

Method for Determining the Rate and Extent of Accelerated Starch Retrogradation¹

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ABSTRACT

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A method to accelerate and quantitate retrogradation of starch pastes using a freeze-thaw cycle (FTC) process and turbidometric analysis has been developed. Using this method and differential scanning calorimetry (DSC), it was determined that the rate of retrogradation in 2.5% waxy maize pastes was inversely correlated to the rate of freezing, and that the thawing temperature affected perfection of the crystallites in retrograded amylopectin. DSC and X-ray diffraction were used to determine whether

the crystallites formed during the FTC process were the same as those formed in starch pastes held isothermally at 4°C. Analysis of retrogradation of pastes of starches from various botanical sources indicated that the method reflects retrogradation in higher concentration pastes. Retrogradation rates were reduced by the addition of sodium dodecyl sulfate. Microstructures of freeze-thaw processed waxy maize and common corn starch pastes were examined.

Retrogradation of gelatinized starch is a crystallization process involving both starch polymers, with amylose undergoing retrogradation at a much more rapid rate than amylopectin. The rate and extent of retrogradation depends on the mole ratio and structures of amylose and amylopectin, temperature, starch-water concentration, method of cooking (time, temperature, amount of shear), botanical source of the starch, and kind, concentration, and time of addition of other ingredients. Several methods have been used to accelerate retrogradation. The purpose of accelerating the process has generally been to test, as rapidly as possible, how it is affected by the variables.

The first study of the effect of temperature on the rate of retrogradation was done by Maquenne (1903), who found that the rate of potato starch paste retrogradation increased as the temperature was reduced. Since the discovery of this temperature dependence, it has been used as a means to accelerate the testing of variables on both retrogradation and staling (believed to be due, at least in part, to amylopectin retrogradation).

Polymer crystal growth theory states that there are three phases to polymer crystallization (nucleation, propagation, and maturation). Slade and Levine (1987) and Marsh and Blanshard (1988) have determined that amylopectin crystallization is a nucleation-limiting process occurring at a temperature above T_g (or T_g' if the starch concentration is <70%) and below T_m (where T_g is the glass transition temperature; T_g' is the glass transition temperature of the maximally freeze-concentrated starch [$\approx -5^\circ\text{C}$], and T_m is the melting temperature of crystalline amylopectin [$\approx 60^\circ\text{C}$])). The maximum rate of nucleation occurs at temperatures slightly greater than T_g (or T_g' depending on concentration), while the maximum rate of propagation occurs at a temperature slightly less than the T_m of crystallized amylopectin. The retrogradation rate of starch pastes held under isothermal conditions should be greatest at a temperature between the optimal temperatures for nucleation and propagation, or $\approx 5^\circ\text{C}$ for a 50% paste (Slade and Levine 1987, Marsh and Blanshard 1988).

Slade and Levine (1987) applied polymer crystallization theory to develop a method to accelerate retrogradation termed temperature-cycling. By cycling between the temperature of greatest nucleation rate and the temperature of greatest propagation rate for set periods of time, rates of both retrogradation of concentrated starch pastes and staling of bread crumb were increased over those which occurred

under optimal isothermal conditions. Temperature cycling has been used to accelerate the staling of bread in the production of croutons (Slade et al 1987) and to test staling of cooked rice (Villareal et al 1993, Perez et al 1993). However, it did not accelerate the production of enzyme resistant starch in wheat starch pastes of lower concentration (10%) (Eerlingen et al 1993).

Woodruff and McMasters (1938) evaluated microstructural changes produced by freezing of starch pastes and were among the first to conclude that freezing causes retrogradation. Albrecht et al (1960) examined the effects of freezing, storage, and thawing on pastes of common corn starch; on a pregelatinized crosslinked waxy maize starch; and on a starch phosphate that was soluble in cold water and produced a paste of unusual clarity. Among their conclusions were that a single freeze-thaw cycle (FTC) had no effect on the degree of retrogradation of the two derivatized starches and that rapid freezing conditions (immersion in liquid N_2) eliminated retrogradation (determined by β -amylase susceptibility). The latter finding confirmed the report of Volz and Ramstad (1952) that β -amylase susceptibility of pastes undergoing an FTC process decreased as the freezing rate increased. FTC procedures are used to retrograde potato starch to improve the properties of processed potato granules (Ooraikul and Hadziyev 1974, Ooraikul et al 1974). They have caused retrogradation-crystallization in 20% potato starch pastes (Eberstein et al 1980), 3% tapioca starch pastes (Mercier et al 1987), and 6% common corn starch pastes (Matsunaga and Kainuma 1986). In a study on the freeze-thaw stability of pastes (30%) prepared from starches of various botanical sources, White et al (1989) followed the development of a staling endotherm using an FTC process. When these pastes were FTC processed 10 times (-10°C for 24 hr, then 25°C for 1.5 hr), they produced similar thermograms and retrogradation enthalpies as did pastes held at 4°C for seven days. In a study of the retrogradation of waxy starches, Shi and Seib (1992), found that 50% waxy maize starch (WMS) pastes held isothermally (for one to four weeks) or subjected to an FTC process (10-40 FTC; -20°C for 22 hr, then room temperature for 2 hr) underwent retrogradation at similar rates and to similar extents. Additionally, 25% and 50% WMS pastes were retrograded at different rates and to different extents (Shi and Seib 1992).

Most of these investigations used differential scanning calorimetry (DSC). To analyze retrogradation by DSC, a starch concentration of >20% is required, and the starch is gelatinized in a calorimeter pan, producing swollen but nondisrupted granules. The pan is then subjected to retrogradation conditions, and the contents are reanalyzed by DSC. The conditions of gelatinization, therefore, are more like those encountered during baking than those encountered during starch cooking for other purposes. The rate and extent of retrogradation during FTC processing of starch pastes of lower concentration could be increased by temperature

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reduction (greater nucleation rates, which could occur during the freezing or the thawing of the paste or while it is frozen) and increased starch concentration due to freeze-concentration. During the freezing, liquid water is removed by crystallization, increasing the concentration of starch. The starch maximally freeze-concentrates to a glassy state, leaving a two-phase system of ice and a glass consisting of ≈70% starch and ≈30% nonfreezable water (Slade and Levine 1987). Because the maximum retrogradation rate occurs at ≈40–60% starch (Longton and LeGrys 1981, Eliasson 1983, Zeleznak and Hosenev 1986), as starch pastes freeze or thaw, the rate of retrogradation should be maximum as the effective paste concentration passes through the 40–60% range.

