THIS BOOK IS OF POOR LEGIBILITY DUE TO LIGHT PRINTING THROUGH OUT IT'S ENTIRETY.

THIS IS AS RECEIVED FROM THE CUSTOMER.

DETERMINATION OF THE DEGREE OF GELATINIZATION OF STARCH

384
1226-5600

by

RAVIPRASAD M. SHETTY

B. Sc., University of Agricultural Sciences, Bangalore, India, 1968

A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

in

Food Science

Department of Grain Science and Industry

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1973

Approved by:

Paul a Leib

Major Professor

LD
2668
T4
1973
S53
C.2
Doc.

TABLE OF CONTENTS

Page

INTRODUCTION

Gelatinization of starch has been familiar to man for a long time, but the changes that accompany the process were not studied until the beginning of this century. Recently, several investigators have tried to define this process and many methods have been developed to measure the changes associated with it.

The degree of gelatinization affects the physical properties of pregelatinized and partially-gelatinized starch foods such as bread, paste products and extruded snacks. Pregelatinized starches have a wide range of industrial applications (1), particularly in foods. In the manufacture of pregelatinized starches, the occasional presence of incompletely gelatinized starch samples is still a problem (2) as it impairs the efficient utility of pregelatinized starches. So a determination of the degree of gelatinization is very useful in quality control of pregelatinized starches. The extent of gelatinization affects the loaf volume and crumb characteristics of bread (3, 4). The elasticity, malleability and softness of paste products, as well as the crispiness and gritty texture of extruded snacks depends on the degree of gelatinization. A precise method to determine the extent of gelatinization is necessary to study the affects of different ingredients and processing variables on gelatinization.

It is an established fact that gelatinized starch is more susceptible to enzymes than raw starch. The extent of gelatinization determines the digestibility of starch to a great extent in animals including humans. Weight gains in animals were found to be related to the degree of gelatinization (5). Since starch gelatinization and starch damage are somewhat similar processes, a method of determining starch gelatinization can very well be

applied to determine starch damage. Several methods for measuring gelatinization are now in vogue, but in the analysis of foods and feeds, methods based on enzyme digestibility are more suitable. We observed that existing enzymic methods suffer from one or more of the following defects: (a) The end products of digestion are heterogeneous; mainly maltose or sometimes a mixture of maltose, oligosacchorides and limit dextrins; (b) The degree of digestion is measured by an analysis of reducing power, a relatively non-specific procedure. The purpose of this investigation was to develop an enzymic method for determining the degree of gelatinization which would overcome the errors cited above by using glucoamylase digestion of gelatinized starch followed by specific determination of D-glucose with D-glucose oxidase. Such a method has now been developed by us and was found to be accurate, precise and sensitive.

REVIEW OF LITERATURE

STRUCTURE OF THE STARCH GRANULES:

The starch molecule is made up of linear and branched glucose polymers known as amylose and amylopectin, respectively. These linear and branched components of individual starch molecules associate themselves by hydrogen bonding to form micelles (6). A starch granule is a micellar aggregate in which these micelles align themselves radially or form areas of varying degree of crystallinity depending on the extent of association. The micelles are intricately interconnected in a three dimentional network into a highly associated structure (6). This high degree of association is responsible for the unique properties of the intact starch granules, such as birefringences, resistance to swelling and insolubility in cold water (despite the fact that the starch molecule is highly hydroxylated) and resistance to enzyme attack. An extensive survey of the work was done to ellucidate the structure of the starch granules and the different types of crystallinity observed in starch was done by Sterling (7). The birefringent properties of the granule were ascribed to the presence of spherulites (special aggregation of needle shaped crystals) by Meyer (8). Kent-Jones and Amos attribute the behavior of intact starch in cold water to its crystallinity (9).

The properties of starch, such as resistance to enzyme attack, remarkably change when its granular structure is lost or damaged. Many investigators tried to explain this peculiar behavior of the intact starch granules in terms of structural intricities which are not yet well understood. It was believed for a long time that intact starch granules are surrounded by membrane, but this theory has been questioned by Leach and Schoch (10) and Badenhuizen (11). Presence of an outer hull which is

different in composition (amylopectin to amylose ratio) than the inner core was another explanation for the unusual properties of the intact starch granule (12, 13). This observation is again a matter of controversy (7).

Most starch granules show a layered structure under the microscope and these layers appear to originate from a distinct spot known as the hilum. This morphological characteristic is believed to have been formed during the growth and development of the starch granule (7, 11, 13, 15). Occasionally, some cracks and crevices are found to originate naturally from the hilum (7). The size and shape of a starch granule is a characteristic attribute determined by its botanical source (16). The granuled size within the same starch might again vary over a narrow or wide range depending on the botanical type (17).

THE PROCESS OF GELATINIZATION:

Gelatinization is a process which is not yet well defined. Earlier workers tried to relate it to changes in starch viscosity, swelling power, and solubility which occur at a much higher temperature than those encountered in the process of gelatinization (17). The loss of birefringence is widely accepted indication of gelatinization (6). A recent definition of gelatinization by Seib (18) seems to be a better explanation of this phenomenon; to quote Seib, "Gelatinization is the irreversible rupture of the native, secondary bond forces in the crystalline regions of a starch granule." He used the word "native" since gelatinization makes the granules much less crystalline but not amorphous and the granules show a new x-ray diffraction pattern (Type V) (19). Apart from water, starch also gelatinizes in several polar organic solvents such as dimethyl sulfoxide (DMSO) (17), aqueous pyridine (21) and ethylenediamine (18). Starch can be solubilized in inorganic solvents

such as liquid ammonia (22) and cold alkali. But the changes that take place in starch during gelatinization in aqueous system are of prime concern to us.

Intact starch granules contain about 10-17% water under normal atmospheric conditions, of which about 10% corresponds to the water of crystalization to form starch monohydrate (6). When starch granules are exposed to a water saturated atmosphere at room temperature, the granules undergo some reversible swelling showing the limited elastic nature of the intermicellar network. They absorb about 40-50% water (on a weight basis) and this process is exothermic. The extent of swelling (9 to 30%) (6), the amount of water held (40-50%) (17) and heats of hydration (17) vary between different botanical types of starches. These variations are attributed to differences in the granular structure of the different starches. According to Ullman (23), cold water is absorbed by the starch granule in one or more of the following forms, viz. water of crystallization, absorbed water or interstitial water. Another explanation is that water is firmly bound as hydrate until free hydroxyl groups are available in the starch molecule for binding (24). A dehydration study on potato and cereal starches suggested that 14% of the water was bound directly, 14 to 30% was absorbed in the intermicellar space of the granules and the remaining water was in free form (25).

The micellar network of granular starch is freely accessible to the entry of water and of most liquids (18). A starch-in-water suspension, when subjected to heating, absorbs a small amount of water without losing its birefringence until it reaches a crucial temperature (18). At this point some granules swell rapidly and irreversibly, losing their birefringent characteristics; the process is known as gelatinization. In gelatinization,

water or a polar solvent in the presence of added heat (or chemical reagent) destroys the native crystalline structure by breaking up the binding forces (18). Gelatinization is therefore an endothermic process (17). As the temperature of a starch in aqueous media is increased, more and more granules undergo the changes described above, until all the granules are gelatinized completely. The entire process of gelatinization takes place over a range of temperature (about 10°C), known as gelatinization temperature range, which varies in different starches. Gelatinization starts in the region of the granule where the associative forces are the weakest (amorphous region); the strength of the associative bonds in this region varies among the different granules belonging to the same botanical type (6). This is why gelatinization takes place over a range of temperature rather than a single temperature. It is also observed that larger granules lose birefringence at a lower temperature than smaller granules (17).

