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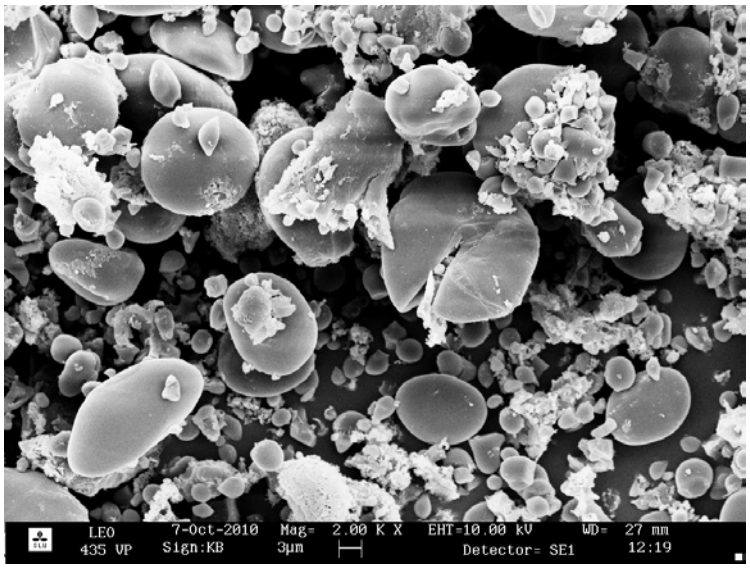

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Abstract

The high viscosity of barley material makes starch isolation problematical using regular methods established for cereals. An adjusted starch isolation method has been set up for barley, based on fractionation and purification. The focus is on attaining truly representative isolates of six flour samples selected for widely differing characteristics within the research program *BarleyFunFood (BFF)*. Beside establishment of the method, this diploma work aspires to serve the BFF with isolated material of sufficient yield valid for further starch characterisation. A pre study was conducted evaluating available wet mixing equipment, experimental conditions and mode of procedure. The yield and purity of isolates of the *BFF* flours were determined to 55.7-72.2% and 61.0-76.4% respectively. Due to an error in the procedure, that partly explains the poor results, one of the samples was isolated de novo resulting in an increase of the yield from 59.7 to 96.2%. Additionally, the amylose-amylopectin ratios were determined to approximately 2/99 in a waxy type, 41/59 in one high-amylose type and 30/70 in remaining varieties. New isolations are suggested to be carried out prior to further analysis of the remaining flours to achieve higher representability.

Keywords*: Cereal starch isolation; Barley starch; Starch recovery; Starch purity; Amylose-Amylopectin ratio; B granules; Chemical deproteinisation; Toluene shaking; Proteinase K*

Sammanfattning

Hög viskositet i kornmaterial gör tillämpning av befintliga stärkelseisoleringsmetoder för spannmål problematiskt. En isoleringsmetod, baserad på fraktionering och rening, har etablerats för korn med fokus på att uppnå högt representativa stärkelseisolat. En förstudie genomfördes för att utvärdera tillgänglig utrustning för våtmixning, experimentella betingelser och generellt tillvägagångssätt. Stärkelse isolerades från sex mjölprover utvalda för sina vitt skilda egenskaper inom forskningsprogrammet *BarleyFunFood* (*BFF*). Utöver etablering av metoden, strävar detta examensarbete till att tjäna *BFF* med isolerat material med tillräckligt högt utbyte för vidare analys. *BFF*-isolatens utbyte och renhet bestämdes till 55,7-72,2% respektive 61,0-76,4%. De låga resultaten berodde delvis på ett inställningsfel under isoleringsproceduren. Ett av proverna (0120) isolerades därför på nytt under korrekta betingelser. Utbytet ökade då från 59,7 till 96,2%. Dessutom bestämdes amylos-amylopektin ratiot till ungefär 2/98 i en vaxig sort, 41/59 i en högamylossort och cirka 30/70 i övriga sorter. För att uppnå högre representativitet på resterande prover föreslås nya isoleringsförsök för vidare analys av stärkelsen i *BFF*-materialet.

Nyckelord: *Cereal Stärkelseisolering; Kornstärkelse; Stärkelseutbyte; renhet; Amylos-amylopektin ratio; B-granuler; Kemisk proteinavlägsning; Toluenskakning; Proteinas K*

Table of contents

Introduction

Barley (*Hordeum Vulgare L.*) has historically been the main bread cereal in Europe and is still of global importance as alimentation, being the fourth largest cereal produced worldwide. Nowadays barley is used mainly as feed and in malting and brewing, making it the second most commonly grown cereal in Scandinavia with a year production of 1,44 million tons (The Swedish Board of Agriculture, 2007). The very small use of barley for food prevails in the form of groats, soup thickener or as ingredient in breakfast cereals, bakery goods and pasta. Barley as part of the human diet has lately been shown increased interest regarding its nutritional aspects. A high content of soluble dietary fibres, as β-glucans is the main contributor to the so called *hypocholesterolemic effect* (lowering of serum cholesterol) imparting health claims to barley consumption.

Covered barley, as originally occurring, with husk tightly cemented to the pericarp are the types always used in preparation of malt. Through breeding development barley also exist in form of several hull-less varieties, suitable for food use, since abrasion causes nutrient loss. The extensive variation of barley genotypes expressing different grain- and spike morphology (two- and poly-row) starch quality and protein characteristics is a result from both induced and spontaneous mutations and offers the opportunity to select particular genotypes for specific uses.

Barley as a source of starch for either food- or nonfood applications is uncommon but has commercial potential, possessing high swelling power, good freeze-thaw stability and unique film-forming properties. Production of ethanol, extraction of β-glucan and preparation of native and modified starch are other applications of barley. Naturally, isolated barley starch also applies to basic research, aiming to elucidate the detailed molecular structure and chemical properties of the starch components.

The over-all goal for this diploma work is to establish and evaluate a laboratory scale starch isolation method based on fractionation and purification of six barley flours, to facilitate future work within the research program *BarleyFunFood (described below)*. Apart from the method set-up, this work aspires to generate starch isolates of adequate yield for further molecular characterisation of starch. Focus has been on reaching the requirements of the *BarleyFunFood* project by acquiring quantitative isolates that highly represents the true distribution of granule sizes. Representability correlates to a quantitative yield, why high yield rather than purity is desired in this isolation trial. Furthermore, a quantitative analysis of amylose and amylopectin of isolates is also included in the study.