Measurements of light-scattering (Paschall and Foster 1952, Foster and Serman 1956) and reduction in transmitted light (Miles et al 1985, Ring et al 1987, Gidley and Bulpin 1989) have been used to follow retrogradation in both low concentration (<2%) starch pastes and solutions of amylose and amylopectin. These methods measure turbidity development which results from molecular associations that occur during early stages of the retrogradation process, before larger-scale organizations that are detected by means such as DSC and X-ray diffraction (Ring et al 1987).

The objective of this work was to develop a simple method to accelerate and quantitate retrogradation in low-concentration starch pastes so that the effects of variables, especially other ingredients, could be evaluated. Initially, the effects of isothermal conditions, temperature cycling protocols (TCP), and FTC processes on low-concentration WMS pastes were evaluated. Results (not given) indicated that the order of effectiveness of the methods in accelerating retrogradation was FTC > isothermal treatment at 4°C > TCP. The reason why TCP were ineffective is unknown. It may be related to the low starch concentration or the high amylopectin content of the starch used. Regardless, this preliminary investigation indicated that a method combining an FTC process with turbidometric analysis would achieve the stated objectives. This article describes optimization of the method and results of the evaluation of the effects of freezing and thawing temperatures (which influence the rates of freezing and thawing), botanical source of starch, and concentration of a water-soluble, known retrogradation inhibitor on the rate and extent of retrogradation.

TABLE I
Analytical Data (%) for Starches Used

Starch	Moisture ^a	Protein ^b	Amylose ^c
Wheat	11.7	0.208	23
Common corn	12.8	0.254	22
Potato	14.8	0.068	21
Tapioca	11.7	0.029	20
Rice	10.7	0.297	16
Waxy maize	12.9	0.168	1.8

^a Approved Method 44-40 (AACC 1995). Average of duplicate analyses.
^b Kjeldahl nitrogen determination (N × 5.7). Average of duplicate analyses.
^c Method of Chrastil (1987). Average of triplicate analyses.

TABLE II
Thermal Analysis of 20% Waxy Maize Starch Pastes

Freeze-Thaw		Staling Endotherm ^a			
(°C)	Cycles	T _o	T _p	T _f	ΔH (J/g)
Paste held at 4°C for 7 days (168 hr)					
...	...	44.1	55.6	66.9	5.1 ± 0.7
FTC processed pastes ^b					
-10/10	20		47.5		
-10/25	20		52.3		
-10/30	20		55.5		
-10/30	10	45.0	54.6	61.6	2.6 ± 0.4
-10/30	20	45.2	55.8	66.4	8.0 ± 0.8

^a Temperatures at onset, peak and final and enthalpy, respectively.
^b FTC = freeze-thaw cycle.

Materials

Waxy maize starch (WMS) (11.7% moisture) and common corn starch (CCS) (10.8% moisture) were obtained from A.E. Staley Mfg. Co. (Decatur, IL), potato starch (12.1% moisture) from Penwest Foods Co. (Englewood, CO), rice starch (11.2% moisture) from Sigma Chemical Co. (St. Louis, MO), wheat starch (9.9% moisture) from Midwest Grain Products, Inc. (Atchison, KS), tapioca starch (12.0% moisture) from National Starch and Chemical Co. (Bridgewater, NJ), sodium azide from MCB Mfg. Chemists, Inc. (Cincinnati, OH), soluble starch (10.2% moisture), and ethylene glycol from J. T. Baker, Inc. (Phillipsburg, NJ), and sodium dodecyl sulfate (SDS) (>99%) from Bio-Rad Laboratories (Richmond, CA). All starches and chemicals were used as received. Analytical data on the starches used are presented in Table I.

General

A UV-visible spectrophotometer (Varian DMS 80) equipped with a programmable cell changer and a printer (Citizen MSP-10) was used to determine total carbohydrate concentration and turbidity (as absorbance). A refrigerated waterbath (Neslab RTE-210) with 50% ethylene glycol was used as a primary freezing bath; a refrigerated waterbath (Lauda RM6) with 50% ethylene glycol was used as the secondary freezing bath (in experiments requiring two waterbaths). A refrigerated bath with 50% ethylene glycol (Lindberg/Blue M Magni-Whirl) was used as a thawing bath. DSC analysis was performed using a differential scanning calorimeter (Mettler DSC30) with a TC10A control center and standard aluminum 40-μL DSC pans (model ME-27331). Using an empty pan as a reference, DSC scans were made from 20–120°C at a rate of 10°C/min. Approved Method 44-40 (AACC 1995) was used to determine moisture content of the starches.

Pastes used in this investigation were prepared by atmospheric cooking (≈100°C) with only mild shear, so complete molecular dispersions were never formed and some crystallization nuclei were, therefore, undoubtedly present in every initial paste. Waterbaths were used to give maximum heat transfer, greater rates of heating and cooling, and maximum temperature control. Polycar-

