

Isolation of Amaranth Starch by Diluted Alkaline-Protease Treatment¹

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ABSTRACT

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Starch was isolated from *Amaranthus cruentus* seeds by different alkaline treatments and combinations of low alkaline steeping and protease treatments. For low alkaline-protease treatments, amaranth seeds were steeped in a NaOH solution (0.05%, pH 12) for 22 hr to loosen the protein matrix and ground. The pH of the ground slurry was adjusted to 7.5 and subjected to a protease (from *Aspergillus sojae*) treatment. The slurry was incubated with 1 or 0.5% of the protease (based on total amount of seeds) for 2 hr at 37°C and 50 rpm. The starch was then isolated by

screening and centrifugation. This method produced starch with a low protein content ($\leq 0.2\%$) and a high recovery ($\approx 80\%$). Amaranth starch isolated by alkaline treatments were also studied by using various concentrations of NaOH steeping solutions and with or without alkaline solution during grinding and washing. The properties of amaranth starch isolated by alkaline and low alkaline-protease treatments were analyzed and compared. The properties of the amaranth starch were also compared with those of normal and waxy maize starches.

Starch is an important ingredient for foods and has many industrial applications. In the United States, 95% of the starch produced is from corn (White 1994). Corn starch has a granular size of 5–20 μm (Jane et al 1994) and is relatively easy to isolate by wet-milling procedures. Recently, starches with novel properties and functionalities have attracted the interest of researchers and industry for expanded applications in food and other industries. Amaranth starch has received attention because of its very small granules with diameters of 1–2 μm (Becker 1989) compared with 3–8 μm of rice starch (Jane et al 1994), the smallest commercially produced starch. The extremely small starch granules of amaranth provide unique properties for many food and nonfood applications, such as fat replacers and paper coatings (Daniel and Whistler 1990, Jane et al 1992b). In addition to small granule size, amaranth starch also displays good freeze-thaw stability and resistance to mechanical shear (Yanez et al 1986, Singhal and Kulkarni 1990).

Amaranth is one of America's most ancient crops with some outstanding agronomic traits. For several reasons it became almost forgotten for many years (Perez et al 1993). In the United States, the role of amaranth as an underexploited plant with promising economic values has been recognized recently by the National Academy of Science. Today amaranth is considered as an alternative crop, and researchers in many parts of the world have focused on improving agronomic features of the plant, the nutritional quality, and processing technology of the seed. Starch is the most abundant component in amaranth seeds. Its content is reported to range from 48 to 69%, depending on the species (Saunders and Becker 1984). Despite the substantial commercial interest in this starch, there is no effective method to isolate the starch from amaranth seeds because of its small granules and relatively high protein content (14–19%) (Williams and Brenner 1995). Amaranth starch can be isolated by many different methods in the laboratory scale. Most of the methods use alkali steeping to remove the protein (Yanez et al 1986, Perez et al 1993, Myers and Fox 1994, Uriyapongson and Rayas-Duarte 1994, Zhao and Whistler 1994). High concentration of alkali damages starch quality and increases the production costs. Therefore, there is a need to develop methods that could improve processing and qualities of amaranth starch and that can be scaled up to industrial production. Removal of protein from starch can be enhanced by the addition

of proteolytic enzymes. Spanheimer et al (1972) reported that a variety of proteolytic enzymes increased protein solubility of maize grits. Steinke and Johnson (1991a,b) tested the feasibility of using steeping in the presence of multiple enzymes and sulfur dioxide to enhance starch separation and reduce steeping time. Du and Jackson (1991) modified the conventional wet-milling process by using enzymes in steeping. Eckhoff and Tso (1991) reported that the addition of protease had a significant effect on starch recovery for both high- and low-temperature-dried corn; however, an approach to modify amaranth wet-milling by using proteolytic enzymes has not been reported.

The objective of this study was to develop new methods that produced high-quality amaranth starch with high recovery and required less chemicals for the process.

MATERIALS AND METHODS

Amaranth seeds were provided by Nu-World Amaranth (Earlville, IA). Protease from *Aspergillus sojae* (Type XIX, 0.35 units/mg of solids), porcine pancreatic α -amylase, and amyloglucosidase from *Rhizopus mold* were purchased from Sigma Chemical Co. (St. Louis, MO). Normal maize and waxy maize starches were purchased from Sigma and Cerestar USA (Hammond, IN), respectively. Other chemicals, all reagent grade, were used without further purification.