The BarleyFunFood project

BarleyFunFood is one of four present multidisciplinary research programs at the Faculty of Natural Resources and Agricultural Sciences at the Swedish University of Agricultural Sciences (SLU). The project was initiated during the 80's and is a collaboration involving SLU, Lantmännen R&D and SW Seed, Svalöf Weibull AB. Research work within plant biology and breeding, food science, chemistry, microbiology and nutrition is exploring the biology of barley and nutritional effects of cereal carbohydrates.

A collection of 250 barley varieties with a wide genetic background have been screened regarding composition using near infrared reflectance techniques. Among the material were both naked and covered, round-shaped and elongated kernels as well as waxy and high-amylose types

represented. Out of the collected varieties, 20 lines were selected for amplification based on phenotype descriptions as well as for interesting carbohydrate composition, interpreted from spectral data. For the possibility to achieve double generations in one year, cultivation was located to the south hemisphere in the village Vilcún, in Chile where the average temperature is around 15˚ C and winters are mild, somewhat resembling Swedish climate conditions. The 20 lines were grown in the winter of 2010. The starch content in these varieties varies between 30 and 60 % and among them are four low-amylose mutants. The selection was narrowed down to five with large differences in carbohydrate composition. The Swedish feed barley variety *Gustav* was also included, resulting in totally six samples.

Barley starch

As in other types of cereal and higher plants, starch constitutes the major energy storage component of barley. Starch molecules are organised in highly ordered layers stored as granules of various shapes formed in amyloplasts in the plant cell. The starch content of barley varies between 47-67 % (Oscarsson et al., 1996; Li et al., 2001; Åman et al., 1985; Frégeau-Reid et al., 2001). As most common starches, the two distinct polymer fractions amylopectin and amylose, with clearly different molecular weight and structural organisation, constitute barley starch. Amylopectin is a large polymer built up of several α-(1→4)-linked D-glucose chains connected to each other through α-(1→6)-linkages arranged in clusters. Three types of unit chains, arranged in clusters, constitute the molecule; the outermost unbranched A-chains connected through their reducing group to B-chains, carrying either A-chains and/or other B-chains; and C-chains carrying all other chains and containing the only reducing group (Eliasson et al., 1987). Amylose is an essentially linear polymer comprising the same residues and type of linkages as amylopectin but is only branched at a few locations. The amylose content is normally 19-30 % of total starch but varies from 0 % in waxy types up to 44 % in high-amylose varieties (Andersson, A, 1999; Fredriksson et al. 1997; Zheng & Batty, 1998). Degradation of starch results in glucose moieties, valid as fuel in all biological systems including the human body.

Barley starch expresses a distinct bimodal distribution of granule sizes with both large lenticular/disc-shaped A-granules (10-30 μm) and small spherical B-granules (<6 μm), similar to wheat and rye (Delcour & Hoseney 2010, 1993; Bathgate & Palmer 1972; Stoddard, 1999). The number of A-granules is lower (10% of total number) than that of B-granules, but these on the other hand represent the major mass (90%) of the starch (Stoddard, 1999; Bathgate & Palmer, 1972) with variations among genotypes, as investigated by Li et al. (2001).

Amylose and amylopectin are not equally distributed in A- and B-granules, as stated by Bathgate and Palmer (1972), there is a higher amylose content of B-granules than of A-granules. Ao & Jane (2006) reported an amylose content of 28.1% in A- and 23% in B-granules. Ao & Jane also proposed structure models of the amylopectins of the A- and B-granule starches based on results from chain length (CL) analysis. A higher degree of polymerisation was found in A-granule amylopectin chains (26.7 units compared to 24.9 units in B granules), which was also verified by Salman et al. (2008), who also report larger lamellar repeat distance of A-granules. The amylose-amylopectin ratio strongly influences the physical properties such as gelatinization temperature, retrogradation rate etc. of starch, as investigated by Fredriksson et al. (1997) and Bathgate & Palmer (1972).

Barley starch isolation

The harder texture and lower water contents of cereals compared to other botanical materials, such as potatoes, makes isolation of cereal starch in general challenging. Cracking and milling of the starchy endosperm, which naturally is a crucial treat, unavoidably results in partial physical

damage of starch granules. Available isolation procedures suitable for wheat and other cereals have not shown to be satisfactory applicable to barley. The major endosperm cell wall component β-D-glucan and the generally high dietary fiber content (14-24% according to Andersson et al., 1999) contribute to high viscosity, which obstructs starch extraction. This adds to the cereal associated difficulty with starch isolation from barley in particular, which has led to the need of developing a customised isolation method.

In Sweden, commercial starch is isolated through alkali extraction, where starch is purified through stepwise fractionation based on size and solubility properties of the different components in increased pH. Generally, starch isolation methods include several centrifugation steps in order to separate the sample into fractions based on mass. On centrifugation, a white layer is produced at the bottom, mainly comprising starch, overlaid by a darker grey-purple coloured layer (generally referred to as the 'brown layer'), containing basically protein. Already reported by Bathgate & Palmer in 1972 there is a tendency for small starch granules to associate with this protein fraction with a content of 1.5 % associated protein in B-granules versus 0.2% in large ones. Hence, there is a considerable loss of small granules on discarding the proteinous layer, which in turn implies a poor representability of the isolated starch. Available isolation methods have been investigated by McDonald & Stark (1988), stressing the importance of retrieving small granules trapped within the proteinaceous layer to obtain representative isolates. The industry on the other hand, employ isolation methods providing high yields to lowest possible cost, where retrieval of B-granules is not prioritised.

Material & Methods

Materials

Barley flours and reference materials- The covered barley (*Hordeum Vulgare*) cultivar *Golf*, with a starch content of 63.8% was used for isolation trials within the pre study. The Major study included six barley types grown in Vilcún (Chile) within the project *BarleyFunFood*; one with shrunken endosperm (0155), one antocyanin-rich (0120), one high-amylose (0228), one lysine-rich (0181), one waxy (0224) variety and one feed variety (0249). All samples had been crushed in a Cemotec 1090 Sample Mill (Tecator) and subsequently milled to flour with a particle size of 500 μm, using a Retsch Type ZM 1. Native starch and commercial potato flour from Lyckeby Stärkelsen (Kristianstad, Sweden) as well as Defatted Barley Starch from ALKO ltd (Helsinki, Finland) were used as reference samples in starch content analysis.

