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Enzymatic Method for Measuring Starch Gelatinization in Dry Products in Situ

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ABSTRACT: An enzymatic method based on hydrolysis of starch by amyloglucosidase and measurement of D-glucose released by glucose oxidase−peroxidase was developed to measure both gelatinized starch and hydrolyzable starch in situ of dried starchy products. Efforts focused on the development of sample handling steps (particle size reduction of dry samples followed by a unique mechanical resolubilization step) prior to the enzymatic hydrolysis using native and fully gelatinized flours of corn and rice. The new steps, when optimized, were able to maximize resolubilization of gelatinized/retrograded starch while minimizing solubilization of native starch in dried samples, thus effectively addressing issues of insusceptibility of retrograded starch and susceptibility of native starch to enzymatic attacks and eliminating the need to isolate starch from dry samples before using an enzymatic method. Various factors affecting these and other steps were also investigated, with the objectives to simplify the procedures and reduce errors. Results are expressed as the percentage of the total starch content. The proposed method, verified by measuring mixed samples of native and fully gelatinized flours of five grain species (corn, rice, barley, oat, and wheat) at different ratios, is simple, accurate, and reliable, with a relative standard deviation of less than 5%.

KEYWORDS: *starch, gelatinization, measurement, enzymatic, in situ, dried*

INTRODUCTION

Starch gelatinization is an important physical, chemical, and biochemical change during processing of starch-containing foods or feeds. The extent of starch gelatinization not only determines the textural and organoleptic properties of processed products[1] but also affects human[2] and animal[3] nutrition through changing enzymatic access to glucosidic linkages and consequent digestibility. Starch gelatinization is characterized by (a) loss of starch granule birefringence, (b) alterations in starch crystalline organization, (c) an increase in viscosity, (d) an increase in dye-binding ability, and (e) an increase in susceptibility to enzyme attack.[1,4] On the basis of these principles, many methods have been described to measure starch gelatinization, including polarization microscopy, X-ray diffraction, amylography,[5,6] differential scanning calorimetry (DSC),[2,7] pulsed nuclear magnetic resonance,[8] absorbance of iodine binding,[9,10] and enzymatic susceptibility.[7,11−18]

However, most of these techniques, including some popular enzymatic methods, are applicable only to purified starch. For processed products, starch has to be isolated first, making the method laborious and/or prone to errors.[5,19] Others, such as the DSC method[2] and biosensor method,[18] can measure gelatinized starch in situ but require costly instrumentation or special devices that are not easily accessible. Still others, such as dye-binding methods, are found to be less reliable.[6] Therefore, there has been a great need to develop a quantitative method for measuring starch gelatinization in processed foods and feeds in situ without using laborious procedures or costly instrumentation.

Starch-containing foods and feeds that have been heated and then cooled often contain substantial amounts of retrograded starch.[4] They also contain some native starch because of partial gelatinization. The objective of the present study was to develop an enzymatic method that can determine starch gelatinization in dried products without the need to isolate starch. In developing an enzymatic method for measuring starch gelatinization, except for Marconi et al.,[18] all previous researchers[7,11−15,17] generally focused on three key steps of the methodology: enzymatic hydrolysis of starch to glucose, assay for D-glucose, and expression of starch gelatinization. The present study was able to achieve the objective by focusing on not only the above three steps but also new steps before the enzymatic hydrolysis. What makes the proposed method in this study unique is that gelatinized/retrograded starch in situ was mechanically resolubilized so that it could react with amyloglucosidase (AGS) to release measurable glucose, while native starch had limited solubilization and thus limited reaction with AGS. The method addressed the issues of retrograded starch insusceptibility as well as native starch susceptibility to enzyme hydrolysis and thus eliminated the need for starch isolation before using an enzymatic method.

MATERIALS AND METHODS

Materials. Seeds of five grain species, barley (CDC Alamo, hulless), corn (yellow dent), oat (Provena, hulless), rice (medium grain, milled into brown rice), and wheat (Brundage, soft white winter), were acquired from local breeders or purchased from a local supermarket. Samples were cleaned and/or screened to remove foreign materials and broken kernels.

Amyloglucosidase (E.C. 3.2.1.3, also known as glucoamylase) from *Aspergillus niger*, 67.4 U/mg lyophilized powder, product no. 10115,

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was purchased from Sigma Co. (St. Louis, MO). The stock enzyme solution (about 3300 U/mL) was made by mixing 1 g of the enzyme preparation with 20 mL of 100 mM sodium acetate buffer, pH 4.75, and stored in a refrigerator. For D-glucose analysis, the Megazyme D-glucose assay kit (K-GLUC), which contained two vials of high-purity glucose oxidase–peroxidase (GOPOD), two bottles of concentrated reagent buffer, and one bottle of D-glucose standard solution, was purchased from Megazyme International Ireland Ltd. (Wicklow, Ireland).

Preparation of Flour/Starch Materials for the Study. *Starch Isolation and Purification.* About 75 g of raw grain flour, finely ground to pass U.S. standard mesh no. 50 (300 μm diameter openings), was mixed with 550 mL of 50 mM NaOH using a mechanical mixer (RW20 digital, IKA Works, Inc., Wilmington, NC) for 10 min at 1500 rpm. The suspension was centrifuged at 4000*g* for 15 min. The supernatant was discarded. The pellet was mixed with 350 mL of water for 10 min and then sieved through a screen (U.S. standard mesh no. 270). The liquid stream passing through the screen was centrifuged at 4000*g* for 15 min. The filmy layer on the top of the pellet was removed. The rest of the pellet was collected and fan dried at room temperature as purified starch.

Preparation of Fully Gelatinized Flour or Starch. Seed samples of five grains (corn, barley, oats, rice, and wheat) were cracked into pieces (particle size about 2.5 mm in diameter) by a Burr coffee mill (Pro Line series, KPCG100, KitchenAid, St. Joseph, MI) at a setting of 6.5. Kernel pieces were soaked in water (1:10 solid to water ratio) overnight in a 1500 mL beaker. The entire content in the beaker was autoclaved at 121 °C for 80 min and then removed and cooled to room temperature while covered. The slurry was mixed at 1500 rpm with the IKA mechanical mixer for 5 min. A portion of the material (about 40 mL) was removed and used for measuring starch gelatinization as a wet sample for comparison with dried samples. The rest of the material was transferred to a larger beaker (4000 mL). While mixing, 95% ethanol was added until a 50% (v/v) ethanol concentration was reached. The mixture was centrifuged at 500*g* for 5 min. The pellet was collected and fan dried in a fume hood. The sample was coarse ground using the Burr coffee grinder and then further dried at 60 °C for 1 h in a forced air oven to drive away trace amounts of ethanol. The autoclaved products were termed "fully gelatinized flour". For purified starch samples, overnight soaking was omitted, but the rest of the procedure was the same. The final product was termed "fully gelatinized starch".

