Rapid Screening Methods of Potato Cultivars for Low Glycemic Trait

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Abstract

Potatoes are a dietary staple consumed by a significant portion of the world, providing valuable carbohydrates and vitamins with minimal fat. Most commercially produced potatoes have a high content of highly branched amylopectin starch, which generally results in a high glycemic index (GI). Consumption of foods with high levels of amylopectin elicit a rapid spike in blood glucose levels, which is undesirable for individuals who are pre-diabetic, diabetic, or obese. Some cultivars of potatoes with lower amylopectin levels have previously been identified and are commercially available in niche markets in some countries but are relatively unavailable in the United States and Latin America. Among Native communities in North America and in the high Andes countries of South America, some foods that include certain potato cultivars, may have been used to help people mitigate what is now defined as the effects of blood sugar and obesity. These cultivars are not widely available on a global market. This study utilizes three independent analyses of potato starch: microscopic examination of granule structure, water absorption, and spectrophotometry of iodine complexes to identify potato cultivars with low amylopectin that are thought to have low GI potential. Differences among cultivars tested were detected by all three types of analyses. Of the 60 potato cultivars evaluated the most promising are Huckleberry Gold, Muru, Multa, Green Mountain, and an October Blue x Colorado Rose cross. Further work is necessary to document the ability of these low amylopectin cultivars to reduce blood glucose spike levels in human subjects.

Keywords

Solanum tuberosum; amylose, amylopectin, diabetes, microscopy; starch granules, starch swelling capacity.

Introduction

Current lifestyles have led much of the general population to become relatively sedentary and to adopt questionable eating habits, including high consumption of foods which appear to drive an alarming rise in the incidence of obesity and Type 2 diabetes worldwide (1-3). This problem is especially severe in low-income populations, where inexpensive high-energy foods tend to be consumed, instead of more expensive foods with more balanced nutrition (4). Potatoes are a popular staple food, available to consumers at a low cost per pound that can be easily stored. Potato starch is also widely used in the food industry as a processed food ingredient (5,6). Most varieties of potatoes have high levels of amylopectin starch, which is highly branched and rapidly digested.

High levels of amylopectin which causes a rapid spike in blood glucose level that occurs after consumption of high glycemic index potatoes (2, 7–9).

Figure 1 (below) illustrates the difference in response of a typical individual's blood sugar levels to high and low glycemic index foods. Most varieties of potatoes are in the high glycemic category of foods that people with prediabetes, diabetes, or obesity should seek to avoid. Not only do high glycemic foods give a large spike in blood sugar levels, the body's response to try to bring the level down tends to cause what is referred to as an undershoot, bringing levels below its desirable amount. This low level of blood sugar tends to stimulate hunger and the desire to consume more high glycemic foods. It follows that high glycemic foods tend to cause a "roller coaster" effect on blood sugar and may cause excessive food consumption (10).

Figure 1: Blood plasma glucose response (mmol/l) from a high versus a low glycemic index (GI) food. The high glycemic food tends to cause an "undershoot" in the blood glucose level below the desirable level, which also tends to stimulate hunger and consumption of additional high glycemic foods (10).

The high glycemic index of widely available potatoes presents a problematic "consumer's dilemma" for individuals and families that may not be able to afford a better-balanced, more favorable diet (2, 7–9). The food industry has addressed this situation, particularly in Denmark and Australia (8,11), by providing and promoting lower glycemic varieties of potatoes that are relatively unavailable in the United States and most other countries. Some native communities in the Andes (Bolivia, Chile, and Peru) reportedly embrace a tradition of providing low glycemic tubers to people with obesity or diabetes (12).

Potato starch consists of two major types of carbohydrates, amylose and amylopectin (13). Amylose, less abundant in most varieties of potato tubers, has a linear repeating glucose 1-4 linked polysaccharide backbone structure that digests relatively slowly. Amylopectin shares the glucose 1-4 linked chains and additionally contains highly branched beta 1-6 side chains that produce a broader structure (8,14–20) that digests much faster than amylose that can present metabolic imbalances. Starch granule synthesis proceeds with the formation of amylose first and amylopectin is layered on the outside of the granules. Typical high amylopectin potato starch granules have a smooth rounded surface with concentric rings, formed by amylopectin (21,22).

Researchers have found that a higher ratio of amylose/amylopectin correlates with loss of surface smoothness in starchy granules (9,23). We noticed when screening potato starch granules, that some potato cultivars showed a highly disrupted starch granule surface, observed under the bright field microscope at 400x, while granules from other cultivars have a smooth appearance. The surface structure of the starch granules might be useful as an initial screen for identifying promising varieties with higher amylose starch content, that would favor a low Glycemic Index (GI) (9,16,20,25,26). In addition, the swelling capacity (water absorption capacity) of the starch has also been reported to correlate with the amylose/amylopectin ratio (18,26–30).