Chemicals - Proteinase K (origin: *Tritirachium album;* activity: 30 U/mg), was purchased from Sigma-Aldrich **(**St.Louis, USA**).** Pullulanase M1 (2.00 U; *origin: Klebsiella planticola;* activity: 699 U/mL), Isoamylase (activity: 520 U/mL), thermostable α-amylase (activity: 3.000 U/mL) and amyloglucosidase (activity: 3.260 U/ml) and Glucose standard solution (100 μg/ 0.1 ml) were obtained from Megazyme (Wicklow, Ireland)

Method

Pre study

A pre-study was conducted in order to find optimal experimental conditions such as sample size, equipment for wetmixing and mixing time as well as to become familiar with the behaviour of the material. Among isolation procedures previously described in the literature, the method by McDonald & Stark (1988) was selected as a basis for this study in order to get a representatitive starch isolate. Some modifications were done in order to adapt for the current sample material.

Ground flours instead of whole kernels was used in this study, which excluded the 17 h steeping, protease XIV incubation of cracked kernels and pestling of digested suspension. Instead, the flours were steeped and wetmixed according to the procedure described below. In addition to scraping off of the brown layer for subsequent proteinase K treatment in separate, incubation of white and brown layer together was also evaluated. The isolation procedure established for the present study included in short the following steps; steeping of flour with inactivation of endogenous enzymes by lowering of pH, wet milling, filtration, enzymatic protein degradation, filtration of fibrous residue, second enzymatic protein degradation and purification with toluene.

Isolation procedure

Flour samples of 10 g, split equally in two were steeped in 0.02 M HCl (10 ml/g) and pH was adjusted to reach below 3 in order to inactivate endogenous enzymes. The suspension was stirred with a magnetic stir bar for 10 minutes before neutralization with 0.2 M NaOH. Samples were mixed with either a Sorwall Omnimixer (Du Ponts Instruments) for 7 minutes, at speed setting 7 or in an Ultra-Turrax (Heidolph Diax 600, 18 mm rod) set to 9500 min^{-1} for 3 minutes. The slurry was smooth already after 2 minutes. Filtration through a Büchner funnel with a 70 μm nylon mesh was performed to roughly separate large particles like fibers, from the crude starch suspension. This fibrous residue according to investigations by Park & Baik (2010) is composed of bran and cell wall material and will also be called the bran fraction. After centrifugation (Heraeus Multifuge 3 s) at 2800 rpm for 10 minutes, supernatants were observed microscopically and showed to be devoid of starch granules. In order to degrade the protein matrix encapsulating some of the starch, mainly B-granules, both crude starch and fiber rich residues were treated with proteinase K (1 mg/sample in 50 ml 0.1 M TRIS-HCl buffer, pH 7.6) in a water bath heated to 25˚ C, for 24 h. Trials also included separation of the brown proteinous layer for proteinase treatment exclusive of the white starchy layer, by repeated scraping off of the brown layer formed during intermediary centrifugations in parallel with co-treatment of both layers. The crude starch obtained from filtration was pooled prior to proteinase treatment, while bran fractions were treated separately due to a proceeding second filtration. The fiber rich residue was filtered through a Büchner funnel with a 70 μm mesh and starch released was pooled to the main starch. Proteinase treatment was repeated once on the pooled material (2 mg/sample). The material was centrifuged at 2800 rpm for 10 min and buffer solution was discarded. The isolate was purified from protein by toluene shaking by suspension in 300 ml 0.2 M NaCl and one volume toluene over night. The interphase was centrifuged and washed with water once, in order to recollect starch and pool it to main isolate. The toluene shaking procedure was repeated once again before samples were dried.

Starch determination

To determine the yield and purity of isolates, starch content was determined according to the method described by Åman et al. (1994). The method was modified concerning the glucose oxidase reaction solution which was replaced with glucose oxidase dehydrogenase and hydrogen peroxide oxidoreductase (GOPOD) from Merck (Bergman & Beving Lab). The reagent volume was changed from 2.00 to 3.00 mL according to standards used at the department, to achieve reliable spectrophotometrical measurements. The use of thermostable alpha-amylase has shown to reduce problems with starch-lipid complexes, incomplete dissolution of starch and retrogradation of amylose (Åman et al, 1994) that could impair the starch content determination.

Samples corresponding to 30 mg starch were dissolved in 15 mL 80 % ethanol in order to rupture granules. The screw cap tubes were placed in a boiling water bath in order to dissolve and extract low molecular weight carbohydrates. After cooling to room temperature the samples were centrifuged (830 rpm/10 min) and the pellet formed was washed twice with 15 mL 80 % ethanol.

Finally, to remove remaining solvent, the test tubes were emptied carefully and inverted on Kleenex for 5 minutes. Samples were incubated with thermostable α-amylase (50 μL in 25 mL acetate buffer) in a boiling water bath for 30 minutes. Tubes were shaken three times during incubation and observed regarding adhering material, lumps and tight capping. Samples were cooled to a temperature of ~ 40 ˚ C before a total degradation to glucose units was assured by treatment with amyloglucosidase (100 μL in 0.1 M acetatebuffer, 140 U/mL) in a shaking water bath at 60˚ C over night. Samples were cooled to room temperature and centrifuged at 830 rpm for 10 minutes. The supernatant was diluted in water to 1:25 and 1.00 mL aliquots were mixed with 3.00 mL GOPOD and placed in a 50˚ C waterbath for 20 min whereafter absorbance was measured at 510 nm using a Novaspec II spectrophotometer (Pharmacia Biotech, Stad, Land). The glucose concentration was determined from a standard curve with a linear correlation stretching from 0.025 to 0.100 mg/mL, and the starch content was calculated according to:

% starch (DM) =([glucose] (mg/mL) *25.15 * 0.9 * 25) / sample weight (DM, mg)

where 25.15 is the total volume of solution in mL, 0.9 is the factor for conversion of free glucose to a glucopyranosyl unit of starch and 25 is the dilution factor. The proportion of starch was calculated based on starch quantity of barley flours. Purity was calculated as mg starch / mg isolate.