Procedure of the Proposed Method. The detailed procedure developed in this study consists of multiple steps (Figure 1).

Sample Particle Size Reduction. Before enzymatic analysis, both native and gelatinized samples had to be ground to reduce the particle size. Dry samples were ground by a coffee grinder (type 203, Krups, Medford, MA) at repeated intervals until all passed through a screen (U.S. standard mesh no. 50). In this study, for comparison with dried samples, a portion (40 mL) of the wet sample slurry after autoclaving was also used. The sample portion was placed in a 250 mL plastic jar (Osterizer), and 110 mL water was added. The mixture was blended for 30 s on the lowest speed using an Osterizer blender.

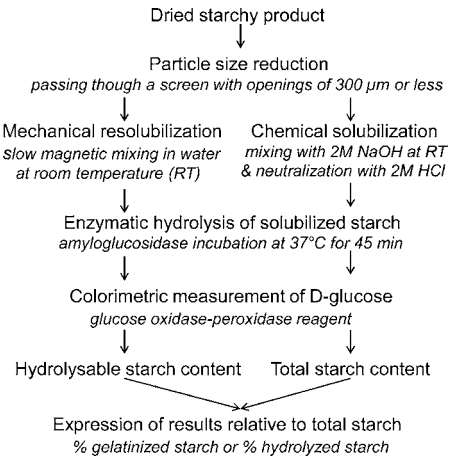

Figure 1. Schematic diagram showing key steps for the proposed method.

Mechanical Resolubilization of Starch in Dry Samples at Room Temperature for Hydrolyzable Starch Measurement. A 20 mg portion of the dry ground sample (flour or purified starch, native or gelatinized) was weighed into a 50 mL plastic graduated centrifuge tube with a conical bottom and a flip cap (these features were important for mixing and fast pipetting later on). To each tube was carefully added an octagonal magnet (5/16 in. × 1/2 in.) with a spinning ring. A plastic rack (24 holes total) holding the tubes (up to 12 maximum) together in the center was placed on the top of a stirrer with a digital speed control. For this study, to investigate the effect of temperature as well, a hot plate/stirrer with both temperature and speed controls, Isotemp, 7.5 in. × 7.5 in. plate size (category no. 11-300-48SHP, Fisher Scientific) was used. While the weighed dry sample was stirred at 100 rpm, 5 mL of deionized water was carefully pipetted into the bottom of each tube. The stirring speed was increased to 300 rpm for 1 min and then decreased to 50 rpm for an additional 69 min (a total of 70 min). Sample tubes in the rack were rotated halfway through to minimize the positional effect of the stirring plate. At the end of stirring, 35 mL of 100 mM sodium acetic buffer, pH 4.75, was added to each tube using a liquid dispenser. The total volume in each sample tube was 40 mL. For wet samples (only for this study), 450 mg of the blended suspension, instead of 20 mg of dry sample powder, was used for solubilization. The rest of the procedure was the same.

Chemical Solubilization of Dry Starchy Samples at Room Temperature for Total Starch Measurement. For measuring the total starch content, another set of dry starchy samples (20 mg each) underwent the same mechanical hydration procedure as for measuring the hydrolyzable starch, except (1) 5 mL of 2 M NaOH was initially added instead of water, (2) at the end of solubilization (total 70 min), 30 mL of the acetate buffer was added instead of 35 mL, and (3) the final mixture was vortexed, 5 mL of 2 M HCL was added, and the sample was vortexed again. The total volume in each sample tube was also 40 mL. This step was to completely solubilize starch in the dry samples.

Enzymatic Hydrolysis of Solubilized Starch to D-Glucose. Following the two concurrent steps of mechanical resolubilization of starchy samples for measuring hydrolyzable starch and chemical solubilization for total starch, each sample tube was vortexed, with the cap on, at a high speed for 10 s, and immediately 2 mL of a sample suspension was pipetted into a 15 mL glass test tube using a 5 mL pipet tip. The procedure was repeated one more time for generating a sample blank for D-glucose measurement using a different 15 mL test tube. A 10 μL volume of the AGS stock solution (33 units) was added to each sample tube except for the sample blank and vortexed for 5 s. Sample tubes in a rack were incubated at 37 °C in a covered water bath for 45 min and vortexed every 15 min for 5 s. At the end of incubation, each test tube was diluted to 10 mL with 50 mM phosphate buffer, pH 7.4 (using a liquid dispenser), and vortexed for 10 s (careful vortexing to avoid spillage).

D-Glucose Measurement. The D-glucose content released from resolubilized/solublized starch in samples by AGS was determined by the Megazyme GOPOD detection procedure that came with the D-glucose measurement kit, but with modification. Chromogen reagent was prepared by diluting 50 mL (one bottle) of concentrated reagent buffer (1 M potassium phosphate, pH 7.4, 0.22 M *p*-hydroxybenzoic acid, and 0.4% (w/v) sodium azide) to 1 L with deionized water, followed by dissolving the content of one vial of GOPOD reagent (also known as glucose determination reagent) in this buffer. The GOPOD reagent should be stored in a brown storage bottle in a refrigerator. From each sample tube or sample blank, 0.4 mL was transferred into a 2.5 mL (4.0 mL to the top edge) cuvette (12.5 × 12.5 × 45 mm), and 1 mL of GOPOD reagent was then added to each cuvette. The D-glucose control consisted of 0.04 mL of 1 mg/mL glucose standard solution, 0.36 mL of the phosphate buffer, and 1 mL

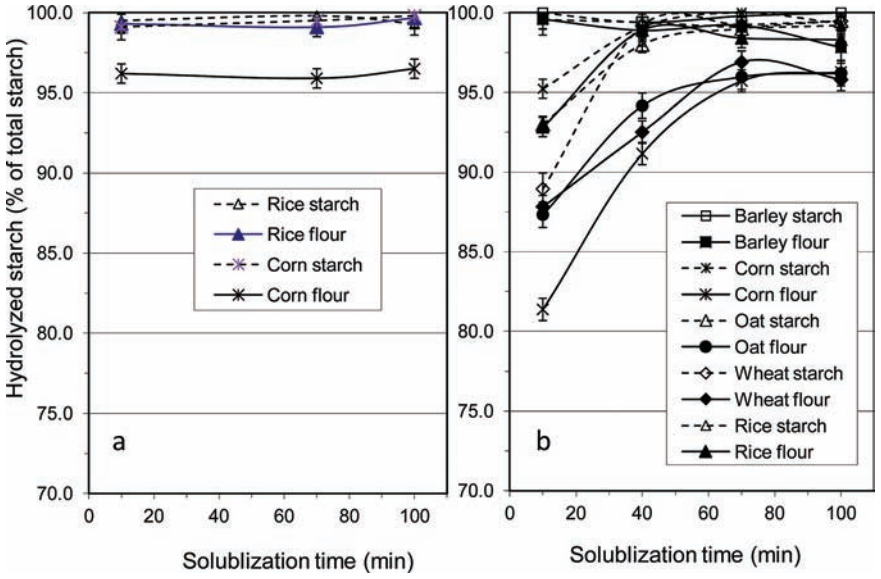

Figure 2. Effect of starch resolubilization time in water before incubation with amyloglucosidase on starch hydrolysis in fully gelatinized flour or fully gelatinized starch isolated from selected grains: (a) wet gelatinized samples, (b) dried gelatinized samples. Resolubilization was carried out by mixing samples (passed through U.S. standard mesh no. 50) in water at room temperature with a magnetic stirring speed of 50 rpm.