Zhu et al. (31), observed that high amylose rice consumption had beneficial health effects on obese rats, by reducing blood sugar levels and body weight, which would presumably also have positive health benefits for humans (2,23,24,31). This might be consistent with beliefs and dietary recommendations about types of plants among Native communities in Peru and Chile, but unfortunately most of their traditional potato germplasm was lost during the colonization of the region (12). However, potato varieties maintained in seed banks or that may already have been commercialized might possess beneficial, low glycemic traits. We focused on screening 60 potato varieties and selections in this study to investigate the relationship between starch granule conformation, swelling power and amylose: amylopectin ratios, seeking to validate rapid screening methods for low GI potential. The favorable lines revealed from this screening study can be used for further agronomic, breeding, and human GI testing.

Methods and Materials

Potato Material Tested

A collection of 60 potato varieties and selections of potato germplasm were obtained from research laboratories of Montana State University, the USDA potato collection (32), commercial seed producers, and private breeder Tom Wagner, Everett, Washington. These varieties were tested for low GI potential using starch granule morphology, water absorption capacity and amylose content, as described below. The potato materials were chosen for their favorable agronomic traits and increased in the MSU greenhouse for testing. Three tubers of each variety or selection were analyzed according to the schematic flow chart shown in Figure 2.

Figure 2: Schematic flow chart illustrating starch analysis of 60 selected potato varieties and selections.

Morphological analysis of starch granules

A cork-borer was used to remove a core (1cm x 1 cm) from the center medullary tissue of tubers for observation. Tubers were assayed several months after harvest and storage. A subsample 5 mm^2 was pressed onto a microscope slide, covered with a cover slip and viewed with a bright field microscope (Nikon Eclipse E440) at 400x magnification and photographed. Starch granules from each variety or selection was numerically graded using a grading scale assessment (GSA). The GSA was numerically graded according to the numerical scale described in figure 2 . Three different tubers of each variety or selection were examined and photographed. Each variety was given more than one rating score if the granules within the picture showed diversity, as explained further in the results section.

Figure 3: Grading scale for starch granule assessment: 0= whole granules with smooth visible hilum 1= whole smooth granules with dots 2= whole smooth granules with surface alterations (wrinkles) 3= Whole granules with large wrinkles 4= Starch granule surface fractures 5= collapsed granule

Water absorption capacity of starch

The swelling capacity (SC) or water absorption capacity (WAC) measures the ratio between weights for the hydrated starch flour gel and dry flour samples. Samples with increased amylose concentrations support lower water absorption after starch jellification (26–28,33–35). WAC was evaluated using a procedure modified from Martin (34). Potato samples were freeze dried and processed into powdered form in a coffee grinder to obtain potato flour consistency. Thirty to forty mg of potato flour was weighed in pre-weighed 2 mL screw top glass tubes and the total weight measured to four significant figures. To each tube 1.5 ml of distilled water was added to the tubes capped, vortexed, placed in a thermo-mixer (Thermo Fisher) at 92 °C, and mixed at 800 RPM for 30 minutes. The tubes were removed and centrifuged at 1000g for 10 minutes at 4° C. The supernatant was aspirated from the tubes, carefully avoiding the gelatinous layer, and the tubes reweighed. The capacity to absorb water was calculated by dividing the flour plus water weight by the dry flour weight.

Spectrophotometric Analysis of Starch Properties

Rundle (36) first reported a marked difference in iodine inclusion between amylose and amylopectin and developed a spectrophotometric method that allows quantification of both components in mixtures. We used this spectrophotometric method, following modifications by Jarvis and Walker (37) and Fajardo, et al (16), to quantify the amylose/ amylopectin ratios. 20 mg of freeze-dried potato flour was suspended in 2ml of 80% ethanol in a 15 ml plastic tube and mixed with a Vortex Shaker for a short period of time. Samples were centrifuged at room temperature for 30-60 seconds at top speed in a clinical centrifuge and the supernatant carefully removed. This washing procedure was repeated twice. The resulting washed starch (pellet) was mixed with 5ml of water and 5ml of 1 M KOH in the same tube and vortexed. It is important to use ultra-pure water in the analysis for reproducible results and the absorbance value of the iodine solution at 550 nm versus solvent is near 0.1, as the iodine solution is light and age sensitive (16). Precision in the preparation of the iodine and the amylopectin/amylose solutions is crucial to getting reliable amylose determinations. Since we are determining the proportion of amylose content rather than the exact amount of amylose in the potato starch, there is no purification step for the isolation of pure starch from the potato powder as presented by other determination methods. One ml of this mixture was transferred into a 50 ml tube, neutralized with 5ml of 0.1M HCl, mixed with 0.5 ml of Lugol solution (37), and adjusted to 50ml final volume with distilled water. The samples were prepared in triplicate and allowed to stand for 15 min at room temperature, before measurement to stabilize the inclusion of the iodine. Each sample was placed in a one cm plastic spectrophotometer cuvette and absorption spectra recorded from 480-800 nm versus a distilled water blank using a Genesis 10 UV Instrument (Thermo Fisher Scientific). The spectra were analyzed utilizing the Vision Lite software (Thermo Fisher).

Figure 4: Spectra of solutions of known concentrations of potato starch polysaccharide mixtures of amylose (referred on the figure as amilosa) and amylopectin. The spectrum marked Yodo is the iodine containing reagent solution blank.