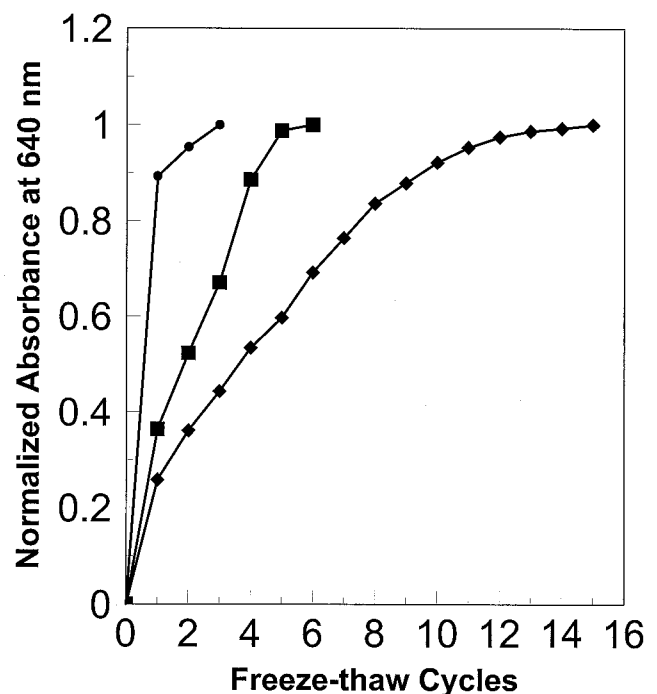


Fig. 1. Normalized turbidity development in a 2.5% waxy maize starch paste as a function of number of freeze-thaw cycles at -15°C/freezer/30°C (●), -10°C/30°C (■), and -20°C/30°C (◆).

bonate centrifuge tubes provided both good thermal shock resistance and transparency for sample viewing. A uniform size of tube and a constant volume of paste is essential for success of this method. WMS was used as one of the starches because amylopectin is the molecule believed to be responsible for long-term quality defects in starch-containing food products. However, the WMS was a commercial product and, therefore, contained the expected, usual contamination with CCS (1–3%), resulting in pastes that should have contained 0.25–0.75% amylose. The 2.5% starch pastes were used for turbidometric analysis because preliminary experiments (Jacobson 1994) indicated that concentrations of >2.5% were too turbid to be measured spectrophotometrically. A thawing time of 1 hr was used because preliminary work (Jacobson 1994) revealed that sample turbidity remained constant after 1 hr. A minimum of 1 hr of freezing time (for samples frozen in waterbaths) was used because samples were completely frozen at that time. Once frozen, samples kept below the freezing temperature had short-term stability (less than three days) to changes that resulted in increased turbidity. This is reflected in curve smoothness, even though some samples were stored overnight during FTC processing, and it is due to freeze-concentration of the starch to a final highly viscous glass. Once in this glassy state, starch molecules lack the molecular motion required to crystallize at an appreciable rate (Slade and Levine 1987). Long-term storage in a frozen state, which was avoided, may result in ice recrystallization and microstructural changes that could effect turbidity measurements upon thawing (Eliasson 1985, Eliasson and Kim 1992, Ferrero et al 1992). All FTC processing was done on the same volume of starch paste in the same size polycarbonate tubes so that the rates of heat transfer and freezing would be as constant as possible. Periodic shear (vortex mixing) was used during thawing to assist resolubilization of noncrystalline molecules, to minimize temperature gradients, and to yield a homogeneous paste that provided consistent turbidometric measurements.

Testing of the reproducibility of results (Jacobson 1994) indicated that triplicate 2.5% WMS pastes required the same number of FTC at $-10/30^{\circ}\text{C}$ for complete retrogradation (precipitation occurred) with <1% variation in normalized absorbance values. Results of turbidometric analysis were expressed in normalized absorbances to allow comparison of results when the factors tested influenced initial paste turbidity (such as the botanical source of the starches). Normalized absorbance was calculated as the $A_i -$

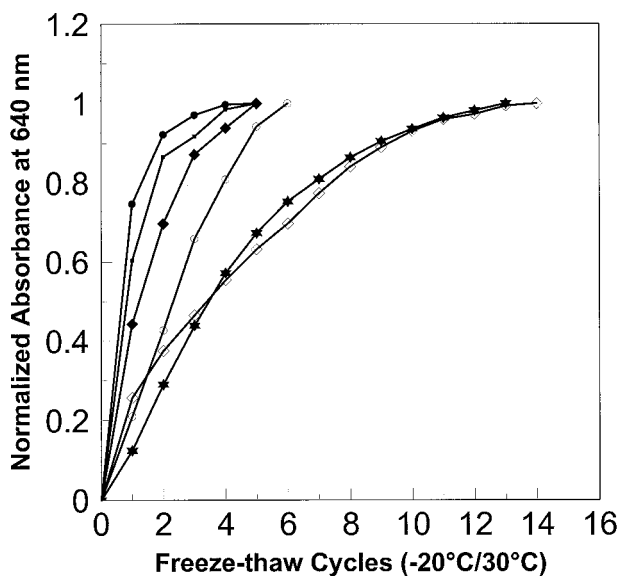


Fig. 2. Normalized turbidity development in 2.5% pastes of various starches as a function of number of freeze-thaw cycles as determined using the developed method. Potato (●), common corn (○), wheat (◆), rice (○), tapioca (★), and waxy maize (◇).

$A_0/A_i - A_0$, where A_0 , A_i , and A_f were the absorbances measured at 640 nm (A_{640}) of the fresh paste, paste after i FTC, and fully retrograded paste (one FTC before precipitation), respectively.

Soluble Starch Determination

To determine the completeness of retrogradation by an FTC process, the amount of starch remaining in solution after centrifugation was determined by the phenol-sulfuric acid assay (Dubois et al 1956). Standards were prepared by sealing 0.1670 g of soluble starch and 100.0 mL of water in a screw-capped plastic bottle and heating the bottle 10 min in a boiling waterbath while stirring slowly with a magnetic stirrer. The paste was then cooled by placing the bottle in a 1-L room-temperature waterbath for 15 min with constant slow stirring (magnetic stirrer). Dilutions of the resulting solution (from 1:10 [$150 \mu\text{g/mL}$] to 1:20 [$7.5 \mu\text{g/mL}$]) were made. Soluble starch concentrations were determined from the standard curve.