of GOPOD reagent. The reagent blank consisted of 0.4 mL of the phosphate buffer and 1 mL of GOPOD reagent. Cuvettes with added reactants were vortexed and incubated at 37 °C for 30 min in the covered water bath. After color reaction, each sample and the glucose control were vortexed and the absorbance at 510 nm was read against the reagent blank by a spectrophotometer (Genesys 6, Thermo Electron Corp., Waltham, MA). The following equation was used to calculate the percentage of starch (as it is basis):

$$\begin{aligned}\text{percentage of starch (as it is basis)} &= (\Delta A - \Delta A_s)F \times \text{FV/SV} \\ &\quad \times 100/W \times 162/180 \\ &= (\Delta A - \Delta A_s)F \times 200/0.4 \\ &\quad \times 100/20 \times 0.9 \\ &= 2250F(\Delta A - \Delta A_s)\end{aligned}$$

where ΔA = absorbance reading of a sample against the reagent blank (use the mean value when duplicate), ΔA_s = absorbance reading of the sample blank against the reagent blank, F = conversion factor from 1 unit of absorbance to the mass (mg) of D-glucose, FV = final volume of the solubilized and enzymatic converted sample solution (40 × 10/2 = 200 mL in this study), SV = sample volume used for the color reaction in the cuvette (0.4 mL in this study), W = sample mass (mg) (20 mg in this study), $100/W$ = factor to express the starch content as a percentage of the sample mass, and 162/180 = adjustment from free D-glucose to anhydrous D-glucose as occurs in starch.

Expression of the Final Results. The hydrolyzable starch following mechanical resolubilization was expressed in two ways, percentage of gelatinized starch and percentage of hydrolyzed starch relative to the total starch content in a test sample, according to the following equations, respectively:

$$\begin{aligned}&\text{percentage of gelatinized starch} \\ &= (\text{hydrolyzable starch content} - \kappa\eta) \\ &\quad /(\text{total starch content} - \kappa\eta) \times 100 \qquad (1)\end{aligned}$$

$$\begin{aligned}&\text{percentage of hydrolyzed starch} \\ &= \text{hydrolyzable starch content/total starch content} \times 100 \\ &\qquad (2)\end{aligned}$$

κ is the hydrolyzable starch content in a native whole grain sample and η is the ratio of total starch content in the test sample over the total starch content of the native whole grain sample. κ is characteristic of each grain species under the defined assay condition. The difference between the two expressions lies in that the percentage of gelatinized starch has a weighted correction factor κ arising from the digestion of native samples by AGS.

Experiments Only for the Method Development in This Study. *Procedure Variation.* In developing the procedure of the enzymatic method described above, several factors, at each of the key steps (Figure 1), were investigated for their effects and optimal conditions. These included (1) particle size of dry flour samples (U.S. standard mesh size nos. 35, 50, and 70, i.e., 500, 300, and 212 μm opening dimension, respectively), (2) starch resolubilization time (10, 40, 70, and 100 min), (3) stirring speed during starch resolubilization (50, 150, and 300 rpm), (4) temperature during starch resolubilization (25, 37, and 47 °C), (5) medium used for starch resolubilization (water or the sodium acetate buffer, 100 mM, pH 4.75), (6) temperature for enzymatic hydrolysis of resolubilized/solubilized starch into glucose (25, 37, and 47 °C), (7) time for the enzymatic conversion (30, 45, and 60 min), (8) enzyme concentration for the enzymatic conversion (5, 10, 15, and 20 μL of the stock solution, corresponding to 16.5, 33, 49.5, and 66 units, respectively), and (9) pH of the acetate buffer for enzymatic conversion (4.50, 4.75, and 5.00). Levels for each of these factors were easily accomplished by modifying the step procedures described above. In addition, moisture, protein, and oil contents of five native and five gelatinized grain flour samples were measured, according to methods described by Han and Liu.[20]

Data Treatments and Statistical Analysis. The experiment was duplicated at the stage of preparing gelatinized flour or gelatinized purified starch. Data were analyzed with the JMP software, version 5 (JMP, a business unit of SAS, Cary, NC). For most factors under investigation, analysis of variance (ANOVA) was conducted on the basis of a complete factorial model or a randomized block model designed for certain steps.

Method Verification. For each of the five grain species, a set of six mixed samples, representing 0%, 20%, 40%, 60%, 80%, and 100% fully gelatinized flour by mass, were made by mixing the appropriate proportions of native and autoclaved flour samples. All mixed samples were tested for two types of starch content, enzyme hydrolyzable starch and total starch, according to the proposed procedures described above, and the results are expressed as percentage of

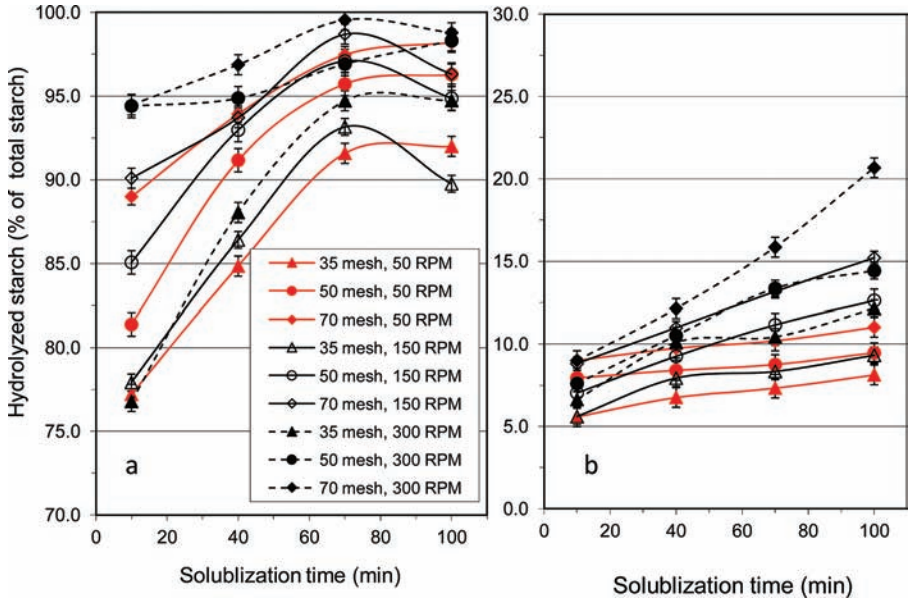

Figure 3. Effect of the particle size of dried samples, magnetic stirring speed, and mixing time during resolubilization in water at room temperature on starch hydrolysis of fully gelatinized corn flour (a) or native corn flour (b) by amyloglucosidase.