Data Analysis

The data obtained was processed using the statistical software Stat Plus: Mac LE 2009.

Results Morphological Analysis of Starch Granules.

We noticed that for most potato varieties and selections, virtually all the granules in each sample were quite similar in appearance. In other cases, there was a high diversity of granular appearance. For these cases, two scores were assigned, as shown in Table 1 with a rough percentage of the granules the scores represented. Figure 5 shows that the granules in the common commercial varieties Russet Burbank and Yukon Gold, known to have a high amylopectin content (72% and 69% respectively), showed very smooth granules within a distribution in the sizes of their granules. The previously shown grading scale in Figure 3 dictated that the granules in Figure 5 fit a GSA rating of 1.

Figure 5: Bright field microscopic photos of smooth starch granules of Yukon Gold (left) and Russet Burbank (right) at 400x. These granules were given a GSA rating of 1.

In contrast, the starch granules in only a few potato varieties showed significant granular surfaces, and had higher GSA values. Huckleberry Gold, Muru , and Mich-Oct have GSA ratings of 5, as shown in Figure 6, where 90-95% of the total granules observed presented collapsed surfaces, the other 5-10% correspond to granules with minor irregularities. The variety Multa graded 4 GSA with 60-65% of the granules presenting a surface fracture level, and the 35-40% corresponded to minor irregularities.

Figure 6: Starch granule images from left to right are Huckleberry Gold, Muru, Multa, Bzura, and Michigan October.

The GSA ratings and percent starch granules in the GSA rating of 60 varieties and selections are listed in Table 1.

Variety or Selection	GSA Rating	% Uniformity
Bjorna	5	55-60
Bzura	5	70-75
Huckleberry Gold	5	100
I 1035	5	90-95
Laram K'anchali	5	75-80
Muru	5	100
Olalla	5	75-80
Arma	4	45-50
Bolivian Blizzard	4	80-85
Bareroot River	4	85-90
I 1036	4	85-90
Multa	4	60-65
Green Mountain	3	60-65

Iker	3	45-50
Juice Valley	3	45-50
Lumper	3	50-55
Marble Gold	3	85-90
Monona	3	35-40
Oct Blue	3	85-90
Oct Blue x Col Rose	3	85-90
Pirola	3	65-70
Biddy Taro	2	65-70
Bison	2	90-95
C97007	2	85-90
Cherry red	2	80-85
Katelidan	2	45-50
Norland	2	75-80
Papa Amorga	2	85-90
Purple Valley	2	65-70
Red Pontiac	2	50-55
Allegany	1	20-30
Anolla	1	35-40
Asun	1	45-50
Bolivian Spring	1	90-95
Russet Burbank	1	90-95
Charlotte	1	90-95
Chella x Bulk Clover	1	90-95

Garnet Chile	1	90-95
Golden Anniversary	1	85-90
Goldra	1	80-85
Leona	1	85-90
Mich Oct x John T	1	85-90
NH x SPG	1	45-50
Nicola	1	45-50
Phytophyter	1	35-40
Picasso	1	45-50
Rush Share	1	45-50
Sangre	1	45-50
Sassy Lassy	1	85-90
Skagit Magic	1	90-95
Violet Butter	1	40-45
X gem	1	60-75
Yukon Gold	1	80—85
Arcilla-597779	0	100
Chelan	0	100
Chipitiquilla	0	100
Dark Red Norland	0	100
Fontenay	0	100
Rose Valley	0	100
Studebaker x Nordic Oct	0	100

Table 1: GSA rating and range of uniformity of starch granules from 60 potato varieties and selections.

Water absorption capacity of starch

During the development of the assay to measure the water absorption capacity (WAC) during starch gel formation, we found that it was essential to handle the samples carefully after the final centrifugation, with minimum vibration, since removing the supernatant was difficult in some samples. The WAC data for 60 varieties and selections, is shown with standard deviations in Table 2. The ten varieties with the lowest water absorption were the same as those with the highest GSA scores. These included Huckleberry Gold, Muru, Bzura, Michigan October, and the F-1 hybrid Michigan October X Colorado Rose (Figure 2). The October Blue x Colorado Rose hybrid was ranked third lowest in its relative capacity to absorb water.

Variety	Water Absorption Capacity WAC		Standard Deviation
Monona	21.6	±	2.3
Chipitiquilla	21.5	±	
Skagit Magic	20.8	±	2.3
Picasso	20.5	±	
Cherry red	20.3	±	1.7
C97007	20	±	1.2
Katelidan	19.4	±	3.9
Purple Valley	18.3	±	4.5
Bison	18.2	±	2.3
Rose Valley	18	±	1.6

Garnet Chili	17.8	±	2.2
Chelan	17.4	±	
Nicola	17.3	±	2.1
Iker	17.2	±	1.6
Phytophyter	17	±	2.1
Dark Red Norland	16.9	±	4.1
Juice Valley	16.4	±	2.4
Bolivian Spring	16.1	±	3.7
Charlotte	15.8	±	2.8
Golden Anniversary	15.7	±	2.8
Goldra	15.7	±	2.7
Pirola	15.7	±	0.9
Asun	15.6	±	3
Anolla	15.5	±	0.9
Laram K'anchali	15.5	±	1.2
I 1035	15.4	±	5
Bjorna	15.4	±	2.1
Sassy Lassy	15.2	±	2.3
Chella x Bulk Clover	15.1	±	1.1
NH x SPG	14.9	±	1.2
Sangre	14.9	±	0.5
Violet Butter	14.7	±	1.3
Arma	14.5	±	2.6