Starch Isolation

Isolation of amaranth starch by alkaline wet-milling. The alkaline wet-milling method was based on that described by Myers and Fox (1994) with some modifications. Amaranth seeds (100 g) were steeped in NaOH solution (1.0, 0.25, 0.10, or 0.05%, 1L) for 24 hr and continuously stirred using a propeller stirrer at room temperature. After steeping, the steep solution was decanted, and the seeds were washed with distilled water. The sample was then milled in an unmodified Osterizer commercial food blender at full speed for 6 min. Distilled water (150 mL) or a NaOH solution of a selected concentration (0.25, 0.10, 0.05, or 0.01%) was added during grinding. The ground slurry was filtered through a nylon screen (30 μm) with additional 600 mL of distilled water or NaOH solutions of different concentrations (0.25, 0.10, 0.05, or 0.01%) for washing the fiber fraction. This fiber fraction was ground in an Osterizer blender at full speed for 4 min, washed with 250 mL of distilled water and filtered through the same nylon screen. The starch was then isolated from the filtrate by using a centrifuge at 6,000 $\times g$ for 20 min. The supernatant was discarded, and the top yellowish layer of protein was removed with a laboratory spatula. The white starch layer was resuspended in distilled water and centrifuged as described above. The process was repeated three to four times. The starch was then dried in a convection oven at 40°C for 48 hr.

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Isolation of amaranth starch by low alkaline steeping combined with protease treatment. In this process, 100 g of amaranth seeds were steeped in 1 L of a 0.05% NaOH solution (pH 12.1) for 22 hr under propeller stirring. The steeping solution was decanted and the seeds were washed with distilled water. Then the sample was blended for 6 min in the Osterizer blender at full speed. Different doses of the enzyme (1 or 0.5%, based on total amount of seeds) were added to the ground slurry (pH 7.5). The slurry was ground in the blender for 1 min at full speed and then incubated in a shaker water bath at 37°C and 50 rpm for 2 hr. After incubation, the slurry was filtered through a nylon screen (30 µm) with additional distilled water for washing the fiber fraction. The fiber fraction was further ground in the Osterizer blender at full speed for 3 min, washed, and filtered through the same nylon screen. The starch was isolated from the filtrate by centrifuging at 6,000 × g for 20 min (1% of protease). For the treatment with a dose of 0.5% of protease, the filtrate was centrifuged at 700 × g for 20 min. The supernatant was discarded, and the top yellowish layer of protein was removed with a laboratory spatula. The starch layer was washed with distilled water two times and was then dried in a convection oven at 40°C for 48 hr.

Starch Content

Starch content in amaranth seeds was determined according to the starch-glucoamylase Method 76-11 (AACC 1995) modified by subsequent measurement of glucose with diagnostic kit no. 15 (Sigma).

Nitrogen Content

Nitrogen contents of isolated amaranth starches and whole amaranth flour were determined by the micro-Kjeldahl method. Protein contents were estimated at N × 6.02 (Breene 1990).

Scanning Electron Microscopy

Scanning electron microscopy (SEM) using a scanning electron microscope (JEOL JSM-35, Tokyo, Japan) was performed at the Microscopy Laboratory of the Department of Botany, Iowa State University. The samples of isolated amaranth starch were suspended in ethanol, and a drop of the suspension was placed on aluminum tape attached to a brass disk. The specimens were coated in a Polaron E5100 sputter with gold-palladium (60:40).

Pasting Properties

Pasting curves of amaranth starches isolated by different methods, whole amaranth flour, and normal and waxy maize starches (8% each, dsb) were determined by using a Brabender Visco-amylograph (model VA-VE, 700 cm-g, Hackensack, NJ), operated with a total weight of 400 g (Smith 1964, Jane et al 1992a). The flour or starch suspension was equilibrated at 30°C and heated at a rate of 1.5°C/min with constant stirring at 75 rpm. Stirring was continued for 30 min while the paste was held at 97.5°C, and the paste was then cooled to 52.5°C (1.5°C/min).