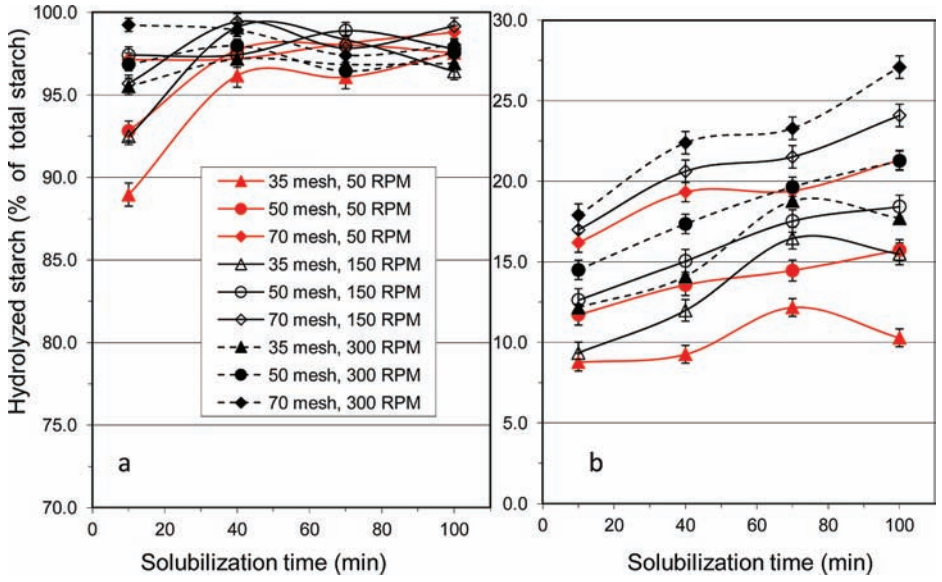

Figure 4. Effect of the particle size of dried samples, magnetic stirring speed, and mixing time during resolubilization in water at room temperature on starch hydrolysis of fully gelatinized rice flour (a) or native rice flour (b) by amyloglucosidase.

gelatinized starch and percentage of hydrolyzed starch, all relative to the total starch content, and plotted against the percentage of gelatinized flour by mass in the sample series.

RESULTS AND DISCUSSION

Need for Starch Resolubilization before the Enzymatic Hydrolysis. When a fully gelatinized grain flour or isolated starch in a slurry was not dried after autoclaving, mixing in water (mechanical resolubilization) for only 10 min followed by 45 min of incubation AGS led to high levels of hydrolysis, as measured by D-glucose released, reaching over 96% of total starch in autoclaved corn and nearly 100% in autoclaved rice, autoclaved corn starch, and autoclaved rice starch (Figure 2a). For these autoclaved wet samples, a further increase in resolubilization time did not improve the value of hydrolyzed starch. The results indicate that gelatinized starch in wet samples was fully soluble and thus available for enzymatic attack without the need for the resolubilization step. However, without sufficient duration of resolubilization before the enzymatic hydrolysis, starch in most fully gelatinized flour or fully gelatinized purified starch samples that were dried following autoclaving showed only partial hydrolysis by AGS (Figure 2b). This indicates that gelatinized starch in dried samples needed to be resolubilized before becoming available for enzyme hydrolysis. The level of resolubilization, expressed as the percentage of hydrolyzed starch with respect to the total starch in the same sample, changed with mixing (resolubilization) time, grain species, and sample type (flour or purified starch) (Figure 2b). In general, under the specific conditions of resolubilization, when the incubation time increased to 70 min, most samples reached a plateau in starch hydrolysis. A further increase in resolubilization time did not cause an additional

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increase, but instead for some samples a slight decrease in values was observed. The reason for the decrease in hydrolyzable starch after an extended duration of mixing is not known. Even at the plateau, many fully gelatinized flour samples did not show 100% enzymatic hydrolysis of starch, indicating incomplete resolubilization of gelatinized starch in dried samples (see further discussion later).

Among dried samples, the effect of resolubilization was most pronounced for corn (Figure 2b). For example, at 10 min of resolublization, only about 82% of the corn starch was hydrolyzed. This value increased to about 96% when the resolublization time was increased to 70 min. In contrast, barley flour was least affected by resolublization. Other species fell between corn and barley samples. Within the same species, starch in flour showed less hydrolysis than purified starch. This is expected since purified starch would resolubilize faster than starch in the flour matrix under the same conditions.

Conditions That Maximize Resolubilization of Gelatinized/Retrograded Starch While Minimizing Solubilization of Native Starch. For further investigating the effect of resolublization on starch hydrolysis, five factors, namely, grain species (corn and rice), flour type (native or fully gelatinized), particle size (U.S. standard mesh nos. 35, 50, and 70), magnetic stirring speed (50, 150, and 300 rpm), and mixing (resolubilization) duration (10, 40, 70, and 100 min) at room temperature, were taken into consideration by a factorial design. The results (Figures 3 and 4) showed that all factors under investigation had significant effects ($p < 0.05$) on starch hydrolysis, as measured by the released D-glucose content and expressed as the percentage of hydrolyzed starch relative to total starch. For dried fully gelatinized corn samples (Figure 3a), the finer the particle size, the higher the value of hydrolyzed starch. The higher the stirring speed, the higher the value also. With an increase in resolublization time, a plateau or a peak was reached at 70 min. A further increase in duration not only failed to improve hydrolysis but also caused some decrease in certain samples. This pattern of effect was also observed with dried flour samples of other species (Figure 2b), as just discussed. From Figure 3a, it appears that the optimal conditions would be no. 70 mesh, 300 rpm stirring speed, and 70 min resolublization time.

