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BRIEF COMMUNICATION

A micro-analytical method for the determination of starch and amylose/amylopectin content in pea seeds

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Abstract

New modifications of the method for pea starch and amylose/amylopectin analysis were tested with small samples size of smooth and wrinkled peas. The milling process of the sample and preliminary extraction with 40 % ethanol were very important and affected markedly the standard deviation.

Additional key words: colorimetric determination, enzymatic determination.

Many protocols for the determination of starch and amylose in different matrices have been published. For starch, in principle, two groups of methods are used: *1*) polarimetric methods in which the starch is quantified as dissolved and partly degraded polymer (*e.g.* the starch determination according to Ewers and the „calcium chloride" method of the AOAC), and *2*) methods in which the starch is fully hydrolysed into glucose and then quantified by measuring the glucose content. These methods comprise four steps: removal of soluble sugars, disintegration of the sample and solubilization of starch, conversion of the starch into glucose, and quantification of the glucose. For determination of amylose, some modifications of methods based on amylose reaction with iodine to form blue coloured complex are used. All the methods discussed above are suitable for determination of starch and amylose/amylopectin in fairly large samples. In contrast, the breeders often need to know the content of starch and amylose in single seeds and even analyse non-destructively (thus retaining full seed viability for further growing).

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Pea starches have unique structure and composition (Colona and Mercier 1984, Wang *et al.* 1998) and it is important to use pure pea starches, amylose, and amylopectin for calibration of the system. One of the main problems encountered was that when using as standards the starches and amyloses from other plant species, *e.g.*, maize and potato, the results obtained were unreliable and often not reproducible.

The objective of this work was to develop methods for accurate, reliable analyses of single pea seeds. For selectivity of the starch determination, we preferred a combination of the enzymatic conversion of the starch into glucose, followed by enzymatic quantification by a standard glucose test kit. The amylose assay then measures colorimetrically the blue amylose-triiodate complex. All the methods formulated below are based on established approaches (Knutson 1986, Carpita and Kanabus 1987) with the appropriate scaling down of the materials and were primarily developed by MacLeod (1994) and Lloyd (1995).

Peas from the Czech National Collection of genus *Pisum* L. held in *Agritec*, Šumperk, Czech Republic, were ground to produce flour using a *Tecator 1093* (*Cyclotec*, Höganäs, Sweden) grinding mill with 0.5 mm sieve. Flour samples were stored under vacuum in a desiccator using P_2O_5 as the drying agent, for at least 24 h before analysis with the aim to eliminate differences in moisture content between samples. Single seeds were sampled non-destructively according to Jones *et al.* (1995).

The flour (5 - 7 mg in 10 cm^3 screw-cap tubes) were extracted in 40 % ethanol to remove soluble sugars (Brunt *et al.* 1997). Then 1 cm^3 of 90 % dimethyl sulphoxide (DMSO) was added to each sample tube. After placing an 8 mm stirring bar into tubes, capped tubes were boiled for 2 h, in a water bath on a stirrer-hotplate, then left stirring continuously overnight (for at least 16 h). On the day of analysis, enzyme mixture was added, consisting of 4 cm^3 acetate buffer (0.1 M, pH 4.5) containing 0.1 cm^3 amyloglucosidase (E.C.3.2.1.3) and 0.01 cm^3 α-amylase (E.C.3.2.1.1). After incubating in a water bath for 30 min at 20 °C, the temperature was increased to 55 °C for a further 2 h to destroy enzyme activity. Two 0.1 cm^3 aliquots from each sample were analysed for glucose content using a glucose test kit (*Boehringer*, Mannheim, Germany), which uses the glucose/GOD-Perid method (Werner *et al.* 1970). Total starch content was calculated according to following equations:

$$\text{starch [\%]} = [(\mu\text{g glucose per 5 mg flour}) \times 0.9]/50, \text{ and}$$

$$\text{glucose } (\mu\text{g per 5 mg flour}) = (c_{st} \times V_{sam} \times A_{sam} \times 5)/(A_{std} \times m)$$

where c_{st} - concentration of glucose in standard [μg cm^{-3}], V_{sam} - volume of sample [cm^3], A_{sam} - absorbance of sample, A_{std} - absorbance of standard, m - mass of sample [mg].

For preparation of pure pea starch seeds (50 g) were soaked in 1 dm^3 of pure distilled water at 1 °C overnight. The softened peas were homogenised in 10 g batches using a domestic blender. The homogenate was passed through a 53 μm sieve to remove fibrous material. The resulting paste was allowed to settle out (30 min) and the top layer of liquid was discarded. The starch was washed with 0.1 M NaCl

solution for 30 min to remove protein contaminants, the liquid layer was discarded. The remaining solid (starch) was filtered through a glass sinter funnel and washed with distilled water. This clean-up process was repeated twice. A final wash and filter with ethanol was made to aid the drying of the starch. The starch was air dried for 1 h using a large Petri dish. The yield of pure starch was approximately 20 g starting from 50 g of round (smooth) peas and slightly less (15 g) from wrinkled seeds.