I 1036	14	±	3.1
Studebaker x Nordic Oct	13.8	±	1.9
Bzura	13.7		
Rush Share	13.6	±	1.1
X Gem	13.6	±	0.8
Leona	13.4	±	1
Bolivian Blizzard	13.2	±	1.7
Olalla	13.2	±	1.6
Bareroot River	13.1	±	3.2
Arcilla-597779	12.9	±	1.2
Allegany	12.9	±	1.9
Green Mountain	12.8	±	1.3
Lumper	12.7	±	1.3
Multa	12.3	±	1.8
Biddy Taro	12.2	±	0.6
Fontenay	12.1	±	1.3
Marble Gold	12	±	1.1
Oct Blue	12	±	
Norland	11.5	±	0.8
Yukon Gold	11.2	±	2.1
Red Pontiac	10.9	±	2
Papa Amorga	10.6	±	0.7
Muru	10.5	±	0.6

Mich Oct x John T	10.4	±	0.3
Oct Blue x Col Rose	9.9	±	0.9
Huckleberry Gold	9.5	±	0.7
Russet Burbank	9.5	±	0.8

Table 2: Water absorption capacity (WAC) and standard deviations of 60 potato varieties and selections.

Determination of Amylose percentage

We encountered a wide range of deviations when we assessed amylose percentage, using the Jarvis and Walker approach (37). Fajardo et al (16) reported that using absorbance changes at the optimum wavelengths can provide highly consistent results. Fajaro, et al (16) found that the maximum absorbance of the amylose-iodine complex was at 620 nm and the maximum absorbance of the amylopectin-iodine complex was at 550 nm. Thus the percent amylose can be determined:

Amylose % = y = 107.7 x -77.4

Where x = A620nm/A550nm, measured against the reagent blank.

We Successfully screened 60 potato varieties in triplicate to present their respective standard deviations. The amylose ratings of 60 potato varieties and selections are shown in Table 3. The potato varieties with a higher concentration of amylose are Green Mountain, Muru, Bzura, Multa, Huckleberry Gold, and the F-1 cross October Blue X Colorado Rose. These samples also had high surface alterations of their starch granules, which supports our initial hypothesis that this granule feature was due to higher amylose.

Variety	Amylose (%)
Green Mountain	60.9
Muru	59.0
Bzura	50.4
Multa	44.7
Huckleberry Gold	44.7
Oct Blue x Col Rose	43.8
Laram K'anchali	43.8
NH x SPG	40.4
Lumper	39.7
Anolla	39.5
Bjorna	38.3
Iker	37.1
Sassy Lassy	37.1
X Gem	36.6
Chipitiquilla	35.7
Red Pontiac	35.0
Bolivian Blizzard	34.7
Phytophyter	34.5
Papa Amorga	34.2
Skagit Magic	34.2
Oct Blue	33.6
Dark Red Norland	33.3
Juice Valley	33.3

Studebaker x Nordic Oct	32.9
Cherry Red	32.8
Marble Gold	32.7
Fontenay	32.5
Nicola	32.3
Leona	32.3
Arma	31.9
Mich Oct x John T	31.8
Arcilla-597779	31.4
Pirola	31.2
Rose Valley	31.2
Violet Butter	30.9
Norland	30.8
Bolivian Spring	30.7
Katelidan	30.7
I 1035	30.4
I 1036	30.4
Yukon Gold	30.4
Olalla	30.0
Biddy Taro	29.8
Bareroot River	29.2
Sangre	28.9
Goldra	28.8

Garnet Chile	28.3
Asun	27.7
Monona	27.6
C97007	27.4
Charlotte	27.1
Chelan	26.6
Rush Share	26.6
Bison	26.2
Golden Anniversary	25.0
Allegany	24.1
Chella x Bulk Clover	24.0
Picasso	23.9
Purple Valley	23.9
Russet Burbank	23,7

Table 3: Amylose content (%) of 60 potato varieties and selections.

Variety	% Amylose Content	Starch Granule Ranking	Water Absorption
Green Mountain	61	3	13
Muru	59	5	10
Bzura	50	5	13
Huckleberry Gold	45	5	9
Multa	45	5	12
Oct. Blue x Col Red	44	3	9
Monona	28	2	22
Picasso	24	1	21

Purple Valley	24	2	18

Table 4: Compilation of traits of seven selected cultivars compared to three cultivars with high amylose content.

Calculation of potential glycemic index based on amylose percentage

Moreira's study in 2012 (38) measured the blood sugar levels in subjects two hours after eating a starchy meal with a known concentration of amylose, and reported a correlation equation where x is the amylose percentage of total starch content and GIp is the GI potential value (GIp). Using this calculation and the amylose content of potato varieties and selections, a GIp can be calculated (Table 5).