Fibrous residues were also analysed for starch content due to observations of white particles remaining in the sieve after filtration and proteinase K treatment. Only fibrous residues with plenty of visible white grains were analysed (samples 0155, 0181, 0224 and 0228) aiming to catch starch that possibly was building up or trapped in these white grains. In this case, samples of 100 and 200 mg respectively were weighed in for analysis.

Dry matter content determination

The dry matter content was determined by oven-drying for 16 hours. Duplicate samples of 0.5 g airdried isolate were dried in a 105 ˚ C using preheated crucibles. Samples were cooled in a desiccator for 1 h before weighed out and dry matter content was calculated.

Scanning electron microscopy (SEM) of granules

In order to investigate the possible degree of damage on starch granules as a consequence of wetmixing, SEM-images with a resolution up to 2000 X were produced. Flour samples mixed using Sorwall Omnimixer (7 min, speed setting 7) and Ultra-Turrax (1, 3 and 5 minutes, speed setting 9500 min^{-1}) as well as untreated control barley flour (variety Golf) was coated with gold/palladium (3:2) in a JEOL JFC-1100 Ion Sputter (Stad, Land) and analysed with a LEO 435VP (10 kV acceleration voltage in high vacuum) at the Electron Microscopy Unit at SLU, Alnarp. SEM gives topographic information about the surface of the sample, producing 3D like pictures of granules suitable for ocular damage scanning.

Major study

Isolation, starch content analysis and dry matter determination

As in the pre-study, barley samples were obtained as milled flours implying the same alterations in the primary steps of the procedure. A flow chart of the procedure is demonstrated in Figure 1. The procedure was conducted essentially as in the pre-study but with the following modifications: The Omnimixer was omitted in advance for the Ultra-Turrax. The Ultra-Turrax mixing was performed with a different rod of same dimensions but different number of bars in

the cutting device, which were reduced from 10 to 8. The distance between the cutting device and the wall of the rod was also less tight. To compensate for this and assure sufficient decomposition, 3 minutes mixing was chosen although 2 minutes had shown to be enough in the pre study. Rubbing of fibrous residue with mortar and pestle was incorporated to procedure of the major study, prior to proteinase K treatment. Separation of white and brown layer was not practised, which were instead *proteinase K* treated together. The sample (BFF 0120) was isolated twice, second time with different Ultra-turrax rod: a 10 mm rod, with tighter cutting device was used, for 3 minutes and higher centrifugation forces, due to poor yields in the first trials. Remaining samples were not reisolated due to time restraints.

The dry matter content as well as the starch content was determined as it was in the pre-study with the exception that centrifugations within the starch content analysis was carried out at 2780 rpm instead of 830 rpm. This setting was incorrect in the pre-study.

Amylose-amylopectin analysis

The ratio of amylose and amylopectin was determined by fractionating debranched starch samples of 200 µL through a CM Sepharose CL-6B column (90 cm x 1.6 cm, flow rate 0.47 ml/min) which separates substances in regard to size. The debranching enzymes isoamylase and pullulanase cleave the branchpoints (1→6 linkages) of the starch molecules leaving only linear chains. This results in a better separation of amylose and amylopectin.
Samples were debranched essentially according to Bertoft et al. (2008). Starch samples (2 mg) were dissolved in 90 % DMSO in a heated water bath with magnetic stirring for 1 hour. Hot water (400 µL) was added followed by further stirring for 10 min. 50 µL 0.1 M Sodium acetate buffer (pH 5.5) was added to samples that were briefly stirred again. After samples had cooled to room temperature, 2 µL pullulanase and 1 µL isoamylase were added and the samples were incubated at room temperature under constant stirring over night. Debranched samples were diluted in 50 µL 5.0 M NaOH and filtered through a 45-µm syringe filter prior to injection (200 µL) on the column and eluted with 0.5 M NaOH. Fractions of 1 ml were collected using an ISCO Foxy Jr (Spectrochrom AB, Sunnansjö, Sweden)
Amylose and amylopectin was detected using the phenol-sulphuric acid method described by Dubois et al. (1956). The original procedure was modified regarding reactants, to 500 µL sample solution, 500 µL of 5 % phenol and 2.5 ml concentrated sulphuric acid. Absorbance was measured at 490 nm on a Colourwave C075000 Calorimeter (WPA, Cambridge, UK) spectrophotometer.