However, the commercially available AGS obtained from *A. niger* is known to act on raw starch also.[14,17] To determine an optimal condition for samples with varying levels of starch gelatinization, samples with native starch had to be taken into consideration also. In fact, most processed foods or feeds are not fully gelatinized due to insufficient heat and/or lack of moisture, and thus contain some native starch. Therefore, in this study the same factors were investigated on native grain flours. In the case with native starchy samples, starch would be solubilized, instead of resolubilized as with heated and then dried samples. Figure 3b shows that the particle size and stirring speed had the same effect on native corn flour as for the gelatinized corn flour. That is, the higher the stirring speed, the more the starch was solubilized. Similarly, the finer the particle size, the more the starch was solubilized. However, for native corn flour, the solublization time did not show a peak or plateau at 70 min as observed with the dried fully gelatinized corn flour. A gradual increase in starch solubilization was observed when the mixing time was increased from 10 to 100 min (and most likely beyond this time). In determining an optimal condition for the solublization, our strategy was to find a condition that could lead to higher starch resolublization in fully gelatinized samples but the least solubilization of starch in native samples. Considering data from Figure 3, resolubilization with a no. 50 mesh particle size, 50 rpm stirring speed, and 70 min duration is considered optimal for dry corn samples. Under these conditions, about 96% of starch in fully gelatinized dried corn flour was resolubilized and then hydrolyzed, but only 7.5% of starch in native corn flour was solubilized and then hydrolyzed.

For dry rice samples (Figure 4), the effects of three factors (particle size, stirring speed, and resolubilization time) generally followed those of dry corn samples (Figure 3). However, for fully gelatinized rice flour (Figure 4a), the effect of resolubilization was less pronounced. For some combinations, particularly those of higher stirring speed and finer particle size, the resolubilization time had little effect. For the rest of the combinations, a plateau was reached at 40 min instead of the 70 min observed for autoclaved corn flour. For native rice flour (Figure 4b), the effects of the three factors were all stronger than those observed with native corn flour (Figure 3b). Considering data from Figure 4, resolubilization with a no. 50 mesh particle size, 50 rpm stirring speed, and 40 min duration is considered optimal for dry rice samples. Under these conditions, about 97% of starch in fully gelatinized dried rice flour was resolubilized and then hydrolyzed, but about 13% of starch in native rice flour was solubilized and then hydrolyzed. However, for a unified procedure, considering both corn and rice samples (Figures 3 and 4), as well as other species (Figure 2), resolubilization with a no. 50 mesh particle size, 50 rpm stirring speed, and 70 min duration is considered optimal. Under these conditions, about 96% of starch in fully gelatinized dried rice flour was resolubilized and then hydrolyzed, but about 14% of starch in native rice flour was solubilized and then hydrolyzed.

The effect of the resolublization temperature (25, 37, and 47 °C) as well as solvent type (water or 100 mM sodium acetate buffer, pH 4.75) was also investigated with both corn and rice samples under a randomized block design. The results showed that an increase in temperature caused a significant increase in starch hydrolysis in native samples but no effect or even some decrease for fully gelatinized samples (Figure 5). Resolubilization for 40 min gave trends similar to those of resolubilization for 70 min. Similarly, with the same particle size, water gave a higher level of hydrolyzed starch than the buffer for gelatinized samples (Figure.6a) but a lower value for native flour samples (Figure 6b). Although a finer particle size gave higher hydrolysis values, the effect of buffer relative to water was the same. Therefore, sample resolubilization at room temperature with water is recommended.

The principle of enzymatic methods for measuring starch gelatinization is based on the increase in susceptibility of gelatinized starch to enzyme attack. Earlier workers reported digestion of samples with β-amylase,[11] AGS,[12] or diastase,[13] followed by measurement of the increase in reducing power of the resulting fragments. Shetty et al.[14] described an improved method to determine starch gelatinization using AGS digestion followed by determination of the released D-glucose through a dual enzyme system, GOPOD. Several later workers who reported enzymatic methods for measuring starch gelatinization generally followed the principle (using AGS) of Shetty et al.[14] with modifications limited to glucose measurement,[15] data interpretation,[7] or both.[16] A combination of amylase−pullulanase followed by reducing sugar measurement was also reported.[17] In spite of differences among the reported

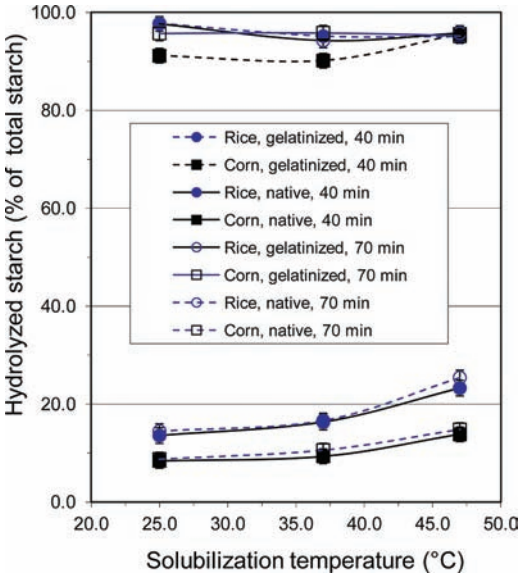

Figure 5. Effect of temperature at different durations during resolubilization on starch hydrolysis by amyloglucosidase in dried rice and corn samples (fully gelatinized or native flour). Resolubilization was carried out by slowly mixing samples (passed through U.S. standard mesh no. 50) in water with a magnetic stirring speed of 50 rpm.

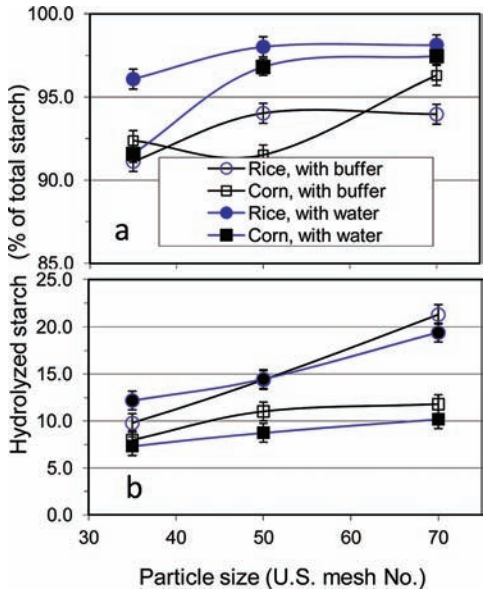

Figure 6. Effect of the incubation medium (water or 100 mM sodium acetate buffer, pH 4.75) during resolubilization on hydrolysis of starch by amyloglucosidase in fully gelatinized flour (a) or native flour (b) with different particle sizes. Resolubilization was carried out by slowly mixing samples at room temperature with a magnetic stirring speed of 50 rpm for 70 min.