Variety	% amylose	GI potential		Variety	% amylose	GI potential
Green Mountain	60.9	(14.69)		Mich Oct x John T	31.8	73.28
Muru	59.0	(8.94)		Arcilla-597779	31.4	74.43
Bzura	50.4	16.93		Pirola	31.2	75.14
Huckleberry	44.7	34.18		Rose Valley	31.2	75.14
Multa	44.7	34.18		Violet Butter	30.9	75.90
Oct Blue x Col Rose	43.8	37.06		Norland	30.8	76.17
NH x SPG	40.4	47.12		Bolivian Spring	30.7	76.65
Lumper	39.7	49.35		Katelidan	30.7	76.66
Anolla	39.5	49.99		I 1035	30.4	77.31
Bjorna	38.3	53.57		Yukon Gem	30.4	77.31
Iker	37.1	57.15		I 1036	30.4	77.51
Sassy Lassy	37.1	57.22		Olalla	30.0	78.72
X gem	36.6	58.62		Biddy Taro	29.8	79.28

Chipitiquilla	35.7	61.45		Bareroot River	29.2	81.13
Red Pontiac	35.0	63.54		Sangre	28.9	81.99
Bolivian Blizzard	34.7	64.46		Goldra	28.8	82.25
Phytophyter	34.5	65.17		Garnet Chile	28.3	83.65
laram K'anchali	34.2	65.81		Asun	27.7	85.70
Skagit Magic	34.2	65.81		Monona	27.6	85.73
Papa Amorga	34.2	65.96		C97007	27.4	86.43
Oct Blue	33.6	67.76		Charlotte	27.1	87.37
Dark Red Norland	33.3	68.68		Chelan	26.6	88.81
Juice Valley	33.3	68.75		Rush Share	26.6	88.89
Studebaker x Nordic Oct	32.9	69.77		Bison	26.2	89.97
Cherry Red	32.8	70.12		Golden Anniversary	25.0	93.81
Marble Gold	32.7	70.58		Allegany	24.1	96.44

Table 5: Predicted glycemic index (GIP) of 52 potato varieties and selections calculated using the equation described by Moreira (38). GIp = Glycemic Index potential.

The GIp for Green Mountain and Muru, the two varieties with the highest percentage of amylose have negative values, which could suggest that such high amylose samples were outside the range of Moreira's equation.

Pearson's correlation coefficient tests were performed to study the possible relationship between granular surface appearance of starch granules (GSA), granule absorption capacity (GAC), and amylose content by spectrophotometric analysis in multiple potato varieties and selections. There was a positive correlation between GSA scores and the amylose content of the potato starch (r^2 = 0.46, p <0.05). There was a negative correlation between GAC and amylose content r^2 = -0.34, p>0.01), confirming the hypothesis that amylose inhibits water absorption by potato starch. These results agree with observations with peas since high amylose peas are more wrinkled and cause a much lower insulin response in humans than smooth peas (41). The wrinkled peas also have a lower capacity to absorb water (42).

Discussion

Nutrition data suggest that consumption of potatoes with higher concentrations of amylose would have a lower Glycemic Index and would be expected to provide a health benefit for people that have prediabetes, diabetes and/or obesity. A lower sugar release during digestion is expected to cause a lower sugar spike and lower release of insulin, due to differences of digestibility of amylose and amylopectin (4,12,31,41,43). Furthermore, high insulin release tends to lead to an undershoot in desirable blood sugar levels (hypoglycemia), stimulating hunger and potential weight gain.

From a sample of over 60 cultivars, we have been able to identify the six-candidate cultivars for further agronomic evaluation for consumer acceptability. Those samples are as follows: Huckleberry Gold, Muru, Bzura, October Blue x Colorado Rose, Multa, and Green Mountain. In addition to the consumer dilemma on choices of affordable healthy foods, there is the Breeders' Dilemma that is the conflict between improved food nutrition that often reduces agronomic yield, which usually drives the choice of varieties (40). To pursue this aim, we identified patterns in properties of potato starch that could be used in rapid screening. By screening of 60 potato lines A pattern was identified in the microscopic appearance of starch granules, confirmed by direct starch content assays, permitting a rapid initial screening of candidate potato varieties for increased amylose contain and potential lower Glycemic Index. This rapid screening system will allow elevation of nutrition as a priority in plant breeding.

This work now will be followed by glycemic index measurements and agronomic fitness (40,41) on the most promising potato varieties.