Agents that can break hydrogen bonds such as salts, alkali and urea were reported to accelerate the process of gelatinization (18) whereas others retard gelatinization by acting as desolvating agents (6). Starch granules continue to swell as the temperature is increased beyond the gelatinization temperature, to several hundred times the original volume. Simultaneously soluble materials leach out of the granule and some of the granules rupture completely. By this process the viscosity and soluble material in the aqueous phase increases (18). Prolonged boiling or mechanical shear will ultimately rupture all the granules. Granules of wheat starch were found to retain their identiy until a temperature of 95° and on further heating in an autoclave at 105°C the identity of granules is completely lost (26). The swelling power, viscosity and pasting properties

are very important characteristics for commercial utilization of starch. These characteristics depend on the associative forces present in the granule which again vary in different starches.

MEASUREMENT OF DEGREE OF GELATINIZATION:

A. General Methods

Degree of gelatinization is not a clearly defined term since there is no single criterion (unit) which exactly reflects the magnitude of gelatinization. The methods used to measure gelatinization can be devided into four categories:

1. Enzymic methods involving selective digestion of the enzyme-susceptible fraction of the starch by one or more of the amylases.

2. Microscopic methods including observation of swelling (27), counting of the birefringent granules (17) and selective staining of the gelatinized granules using dyes such as Congo red (28).

3. Methods based on physical properties of starch pastes such as swelling, turbidity, viscosity and solubility (1, 17, 29).

4. Other specialized techniques including x-ray diffraction (based on measuring crystallinity) (17), nuclear magnetic resonance spectrometry (based on mobility of water in starch-water systems) (30) and differential scanning colorimetry (based on measuring heat absorbed in the endothermic process of gelatinization) (31).

The most sensitive and popular methods of measuring gelatinization of starch are those based on birefringence and susceptibility to enzymes. These two methods involve different criteria: the former is a measure of the number of granules which are gelatinized and the latter measures the sum of the extent to which individual granules have gelatinized. The first

criterion may not be a true measure of the degree of gelatinization because; (a) a granule that is counted as ungelatinized because it is birefringent may be birefringent only on a portion of the granule and the size of this portion may vary among the individual granules belonging to the same starch (17); (b) even though the granules vary in size, equal consideration is being given to large and small granules, which may have different rates of gelatinization.

The method based on enzyme susceptibility on the other hand is a better measure of the degree of gelatinization since the enzyme hydrolyzes the gelatinized fraction of the starch much faster than intact starch (32). This explains why the results from birefringence and enzyme susceptibility are correlated but not exactly comparable (32, 33). In the analysis of foods and feeds, the birefringence method is difficult to apply because starch granules are difficult to count in heterogeneous and colored media and because starch cannot be easily separated from other components in the cooked material (18). The enzymic method is therefore widely used in the analysis of foods and feeds and it also has an additional advantage of simulating the in vivo digestibility of starch (18).

B. Enzymic Methods

Native starch granules are relatively resistant to enzyme attack, possibly because the large enzyme molecule cannot act upon the highly associated starch due to steric hinderence. This compact structure is broken in the process of gelatinization allowing the large molecular weight enzymes to act upon the polymer molecules. In partially gelatinized starches amylolytic enzymes selectively attack and depolymerize the exposed and disassociated starch molecules.

The damage to some starch granules that occur during grain milling also increases the susceptibility of starch to enzyme attack in the damaged area. Starch damage is different from gelatinization because the crystallites are normally unbroken in the former due to their very small size (18). Increased enzyme susceptibility is therefore mainly due to exposure of more surface (18). So most of the methods of measuring starch gelatinization based on enzyme susceptibility are also applicable with little modification for determination of starch damage (4).

The methods of measuring gelatinization based on enzyme susceptibility commonly employ one of the two amylases; β-amylase (33-36) and glucoamylase (37-39) or a mixture of amylases containing mainly α- and β-amylase such as diastase (2, 32, 40). In all enzymic methods, except one case (2) mentioned above, fragments resulting from digestion are assayed by measuring the increase in reducing power. Methods based on glucoamylase digestion are inherently more accurate compared to ones based on α- and β-amylases for four reasons:

1. Glucoamylase provides an absolute estimate of degree of gelatinization since it can cleave both α-1,4 and α-1,6 bonds in starch giving almost quantitative conversion of starch to glucose (41). On the other hand, α- and β-amylases are not capable of cleaving α-1,6 branch points in starch molecules, yielding α-1,6 linked oligosaccharides including limit dextrins in addition to maltose (42, 43).

2. All enzymic methods of measurement of starch gelatinization are based on measuring the number of sugar molecules released during digestion. Glucoamylase converts gelatinized starch to

a much larger number of molecules (D-glucose) than α- or β-amylase (predominantly maltose and limit dextrins). In this manner, the degree of gelatinization is measured by larger differences between larger numbers when glucoamylase is used.

3. The amount of reducing oligosaccharides released by α-amylase and β-amylase is dependent on the molecular weight of the amylopectin fraction of the gelatinized starch (42, 43). Whereas the D-glucose released by glucoamylase is independent of molecular weight. This is particularly important where starch is cooked at low pH (44).

4. The sensitivity and accuracy of the glucoamylase method can be increased by taking advantage of a very precise specific and sensitive method for measuring the D-glucose released using glucose-oxidase enzyme.

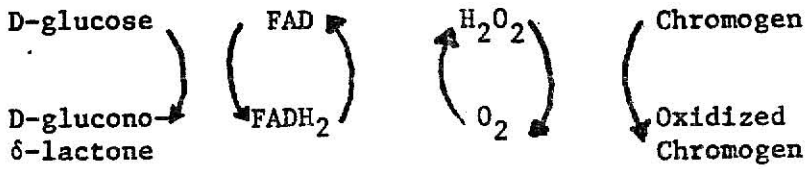
D-GLUCOSE DETERMINATION USING GLUCOSE OXIDASE:

D-Glucose determination is performed most often on blood and other biological fluids; therefore many of the methods for determining D-glucose are designed to suit this purpose. For the sake of expediency such analyses are being performed with procedures which are relatively non-specific for D-glucose (45); the most popular ones are those based on either ferricyanide (46) or cupric ion (47) reduction in alkaline medium. Such oxidation reduction systems are not only capable of oxidizing D-glucose but many other compounds containing an oxidizible group.

Recently a number of procedures have been developed to determine D-glucose with phenols in strong acid solution; for example anthorone reaction (48) and phenol sulfuric acid reagent (49). These procedures in

addition to being nonspecific for D-glucose, have handling problems due to the high acid concentration employed.