Effects of Freezing Temperatures

WMS paste (2.5%, w/w) was prepared by combining 2.1237 g of WMS and 72.88 mL of 0.02% NaN_3 solution (to prevent microbial growth) in a 125-mL screw-capped plastic bottle. The sealed bottle was placed in a 100°C waterbath for 20 min, and the contents were stirred slowly (magnetic stirrer). The paste was cooled 10 min in a $25 \pm 2^{\circ}\text{C}$ waterbath with slow stirring. Into three 28-mL polycarbonate centrifuge tubes (Oak Ridge) were placed 20.0 mL of paste. The tubes were sealed, and the contents were frozen by placing the tubes in either -20 or -10°C waterbaths or a freezer (-15°C). The remaining unfrozen paste was placed under slight vacuum to remove air bubbles, and initial turbidity was determined by absorbance at 640 nm (A_{640}) (Miles et al 1985) in triplicate. Centrifuge tubes containing paste samples were kept in the respective waterbaths for at least 1 hr, while the third tube was kept in the freezer for at least 12 hr. Frozen pastes were thawed by placing the tubes in a 30°C waterbath for 1 hr with vortex mixing

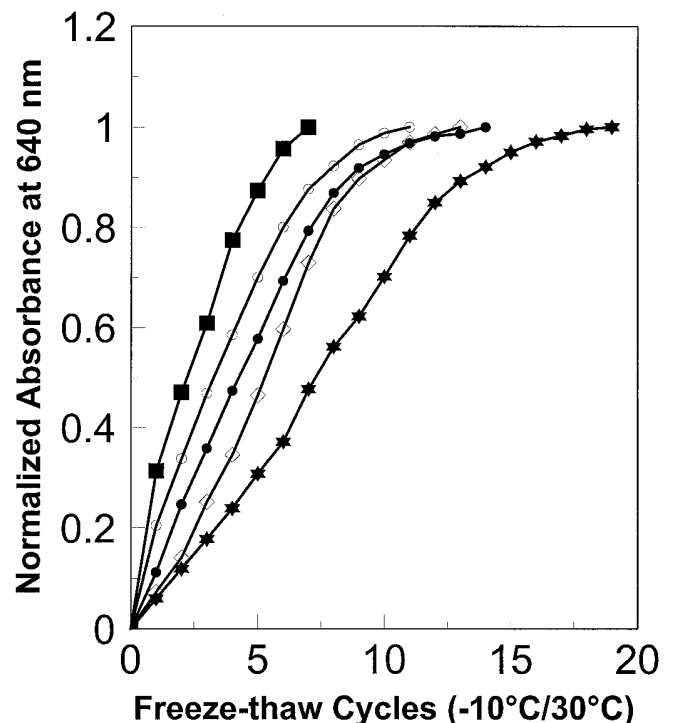


Fig. 3. Normalized turbidity development in a 2.5% waxy maize starch paste in the presence of various concentrations of SDS (mmol of SDS/g of starch) as determined using freeze-thaw cycle and turbidometric analysis. Control (■), 0.013 (○), 0.027 (●), 0.053 (★), and 0.027 (◇) after gelatinization. Note: 0.027 mmol of SDS/g of starch provides the same molar ratio of surfactant to starch as 1 g of SSL/100 g of starch (i.e., 1% SSL based on the weight of the starch).

for 10 sec every 15 min. Pastes were placed briefly under vacuum to remove bubbles, and paste A_{640} was determined in triplicate. The samples were then returned to their respective tubes and re-frozen as before. Cycles of freezing, thawing, and absorbance determinations were repeated until precipitation occurred. The mixtures were then centrifuged 15 min at $800 \times g$. The supernatant was diluted 1:100, and the total carbohydrate (soluble starch) concentration was determined in triplicate.

Effects of Thawing Temperature

Three WMS slurries were prepared by combining 4.5305 g of starch and 20.0 mL of 0.02% NaN_3 solution in 28-mL polycarbonate centrifuge tubes and vortex mixing the sealed tubes to disperse the starch. To gelatinize the starch homogeneously at this high concentration (20 wt%, db), the tubes were placed 1 min in a boiling waterbath, vortex mixed 10 sec, placed back in the boiling waterbath for 1 min, vortex mixed 10 sec, then finally placed back in the boiling waterbath for 18 min. Samples were cooled by placing the tubes in a $25 \pm 2^\circ\text{C}$ waterbath for 5 min. The resulting

pastes were frozen by placing the tubes in a waterbath at -10°C for at least 1 hr, and then thawed by placing the tubes in baths at 10, 25, or 30°C . The FTC process was repeated 10–20 times until DSC analysis showed paste staling endotherm enthalpies $>1 \text{ J/g}$. For DSC, paste samples (10–12 mg) were weighed accurately into preweighed pans which were then sealed. The staling endotherm onset (T_o), peak (T_p), and final (T_f) temperatures and enthalpies were determined (uncorrected for dry starch weight).

Comparison of Isothermal Conditions and the FTC Process

Two WMS slurries (20%, w/w) were prepared by combining 4.5305 g of WMS and 15.47 mL of 0.02% NaN_3 solution in 28-mL polycarbonate centrifuge tubes and immersing the sealed tubes 20 min in a boiling waterbath, with mild agitation for the first 3 min to gelatinize the starch homogeneously. Tubes were cooled by placing them 10 min in a 1-L room-temperature ($25 \pm 2^\circ\text{C}$) waterbath. One tube was held at 4°C in a cold room, while the other tube underwent the FTC process by freezing at least 1 hr in a -10°C waterbath and thawing 1 hr in a 30°C waterbath. After