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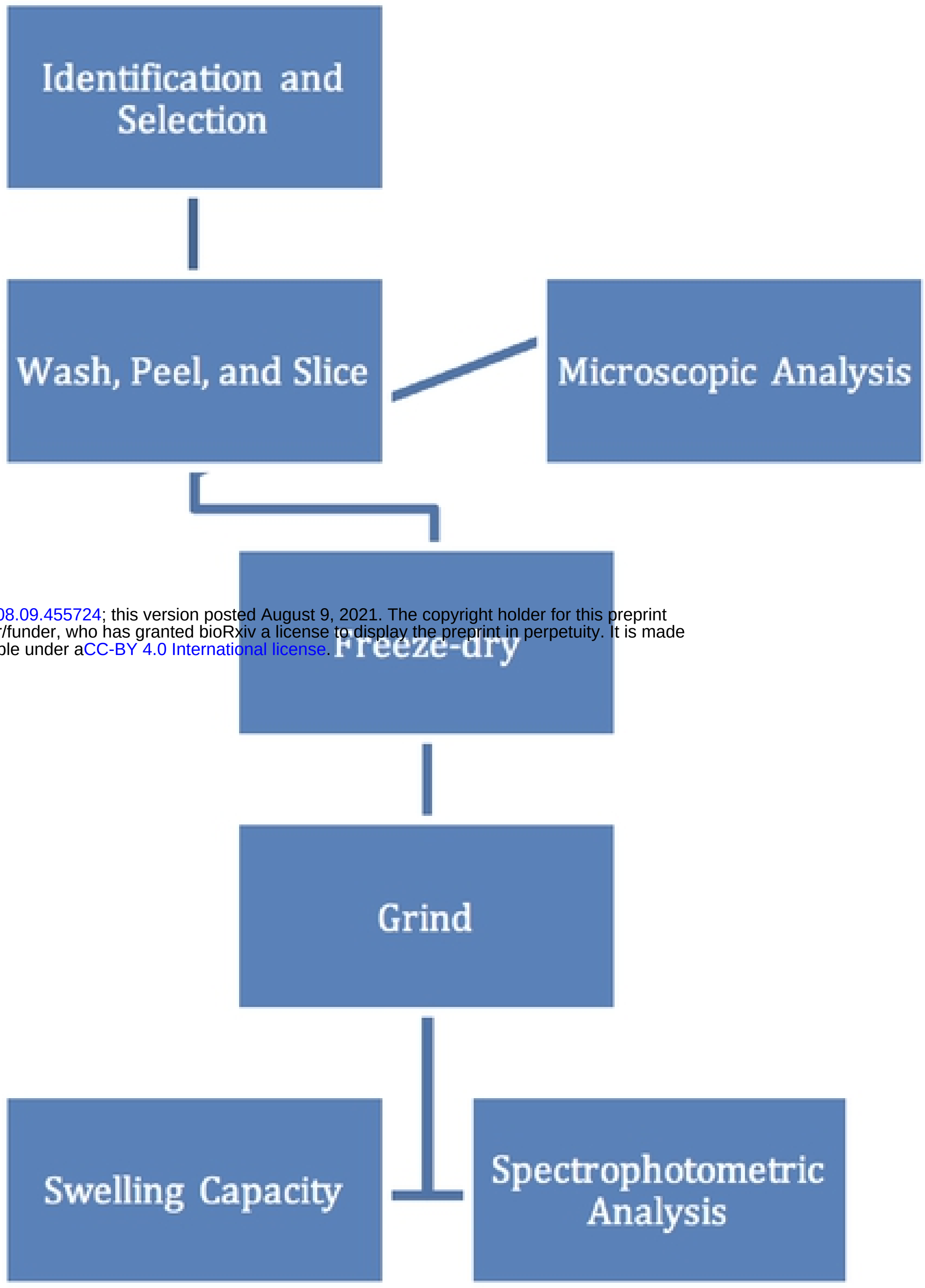


FIG 2

Figure 2

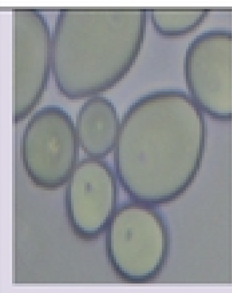
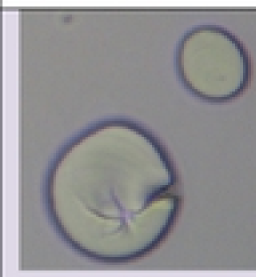
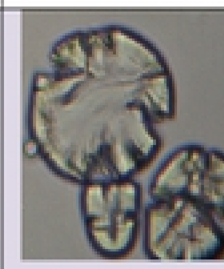
Description	Whole smooth granule, halo visible	Whole smooth granule with points	Whole smooth granule with wrinkles	Whole granule with surface alteration	Granule with fracturing	Collapsed granule
Images						
Ranking	0	1	2	3	4	5