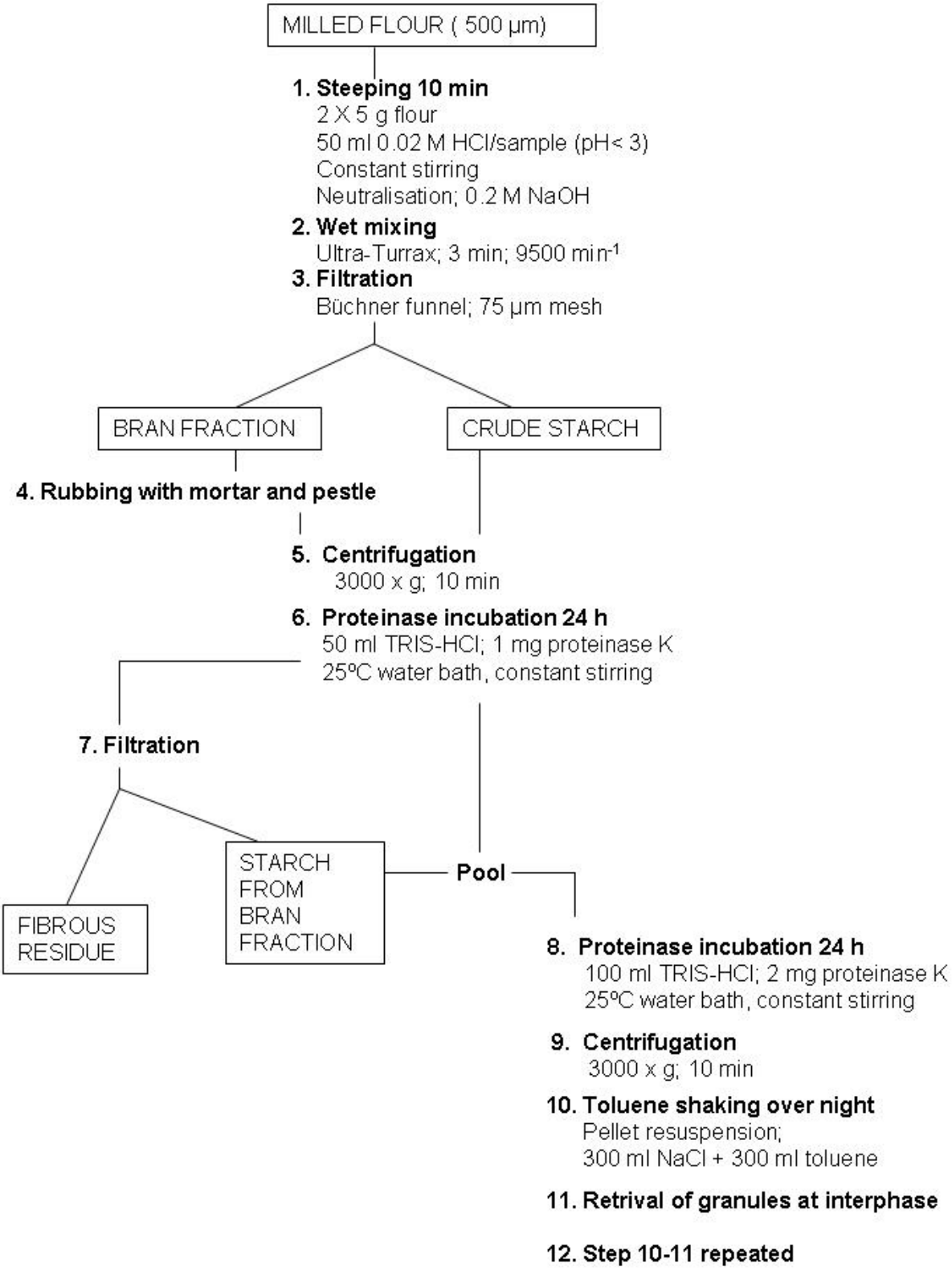


Fig 1. Procedure for starch isolation and purification from barley flours

Results

Pre-study

Isolates of up to 5.3 g (DM basis) was extracted from 10 g control barley sample using the Ultra-Turrax for wetmixing. Results of starch yield and purity of isolates are presented in Table 1. Separation of the brown proteinous layer resulted in a lower starch yield as well as a lower purity of the starch compared to co-incubation together with the white starchy layer. Moreover, the procedure is time consuming and implies repeated centrifugation steps. Continuously, the brown and the white layer were incubated together.

Yield and purity

The use of Ultra-Turrax versus Omnimixer did not have a considerable impact on the yield nor the purity. However, pictures from the electron microscopy observations indicate a higher amount of damaged starch granules in the sample mixed with the Omnimixer. An initial sample weight of 5 g shows tendencies to give a higher yield and purity than 10 g samples, which can be explained by size of the filtration funnel being more suitable for a smaller amount of material. 10 g samples were nevertheless used for BFF samples, in order to end up with a sufficient amount of starch isolate for further analysis. These were however filtrated in portions of 5 g at the time. The starch derived from the fiber rich residue was kept separated during all analysis which made a calculation comparison possible, see Table 1. Tendencies towards a higher yield but no significant effects on purity were shown when fiber-derived starch was included to total yield.
Since further analysis requires high representability, which is attained by high yields rather than purity, the fiber-derived fractions were hereafter chosen to be included during the procedure. Hence, the starch extracted from the fibrous residue/bran fraction was pooled to the crude starch prior to second proteinase K treatment.

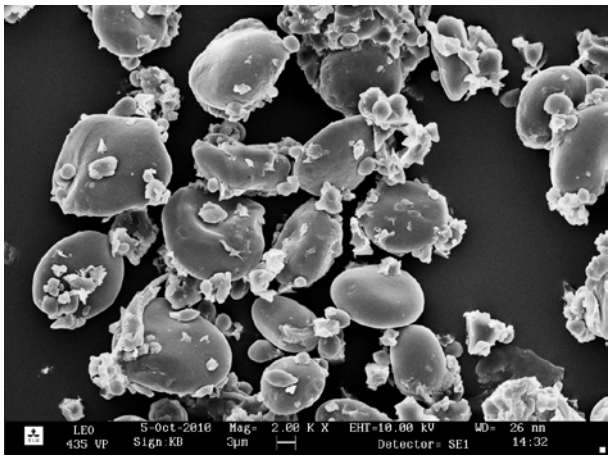
Table 1. Yield and purity of isolated starch from Golf (63.8 % starch) employing Sorwall Omni-mixer, in comparison to Ultra-Turrax wet mixer.

	Starch yield (%)		**Purity (%)**	
Sorwall Omni-mixer	Bran fraction included	Bran fraction excluded	Bran fraction included	Bran fraction excluded
Separated (10 g)	83.3	82.0	94.1	95.0
Co-treated (10 g)	90.2	89.3	97.8	98.4
Ultra-Turrax				
Co-treated (5 g)	83.9	78.9	88.2	91.6
Co-treated (10 g)	74.8	68.6	82.9	82.9

Degree of damaged starch granules

SEM-images of wetmixed flour samples are to be viewed in Figure 1. Damaged granules appear to some extent in all samples but only to a substantial degree in the samples mixed in the Omnimixer (Figure 1c). The treatment time is longer in this case but more crucially, a lot of heat is quickly developing in the closed container during mixing, which could harm the starch. The Omnimixer was further omitted in advance for the Ultra-Turrax.