Thermal Properties

Gelatinization and retrogradation properties of amaranth starch samples were determined by using a differential scanning calorimeter (DSC-7, Perkin Elmer, Norwalk, CT) and compared with those of normal and waxy maize starches. Amaranth starch and other tested starches (2 mg each, dsb) were weighed in aluminum pans, mixed with distilled water (≈6 mg), and sealed. The samples were heated at 10°C/min over a temperature range of 25–100°C. Retrogradation properties of the samples were determined by following the method of Jane et al (1992a).

Gel-Permeation Chromatography

Molecular weight distributions of amaranth starches isolated by both alkaline and low alkaline-protease treatments were analyzed by gel-permeation chromatography (GPC) using a Sepharose CL-

2B column following the procedure of Jane and Chen (1992). Starch (1 g) was wetted with 10 mL of water and suspended in dimethyl sulfoxide (DMSO) (90 mL) under mechanical stir and heated in a boiling water bath for 1 hr and then stirred for 24 hr at 25°C to prepare a starch solution (1%). An aliquot (2 mL) of the starch solution was mixed with absolute ethyl alcohol (8 mL) to precipitate the starch, followed by centrifugation. The precipitated starch was redissolved in boiling water (10 mL) and stirred for 30 min, and the mixture was filtered to remove the insoluble residues. The supernatant (5 mL) then was injected into a 2.6- × 85-cm column packed with Sepharose CL-2B gel (Pharmacia Inc., Piscataway, NJ). The column was eluted with a solution containing 25 mM NaCl and 10 mM NaOH in the ascending direction. The flow rate was 0.5 mL/min. Fractions of 4.8 mL were collected. The total carbohydrate (anthron-sulfuric acid method) and blue value (iodine binding) of each fraction were analyzed by using an Autoanalyzer II (Technicon Instruments Corp., Elmsford, NY) at 630 and 640 nm, respectively (Jane and Chen 1992).

Amylose Content

To determine amylose content, iodine affinity of defatted starch was measured by using a potentiometric autotitrator (702 SM Titrino, Brinkmann Instrument, Westburg, NY). The analysis was done following the methods of Schoch (1964) and Kasemsuwan et al (1995). Iodine affinity analysis of the sample was replicated three times.

RESULTS AND DISCUSSION

Whole *A. cruentus* grain used for starch isolation contained 60% ± 1 starch and 15.9% ± 0.1 protein. The starch and protein contents of whole grain fell within the range of values reported in the literature (Saunders and Becker 1984, Yanez et al 1986, Uriyapongson and Rayas-Duarte 1994).

Comparison of Laboratory Wet-Milling Procedures

Performance of each amaranth starch isolation method was evaluated on the basis of recovery (ratio of extracted starch to the starch content of the grain), yield (ratio of extracted starch to amount of grain), and purity of starch (protein content of starch). High starch recovery, high starch yield, and low protein content in starch are indicators of good wet-milling.

The data in Table I showed starch recoveries, starch yields from whole grain, and protein content in starch isolated by using 1 and 0.25% NaOH solutions (pH 13.2 and 12.7, respectively) for steeping of amaranth seeds. The data showed that a greater amount of starch with lower protein content was recovered from

TABLE I
Protein Contents and Recoveries of Amaranth Starch Isolated by Using Different Concentrations of NaOH Solution for Steeping Amaranth Seeds

NaOH Concentration (%)	Protein Content (%)	Starch Recovery (%)	Starch Yield (%)
0.25	0.40 ± 0.12	72.4 ± 5.4	43.6 ± 3.3
1.00	0.08 ± 0.00	79.4 ± 1.1	47.8 ± 0.7

TABLE II
Comparison of Protein and Recovery of Amaranth Starch Isolated by Using 0.05% NaOH Steep and Different Doses of Protease Treatments of Amaranth Seeds

Protease (%)	Protein Content (%)	Starch Recovery (%)	Starch Yield (%)
0 ^a	0.22	71.8	43.2
0.5	0.18 ± 0.05	79.8 ± 0.2	48.0 ± 0.1
1.0	0.10 ± 0.00	83.5 ± 0.3	50.3 ± 0.2

^a 0.05% NaOH for steeping and washing.