Fig 3

Figure 3

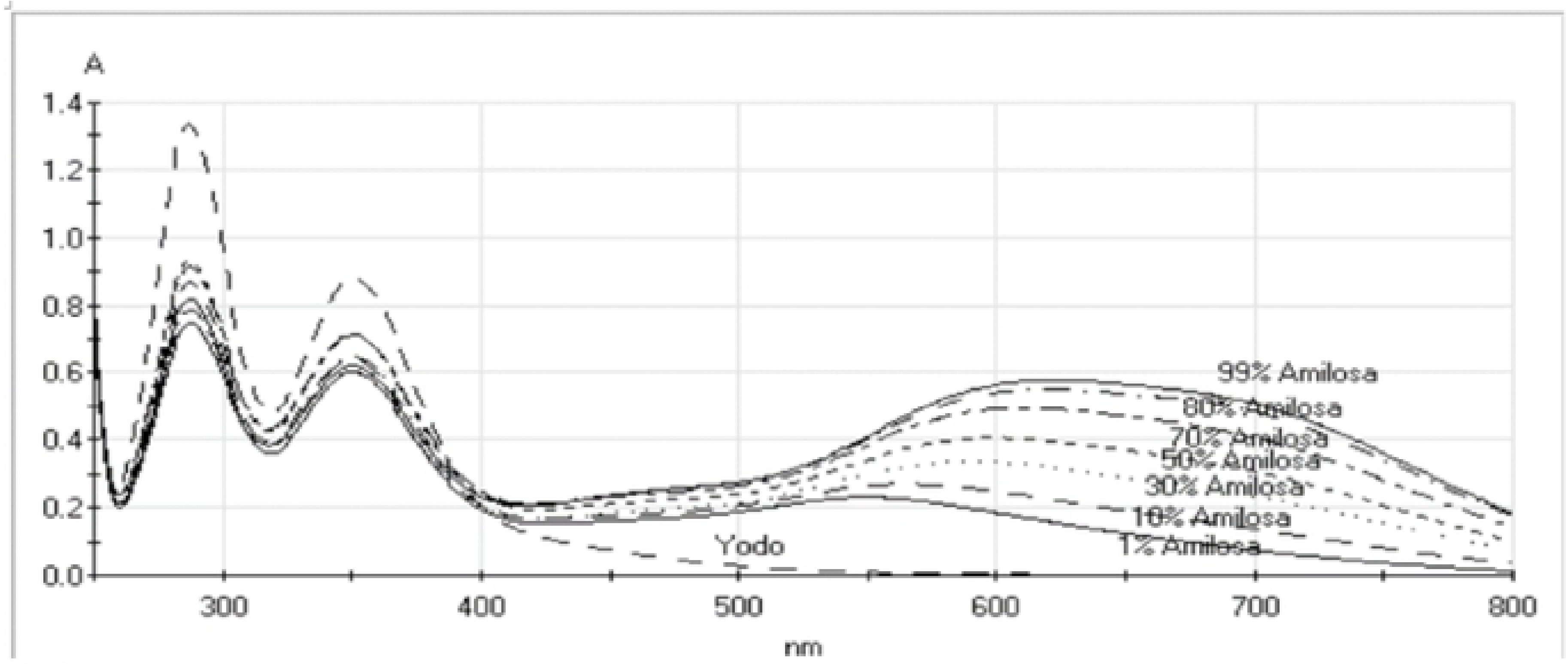


Fig 4

Figure 4

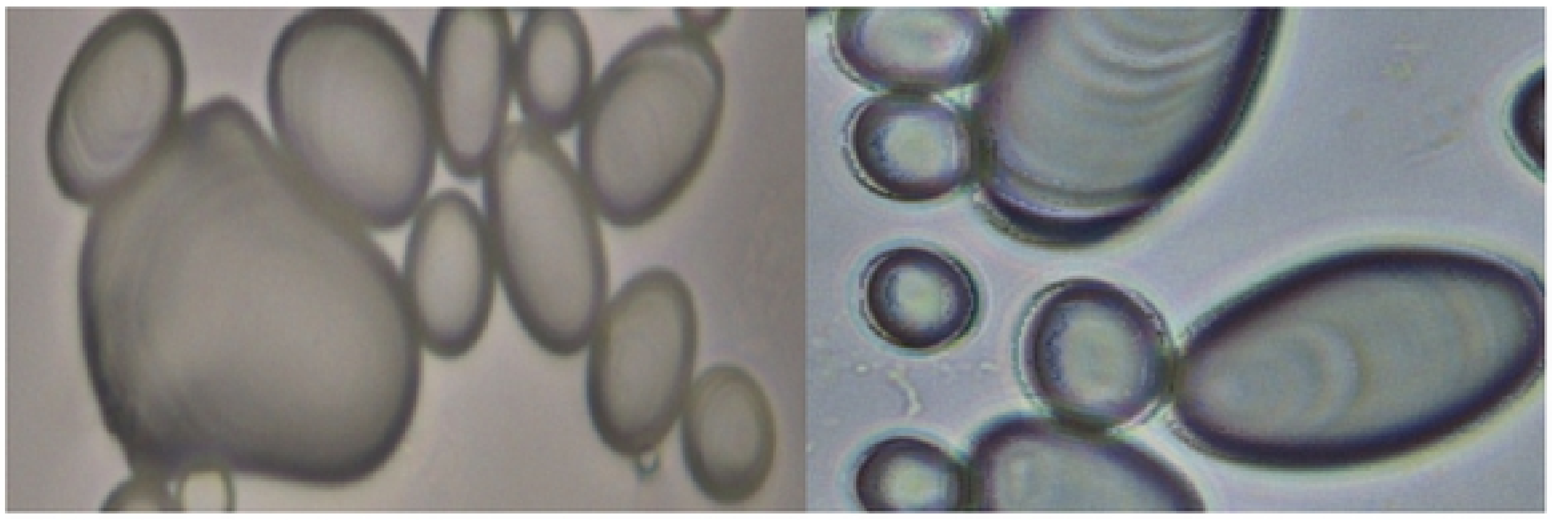


Fig 5

Figure 5

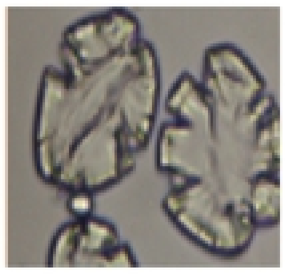