(a)



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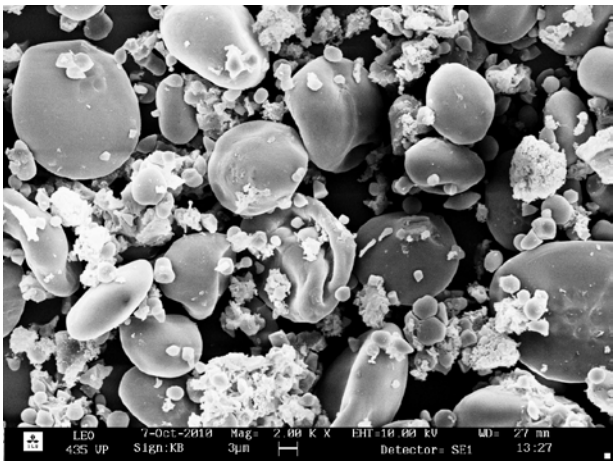

(c)

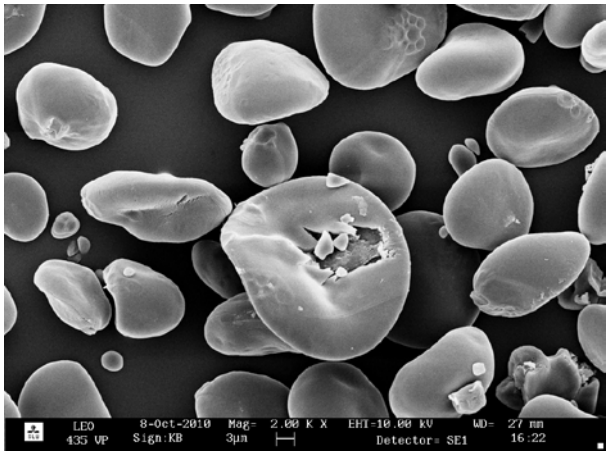

Figure 1. Scanning electron microscopy image of starch granules of (a) untreated control barley flour type *Golf,* (b) wetmixed using Ultra-Turrax for 3 min (9500 min^{-1}) and (c) wetmixed using Sorwall Omnimixer.

Major study

The isolation conducted according to the experimental conditions chosen by means of the pre-study, resulted in material mass of approximately 2.9 -5.0 g (DM basis).

Yield and purity

Starch recovery varied around 50-60% and purity of isolates around 60-75%, presented in Table 2. The isolate yield attained from sample BFF 0120 increased from 4.54 g to 4.95 when centrifugation speed was set to 4350 rpm instead of 2800 rpm and the starch recovery increased with 36.5%.

Table 2. Yield and purity of isolated starch from BFF samples. All data is reported on dry matter basis and represent the mean of two determinations.

	Starch yield (%)	Purity (%)
120*	96.2	90.3
120	59.7	61.0
155	55.7	63.7
181	58.9	76.4
224	72.2	74.7
228	70.0	70.0
249	59.6	73.1

* Additional isolation trial with centrifugation force set to 4350 rpm (3000 x g). All other samples were centrifuged at 2800 rpm (1300 X g) throughout the isolation and purification procedure.

Compared to control barley Golf in the pre-study, all BFF samples except for 0224 were darker in colour and had a rougher and more sandy texture that more readily let filter water rinse through. Crude starch suspensions (except for 0224) were darker towards grey-purple in shade than Golf. With some variations, the BFF samples contained quite long, not easily breakable fiber particles, again with an exception for sample 0224. An interesting observation made was small white grains that did not fit through the 70 um sieve. These were present to some extent also in Golf, but showed to be very difficult to break both mechanically and enzymatically in the BFF samples. A great reduction was expected upon proteinase K treatment with theories on proteins agglutinating starch into lumps. Generally, a second filtration of the fibrous residues after proteinase K treatment did not result in a substantial amount of additional starch, even though the filtrate appeared fairly white. Finally, an apparently substantial amount of white lumps ended up in the fibrous residues, which subsequently were analysed regarding starch content. An exception regarding the white grains is sample 0249, the feed variety, which gave an opaque white filtrate and few white grains in the fibrous fraction. The behaviour of 0249 resembles a lot to that of control barley type Golf in the pre study. For example, material is readily washed from white solution and loses volume on filtration. These samples also require more stirring during filtration as water does not run through material as easily as for other BFF samples. The proteinase treatment of fibrous residues seemed to have most effect on sample 0120, where the difference in amount of visible white grains was significant after treatment. The highest recovery was obtained from sample 0224, which was the only naked variety. In sample 0228, less white grains were observed in sample 0228 compared to 0155, 0181 and 0224, and also, a relatively high yield was obtained The fibrous residues showed various contents of starch stretching between 27-155 mg (3-13% of fraction weight) These values did not correspond with the total content of dietary fiber of flours (not presented), which was expected to affect the starch recovery from these fractions. The fiber residue from the second isolation trial on sample 0120 (4350 rpm) had a starch content of only 0.8%.

Fibrous residues from samples 0224 and 0155 contained the highest amount of white grains after proteinase treatment. Suspected insufficient amount of enzyme perhaps due to composition differences led to a second treatment with a double amount of proteinase K separate from the crude starch fraction. The white lumps remained in both samples but had decreased somewhat in sample 0155, which also gave a whiter filtrate and slightly better pellet on centrifugation.

White lumps (0155) were soaked, mashed and viewed with a microscope. They were shown to contain granules appearing mostly in clusters surrounded by bag-like structures but also free in solution. Strains of connected single granules were also present. Size of granules was large but slightly smaller than A-granules of control barley *Golf*. Concentric rings appeared on some of the granules and all turned dark blue on iodine staining.

With intentions to rupture the white lumps in fibrous residues these were exposed to further mechanical treatment. Mixing on Ultra-Turrax at highest possible velocity did not reduce the sandy, grainy appearance (0224) and did not result in much of a pellet after centrifugation. On the other hand, rubbing fibrous material with mortar and pestle prior to proteinase treatment, showed to have an effect. The lumps were not apparently ruptured on rubbing but a visual effect was reached subsequent to enzymatic treatment. Although a somewhat grainy sense still prevailed, the size of the white lumps was heavily reduced. The filtrate however was not very opaque and did not produce a proper pellet on centrifugation. Sediment from this supernatant showed to contain granules on microscopic observations. Granules were mainly intact, freely moving large and small, as well as appearing in bag-like structures that partly were empty.

Amylose and amylopectin ratio

The amylose and amylopectin ratios are presented as percental weight curves obtained from absorbance measurements, in Figure 2. The waxy type, sample 0224 showed the highest amylopectin content and the high amylose type showed the highest amylose peak, as expected. Amylose contents of all samples are also presented as figures in Table 3.