The specific determination of D-glucose became possible with the discovery by Muller (50) of D-glucose oxidase (Notatin) an enzyme specific for D-glucose. He showed that glucose oxidase catalyzes the oxidation of β-D-glucose by molecular oxygen to D-gluconic acid. Franke and Lorenz (51) discovered that hydrogen peroxide is simultaneously produced in this reaction. The first practical application of glucose oxidase in the estimation of D-glucose in urine was done by Keston (52) and Comer (53). This colorimetric analysis, which employed simultaneous use of two enzymes, follows the sequence of reactions shown below:



The enzyme glucose oxidase specifically catalyzes the oxidation of β-D-glucopyranose to D-glucono-δ-lactone, which undergoes hydrolysis to gluconic acid. The enzyme glucose oxidase is a flavoprotein, containing the flavine adenosine dinucleotide (FAD) moiety and its systematic name is "β-D-glucose: O_2 oxidoreductase" (E.C.1.1.3.4). Oxidation of D-glucose is simultaneously accompanied by reduction of FAD. The reduced FAD reacts with atmospheric oxygen to generate hydrogen peroxide which, in the presence of peroxidase, oxidizes the colorless reduced form of the chromogen (ortho dianisidine or ortho tolidine) to its colored, oxidized form. The intensity of color so produced is directly proportional to the D-glucose concentration originally present (54-56).

The specificity of the enzyme for β-D-glucose was established by Keilin and Hartree during their study of the action of the enzyme on 50 sugars and their derivatives (57) and on all eight aldohexoses (58). Glucose oxidase was found to oxidize 2-deoxy-D-glucose (59, 60); therefore analysis of glucose cannot be done in presence of the latter. Several investigators examined the different factors that influence the accuracy of glucose determination using glucose oxidase such as temperature of incubation, pH of the reaction media, acidification of the digest (61, 62), stability of color formed and optimum concentration of glucose oxidase and peroxidase (63). The use of concentrated sulfuric acid in glucose analysis using glucose oxidase was proposed by Wasko and Rice (64) and McComb et al. (60) to stabilize the color formed due to oxidation of the chromogen and to increase the sensitivity of the glucose assay. Glucose assay, using glucose oxidase, was inapplicable to glucose solutions containing α-1,4 linked glucans and oligosaccharides because of contamination of all but the most highly purified commercial glucose oxidase preparation with carbohydrases (65). This problem has been overcome by development of buffer systems containing Tris-(hydroxymethyl)aminomethane (65, 66). Which was found to inhibit the activity of carbohydrases.

Teller (59) developed a preparation known as glucostat [a commercial reagent containing glucose oxidase, peroxidase and chromogen (67)] for analyzing glucose in serum and plasma. In replicate blood glucose analyses using the glucostat method (59), Field and Williams (68) reported a coefficient of variation of ± 1.5% at 0.5 to 10 mg/ml glucose concentration.

Very recently Banks and Greenwood (69) made a detailed comparative study of the popular buffer systems developed for analyzing glucose using

glucose oxidase enzyme. They also examined various factors that influence the accuracy, precision and sensitivity of the glucose oxidase system and reported a coefficient of variation of ± 0.5% at 20 µg/ml glucose concentration.

MATERIALS AND METHODS

MATERIALS:

Reagents:

9:1 Dimethyl Sulfoxide (DMSO)-Water (V/V) Solution:

It was prepared by mixing one part of water with nine parts of absolute DMSO (reagent grade) in a volumetric flask. Addition of DMSO was done while the volumetric flask was immersed in cold water to absorb the heat generated during mixing. The contents of the flask were allowed to equilibrate to room temperature and the volume was adjusted with DMSO. Since DMSO is very hygroscopic, the solution was prepared fresh daily.

Citrate Buffer:

Solution a: Citric acid (21.01 g) was dissolved in water (2 ℓ) to obtain a 0.05M solution.

Solution b: Sodium citrate (29.41 g) was dissolved in water (2 ℓ) to obtain a 0.05M solution.

Citrate buffer (0.05M pH 4.8) was prepared by mixing one volume of 0.05M aqueous citric acid (Solution a) with approximately 1.2 volumes of aqueous sodium citrate (Solution b). The pH was adjusted to 4.8 (using a pH-meter) by addition of solution (a) or (b) as required.

Tris/ HCl Buffer:

Buffer was prepared by dissolving Tris-(hydroxymethyl)-aminomethane (61 g) in 5M aqueous hydrochloric acid (80 ml) and the solution was diluted to 1 liter with water. The buffer was warmed to 37°C and its pH was adjusted (pH-meter) to 7.0 by the addition of hydrochloric acid.

Glucose Stock Solution:

A standard solution of D-glucose was prepared by dissolving the anhydrous sugar (1 g) in water (100 ml) saturated with benzoic acid (~2.4 g benzoic acid crystals dissolved in 100 ml water) in a volumetric flask. This solution has indefinite shelf life (69). Glucose standards (eg. 50 µg/ml) for analytical purposes were prepared by appropriate dilution of the stock solution with distilled water. At this dilution, the benzoic acid present was found not to interfere in enzymic glucose assay (69). Anhydrous glucose was prepared by vacuum drying of reagent grade anhydrous D-glucose over phosphorus pentoxide for at least 12 hours. Drying was carried out in a drying pistol using ethanol vapors (about 78°C).

Starch:

Prime Wheat Starch:

Starch was isolated from soft red winter wheat by wet milling (70) and purified according to the specifications of Fellers et al. (71). Wheat kernels (~500 g) were soaked in approximately 1 liter water overnight at 5°C. The steep water was drained and the soaked kernels were ground with fresh water in a Waring blender at high speed for three minutes. The ground mass was filtered through a 100-mesh sieve (stainless steel wire screen) and the liquid slurry that passes through the screen was collected. The residue on the screen was blended again in a Waring blender at high speed for three minutes, and the ground mass was sieved as before. The filtered, liquid slurry was combined, and the excess water present in the slurry was removed either by centrifuging or by allowing the solids to settle over a period of one hour. The turbid supernatant liquid was carefully removed without disturbing the settled mass. The settled mass was in the form of a slurry

and it was centrifuged. Centrifuging was done conveniently in plastic centrifuge bottles whose upper portion had been cut off to enable scraping the top layer of the sediment. The visible brown layer on the top of the white starch sediment was carefully scraped off using a bent spatula.

Further separation of gluten from starch was done by following the specifications of Fellers et al. (71). They reported that an easy and efficient separation of gluten from starch was obtained when a starch slurry with solids to water ratio of 1:1.5, was centrifuged at low speed (1000 r.p.m.) for a short time (5 min.). In our work, the approximate weight of the solids was estimated by weighing the sediment after the first centrifugation and the corresponding amount of water (or a little more as required to obtain a manageable slurry) was added. The turbid supernatant solution was decanted. The thin brown film deposited on the sedimented starch was washed off by directing a jet of water gently on the surface. The sedimented starch was reslurried and recentrifuged (3 or 4 times) as before until no visible layer of gluten was on top of the white sedimented mass of prime starch.

The purified wet starch was dispersed in enough water to obtain a 2% starch suspension. The suspension was stirred to disperse the starch granules uniformly. The suspension was freeze dried and the dried sample stored under constant humidity. This starch had 4.5% moisture, 0.7% protein, 0.06% fat and 0.02% ash.