enzymatic methods, some small and some large, they all have a common feature; that is, in developing an enzymatic method for measuring starch gelatinization, these previous researchers[7,14,15,17] focused on three key steps of the methodology, enzymatic hydrolysis of starch to glucose, assay for D-glucose (or reducing sugar), and expression of starch gelatinization, but neglected the steps before the enzymatic hydrolysis. As a result, most of the methods are generally applicable to purified starch only and cannot measure starch gelatinization in situ. When dealing with dry processed samples, starch had to be isolated first, making some of these methods laborious and prone to errors.[5,19]

The enzymatic method described in this study followed the principle of Shetty et al.[14] in that it used both AGS for enzymatic hydrolysis of starch to D-glucose and GOPOD for glucose measurement, although detailed procedures of these steps varied significantly. However, what sets the present study apart from the previous ones on enzymatic methods for measuring gelatinized starch is that before the enzymatic hydrolysis with AGS two new steps (sample particle reduction and slow magnetic mixing in water (mechanical resolubilization of starch)) were added. On the basis of the results of Figures 2−4, without a sufficient mixing time to allow resolubilization of starch in dried autoclaved samples, even through the rest of the steps were kept the same, the level of hydrolyzable starch, based on the measured amount of D-glucose released relative to total starch, was much less than expected.

According to Copeland et al.,[4] when starch granules are heated in the presence of water, they lose their crystallinity and structural organization and become gelatinized: "On cooling, the disaggregated starch molecules form a gel and then retrograde gradually into semi-crystalline aggregates that differ in form from the native granules. Thus, starchy foods or feeds that have been heated in some ways and then cooled often contain substantial amounts of retrograded starch." We believe that adding the two new steps, sample particle reduction and hydration with low-speed stirring, helped resolublization of both gelatinized and retrograded starch and made both types of starch readily susceptible to AGS attacks. At the same time, the optimized condition selected at the resolubilization step helped minimize susceptibility of native starch to AGS attacks. Thus, the two new steps effectively eliminated the need for starch isolation before using the enzymatic method, making it possible to measure starch gelatinization in situ of dry processed samples.

The mechanical resolubilization procedure described in this study is rather unique and innovative. It features room temperature hydration of powdery samples in water, with selection of a proper size of test tubes, a proper size and shape of magnetic stirring bars, a proper stirring speed (50 rpm), and a proper duration (70 min). By using a test tube rack and a proper magnetic stirring plate, the procedure can handle multiple samples at once and thus free up hands for other work while the samples are being hydrated. When using the method, it is very important to follow the detailed procedures without substantial deviation, including the sample weight, the liquid volume used at each step, the final volume at the end of resolubilization, proper selection of tubes, stirring bars, and stirring plates, etc. It should be pointed out that even with the unique procedure of mechanical resolubilization, it was difficult to obtain complete resolubilization of gelatinized or retrograded starch in dried samples, as evidenced by the observation in Figures 2−4 that autoclaved flours of several grain species did not reach 100% enzymatic hydrolysis at the plateau. This also indicates complexity in dealing with gelatinized starch in dried samples.

Determination of Optimal Conditions for the Enzymatic Hydrolysis. After starch in powdery samples was optimally resolubilized through slow magnetic stirring in water at room temperature, the next step was enzymatic hydrolysis of resolubilized starch into D-glucose by incubation with an AGS preparation. For optimizing this step, several factors, including

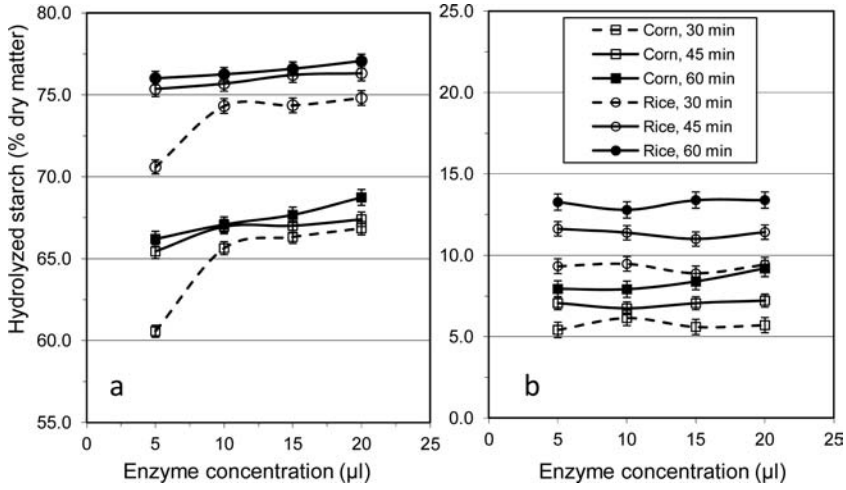

Figure 7. Effects of the enzyme concentration and time during incubation of resolubilized starch samples with AGS at 37 °C on starch hydrolysis in fully gelatinized corn or rice flour (a) and native corn or rice flour (b). A 1 μL volume of stock AGS solution contained about 3.3 units. Before this step, starch resolubilization was carried out by mixing samples (passed through U.S. standard mesh no. 50) in water at room temperature with a magnetic stirring speed of 50 rpm for 70 min.

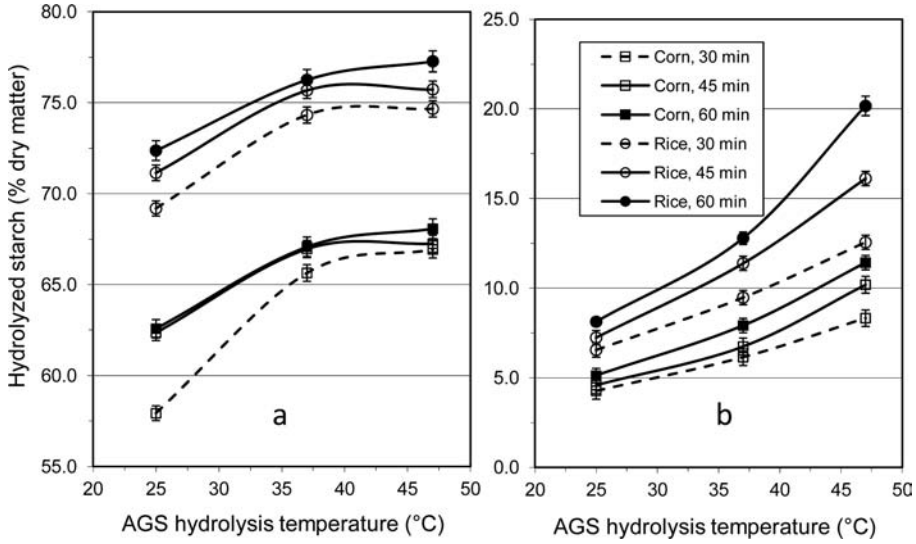

Figure 8. Effects of temperature and time during incubation of resolubilized starch samples with 33 units (10 μL of stock solution) of AGS on starch hydrolysis in fully gelatinized corn or rice flour (a) and native corn or rice flour (b). Before this step, starch resolubilization was carried out by slowly mixing samples (passed though U.S. standard mesh no. 50) in water at room temperature with a magnetic stirring speed of 50 rpm for 70 min.