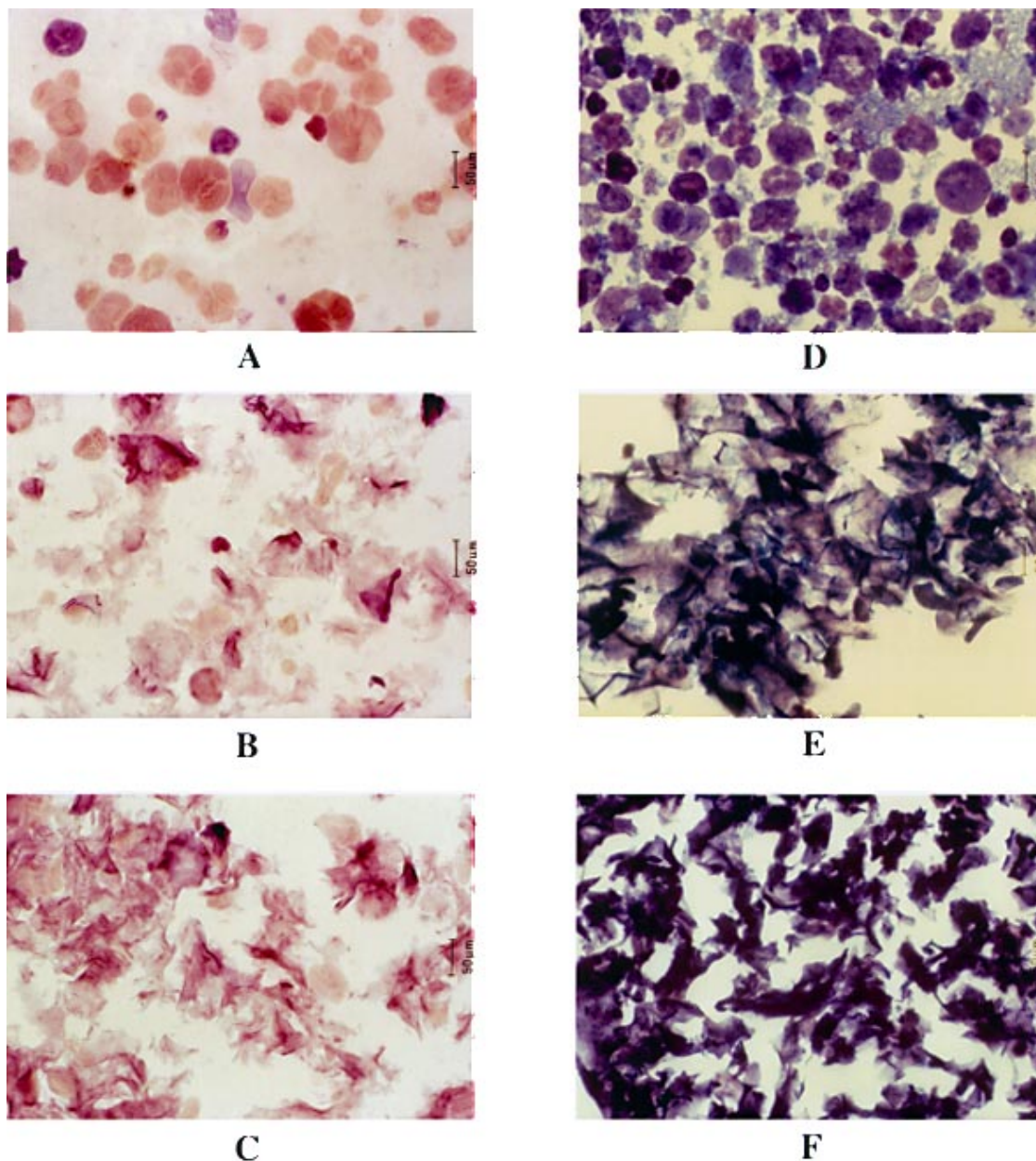


Fig. 4. Photomicrographs (50 \times) of 2.5% starch pastes. **A**, Waxy maize starch, fresh. **B**, Waxy maize starch, three freeze-thaw cycles ($-10^\circ/30^\circ\text{C}$). **C**, Waxy maize starch, seven freeze-thaw cycles ($-10^\circ/30^\circ\text{C}$). **D**, Common corn starch, fresh. **E**, Common corn starch, one freeze-thaw cycle ($-10^\circ/30^\circ\text{C}$). **F**, Common corn starch, three freeze-thaw cycles ($-10^\circ/30^\circ\text{C}$)

10 and 20 FTC for the processed paste and seven days for the paste held at 4°C, samples (10–12 mg) from each resulting paste were weighed accurately into five preweighed DSC pans, which were then sealed with preweighed lids. The resulting staling endotherm onset (T_o), peak (T_p), and final (T_f) temperatures and enthalpies were determined. Pan lids were punctured, and pan contents were dried in a vacuum oven at 100°C and <25 mm Hg pressure to constant weight. Dry weights of the samples were used to correct the enthalpies.

Effect of the FTC Process on X-ray Diffraction Patterns

The WMS paste used to determine the effects of a thawing temperature of 30°C for 30 FTC using temperatures of -10°C for at least 1 hr and 30°C for 1 hr. A 5-g sample was removed from the paste and placed in a screw-capped culture tube. The moisture in the sample was removed by solvent exchange using a modification of the method of Roulet et al (1988), which was reported to stop retrogradation without modifying crystallites. Acetone (20 mL) was added to the sample, and the sample was vortex mixed quickly. After standing at room temperature for 10 min, the sample was centrifuged 4 min at 1,000 × *g* and the supernatant decanted. Solvent exchange was repeated three times. The resulting solid was dried 12 hr in a vacuum oven at 40°C and <25 mm Hg pressure, crushed to a powder, and placed in a quartz capillary tube. X-ray diffraction patterns of the powder were recorded on film at 100% rh using Ni-filtered CuK α radiation (generated using a Picker Microfocus X-ray generator) and a pin-hole camera. Diffractograms were prepared from the resulting powder patterns using a microdensitometer (Joyce, Loebel & Co.) with a 0.79 Å maximum optical wedge.