Gelatinized Wheat Starch

Prime starch was gelatinized by heating a 1 to 2% suspension of starch in a pressure cooker at 120° for one hour. The gelatinized starch solution was mixed slowly in four volumes of anhydrous methanol in a Waring blender

at high speed. The precipitated starch was centrifuged. The sediment was reblended with more methanol and centrifuged. The blending and centrifuging was repeated three or four times. After the last blending, the precipitate was filtered and the residue was dried in a desiccator over phosphoros pentoxide under vacuum and stored at constant humidity.

Starch Standards With Different Degrees of Gelatinization

Mixture of gelatinized and prime starch (with known degrees of gelatinization) were obtained by mixing weighed quantities of pregelatinized and prime starch (total starch weight 100 mg). Both prime and gelatinized starches had the same moisture level.

Enzymes:

Glucoamylase:

The glucoamylase used, unless otherwise stated, was a commercial preparation (Takamine Diazyme, Miles Lab., Elkhart, Ind.) from Aspergillus niger. Solutions of glucoamylase were prepared by mixing (∿85 mg) the commercial powder (1.9% nitrogen, Kjeldahl) with (∿50 ml) 0.05M citrate buffer (pH 4.8). Insoluble material was eliminated by filtering through glass fiber filter paper. The filter paper was washed a couple of times with fresh (∿50 ml) citrate buffer to extract the soluble protein remaining on the filter paper. The enzyme solution so prepared when made up to a volume of 100 ml contains approximately 2 I.U. of enzyme activity ml^{-1} of the solution. The specific activity of the enzyme preperation was determined by measuring (72) the D-glucose released by the enzyme from a large excess of soluble starch; (see Methods) and was found to be 12.0 μmole $min.^{-1}(mg.^{-1}$ $protein)^{-1}$. Protein was obtained from Kjeldahl nitrogen analysis using the

factor 6.25. In experiments on the digestion of granular wheat starch, glucoamylase from Rhizopus niveus (Miles Laboratories, Elkhart, Ind.) was also used. This twice recrystallized, freeze dried enzyme was not readily soluble in 0.05M citrate buffer. A solution of the freeze dried enzyme was made by suspending it in cold buffer (5°C) followed by homogenizing in Potter-Elvehjem homogenizer). The specific activity of the latter, crystalline enzyme was given by the supplier as 20 μmole $min.^{-1}(mg\ protein)^{-1}$.

Glucostat Reagent:

Glucostat (Worthington Biochemical Corp., Freehold, N. J. see p. 12) is supplied in two separate vials. The bigger vial contains a mixture of two enzymes; glucose oxidase and peroxidase. The smaller vial contains the chromogen (ortho-dianisidine). "Glucostat X4" is a commercial brand of glucostat which contains four times as much enzyme and chromogen as a "Glucostat Regular". Glucostat reagent (Worthington Biochemical Corp., Freehold, N. J.) was prepared by mixing the contents of one vial of "chromogen for Glucostat X4" with 4.0 ml of a 20% ethanolic solution of the surfactant Triton X-100 (Mann Research Laboratories, New York, N. Y.). The mixture was dissolved in 400 ml of 0.5M Tris-HCl buffer (pH 7.0) which contained the contents of one vial of "glucostat X4".

METHODS:

Determination of D-Glucose:

D-Glucose was determined by either a modified Worthington procedure (72) or by one of the two procedures described below. Method A was used in determining the total starch content of samples, when digests contained 40-120 μg ml^{-1} of D-glucose. Method B was used to determine D-glucose in the glucoamylase digests of partially gelatinized samples, when the digests

contained lower concentrations of D-glucose (6-18 μg ml^{-1}) than those assayed by Method A.

Method A. An aliquot (1.0 ml) of a solution containing[a] 40-120 μg ml^{-1} of D-glucose in absolute ethanol: 0.05 M citrate buffer: 90% DMSO (10:5:1, v/v) was incubated at 37 ± 0.5°C with Glucostat reagent (10.0 ml). After one hour the reaction was terminated by the addition of 0.5 ml of 4M aqueous hydrochloric acid with thorough mixing. The clear solution was allowed to cool to room temperature and the absorbance was measured in 1 cm cells at 400 nm using a reagent blank and a Beckman DU Spectrophotometer. Analyses were done in duplicate, and each analytical run included the analysis of a standard solution of D-glucose (50 μg ml^{-1}).

Method B. An aliquot (3.0 ml) of a solution containing 6-18 μg ml^{-1} of D-glucose in absolute ethanol:0.05M citrate buffer (10:5, v/v) was incubated at 37° ± 0.5°C with Glucostat reagent (3.0 ml). After one hour the reaction was terminated by adding 0.15 ml of 4M aqueous hydrochloric acid. The remainder of the procedure was identical to that used in Method A.

Determination of Specific Activity of Glucoamylase:

Specific activity of glucoamylase was determined as described by Lineback et al. (72). Glucoamylase solutions containing 80-160 μg protein ml^{-1} (protein = 6.25 x Kjeldahl nitrogen) were prepared in 0.05M citrate buffer, pH 4.8. (see p.17). The substrate used was a 4% solution of

---

[a]The presence of 5.6% dimethyl sulfoxide in the solution of D-glucose being assayed had no effect on the color produced during incubation with Glucostat reagent.

soluble (lintner) starch in 0.05M citrate buffer, pH 4.8. Frequently starch particles aggregate if they are not dispersed uniformly in the buffer prior to dissolution. This problem was overcome by first wetting the starch with small amounts of buffer, followed by mixing as additional buffer was added. The starch suspension was heated with constant stirring, using a magnetic stirring bar, until a clear solution was obtained (around 95°C).

Enzyme activity was assayed by incubating aliquots (1 ml) of the enzyme solution with starch solution (3 ml) at 30°C for 5, 10, 15, 30 and 60 minutes. Enzyme hydrolysis was stopped after these intervals by the addition of 5.0 ml of absolute ethanol. The precipitated starch and protein were removed by centrifugation. The supernatant solution was analyzed using Method A (see p.19). One unit (IU) of glucoamylase activity was defined as the amount of enzyme required to liberate 1 μmole of glucose per minute at 30°C after 60 min. incubation period under the reaction conditions defined above (72). Specific activity was expressed as units per mg protein, determined on the basis of Kjeldahl nitrogen.

Proximate Analysis of Starch:

Moisture:

Moisture was determined according to AOAC Method 14,004 (72a) The method involves heating a known amount of sample (1 g) at constant temperature (130°C) for one hour.

Protein:

Protein was calculated as Kjeldahl Nitrogen, determined by AOAC method 2.049-2.051 (72a) multiplied by 5.7.

Fat:

Fat was determined by a procedure followed by Libby (73) which involved fat extraction by an equivolume mixture of butanol:methanol:benzene (1:1:1) in a Soxhlet extractor. Starch (~50 g) of known moisture content was weighed into a thimble, and the thimble was placed inside a Soxhlet extractor. The volume of the solvent mixture used for extraction was adjusted so that all three solvents were evaporated in the process of extraction (volume slightly in excess of the siphoning volume of the extractor). After about 16 hours of extraction, the extract was evaporated to dryness. The dried material was re-extracted with chloroform by rinsing the flask several times with chloroform followed by filtration through Whatman No. 4 paper. The filtrates were collected in a flask of known weight and evaporated to dryness. This material was oven dried at 120°C, for one hour and weighed. The weight gain represents the lipid content present in the original sample (50 g).

