within laboratories but reproducibility between laboratories was low. ZHU et al. (2008) compared various amylose determination methods from different starch sources (corn, rice, wheat, and potato) to conclude, that each amylose determination method has its benefits and limitations. A two-wavelength iodine binding procedure seemed to be the most precise and generally applicable method. In a study of HU et al. (2010) the colorimetric iodine-potassium-iodide and the enzyme-based Megazyme methods (GIBSON et al., 1997) for amylose-detection in cereal grains were compared. Amylose detection

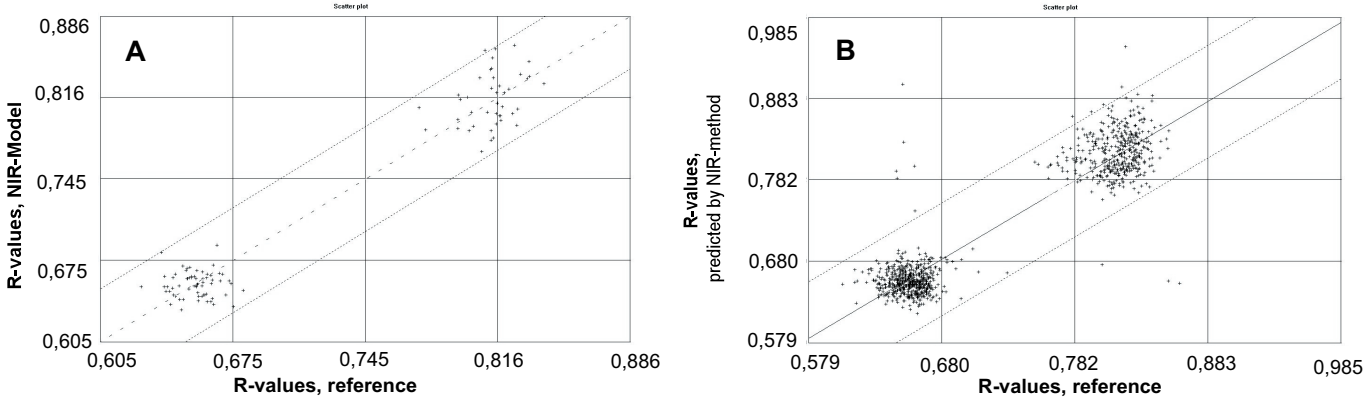

Fig. 3: A: Correlation between NIT-spectra data and chemical analysis data (R-values) determined by the two-wavelength colorimetric method in single kernels.
B: Scatter plots of the analytical determined and predicted R-values for the MPLS-model using NIR.

accuracy, using the iodine-potassium method, was comparable with the Megazyme method in samples with a low to medium amylose content. Another problem in amylose determination is the dissolution of the starch components. MAHMOOD et al. (2007) discussed that the solvent may also affect the R-values. They came to the conclusion that the cold sodium hydroxide method is an accurate method for a preliminary screen of a large number of samples. Therefore, in this study the dissolution of samples with sodium hydroxide and the dual wavelength iodine binding technique was chosen and modified in order to use it as a standard method to determine the amylose content in single kernels of waxy and normal barley grains. Based on this standard method a non destructive NIR-method was developed.

DELWICHE and GRAYBOSCH (2002) used Near Infrared Spectroscopy to identify waxy wheat, and differentiate them from non-waxy, partial waxy and wild-type phenotypes, but a further differentiation among the non-waxy types was difficult.

DELWICHE and GRAYBOSCH (2007) used ground material and scanned different lines in NIR reflectance. Ground material was also used by LEE et al. (2007) for rapid prediction of amylose content of polished rice by Fourier Transform Near-Infrared Spectroscopy. Near infrared transmittance (NIT) technique for predicting maize seed composition in single kernel was described by BAYE et al. (2006) for the determination of the protein, oil and starch content. Up to now the prediction of the amylose content in whole grain maize (CAMPBELL et al., 1997) was only applicable to a sample containing many grains, but not to a single grain. DOWELL et al. (2009) used an automated single kernel near-infrared sorting system to separate single waxy wheat kernels from segregating breeding lines.

As shown in this paper the NIT-technique can also be successfully used in the classification of single kernels of waxy and non-waxy barley varieties, thereby facilitating selection for the amylose content in large numbers of grain samples as this has been demonstrated already for maize (CAMPBELL et al., 1999) and wheat (DOWELL et al., 2009). Besides this, this method is well suited to detect contamination of normal and waxy genotypes during transport and storage. However, the developed method is too vague for a further differentiation of single kernels within waxy and non waxy barley genotypes.

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