Application of the Developed Method to Compare Retrogradation of Starches from Various Botanical Sources

Common corn, waxy maize, potato, tapioca, rice, and wheat starch pastes (2.5%, w/w) were prepared by combining the appropriate amount of starch and 0.02% NaN₃ in 60-mL screw-capped plastic bottles and heating the sealed bottles 20 min in a 100°C waterbath with slow stirring (magnetic stirrer). The pastes were then cooled 10 min in a 25 ± 2°C waterbath with slow stirring. The initial turbidity of the pastes was determined in triplicate as A₆₄₀ following removal of air bubbles by placing the pastes briefly under vacuum. Portions (20 mL) of each paste were placed in 28-mL polycarbonate centrifuge tubes. The tubes were sealed, and the samples were frozen by placing the tubes in a -20°C waterbath. After at least 1 hr at -20°C, the pastes were thawed by placing the tubes in a 30°C waterbath for 1 hr with vortex mixing for 10 sec every 15 min. After placing the thawed pastes briefly under vacuum to remove air bubbles, A₆₄₀ was determined in triplicate, after which the pastes were returned to their respective tubes. Cycles of freezing, thawing, and absorbance determinations were repeated until precipitation occurred. Samples were then centrifuged at 800 × *g* for 15 min, and the carbohydrate contents of the supernatants were determined.

Application of the Method to Determine Effects of Retrogradation Inhibitors on 2.5% WMS Pastes

Four WMS slurries (2.5%, w/w) were prepared by placing 1.1326 g of WMS and 38.87 mL of 0.02% NaN₃ in 60-mL screw-capped plastic bottles. SDS (a compound known to inhibit retrogradation) (Gudmundsson and Eliasson 1990) was added in the same mole ratio to starch as that given by 0.5, 1.0, and 2.0% sodium stearyl lactylate (SSL) based on starch (0.013, 0.027, 0.053 mmol per gram of starch) to three of the bottles. The sealed bottles were placed in a boiling waterbath for 20 min with slow stirring (magnetic stirrer). To differentiate the effects of SDS addition before and after gelatinization, 7.7 mg of SDS was added to the fourth bottle containing gelatinized paste, and the contents of the bottle were stirred rapidly (magnetic stirrer) for 20 sec. The

bottles were placed 10 min in a 25 ± 2°C waterbath to cool. Paste samples (20 mL) were placed in 28-mL polycarbonate screw-capped centrifuge tubes. The tubes were sealed and placed in a -20°C waterbath. Initial A₆₄₀ of the remaining paste samples was determined in triplicate following removal of air bubbles by placing the tubes briefly under vacuum. After at least 1 hr at -20°C, the samples were thawed by placing them 1 hr in a 30°C waterbath with vortex mixing for 10 sec every 15 min. A₆₄₀ of the thawed pastes was determined in triplicate, and the pastes were returned to their respective tubes. Cycles of freezing, thawing, and absorbance determinations were repeated until precipitation occurred. Samples were then centrifuged 15 min at 800 × *g*, and the carbohydrate contents of the supernatants were determined.

Microscopy

CCS and WMS pastes (2.5%, w/w) were prepared and FTC processed at -10°C and 30°C as described earlier. Samples of CCS paste (100 mL) (fresh and after 1, 2, and 3 FTC) and WMS paste, (fresh and after 1, 3, and 7 FTC) were gently mixed with 10 µL of an iodine stain solution (7.5% KI + 7.5% I₂). The resulting stained mixtures were viewed and photographed using a photomicroscope (Olympus Vanox). Fresh pastes were cooled before the iodine stain was added, but the process was done as rapidly as possible.

Effects of FTC on the Turbidity of Suspensions of Granular Remnants and Leached Material

CCS paste (2.5%, w/w) was prepared by combining 1.1213 g of starch and 38.9 mL of 0.02% NaN₃ solution in a 60-mL, screw-capped, plastic bottle. The sealed bottle was heated 20 min in a 100°C waterbath with slow stirring (magnetic stirrer). The paste was then cooled 10 min in a 25 ± 2°C waterbath with slow stirring and centrifuged 5 min at 2,500 × *g*. The supernatant was decanted into a graduated centrifuge tube (supernatant volume 20 mL). Boiling water (20 mL) was added to the pellet and the mixture was vortex mixed quickly. The suspension was centrifuged 2.5 min at 2,500 × *g* and the supernatant was combined with the previously prepared supernatant (total supernatant volume 40 mL). Water (20 mL) was added to the pellet and the mixture was vortex mixed gently, and 100 µL of the suspension was combined with 10 µL of an iodine solution (3% I₂ + 3% KI). Mixing was effected by tapping the tube. The resulting stained mixtures were viewed by light microscopy to determine the extent of separation of ghost and extra-ghost material.

Supernatants (leached material) and pellet (swollen granule) suspensions (20 mL) were transferred to 28-mL polycarbonate screw-capped centrifuge tubes, and tubes were placed in a -10°C bath. Initial A₆₄₀ of the remaining suspensions were determined in triplicate. After at least 1 hr at -10°C, the tubes were thawed by placing them in a 30°C waterbath for 1 hr with vortex mixing for 10 sec every 15 min. The A₆₄₀ of the thawed suspensions were determined in triplicate. Samples of the suspensions were stained and viewed microscopically as before to determine the effects of the FTC process.

RESULTS AND DISCUSSION

The effect of freezing rate on turbidity development was originally tested by placing identical WMS pastes in the freezer and in two waterbaths at -10 and -20°C. The rate of turbidity development was greatest in the sample frozen in the freezer at -15°C, followed by the sample frozen in the -10°C waterbath. The least rate of turbidity development was found in the sample frozen in the -20°C waterbath (Fig. 1). Because the freezer is less efficient at removing heat than is a waterbath, the rate of freezing in the freezer should have been slower than that in the waterbaths. In addition, the freezing rate of samples frozen in the waterbaths should decrease with increasing temperature. Therefore, the rate of turbidity development increased with decreasing freezing rate,

in conformance with earlier reports (Volz and Ramstad 1952, Albrecht et al 1960). Thus, the retrogradation rate can be varied by varying the rate of freezing.

After turbidometric analysis, the completely retrograded pastes (pastes undergoing FTC to the point that precipitation occurred) were centrifuged, and the total carbohydrate content of the supernatant was measured to determine the extent of retrogradation. Results indicated that 1.72 [± 0.01] mg/mL, 3.33 [± 0.06] mg/mL, and 5.02 [± 0.06] mg/mL of starch remained in solution after complete retrogradation of samples FTC processed at -15°C (freezer) / 30°C , $-10/30^{\circ}\text{C}$, and $-20/30^{\circ}\text{C}$, respectively. These results indicate that slower freezing rates resulted in more starch molecular associations and precipitation. Intuitively, this can be explained by the fact that, during slower freezing, the starch paste is at a temperature near that of maximum nucleation for a longer time, allowing more molecular associations to occur. Propagation can then occur either when the paste is freeze-concentrated or, perhaps more likely, during thawing.