Fat determination was not considered important in isolated starch, since the fat content was too low (around 0.1%) to significantly affect the accuracy of our method for determining gelatinization.

Ash:

Ash content was determined by AOAC method 14.006 (72a).

Starch:

From these values, the theoretical starch content in a sample was calculated as follows:

$$\text{Theoretical Starch Content \%} = 100 - \underset{\text{Moisture}}{\%} + \underset{\text{Protein}}{\%} + \underset{\text{Fat}}{\%} + \underset{\text{Ash}}{\%}$$

Determination of Total Starch:

Wheat starch (75-100 mg) sample was weighed accurately into a 100 ml volumetric flask containing a magnetic stirring bar of known volume. The flask was placed in a water bath (55°C) on a magnetic stirring plate with heater and 90% DMSO (preheated to 55°C) was added to volume with stirring. After stirring 5 min. at 55°C, the flask was placed in a water bath at 37° ± 0.5°C, and the contents allowed to equilibrate to temperature (~10 min.). The volume of the starch solution was readjusted by adding 90% DMSO at 37°C. After the mixture was thoroughly stirred, an aliquot (1.0 ml) was pipetted quickly into a test tube (18 x 120 mm) containing glucoamylase (10 IU) in 5.0 ml of 0.05M citrate buffer (pH 4.8). The tube was incubated at 37° for 30 min. Ethanol (10.0 ml) was then added, and the tube was cooled briefly in an ice-water bath and centrifuged. The supernatant was warmed to 37° and a 1.0 ml aliquot was assayed for D-glucose using Method A.

$$\text{Percent Total Starch (T)} = \frac{(\text{Glucose released})(0.90)}{(\text{Sample weight})} \times 100$$

Determination of Digestible Starch:

Predetermined amounts of gelatinized wheat starch and/or prime starch were weighed (together) into 125 ml erlenmeyer flasks; each standard sample contained a total of 100 mg of starch with 0, 5, 10, 25, 50, 75, or 100% of the total as gelatinized starch. Five-hundred milligrams of silica gel (Grade, plain; Warner-Chilcot Laboratories, Richmond, California) was added and mixed well with the starch to facilitate wetting of the polysaccharide with buffer. A solution of glucoamylase (500 IU) in 50 ml of 0.05 M citrate buffer (pH 4.8) at 37° was added with mixing. After incubation at 37° for

30 min. an aliquot (5.0 ml) was removed from the reaction mixture and added to absolute ethanol (10.0 ml) in a test tube (18 x 120 mm). The tube was chilled in ice-water to facilitate precipitation of protein and undigested starch. Following centrifugation the supernatant was warmed to 37° and assayed for D-glucose using Method B. If the supernatant contained too high a concentration of sugar, it was diluted with absolute ethanol:citrate buffer (2:1, v/v).

$$\text{Percent Starch Digested by Glucoamylase} \quad (X) = \frac{\dfrac{(\text{Glucose released}) \ 0.90}{\text{Sample Weight}}}{T} \times 100$$

Susceptibility of Granular Starch to Glucoamylase:

Digestion:

Prime wheat starch (100 mg) was exposed to 500 IU of A. niger glucoamylase (Takamine Diazyme) or R. niveus glucoamylase dissolved in 0.05M citrate buffer (50 ml, pH 4.8) at 37°C. Toluene (0.5ml) was added to prevent microbial contamination, and each reaction mixture was stirred gently with a magnetic stirring bar. Aliquots (5.0 ml) were removed periodically and added to 10.0 ml of absolute ethanol. Aliquots were removed after 0.5 (to get variable B) 1, 8, 16, 32 and 64 hrs. of incubation. After centrifugation the supernatant was assayed for D-glucose using Method B (see page 19). A total starch determination (T_1) of the prime starch is necessary for calculating the percent digestibility of granular starch if prime starch has a different proximate analysis than the starch sample used for measuring percent gelatinization.

$$\text{Percent Granular Starch Digested by Glucoamylase} \quad (B) = \frac{\dfrac{(\text{Glucose released after 0.5 hr. incubation period}) \ 0.9}{\text{Sample weight}}}{T_1} \times 100$$

Scanning Electron Microscopic Observation of Digested, Intact Starch:

The sediment in the centrifuge tube from the digestion described above was washed three times with water, three times with ethanol, and dried in a desiccator over calcium chloride under vacuum. The sedimented starch samples were mounted on stubs using double-backed Scotch tape, and were coated in vacuum with approximately 2 p.m. of gold. The coated specimens were viewed in a ETEC auto-scanning electron microscope operating at 10-kv accelerating potential. Photographs were recorded on Polaroid film (Type 55 P/N, Polaroid Corp., Cambridge, Massachusetts).

RESULTS AND DISCUSSION

The percentage gelatinization of starch in a food or feed may be calculated by the equation: (see Appendix)

$$Y = \frac{100(X-B)}{100-B} \qquad (I)$$

where Y is the percent gelatinization, X is the percent of starch removed from a sample by glucoamylase digestion for 30 minutes, B is the percent digestion of the sample's intact granular starch by glucoamylase in 30 minutes and 100 represents the total starch content in the sample. Since the digestion of starch by glucoamylase is to be measured by the amount of D-glucose released into the digest medium, it follows from equation (I) that the accuracy of a glucoamylase-glucose oxidase procedure for determining gelatinization depends on the following factors: (a) the accuracy of the assay procedure for D-glucose; (b) the reliability of the method used to determine total starch; and (c) the percent digestion of intact granular starch in a sample. Each of these factors has been examined individually in the following discussion.

GLUCOSE ANALYSIS:

The important factors that affect the accuracy of glucose-oxidase systems as pointed out by Banks and Greenwood (69) are: choice of buffer solution; temperature of incubation; pH of the system; period of incubation; and amount of acid added to stop the reaction. Each variable in the analysis of D-glucose in starch hydrolyzate was studied and will be discussed individually.

Choice of Buffer Solution:

A buffer system in which glucose oxidase enzyme gives maximum specificity, highest sensitivity and rapid completion of glucose oxidation was desired. The buffer systems commonly employed for glucose oxidase are:

(1) Tris-HCl

(2) Tris-HCl-glycerol

(3) Tris-phosphate-glycerol

(4) Phosphate

(5) Phosphate-glycerol

Banks and Greenwood (69) found Tris-HCl buffer to optimally satisfy the three conditions mentioned above and was suitable for our purpose. Phosphate buffer, even though it enabled maximum rate of glucose oxidation, lacked specificity. Glycerol systems lowered the sensitivity of the assay considerably (69).

Temperature of Incubation:

The temperature of incubation was increased to 37°C, since maximum reactivity and rapid completion of reaction was attained at this temperature (61). Variation in temperature during incubation should be minimized (± 0.2°C) in order to get reproducible results, especially since pH of the Tris buffer is highly temperature dependent (69).