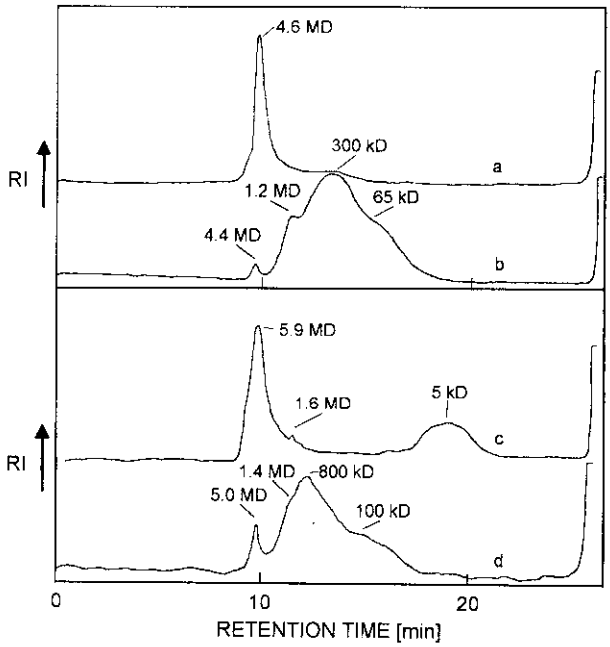

Fig. 1. Size exclusion chromatograms of amylopectin (*a,c*) and amylose (*b,d*) isolated from smooth (*a,b*) or wrinkled (*c,d*) peas.

For preparation of pure amylose a concentrated slurry of pea starch was prepared by adding 30 g of starch to 40 cm^3 of distilled water. The slurry was poured into 3 dm^3 of distilled water at 70 °C, making a final concentration of 2 % (m/v). The mixture was kept at 70 °C with gentle stirring and stream of nitrogen gas passing through the mixture for 1 h. The mixture (still warm) was centrifuged at 2 000 *g* for 20 min. The supernatant was then carefully decanted (without disturbing the particulate fraction which is discarded) and filtered through a glass sinter funnel. Butan-1-ol was added to produce a final concentration of approximately 10 % (v/v). After thorough mixing the solution was allowed stand overnight. The amylose precipitate was recovered by centrifugation. Solid amylose was prepared by washing the precipitate with ethanol and centrifuging. The powder was allowed to air dry naturally.

For preparation of pure amylopectin pea starch (10 g) was dissolved in 300 cm^3 of 90 % DMSO with continuous stirring overnight. The solution was transferred to a large conical flask and an excess of ethanol (≅ 300 cm^3) was added. The resulting

dense glutinous precipitate was stirred with a glass rod and left to settle for 4 h. The mixture was filtered through a glass sinter funnel and washed three times with excess ethanol (100 cm^3). The residual degranular starch (mainly amylopectin) that was left on the filter was collected and dried. Degranular starch (200 mg) was dissolved in 10 cm^3 distilled water and then 2 cm^3 each of butan-1-ol and iso-amylalcohol. The solution was gently mixed and left overnight. The solution was centrifuged and the supernatant recovered (the solid material is mainly amylose). The liquid volume was reduced using a rotary evaporator to about one third of the initial volume. Solid amylopectin powder was prepared by precipitation with ethanol, centrifugation and then air dried.

Table 1. Purity of isolated standards determined by size exclusion chromatography. Fractions [kD] are given in parentheses.

Pea	Sample	Content of amylose [%]	Content of amylopectin [%]
Smooth	amylose	98.0 (1 200 - 65)	2.0 (4 400)
	amylopectin	0	100.0 (4 600)
Wrinkled	amylose	92.2 (1 400 - 100)	7.8 (5 000)
	amylopectin	28.8 (5)	71.2 (5 900 - 1 600)

For determination of amylose content in pea flour 5 - 7 mg of flour in 10 cm^3 screw-cap tubes (in duplicate) were extracted in 40 % ethanol to remove soluble sugars (Brunt *et al.* 1997). Then 1 cm^3 of 90 % DMSO was added to each sample tube. Capped tubes were boiled for 2 h, in a water bath on a stirrer-hotplate, then left overnight (16 h), stirring continuously. 4 cm^3 of 100 % ethanol was added to each tube and left for 10 min to allow the starch to precipitate. Next the tubes were centrifuged at 2 000 *g* for 15 min and the supernatant was then discarded. The starch pellet was dissolved in 5 cm^3 90 % (v/v) DMSO with 0.06 M iodine solution. This was prepared by dissolving 0.7615 g of iodine into 50 cm^3 of DMSO solution, before returning to the bulk 90 % DMSO to make up 1 000 cm^3 of the reagent. The samples were boiled in a water bath for 2 h with continuous stirring and then left at room temperature, stirring overnight. Two 0.5 cm^3 aliquots were taken from each sample and diluted with 8 cm^3 distilled water, and well mixed. The solution was left for 1 h at room temperature to allow the blue amylose-triiodate complex to form and stabilise, which is then measured colorimetrically. Absorbance readings of the samples were made in a spectrophotometer (*Spekol 221*, *Carl Zeiss*, Jena, Germany) at a wavelength of 600 nm.