DSC was used to determine the thawing temperature that would create a retrogradation endotherm T_p similar to that which would occur if the WMS underwent retrogradation under isothermal conditions at 4°C . Preliminary work (results not given) on the effects of thawing temperature on turbidity development showed that, over the limited range of $25\text{--}40^{\circ}\text{C}$, the effect of thawing temperature on turbidity development was not significant. Because thawing temperature did not affect turbidity, its effect on the peak temperature of the retrogradation (or staling) endotherm was examined. As the thawing temperature is increased, the degree of crystal perfection of the retrograded amylopectin (responsible for staling endotherm formation) should also increase, resulting in a shift of the staling endotherm peak temperature (T_p). For this analysis, 20% WMS pastes were used, as this is near the minimum concentration for staling endotherm detection by DSC (Longton and LeGrys 1981, Zeleznak and Hosenev 1986). While it was, therefore, as close to the concentration used for turbidity measurement as could be used effectively, it was $8\times$ the 2.5% concentration used to determine the degree of retrogradation using turbidity measurement. Results (Table II) indicated that the T_p increased with increasing thawing temperature due to greater crystal perfection. A thawing temperature of 30°C yielded T_p values for WMS pastes similar to the T_p of 54°C given by amylopectin pastes held isothermally at 4°C (Ring 1985, Ring et al 1987) and the 55.6°C temperature determined in this work (Table I) and, therefore, was used in further work.

Identical samples of 20% WMS paste FTC processed at $-10/30^{\circ}\text{C}$ or held at 4°C then analyzed by DSC to determine whether FTC processed or isothermally held 20% WMS pastes retrograde faster. The results (Table II) indicate that the freezing at -10°C effected retrogradation in a 20% WMS paste much faster than did aging at 4°C . (Considering that it takes a minimum of 2 hr to complete one FTC, the minimum time required for 10 and 20 FTC would be 20 and 40 hr, respectively.) Additionally, the similarity of the T_o , T_p , and T_f values of the staling endotherms produced by isothermal retrogradation and the FTC process indicated that the amylopectin crystallites that were formed by the FTC process had thermal properties similar to those formed under isothermal conditions.

An X-ray diffraction powder pattern of a WMS paste retrograded by the FTC process was prepared to determine whether the WMS crystallites that result from FTC were the same as those that resulted from isothermal retrogradation. The resulting powder pattern (Jacobson 1994) was similar to the one obtained by Ring (1985) from a 20% amylopectin gel held at 2°C for 62 days, although the pattern obtained from isothermally retrograded WMS gel contained a greater number of defined rings. A diffractogram was consistent with a B-type pattern (Ring 1985). A large peak at $2\Theta = 20.0^{\circ}$ may be due to the presence of a V-pattern (d spacing of 4.4 \AA) that could have originated from lipid-complexed starch

(possibly native starch lipids with contaminant amylose) (Zobel 1988). These results, along with those from DSC, indicated that the amylopectin crystallites from the FTC process are very similar, if not identical to those that form under isothermal retrogradation conditions. Matsunaga and Kainuma (1986) found that CCS paste undergoing an FTC process resulted in the development of crystallites that created a B-type X-ray pattern typical of isothermally retrograded CCS pastes (Miles et al 1985).

The effect of the FTC process on the rate of turbidity development in 2.5% pastes prepared from starches from various botanical sources as determined using the freeze-thaw method was evaluated. Results (Fig. 2) indicated that the order of starch retrogradation rates under the FTC conditions used were: potato > CCS > wheat > rice > tapioca > WMS. These results are similar to those obtained at 2% concentration using gravimetric (Whistler 1954) and turbidometric methods (Jacobson et al 1997) after isothermal storage with the exception of potato starch, which fell between rice and tapioca starches in the results of those investigations. Retrogradation rates obtained from starch pastes undergoing the FTC process seem to most closely resemble those found when very high starch concentrations were used (e.g., as determined by DSC at 40% concentration) (Roulet et al 1990) where rates followed the order potato > wheat > rice > tapioca > modified WMS; at 30% concentration, the order was pea > potato > CCS > wheat (Orford et al 1987). Retrogradation rates in 33% gels as determined by nuclear magnetic resonance followed the order: mung bean > potato > CCS > rice > sago > waxy rice (Teo and Seow 1992). This difference in the order of retrogradation rates of the various starches at high and low concentrations (especially in the case of potato starch) may reflect differences in phenomena detected by the methods, or it may be due to differences in concentration dependence for potato starch retrogradation versus other starches. Because the different methods of analysis gave similar results (except those for potato starch pastes), the variation in order is likely to be due to differences in starch concentration-dependence. The similarity of the results obtained by the FTC process on 2.5% pastes and those obtained by other methods on 30–40% pastes can, therefore, be explained by freeze-concentration which produces starch concentrations of from 2.5 to 70% continuously over the time period in which freezing occurs.

The concentration of starch remaining in solution after complete retrogradation of pastes by the FTC process was 1.17 [± 0.01] mg/mL for potato starch, 0.50 [± 0.01] mg/mL for CCS, 1.79 [± 0.06] mg/mL for wheat starch, 0.62 [± 0.01] mg/mL for rice starch, 5.67 [± 0.05] mg/mL for tapioca starch, and 6.00 [± 0.08] mg/mL for WMS. These results show that the amount of dissolved starch remaining after complete retrogradation by the FTC process varies with the botanical source of the starch and is not a function of the amylose-to-amylopectin ratio, although it is likely a function of their fine structures or a combination of the ratios and structures. Total carbohydrate analysis of the WMS pastes frozen at different rates revealed that pastes requiring more FTC to reach the end-point (precipitation in the cuvette) also had a higher concentration of soluble starch molecules at the end-point.