Hydrogen ion Concentration (pH):

The activity of glucose oxidase is very sensitive to changes in pH (56). Since the pH of tris buffer is temperature dependent and addition of Glucostat reagent and starch hydrolyzate lowers the pH of the Tris buffer system,(as seen in Table I) it is necessary that the pH of the Tris buffer be adjusted to 7.0 at 37°C.

TABLE I

EFFECT OF TEMPERATURE ON pH

TEMPERATURE	TRIS BUFFER	TRIS BUFFER + GLUCOSTAT REAGENT	COMPLETE ASSAY MIXTURE
25°	7.34	7.28	7.27
27°	7.28	7.22	7.20
29°	7.22	7.15	7.13
31°	7.16	7.11	7.07
33°	7.11	7.07	7.04
34°	7.08	7.04	7.02
35°	7.05	7.02	7.00
36°	7.03	7.00	6.97
37°	7.00	6.97	6.94
38°	6.98	6.93	6.90
39°	6.95	6.90	6.88
40°	6.90	-	-

Incubation Period:

This is determined by the temperature of incubation and the buffer system used (69). For Tris-HCl buffer system at 37°C, (the incubation temperature employed in our work) the reaction was nearly completed after one hour. The reaction could be stopped earlier (15 or 30 min.) if timing of incubation, pH and temperature could be accurately controlled; however minor variations in any one of the above factors will introduce significant variations in the results. The effect of minor variations in temperature, pH and timing of incubation period are made negligible by prolonging the incubation period to 60 min. Therefore, by sacrificing the rapidity of the assay to a small extent, a substantial improvement in the precision of the analysis was obtained (see Table II, page 29).

Amount of Acid Added to Stop the Reaction:

The acidification of the digest serves three purposes: a) it stops the enzyme reaction, b) it clarifies the solution, and c) it yields a color stable for more than 24 hours (69). The procedure of Worthington Biochemical Corp. (67) recommends the addition of two drops of 4M HCl for this purpose.

It could be observed from the data in Table III that in the region of three to five drops, a slight variation in the volume of acid added can introduce significant variations in absorbance. So 12 drops of acid was chosen as the amount to be used in this study, since little effect on the absorbance was observed between 9 to 14 drops. Twelve drops was equivalent to 0.5 ml, since the average size of the drop delivered was 0.042 ml. Hence we chose to add 0.5 ml of acid per 10 ml (Method A) and 0.15 ml per 3 ml of glucostat reagent (Method B).

TABLE II

PRECISION OF TWO ASSAY PROCEDURES

FOR D-GLUCOSE USING GLUCOSE OXIDASE/PEROXIDASE

Absorbance[a]							
Worthington Procedure with Tris Buffer[b]				Modified Procedure[c]			
glucose µg[d]							
50	75	100	150	40	80	120	160
0.197	0.309	0.428	0.650	0.178	0.360	0.542	0.717
0.200	0.310	0.430	0.640	0.178	0.359	0.542	0.720
0.197	0.320	0.440	0.640	0.178	0.358	0.543	0.720
0.199	0.308	0.430	0.635	0.177	0.360	0.543	0.719
Mean Absorbtivity[e]				Mean Absorbtivity			
4.18				4.488			
Standard Deviation[f]				Standard Deviation			
0.15				0.028			
Coefficient of Variation				Coefficient of Variation			
3.6%				0.62%			

[a]Measured at 400 nm in a 10 mm cell.

[b]See reference 72.

[c]This work: Method A.

[d]D-Glucose in 1.0 ml of ethanol:citrate buffer : 90% DMSO (10:5:1: v/v) added to Glucostat reagent (10.0 ml).

[e]Mean of sixteen individual absorbtivity values, where absorbtivity equals absorbance divided by the product of cell-path length (cm) and concentration (mg ml^{-1}).

[f]Standard deviation of mean absorbtivity.

TABLE III

EFFECT OF ACIDIFICATION (4N HCl)

No. of Drops	Absorbance 1[a]	Absorbance 2[a]	No. of Drops	Absorbance 1[a]	Absorbance 2[a]
0	.287[b]	.285[b]	12	.168	.166
1	.282[b]	.281[b]	13	.169	.166
2	.278[b]	.277[b]	14	.169	.166
3	.277[b]	.278[b]	15	.167	.164
4	.150	.149	16	.166	.164
5	.172	.169	18	.165	.162
6	.171	.169	20	.164	.158
7	.174	.172	22	.164	.161
8	.174	.172	25	.160	.151
9	.171	.169	30	.156	.153
10	.170	.168	40	.150	.147
11	.169	.167	60	.135	.130

[a]Absorbance values for two different samples (1 and 2).

[b]Solution was turbid.

1 Drop = 0.042 ml

12 Drop = 0.504 ml

Some minor factors which, if neglected,will introduce significant errors in the results are:

1. Storing freshly prepared Glucostat reagent at 5°C for at least 12 hours before use (69) improved the reproducibility of our results.

2. Since Tris-HCl buffer has a very low buffering capacity at pH 7.0, analysis of glucose in solutions which were acidic or basic was not possible. Therefore, a neutralization step to adjust the pH between five to nine was found necessary in order to use Tris-HCl buffer with the glucose oxidase system. A solution of D-glucose in 0.1M sodium sulfate (obtained by acid hydrolysis of starch with sulfuric acid and subsequent neutralization of excess acid with Sodium hydroxide) gave the same absorbance as a solution of the same concentration of glucose in water.

3. The glucoamylase used in starch hydrolysis was completely precipitated by addition of ethanol and the precipitate was removed by centrifugation. The presence of the enzyme in a starch hydrolyzate due to incomplete precipitation and/or inadequate centrifugation was found to interfere in the glucose assay using glucose oxidase.

4. Dehydration of the reagent-grade, anhydrous glucose available commercially was found inadequate. At least 12 hours of vacuum drying at 60-70°C was found necessary. The dried sample should be weighed soon after cooling as it tends to reabsorb moisture on exposure to the atmosphere.

5. Glucose stock solution could be easily preserved indefinitely by preparing the stock solution in water saturated with benzoic acid (69). Benzoic acid was found not to interfere with the glucose oxidase system; its concentration will be quite low in diluted standard solutions.

6. Freshly prepared stock solutions from dry D-glucose should be kept

at room temperature for at least 12 hours to mutarotate and establish an equilibrium.

7. A solution of the same composition (66.6% ethanol, 1% DMSO, 0.15M citrate buffer) as the starch hydrolyzate should be used as a blank and to prepare glucose standards, since glucose solutions in ethanol and citrate buffer were found to give higher absorbances than the same concentration of glucose in water. The effect of 1% DMSO on absorbance however was negligible.

In the early stages of our investigation we assayed for D-glucose using the Worthington procedure (67) except that Tris-HCl buffer was substituted (65, 72) for phosphate buffer (pH 7.0). This procedure gave too great a variability for our purpose. However, we were able to greatly improve the precision of the D-glucose assay by incorporating four changes as suggested by Banks and Greenwood (69). These changes were: (a) Tris-HCl buffer (pH 7.0) was prepared at 37°C; (b) the glucose oxidase-peroxidase catalyzed reaction was allowed to proceed to completion (incubation for 1 hr. at 37°C); (c) increased amounts of mineral acid (0.5 ml) were used to terminate the reaction; and (d) freshly prepared Glucostat reagent was aged 12-24 hours before use. Data in Table II are typical of results obtained using the Worthington procedure and Method A (see Methods) of this study. When the individual absorbance values in Table II are converted to a common basis, the coefficient of variation of the sixteen absorbtivity values obtained using the Worthington procedure is approximately six times greater than the coefficient of variation obtained using Method A.