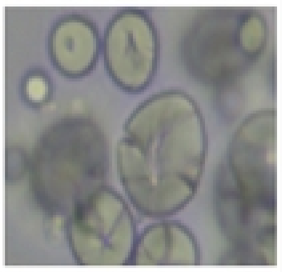
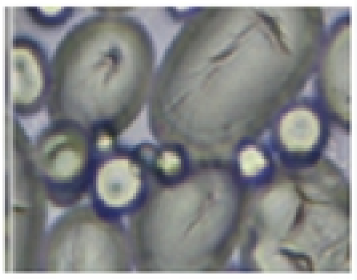
Type	Huckleberry	Muru	Multa	Bzura	Mich. October
Imag e					

Figure 6

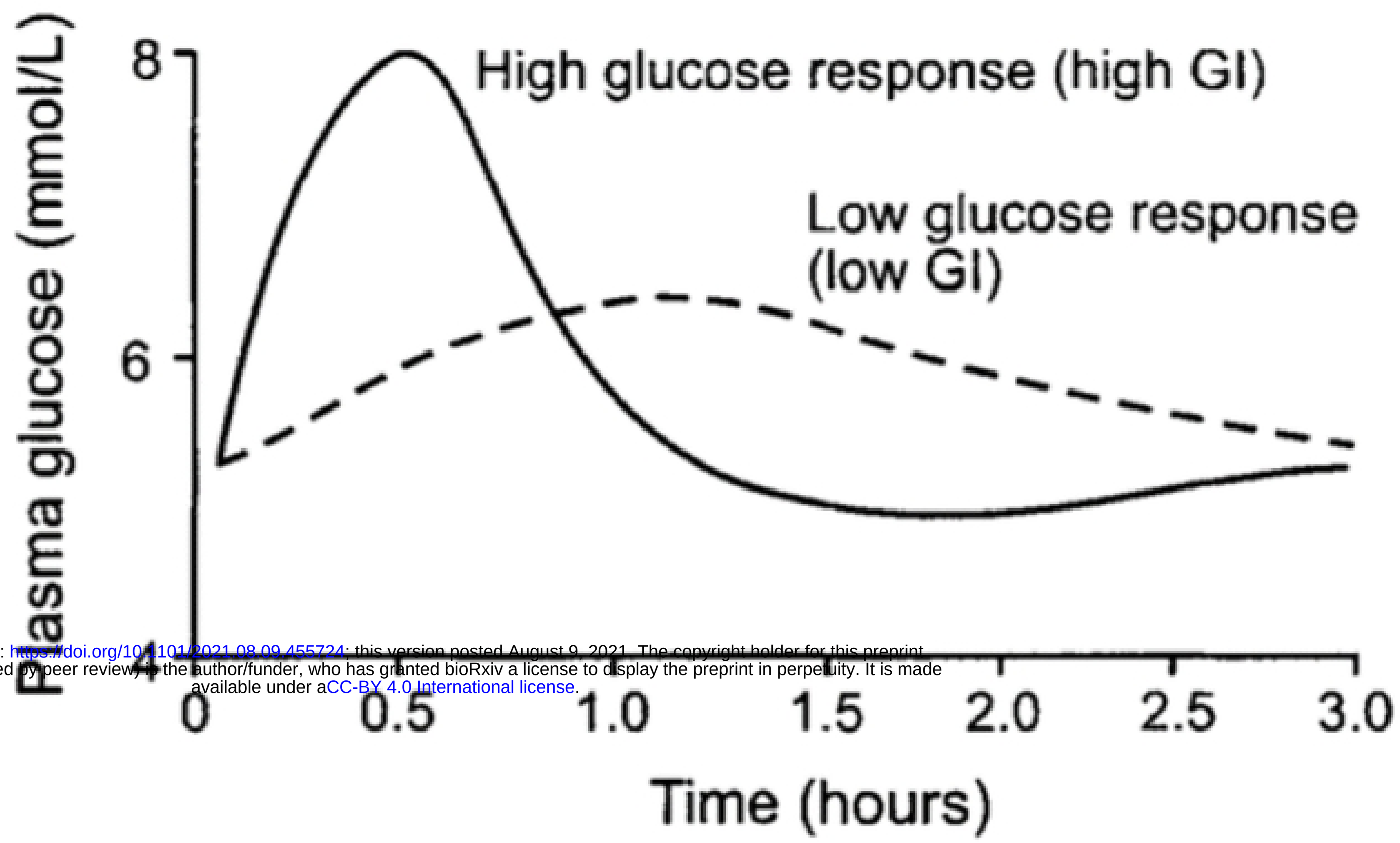


FIG 1

Fig 2

Figure 1