the enzyme concentration, reaction temperature and duration, and buffer pH, were investigated, again using both native and gelatinized corn and rice samples. The results showed that when the incubation temperature was held at 37 °C, the enzyme concentration affected starch hydrolysis (based on the percentage of hydrolyzable starch measured relative to the total dry sample mass) of gelatinized rice and corn flours up to 10 μL of the stock solution (equivalent to 33 units) (Figure 7a). At a given enzyme concentration, the incubation time had a significant effect up to 45 min. For native flours, the enzyme concentration had little effect, but the reaction time had a significant effect for the range studied (Figure 7b). Therefore, 10 μL (33 units) of enzyme concentration was used for the proposed method.

The results also showed that at the 10 μL enzyme concentration a longer incubation and higher temperature led to higher starch conversion, but native and gelatinized samples showed different trends (Figure 8). For gelatinized samples (Figure 8a), the increase in starch hydrolysis between 25 and 37 °C was much larger than that between 37 and 47 °C. The differences among different reaction times were largest at 25 °C and smallest at 47 °C. For samples with a longer reaction (45 or 60 min), 37 °C appeared to give a plateau. In contrast, for native flour samples (Figure 8b), the increase in starch hydrolysis between 25 and 37 °C was much smaller than that between 37 and 47 °C. The differences among reaction times were largest at 47 °C but smallest at 25 °C. No plateau was observed. In terms of the buffer pH, between 4.50 and 5.00, there was a slight but insignificant difference in starch hydrolysis of both gelatinized and native flour samples (data not shown). Overall, for enzymatic conversion, a combination of 37 °C, 45 min, and a buffer pH of 4.75 is recommended.

Besides investigation of the above factors affecting enzymatic hydrolysis, we also looked at other minor aspects, with an effort to reduce experimental errors. In transferring the resolubilized starch liquid sample into a test tube for enzyme hydrolysis, we

Table 1. Moisture, Protein, Oil, and Total Starch Contents of Five Native and Five Fully Gelatinized Grain Flours[a]

		native				gelatinized			
species	variety and/or feature	moisture	starch	protein	oil	moisture	starch	protein	oil
barley	CDC Alamo, hulless	9.7	50.3	17.3	2.8	7.6	55.1	14.9	2.5
corn	yellow dent	7.5	70.2	7.5	3.4	5.9	70.2	7.3	2.8
oat	Provena, hulless	9.5	53.3	16.3	6.1	7.2	57.1	15.2	3.5
rice	medium grain, milled to brown rice	10.9	78.4	6.9	3.0	6.3	77.9	6.5	2.9
wheat	Brundage, soft white winter	7.7	60.5	13.4	2.3	7.6	65.3	12.0	2.0

[a]Means of duplicate measurements. Starch, protein, and oil contents are expressed on a percentage of dry matter basis.

found that it was essential to use 5 mL pipet tips to avoid particle adhesion and tip blockage. It was also important not to pipet the sample from the bubble area in the tube after vortexing and not to use the same pipet tip for different samples.

Xiong et al.[16] investigated the effects of the buffer pH (4.45, 4.50, and 4.55) and frequency of shaking during AGS incubation at 40 °C for 60 min on starch hydrolysis and found that the pH did not influence glucose release but with up to three shakings the glucose values increased to a plateau. They also found that coarse grinding consistently gave lower glucose values and a higher coefficient of variation than did fine grinding. In the present study, we investigated a relatively larger range (4.50−5.00) of the buffer pH for AGS incubation and confirmed that the buffer pH had a minimal effect on starch hydrolysis. Although we did not investigate the shaking frequency effect, we recommended vortexing every 15 min for 5 s. As for the particle size effect, the present study was conducted at the resolubilization step, which was the step prior to the AGS incubation, while Xiong et al.[16] investigated the effect directly at the enzyme incubation stage since they did not have the resolubilization step. Both studies, however, showed the effect of the sample particle size on starch hydrolysis. Our recommendation is that dry samples be finely ground to pass a screen with a 300 μm opening dimension (U.S. standard mesh no. 50), much finer than the screen size (1 mm) recommended by Xiong et al.[16]

D-Glucose Measurement. The D-glucose measurement kit from Megazyme is based on a dual enzyme system, glucose oxidase−peroxidase. This is the most commonly used method for measuring D-glucose.[14,21] Glucose oxidase converts glucose into equal molar amounts of gluconic acid and hydrogen peroxide. The latter is decomposed by peroxidase in the presence of a chromogen to form a light-absorbing complex suitable for colorimetric analysis. Although the kit came with a protocol in detail, we modified the procedure to reduce the total assay volume (from 3.2 to 1.4 mL). Thus, one kit could perform as many as 1500 assays instead of 500. We also included sample blanks since we did not wash all our samples (such as native flour and native starch) with an alcohol solution.

Total Starch Measurement. For measuring total starch content, a chemical reagent, such as dimethyl sulfoxide (DMSO),[14] an alkali solution,[15,18,22] or an enzyme preparation,[22,23] is usually used to solubilize starch in a sample as completely as possible immediately before AGS hydrolysis. Among the methods of using an alkali solution (NaOH or KOH), there are variations in alkaline concentrations, from 0.25 M[15] to 2.00 M.[22] Shetty et al.[14] stated that the reliability of the method used to determine total starch was one of the three key elements for the accuracy of their enzymatic procedure for determining starch gelatinization. In this study, since the results of the proposed method are to be expressed in relation to the total starch content in a sample, the reliability of the total starch method is also very important. Accordingly, we used 2 M NaOH as a reagent to completely solubilize starch in samples for total starch measurement since it was reported to give a higher value than an enzymatic method.[22] The total starch content, along with moisture, oil, and protein contents, of five native and five gelatinized grain flours is shown in Table 1. These components varied among samples. For the same species, differences existed between native and gelatinized samples, apparently due to the procedure of autoclaving and subsequent washing with alcohol applied to gelatinized samples.

Expression of the Results. In the proposed method, two types of starch were measured. One was enzyme hydrolyzable starch. It was the starch content in a test sample measured after the sample underwent the mechanical resolubilization that had a limited effect on native starch, followed by enzymatic hydrolysis of solubilized/resolubilized starch to glucose and D-glucose measurement. This type of starch can also be considered as enzyme susceptible starch or digestible starch. The other type was the total starch, which was measured after a sample underwent chemical solubilization that could completely solubilize starch in any type of sample, followed by the same steps of enzymatic hydrolysis and D-glucose measurement as for hydrolyzable starch. The results can be expressed in two ways, percentage of gelatinized starch and percentage of hydrolyzed starch, using eqs 1 and 2, respectively. Both expressions have a relevance to the total starch content, but the difference lies in that the former has a weighted correction factor (κ) arising from measurable but limited digestion of native starch by AGS.