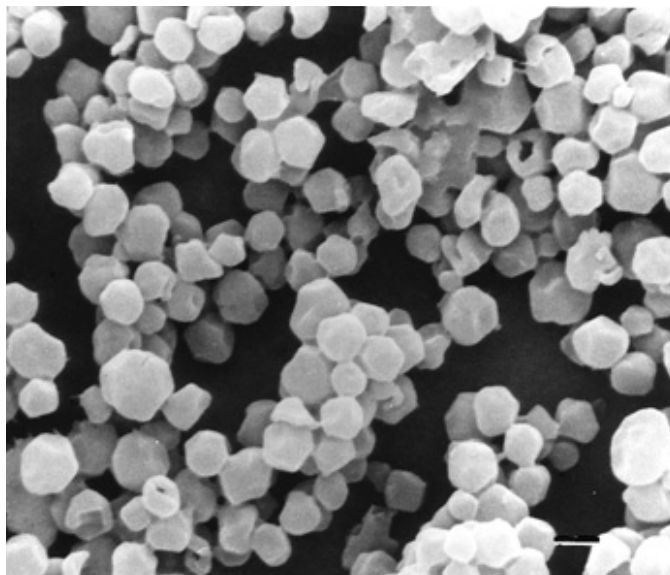


Fig. 1. Scanning electron micrograph of starch isolated from amaranth seeds by low alkaline-protease treatment (5,400 \times). Bar = 1 μ m.

amaranth seeds steeped in a NaOH solution of higher concentration. The greater starch yield from amaranth seeds steeped in more concentrated alkali is expected because the glutenins, the major protein fraction in amaranth grain, ranging from 42 to 46% of the total protein, are soluble in alkaline solution. Lower concentrations of NaOH for steeping of amaranth seeds (0.25 and 0.10%) significantly decreased starch recovery from 79.4% for 1% NaOH steep to 72.4 and 68.1% for 0.25 and 0.10% NaOH, respectively. In addition, the protein content of the isolated starch was high (1.62%), especially when a 0.10% NaOH solution was used for steeping. The higher protein content in the isolated starch is attributed to the decrease in protein solubility in the lower alkaline solution.

To improve starch recovery, starch yield, and starch purity by milling with lower concentrations of alkali for steeping, NaOH solutions of different concentrations were added during grinding and washing steps. Addition of 0.25 and 0.05% of NaOH solution during grinding and washing of amaranth seeds steeped in 0.25% NaOH solution gave similar yields (48.0 and 48.3%, respectively) and starch purities (protein contents 0.08 and 0.15%, respectively) as high alkali steeping (1% NaOH solution) (Table I). Similar results were also obtained by additions of 0.10 and 0.05% NaOH solution during grinding and washing of amaranth seeds steeped in 0.1% of NaOH solution (yields 48.9 and 48.2%, respectively; protein contents 0.15 and 0.19%, respectively). Higher recoveries were expected because addition of diluted alkali solution during grinding and washing helped to remove protein and improve starch isolation. But the addition of 0.01% NaOH solution during grinding and washing of amaranth seeds steeped in 0.25, 0.1, or 0.05% NaOH solution and the addition of 0.05% of NaOH during grinding of the seeds steeped in 0.05% NaOH (Table II) solution resulted in poor yields (45.4, 44.8, 46.9, and 43.2%, respectively) and higher protein contents of isolated starch (0.20, 0.45, 1.88, and 0.22%, respectively). The starch recoveries and yields obtained in this study by alkaline wet-milling treatments were much higher than those obtained by similar methods published recently (Uriyapongson and Rayas-Duarte 1994, Myers and Fox 1994, Zhao and Whistler 1994).