To further demonstrate the improved reliability and sensitivity of our modified glucose assay procedure (Method A), a standard solution of D-glucose was analyzed over a 5-day period using a single supply of Glucostat reagent.

The data in Table IV indicate that only one determination of a D-glucose standard is required to calibrate a new supply of Glucostat reagent, if the reagent is stored at 5°C in the dark and used within five days. Furthermore, the reproducibility of Method A was ± 0.3% at a concentration of 120 μg ml^{-1} (Table IV); Banks and Greenwood (69) reported a reproducibility of ± 0.5% at 20 μg ml^{-1}.

A more sensitive method for determing D-glucose than Method A was also employed in this investigation. By adjusting reaction volumes (changing the ratio between starch hydrolyzate and Glucostat reagent from 1:10 to 1:1) we found Glucostat reagent could be used to assay solutions containing 6-18 μg ml^{-1} of D-glucose (method B). Ten separate analyses of a standard D-glucose solution (10 μg ml^{-1}) were performed over a 7-day period. Method B was found (Table V) to give good reproducibility; the coefficient of variation for 10 μg of D-glucose was found to be 0.5%.

These results show that the glucose oxidase-peroxidase assay procedure provides a very accurate and sensitive measure of D-glucose. That is essential to the success of using glucoamylase to measure gelatinization for small amounts of material.

TOTAL STARCH DETERMINATION:

In order to measure percent gelatinization in any sample, one must first know the total starch content (gelatinized and non-gelatinized) of the sample. Since starch is a glucose polymer, it on hydrolysis should yield glucose. Therefore by measuring the amount of glucose released by hydrolysis of starch one should be able to estimate the total starch content in a sample.

But actually, starch estimation is not so simple as explained above because of the following two reasons; (a) a method is required which yields

TABLE IV

REPRODUCIBILITY OF A MODIFIED PROCEDURE (METHOD A) FOR ASSAY OF D-GLUCOSE[a]

	Average of Duplicate Determination									
Trial	1	2	3	4	5	6	7	8	9	10
Absorbance	.542	.545	.541	.542	.543	.542	.540	.542	.543	.544

Mean, 0.542; standard deviation, 0.0014; coefficient of variation, 0.26%

[a]The amount of D-Glucose was 120 µg in 1.0 ml of ethanol:citrate:buffer: 90% dimethyl sulfoxide (10:5:1; v/v). Analyses were performed over a period of five days using Method A (see Materials and Methods) and a single preparation of Glucostat reagent (stored at 5°C).

TABLE V

REPRODUCIBILITY OF A SENSITIVE ASSAY PROCEDURE (METHOD B) FOR D-GLUCOSE[a]

	Average of Duplicate Determinations									
Trial	1	2	3	4	5	6	7	8	9	10
Absorbance	.296	.295	.295	.294	.297	.294	.296	.295	.293	.298[b]

[a]The amount of D-Glucose was 10 µg in 1.0 ml of ethanol-citrate buffer (2:1; v/v). Analyses were performed over a period of seven days using Method B (see Materials and Methods) and a single preparation of Glucostat reagent (stored at 5°C)

[b]Mean, 0.295; Standard deviation, 0.0015; coefficient of variation, 0.5%.

100% conversion of starch to glucose, and (b) an accurate, specific and precise technique is needed to measure the amount of glucose released. The latter requirement has been fulfilled by our modified enzymatic method (Method A, page 19) based on oxidation of glucose using glucose oxidase.

Theoretically the first requirement can also be met by using glucoamylase, an enzyme capable of converting starch completely to glucose. Actually the quantitative conversion of starch to glucose by glucoamylase was found to depend on several factors such as the source of the enzyme, and the botanical source of the starch substrate (41), and the method of dissolving the starch prior to enzymic digestion. Marschall (74) reported that purified glucoamylase doesn't give quantitative conversion of starch to glucose without the presence of endo acting enzyme such as α-amylase.

To determine total starch we used the method described by Libby (73) (see Method G). We chose this procedure principally because aqueous DMSO can be used to determine starch in cereal grain (75). In Libby's procedure starch is dissolved in 90% DMSO, and the solution is diluted with buffer (pH 4.8) so that the concentration of dimethyl sulfoxide in the glucoamylase digest is 15% by volume. Lineback and Sayeed (76) had previously shown the rate of starch digestion by glucoamylase is unaffected by the addition of up to 18% by volume of dimethyl sulfoxide. The D-glucose released during digestion is subsequently assayed using Glucostat reagent (Method A). In the initial stages of our work, a 93% conversion of starch to glucose was obtained with A. niger glucoamylase using prime wheat starch as a substrate. This incomplete hydrolysis must be attributed to one or more of the components involved in the hydrolysis: starch, enzyme or solvent (DMSO). These components and their possible relation to the incomplete hydrolysis will be discussed individually.

Starch:

Abnormal linkages between glucose units if present in starch molecule may act as an obstacle for enzyme action.

Molecular aggregates may form during the process of drying starch which may be inaccessible to attack by enzyme. This error is eliminated by changing the drying procedure. Instead of drying the starch by repeated methanol extraction after gluten separation, a dilute suspension of starch (2 to 3% solids) was freeze dried. Under these conditions the starch is dispersed in the form of very fine particles, which when subjected to freeze drying may remain as discrete particles without aggregation.

The possible presence of hemicelluloses or other non-starch components was checked by analyzing the starch hydrolyzate obtained by acid hydrolysis for the presence of pentoses. A paper chromatogram, spotted heavy enough to detect the presence of less than 1% of D-xylose, failed to show any indication of the presence of pentoses.

Incomplete solubility of starch in 90% DMSO may be responsible for incomplete hydrolysis. Libby (73) reported that maximum solubility of starches in 90% DMSO, under the conditions used for starch dissolution (i.e. 55°C for 20 min.), is 0.1% by weight. Therefore less than 100 mg of starch was dissolved in 100 ml of 90% DMSO and the mixture was constantly stirred throughout the dissolution time (2 min.) at 55°C.

Enzyme:

Transglucosylase activity present in the commercial glucoamylase may be responsible for incomplete conversion to glucose. Transglucosylase activity was assayed by paper chromatography of starch hydrolyzates to detect the presence of oligosaccharides. A very faint spot with the mobility

of oligosaccharides appeared on the chromatogram, and was estimated to represent less than one percent of hydrolyzed material by quantitative spotting of increasing concentrations of hydrolyzate. This possiblity was further checked by hydrolyzing a wheat starch sample with a commercially purified (twice recrystallized) glucoamylase enzyme (Rhizopus niveus) reported to be free from transglucosylase activity (74, 77). It was reported by Marshall (74) that a highly purified glucoamylase needs an endo enzyme to give quantitative conversion of starch to glucose. Therefore, this purified glucoamylase (Rhizopus niveus)was mixed with small amounts of commercially purified pancreatic α-amylase (78) free of transglucosylase activity, in order to assist the quantitative conversion of starch to glucose. No increase in percent conversion of starch to glucose was observed.