To determine the effect of retrogradation inhibitors on retrogradation effected by the freeze-thaw method, SDS was used because of its ability to form clear solutions rather than turbid suspensions, as compared to SSL which is widely used in food, especially bakery, products. The restriction of using only additives whose solutions remain clear is a limitation of this method as a means of testing retrogradation inhibitors or other ingredients. Results (Fig. 3) show that SDS was effective in inhibiting turbidity development of a 2.5% WMS paste during the FTC process and that the rate of turbidity development decreased with increasing SDS concentration. These results indicate that combining FTC with turbidometric analysis is a simple and effective means for the accelerated testing of retrogradation inhibitors (provided that they form clear

solutions). The soluble starch concentration in solutions upon complete retrogradation were 4.31 [± 0.06], 5.12 [± 0.04], 7.69 [± 0.05], and 9.98 [± 0.04] mg/mL for pastes with 0, 0.013, 0.027, and 0.053 mmol SDS/g of starch, respectively, clearly indicating an inverse relationship between SDS concentration and the amount of starch precipitate present at the end-point of the FTC process. When 0.027 mmol SDS/g of starch was added after, versus before, gelatinization, one less FTC was required before precipitation occurred (13 vs. 14 FTC when added before gelatinization), but initially retrogradation was inhibited to a greater extent. In addition, 5.68 mg/mL [± 0.01] versus 7.69 mg/mL [± 0.05] of soluble starch remained in pastes when SDS (0.027 mmol/g of starch) was added after, versus before, gelatinization, respectively. These results indicate that the time of SDS addition relative to gelatinization may have a small effect on the resulting rate of retrogradation, with SDS added before starch gelatinization showing a slightly greater retrogradation inhibiting effect.

To determine the effects of FTC on the microstructure of starch pastes, 2.5% WMS and CCS pastes were subjected to FTC processing at $-10/30^{\circ}\text{C}$. After one FTC, when retrogradation was approximately half complete, and at the end-point of turbidity development, samples were removed, iodine-stained, and examined by light microscopy (Fig. 4). In the WMS sample, swollen granules (along with some blue-staining, contaminating CCS granules) and lightly stained red interstitial regions, probably consisting of leached amylopectin, were clearly seen (Fig. 4A). After three FTC, many of the swollen granules were ruptured or disintegrated (especially contaminating CCS granules), leaving a small fraction of swollen granules and granular fragments (Fig. 4B). In addition, extragranular material had begun to aggregate with a subsequent loss of background haze (reddish). After seven FTC, the swollen granules and fragments that remained were as numerous as after three FTC, but they were now associated with extragranular aggregates that were larger and more intensely stained and had a definite sheet-like character, possibly the result of freeze-concentration of the amylopectin into lamella between ice crystals and subsequent association of the material in this state (Fig. 4C). In addition, the reddish haze present in the fresh samples and those subjected to only a few FTC was almost absent, indicating a reduction in soluble amylopectin.

The microstructure of fresh CCS paste was a composite of swollen granules with interstitial leached amylose present as lightly blue-stained aggregates (Fig. 4D). After only one FTC, the swollen granules were ruptured, only small granular fragments remained, and the leached interstitial amylose seen in the fresh paste was absent (Fig. 4E). Granular fragments, distinguishable as red-colored regions, were entrapped in large, blue-stained lamella similar to those seen in completely retrograded WMS paste after seven FTC. After three FTC, the CCS paste microstructure was dominated by blue-stained sheets; no granular fragments nor leached amylose could be seen (Fig. 4F). With continued FTC processing, the background color lightened, probably due to reduction in soluble starch.

Turbidity associated with a starch paste is due to: 1) the number and size of molecular associations that reflect or scatter incident light, and 2) the number and nature of swollen granular remnants that refract incident light (Craig et al 1989). Because the decreased transmittance (increased absorbance) that resulted from freezing and thawing could have been due to microstructural changes in swollen granules (i.e., the rupturing of swollen granules by FTC processing could have changed the refractive properties of the paste and effected changes in scattering and refraction), the turbidometric contribution of the changes to swollen granules resulting from FTC processing was determined. Swollen granules were separated from a 2.5% CCS paste by centrifugation, and changes in turbidity within the supernatant and the resuspended pellet were evaluated separately after one FTC. Initial A_{640} of the swollen granules and leached material was

1.687 [± 0.004] and 0.468 [± 0.003], respectively. After one FTC at $-10/30^{\circ}\text{C}$, the absorbance of the swollen granules and leached material increased to 2.078 [± 0.006] (ΔA_{640} 0.391) and 2.361 [± 0.009] (ΔA_{640} 1.893), respectively. The much greater increase in the turbidity of the leached material versus the suspension of swollen granules indicated that the changes in turbidity resulting from freezing and thawing is due primarily to molecular association of the leached material and to a much lesser extent on microstructural changes in the granule remnants.

SUMMARY

Presented is a simple method to accelerate and quantitate retrogradation that does not require special equipment. Acceleration of retrogradation can be controlled by controlling the freezing rate. The greater the rate of freezing of 2.5% WMS pastes, the lower the rate of retrogradation. Higher thawing temperatures (20% WMS pastes) result in higher staling endotherm peak temperatures (reflecting greater crystallite perfection), with a thawing temperature of $\approx 30^{\circ}\text{C}$ producing staling endotherms with peak temperatures similar to those that occur on isothermal storage at 4°C . X-ray diffraction confirmed that the crystallites that resulted from FTC processed WMS paste were almost identical to those that form during isothermal storage at 4°C .

Using a method that combined FTC and turbidometric analysis to accelerate and quantitate retrogradation, respectively, the rate of retrogradation of 2.5% pastes of starches from various botanical sources followed an order similar to that found using other methods and much higher starch concentrations. Using the new combined FTC-turbidometric method, higher concentrations of SDS gave greater inhibition of retrogradation of 2.5% WMS pastes.

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