Table II shows results obtained by a method using less concentrated NaOH (0.05% solution) for steeping and followed by a protease treatment. The yields of starch with these alkaline-protease treatments were better than those with alkali alone. During the protease treatment, the enzyme hydrolyzes the protein matrix surrounding the starch granules and facilitates the separation of

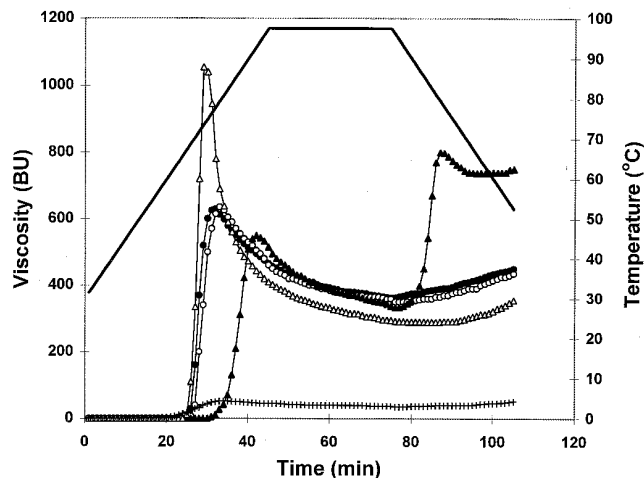


Fig. 2. Pasting properties of various starches and whole amaranth flour as measured by Brabender Viscoamylograph. Preparations and purities of amaranth starches as in Table II. Amaranth starch (0.05% NaOH, 0.5% protease) (●), amaranth starch (0.05% NaOH for steeping and washing) (○), normal maize starch (▲), waxy maize starch (Δ), amaranth flour (+), temperature (solid black line).

starch by washing with water. The starch yields, starch recoveries, and starch purity were also good with low concentration protease (0.5%) treatments. The change of centrifuging force from 60,00 to 700 \times g did not affect starch recovery and yield, but changing centrifugation force from 700 to 200 \times g resulted in lower starch recovery (from 79.8 to 70.8%) and yield (from 48.0 to 42.7%).

Properties of Isolated Starch

Diameters of amaranth starch granules were in the range of 0.5 to 1.5 μ m and had a polygonal shape similar to that of other amaranth cultivars (Saunders and Becker 1984). The method of isolation did not affect the size and shape of the granules. A scanning electron micrograph of amaranth starch isolated by low alkaline-protease treatment is shown in Fig. 1.

Pasting properties. Pasting properties of amaranth starches isolated by alkaline and low alkaline-protease treatments were compared with those of normal and waxy maize starches (Fig. 2). Pasting and peak temperatures of amaranth starches were lower than those of normal maize starch, but higher than those of waxy maize starch. Amaranth starch isolated by alkaline treatment (NaOH 0.05% for steeping and washing) had higher pasting and peak temperature and peak viscosity than those isolated by low alkaline-protease treatment. Set-back viscosities of both isolated starches were almost the same and similar to that of waxy maize starch. Whole amaranth flour showed very low values for all the tested properties.

Thermal properties. Thermal properties of amaranth starches isolated by both alkaline and low alkaline-protease treatments, and normal and waxy maize starches are shown in Table III. The thermal transition temperature and enthalpy changes of native and retrograded starch isolated by low alkali-protease treatment were lower than those isolated by alkaline wet-milling. The DSC thermal properties of amaranth starches isolated by low alkaline-protease treatment were very similar to those of normal and waxy maize starch. The DSC thermal transition data of whole amaranth flour were between those of starches isolated by alkaline and by low alkaline-protease treatment. The enthalpy change of flour was substantially lower than those of starch samples. This was attributed to the high protein content and other components in the flour.

Molecular weight distribution. Gel-permeation chromatograms of amaranth starch did not show any difference in molecular weight distribution between the starches isolated by the two methods. GPC profiles of amaranth starches isolated by both alkaline (1% NaOH) and low alkaline-protease (1% protease) treatments