$$\text{Amylose [\% in starch]} = A \times [(A_{sam} \times 5)/(m \times 0.01 \times \text{starch})] - B$$

A, B - obtained constants from calibration curve of amylose, A_{sam} - absorbance of sample, m - mass of sample [mg], starch - content of starch [%]. For calibration mixtures (m/m) of varying proportions of pure pea amylose and amylopectin were made covering the range that these compounds might be found in natural pea flour. For round pea seeds, amylose contents of 0 - 40 % and for wrinkled seeds amylose

contents of 50 - 100 % are required. The standard mixtures were dried, stored under vacuum and analysed for amylose content as previously described.

Size exclusion chromatography was used to determine the purity of prepared amylose and amylopectin from both smooth and wrinkled peas. The equipment used consisted of an isocratic pump and differential refractometer (*HPP 5001 & RIDK 101, Laboratory equipment*, Prague, Czech Republic), two columns (250 × 8 mm) packed with *Separon Hema Bio 1000* and *Hema 100*, connected one after the other, particle size was 10 μm. Two pre-columns (30 × 3 mm) packed with *Separon Hema Bio 1000*, particle size 20 μm, were installed in front of the main columns. The eluant was pure deionized water at a flow rate of 0.8 cm^3 min^{-1}. A sample volume of 0.2 cm^3 was injected onto the columns, all analyses were conducted at laboratory temperature. Dextrans were used as molecular mass calibration standards. Samples were prepared by dissolving 100 mg of sample in 1 cm^3 water, after 1 h 9 cm^3 DMSO was added. The samples were left overnight at 5 °C then each sample was filtered through a 0.45 μm membrane filter and finally injected directly on the set of columns for analysis.

The presence of amylose and amylopectin in small samples from wrinkled and smooth peas was controlled by size exclusion chromatography (Fig. 1). Amylose and amylopectin have similar rotations, $[\alpha]_D$ being approximately +200° in water, +163° in 1.0 M NaOH and +175° in DMSO (Stephen and Zobel 1996), therefore purity of prepared standards was calculated as quotient of peak areas (Table 1). For smooth pea we obtained pure amylopectin (100 %) and almost pure amylose (98 %). Purity of amylose and amylopectin of wrinkled pea was not so good (Table 1) due to a different structure and composition of starch from wrinkled peas, but it could be used for creation of calibration curve in range of 29 - 92 % amylose in starch. Most cultivars of wrinkled peas cover this range. Calibration curves were done from these standards (absorbance per 5 mg starch against % amylose) in ranges 0 - 36 % amylose in starch for smooth peas and 29 - 92 % amylose in starch for wrinkled peas (calibration curves were $y = 71.113 x - 22.87$ and $y = 63.494 x - 5.26$ for smooth and wrinkled peas, respectively).

We studied the effect of particle size (0.5 or 1 mm milling sieves used for disintegration, 66 μm sieve used for sieving flour) and extraction of soluble sugars by 40 % ethanol on the reliability of the method for determination of starch in smooth peas (Table 2). Best results with smaller standard deviation (SD) were obtained with disintegration using 0.5 mm sieve. After sieving trough the 66 μm sieve, SD was almost the same as by milling with 0.5 mm sieve, but we found higher starch contents

Table 2. Starch contents in smooth pea determined by different methods of starch determination (A - method without extraction, B - classical method, C -flour was sieved through a 66 mm sieve).

Milling sieve	0.5 mm			1 mm		
	A	B	C	A	B	C
Mean	49.30	46.50	52.80	45.90	41.90	51.70
SD	1.13	0.92	0.89	1.77	1.99	0.84

by this procedure, probably because a part of the total mass (fibre) was left on the sieve and thus was absent in the analysed sample. Hence more accurate were the results without sieving. Extraction by 40 % ethanol yielded more accurate lower results, because samples did not contain soluble sugars. Due to small size of sample used for analysis the described method is suitable for analysis of single seeds. Relative error of determination in 10 tests was 3.6 % for starch and 6.5 % for amylose.

The method was used for calibration of *PerCon Inframatic 8100* (*Perten Instruments*, Huddinge, Sweden) (Urban and Hýbl 1999) and for screening of 393 genotypes of pea for starch and amylose content (Hýbl *et al.* 1998a,b).

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