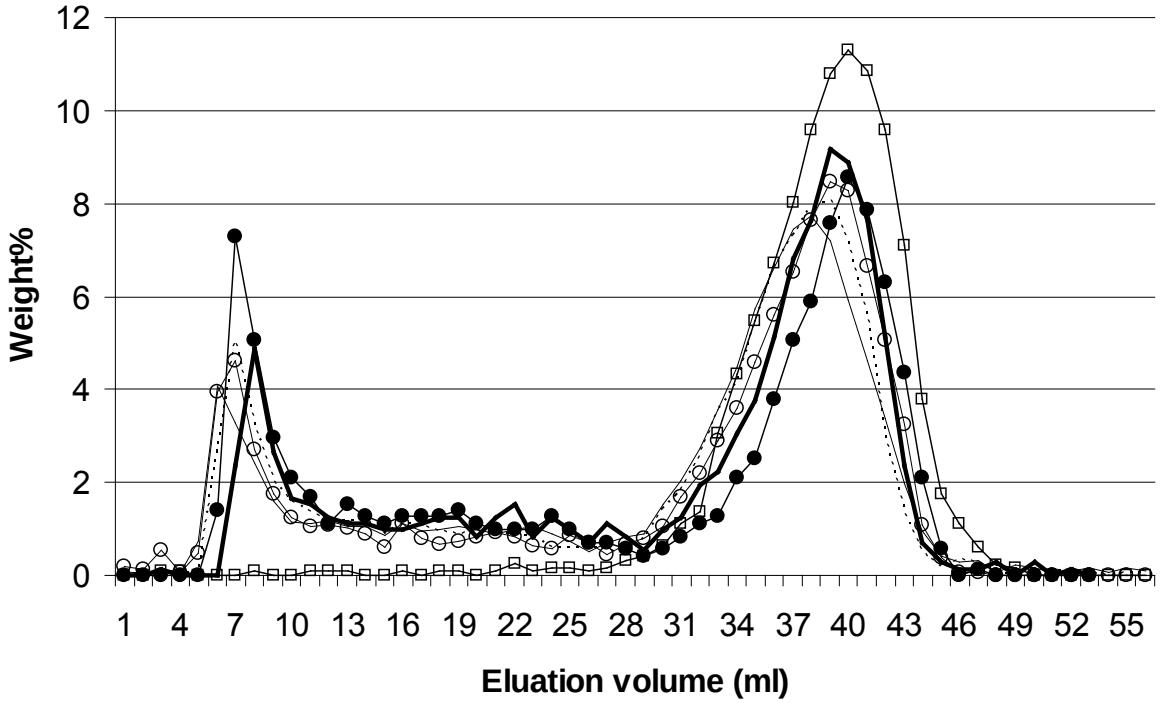

Figure 2. Amylose-Amylopectin ratio of debranched starch isolates BFF 0120 (····), BFF 0155 (—), BFF 0181 (▬), BFF 0224 (□) , BFF 0228 (•) and BFF 0249 (○) separated on a size exclusion CL6B column.

Table 3. Average amylose content showing duplicate results in brackets in cases of poor agreement.

	Amylose content (%)
0120	30 (27-33)
0155	31
0181	33
0224	2 (0-4)
0228	41
0249	30 (26-33)

Discussion

An isolation method for barley flours has been developed with some adjustments done to optimise isolation for the current material. A large volume of rinsing water is of great necessity in order to wash out all soluble starch from the viscous material (the bran fraction) during filtration. As a minimum, 100 ml/g starting material was used and showed to be sufficient in order to finally achieve a nearly water clear filtrate. Small portions of the slurry should in a systematic manner be washed with continuous stirring with certain precautions to avoid contamination of crude starch suspension with fibrous material. The slurry is suggested to be applied using a 5 mL pipette with a tip shortened by approximately 1 cm to create a larger aperture to prevent stuffing, as well as to successively remove washed fibrous material from sieve.

All centrifugations during the isolation procedure were carried out at 2800 rpm instead of 8800 rpm due to a calculation mistake (incorrect unit conversion). As learned from trials in this study as well as from literature, sedimentation alone produces a pellet like deposit covered by a 'supernatant' containing barely small granules, proposing that large ones will sediment without centrifugation. The mistake is severe in view of the fact that the goal for this isolation was to

retrieve as much of small granules as possible, and these seem to have ended up in the supernatant, which was discarded. Despite incorrect setting, starch formed pellets on centrifugation but these more or less tended to resolve a little at end of decantation. Several centrifugation steps have presumably resulted in a substantial loss of starch upon decantation of supernatant. In early work reported in the literature, starch granules were fractionated only by sedimentation, which can be sufficient for recovering most of the large granules. Hence counts were made on a low magnification microscope it is possible that small granules remained undetected. Low experience in observing starch is as well a factor.

The poor retrieval of starch in BFF samples, most noticeable when recollecting starch from fibrous fraction and washing of interphases, where pellet production was poor or absent, can partly be explained by the incorrect centrifugation settings. There might also be unknown characteristics of these not fully explored varieties that cause unusual behaviour on extraction. The varieties included in the BarleyFunFood project have been selected to represent a wide range of different characteristics. These differences in composition could account for these samples not showing behaviours matching those of regular barley cultivars. As presented in Table 2, the higher centrifugation force (4350 rpm) used in the additional isolation of sample 0120, has a heavy impact on starch yield. The addition of a pestling step of fibrous residue is also assumed to contribute to the higher yield, although it remains uncertain to what degree. The fibrous residue from sample 0120 in this trial had the lowest starch content of all fiber residues but it must be mentioned that the fibrous residue of the same sample was discarded in the major study. Hence, no comparison is possible. The reason for not running centrifugations on stipulated force settings was the second time lack of equipment. To compensate for this, longer centrifugation times could improve pellet formation. As an example do Park & Baik (2010) use only 1500 x g, but for 15 min. At Lyckebystärkelsen 3000 x g is used for 3-5 min. As already stated, the industry does not prioritise representability.

The pellets formed even on low centrifugation speed were relatively firm. Pellet stability seemed to be affected by the degree of starch purity, as protein and fiber impurities tended to disturb a proper pellet formation. Quick resolubilisation of starchy material correlated to weak pellets and vice versa was noted during the drying steps. As focusing on yield rather than purity, high levels of impurities in crude starch could in turn decrease the yield more as combined with the low centrifugation force.