TABLE III
Thermal Properties^a of Starches and Whole Amaranth Flour

Sample ^b	T _o (°C)	T _p (°C)	T _c (°C)	ΔH (J/g)
Gelatinization				
Amaranth starch (alkaline treatments) ^c	70.6 ± 1.0	74.3 ± 1.1	82.9 ± 1.4	15.0 ± 1.6
Amaranth starch (low alkaline-protease treatments) ^d	65.5 ± 0.9	69.2 ± 0.9	77.7 ± 1.1	14.4 ± 0.7
Normal maize starch ^e	64.7 ± 0.4	70.8 ± 0.4	78.6 ± 0.2	12.4 ± 0.2
Waxy maize starch ^e	64.0 ± 0.3	69.0 ± 0.4	76.7 ± 0.9	15.0 ± 0.4
Whole amaranth flour ^e	68.0 ± 0.9	72.4 ± 0.1	77.9 ± 0.1	2.0 ± 0.2
Retrogradation ^f				
Amaranth starch (alkaline treatments) ^c	45.9 ± 1.5	54.0 ± 0.8	62.5 ± 1.7	6.6 ± 1.1
Amaranth starch (low alkaline-protease treatments) ^d	45.2 ± 1.7	52.8 ± 1.1	60.0 ± 0.8	5.1 ± 1.0
Normal maize starch ^e	43.3 ± 0.2	51.8 ± 0.0	61.7 ± 0.3	7.6 ± 0.1
Waxy maize starch ^e	45.0 ± 1.2	53.7 ± 0.3	62.5 ± 0.5	9.8 ± 0.2
Whole amaranth flour	nd ^g	nd	nd	nd

^a T_o, T_p, and T_c = onset, peak, and complete temperature, respectively. ΔH = enthalpy change.

^b Starch samples (≈2 mg, dsb) and distilled water (≈6 mg) used for analysis.

^c Mean ± standard deviation calculated from three replicates of 19 samples isolated by alkaline treatments (1.0, 0.25, 0.10, and 0.05% NaOH).

^d Mean ± standard deviation calculated from three replicates of 15 samples isolated by low alkaline-protease treatments (0.05% NaOH).

^e Mean ± standard deviation calculated from three replicates.

^f After storage at 4°C for 28 days.

^g Not detectable.

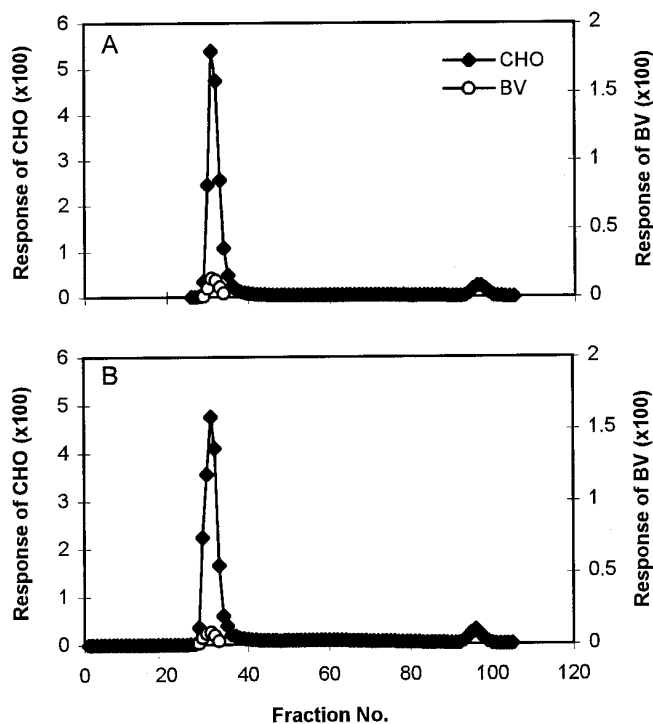


Fig. 3. Sepharose CL-2B gel permeation profile of starch isolated from amaranth seeds. A, alkaline treatment (1% NaOH); B, low alkaline-protease treatment (1% protease). CHO = total carbohydrate, BV = blue value. Glucose used as marker.

are shown in Fig. 3. All the chromatograms showed no amylose in the starch, indicating a waxy variety.

Amylose content. Amylose content of the isolated amaranth starch was determined by measuring iodine affinity. The iodine affinity and apparent amylose content of the isolated amaranth starch were 0.4 and 2.1%, respectively. These were attributed to long branch chains of amylopectin. Amaranth starch amylose contents determined by iodine potentiometric titration and GPC are in reasonably good agreement.

CONCLUSION

This study showed that amaranth starch could be isolated with 0.2% protein content by a low alkaline steeping followed by protease treatment. The recovery of this method is ≈80% of the total

starch. This method has been scaled up for pilot plant processing for amaranth starch production. The method requires much less NaOH for the processing, which reduces production costs of the starches. In addition, the method produces starch with better quality and recovery.

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