In calculating the percentage of gelatinized starch, Shetty et al.[14] and Chiang and Johnson[15] both used a correction factor (κ) to take into consideration limited hydrolysis of native starch. However, in the present study, the correction factor κ is weighted by introducing the η value. In each of these studies, the correction factor κ can only be determined under a defined assay condition. Furthermore, even under the same assay condition, the value changed also with the grain species.[24] In the present study, for each grain species, a new κ value had to be determined using a native flour of that species. Accordingly, κ values for barley, corn, oat, rice, and wheat were found to be 10.2%, 6.3%, 5.4%, 11.6%, and 5.4% (of the sample mass), respectively. Furthermore, when the η value (the ratio of total starch in a test sample to the total starch of a native whole grain sample) in eq 1 becomes small (e.g., <0.4), the values of eqs 1 and 2, that is, percentage of gelatinized starch and percentage of hydrolyzed starch, approach each other. In this case, for simplicity, eq 2 can be used to estimate the percentage of gelatinized starch.

There are other ways to interpret the results. Di Paola et al.[7] interpreted their data by plotting the initial velocity of the enzymatic reaction as a function of the temperature of treating

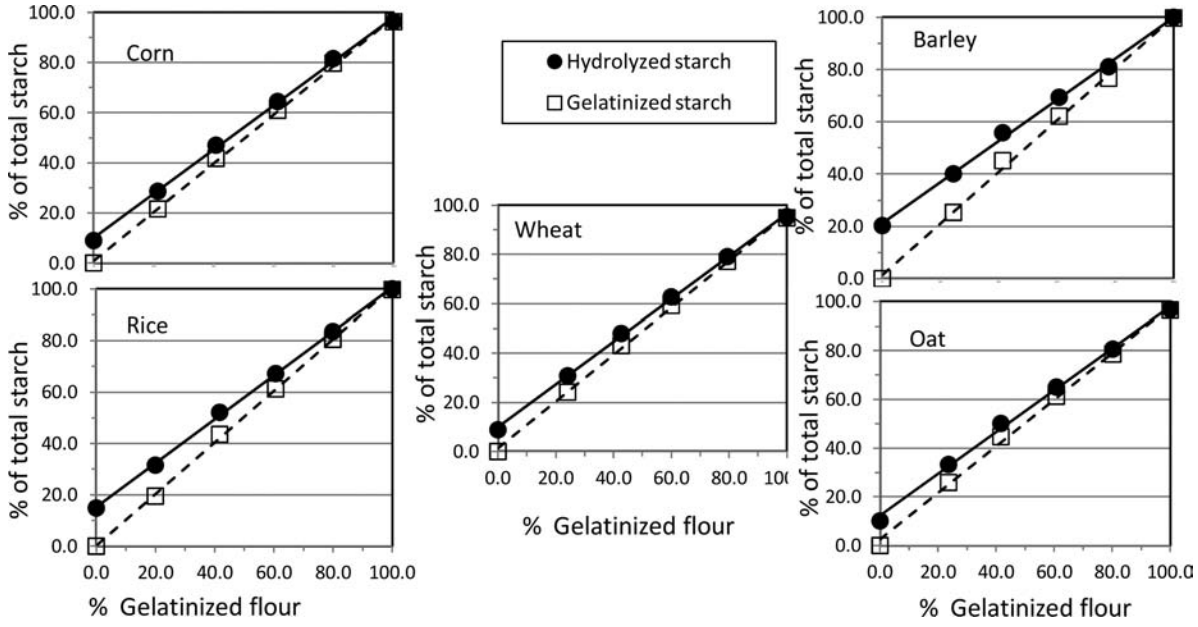

Figure 9. Levels of gelatinized starch or hydrolyzed (hydrolyzable or susceptible) starch in flours of five grain species, as determined by the proposed method and expressed as a percentage of total starch, in sample series containing increasing levels of gelatinized flour by mass.

an aqueous suspension of maize starch (from 25 to 95 °C). Xiong et al.[16] determined enzymatically released glucose from a mixture of fully gelatinized and raw cereal grains, plotted the data into a standard curve, and used the curve to express the degree of starch gelatinization of unknown samples.

Method Verification. To determine the accuracy and reliability of the proposed method, we prepared a series of flour sample mixtures containing 0%, 20%, 40%, 60%, and 100% fully gelatinized flour by mixing native and dried autoclaved flour samples at varying ratios for each of five grain species. This has been a common way to verify a new method developed for measuring starch gelatinization.[9,14,15] The results show that, with each flour sample, there was a strong linear relationship between hydrolyzed starch (expressed as a percentage of the total starch) and the percentage of fully gelatinized flour by mass in the sample series (Figure 9). There was also a strong linear relationship between gelatinized starch (expressed as a percentage of the total starch) and the percentage of fully gelatinized flour by mass in the sample series. Thus, the agreement between measured values of gelatinized starch and the theoretical values was excellent. This applied to all five grain species used in this study. Equally important is that for all the duplicate measurements the relative standard deviation was under 5%, mostly in the range of 2−4%, indicating excellent repeatability of the proposed method.

As shown in Figure 9, the difference between hydrolyzed starch and gelatinized starch is that the line for gelatinized starch always crossed at 0 on both the *x*- and *y*-axes while the line for hydrolyzed starch always intercepted the *y*-axis at a positive value. This is determined by the difference in definition between the two types of starch. Native starch, like gelatinized starch, was also susceptible to AGS attack but on a limited scale (Figures 3b and 4b). Thus, it always showed some value of hydrolyzed starch, which is the *y*-axis intercept value. Since this value relates to susceptibility of native starch to AGS attack, it varied with the grain species. Gelatinization of starch is historically defined as a heat-induced change. Since native starch in raw grain flour had not been subjected to any heat treatment, we attributably set its gelatinized starch value to 0 through a weighted correction factor as shown in eq 1. As the percentage of gelatinized flour by mass in the sample series increased, the two types of starch approached each other and almost reached the same value when the *x*-axis value increased to 100% (Figure 9).

In conclusion, the enzymatic method developed in this study has an advantage over many previously reported methods in that it can measure starch gelatinization of dried processed samples in situ, without a need for expensive instruments or unique devices. Optimal conditions were determined for simplicity, accuracy, and reliability using native and fully gelatinized grain flours.

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Notes

The authors declare no competing financial interest.

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