Most likely, the low centrifugation force setting contributed to the poor starch retrieval from the fibrous residues as well. The fibrous residues produced very weak pellets causing material loss on decantation. The correct centrifugation force would presumably overcome this problem. Starch appearing in the supernatant was assumingly consisting of mostly small or broken granules, not heavy enough to sediment by weight. Removal of damaged granules was desired whereas removal of B-granules was not.

One of the challenges within this work was to maximize isolation of B-granules, to optimise chances for attaining representative starch samples. This could be the strongest motive for disapproval of extracted material for further analysis.

Another possible explanation of poor yield is an insufficient proteinase K activity due to inconsistent stirring during incubation. Light plastic tubes tending to float in water bath made handling of multiple samples difficult. At times, double samples showed tendencies that unequal treatment regarding reduction of white lumps in fibrous material had occurred, suggesting

discontinuation in stirring or perhaps properties with the material that somehow hinders enzyme activity. Assurance of secure water bath arrangement should promote sufficient enzyme action.

Since amylose-amylopectin ratio was determined on material isolated with incorrect centrifugation force settings, these results are not completely reliable. Due to poor retrieval of B-granules, containing a higher proportion amylose than A-granules, the correct true amylose content is probably higher.

As the scraping-off procedure showed to be troublesome regarding purity and showed indications of lower yields it was removed from procedure. The amount of enzyme needed could on the other hand be reduced by separate treatment of proteinous layer, but this requires that untreated starch is devoid of protein impurities, which showed to be difficult. The difficulties in scraping off brown layer could perhaps, as well as the entire fractionation process be impaired by the incorrect centrifugation force setting resulting in infirm pellet fractions.

In the starch content determination within the pre-study, all centrifugations were incorrectly carried out at 830 rpm (115 x g) instead of 2780 rpm (1290 x g). On ethanol washing this is expected to cause some loss of starch, mainly B-granules, contributing to unrepresentative preparations. On centrifugation after enzymatic treatment where supernatant is diluted prior to spectrophotometric detection, this could imply impurities present in sample that possibly could disturb absorbance properties.

In order to assure maximum retrieval of starch associated with fibers, both mechanical and enzymatic treatment of the bran fraction seemed to be required. Rubbing fibrous residue with mortar and pestle after filtration showed to be efficient. Addition of starch amount derived from fibrous residues onto starch weight in main isolate, the yield only increases by 1-3 %. Conclusively, the non-success in retrieving the starch from the white grains is not the only cause of low yield. The samples containing the highest starch contents in fibrous residues were not the same as those showing the highest visible amount of white lumps, which could be regarded as a verification of this.

Shaking with toluene and saline mixture is a time-consuming procedure that implies handling of big quantities of health harming chemicals and is not practiced in commercial starch isolation (Lyckebystärkelsen). To optimize for a quantitative yield *and* a fair purity, this toluene shaking was included in this study as it effectively purifies starch suspension from protein (McDonald and Stark. 1988).

Starting with whole kernels instead of flours would have enabled for an extended steeping process but is not judged to impair inactivation of enzymes, as amylases. Prolonged steeping also serves to hydrolyse viscous material which could have been an advantage in the isolation procedure. It also acts to soften the protein-starch matrix. However, in the procedure set up in this study the wetmixing should account for this activity on the material. The use of ground flour has been an object for criticism as it requires a harsher milling that possibly can result in less pure starch and can also damage the granules. However, SEM pictures of granules after both milling and wetmixing shows acceptable amount of damaged granules.

When washing from solvents and when drying, it is beneficial to use enough liquid to resolve material but still as little as possible to allow for a quick flow over pellet without resolving it partly in the end of decantation. It is also an advantage to run centrifugations in tins without the

lid on, since opening of tins can initiate resolution of pellet. It is of great importance to handle the tins with care to prevent this.

The differences in mixing rod dimension and tightness of cutting device makes the choice of rod in combination with mixing time suggested for further investigation,

In a recent study from 2010, Park & Baik demonstrate how recovery and purity of starch are affected by the temperature of fractionation water. It was shown that the optimal temperature for a maximum starch recovery was obtained with 40ºC and 60 ºC water for waxy and regular barley respectively, without negative effect on purity. This is explained by increased solubility of fibers weakening the bonds to entangled starch and protein. The yield obtained in this study is higher than for Park & Baik, but the isolation procedure employed by Park & Baik is adapted for industry in that sense that it does not use chemicals. The use of heated water during screening would presumably not affect the recovery rate as much in the method used in this study, due to the inclusion of proteinase treatment, toluene shaking and pestling.

Park & Baik also states that starch recovery is increased by isolation preceded by abrasion of kernels. Removal of bran prior to isolation however bring about removal of aleurone layer comprising a substantial amount of the β-glucan content (Bacic & Stone, 1981) which would impair fiber, protein, vitamin and mineral analysis. Abrasion might as well cause loss of endosperm material and thereby starch. Furthermore, isolation in this study was performed on whole kernels already milled to flour.

Dietary fiber degrading enzymes could be used to improve starch recovery as reported by Zheng & Bhatty (1998). Enzymes are however not absolutely pure and could contain contaminating amylases causing starch hydrolysis.

Conclusions

Primary isolation carried out using the incorrect centrifugation force settings did not result in a sufficient yield so as to assure starch material representing the origin flours. Supplementary isolation of sample 0120 at a higher centrifugation force indicates a great improvement regarding yield and could be regarded as representative. For further studies of samples 0155, 0181, 0224, 0228 and 0249 new isolations are suggested according to the method used for the second isolation trial of 0120.

Scraping off the brown proteinous layer for separate proteinase K treatment was shown difficult regarding maintaining purity of the starchy layer, which tended to resolve and mix into proteinous layer on scraping. Results also show that the total yield was lower in this case why this step is suggested to be omitted.

Prior to proteolytic treatment, rubbing with mortar and pestle was required to rupture white grains is efficient for a quantitative yield. Pestling of fibrous residue ought to be incorporated to the procedure.

Starch granules appear to be sensitive to heat exposure whether as not that sensitive concerning mechanical violence.

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