Time of Hydrolysis:

Insufficient time of hydrolysis can cause incomplete conversion. Approximately one percent increase in percent hydrolysis was obtained when starch was hydrolyzed for a longer period of time (18 hours) which agrees with the observation of Pazur and Ando (79).

Therefore the factors due to starch, enzyme, or length of incubation period discussed above may not be responsible for the incomplete digestion (93%) of wheat starch.

Solvent (DMSO):

The effect of solvent did account for the incomplete hydrolysis observed in our study. Incomplete hydrolysis, a source of error in determining T (Total starch) in equation I was found to be associated with the dissolution of starch in 90% DMSO at 55°C. For unknown reasons, prolonged heating of

starch in this solvent decreases its digestibility by glucoamylase. Libby (73) alluded to this phenomenon but did not present data on its magnitude. On the other hand, Leach and Schoch (20) reported solutions of starch in 90% DMSO were stable over a period of months. The curve in Fig. 1 shows the digestibility of starch by glucoamylase (10 IU per mg of starch) decreased linearly (about 5%) as the period of dissolving starch in 90% DMSO at 55°C was increased from 5 min. to 30 min.

The amount of glucoamylase we used to determine total starch was determined by the amount needed to quantitatively digest the starch in a sample in 30 min. We found 10 IU or 15 IU of glucoamylase gave almost a quantitative (98.0%) yield of D-glucose from 1 mg of starch in 30 min. at 37°C. Increasing the incubation period to 18 hr. increased the conversion to 98.5% D-glucose. The slightly lower than quantitative hydrolysis of starch to D-glucose, is not a serious problem since total starch (T) appears in the denominator of equation I. The theoretical starch content as determined by proximate analysis (see Methods) serves as a check on the value obtained by total starch determination.

SUSCEPTIBILITY OF INTACT STARCH TO GLUCOAMYLASE:

The accuracy of our method to determine the extent of gelatinization is affected by the degree of selectivity of glucoamylase for gelatinized starch compared to prime starch. The literature contains conflicting reports concerning the action of glucoamylase on intact granules. Macrae and Armstrong (80) claimed a commercial glucoamylase from A. niger (Agidex, Glaxo Laboratories, Greenford, Middlesex, England) did not attack raw starch, and Manners (81) stated glucoamylase has only limited action on starch granules. On the other

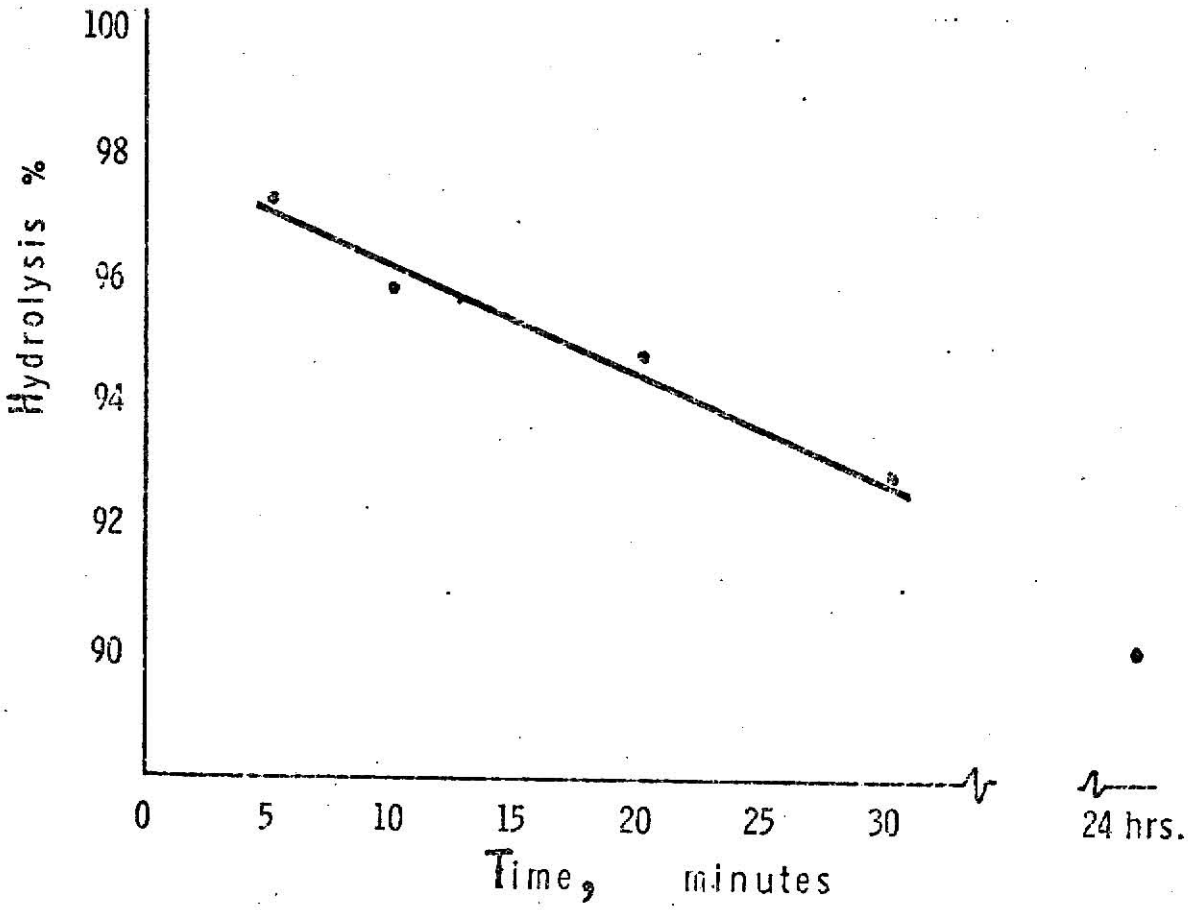


Fig. 1. Digestibility of starch after dissolution in DMSO-water (9/1) at 55°.

hand, Evers (82) presented scanning electron microscopic evidence of attack by Agidex. However, it is possible the attack observed by Evers is due to the presence of α-amylase in commercial preparations of glucoamylase from A. niger (74, 77). It has been well established that α-amylase attacks granular starch (83).

A group of Japanese workers (37) reported a commercial glucoamylase from an Endomyces species gave 6.1% and 1.5% digestion of native corn starch and potato starch, respectively, when 25 mg of starch was treated with 6 IU of enzyme for 1 hr. at 37°. Again other workers (83) had previously shown this commercial enzyme preparation contained an α-amylase which was very difficult to separate from glucoamylase. Leach and Schoch (84) also used an impure glucoamylase to partially digest granular corn starch.

We therefore decided to examine the digestibility of intact wheat starch not only with Takamine Diazyme, which we used to determine gelatinization, but also with R. niveus glucoamylase, a crystalline enzyme which contains no α-amylase (74, 77, 85).

We found[a] both glucoamylases extensively digested wheat starch (Table VI). Therefore, pure glucoamylase can indeed attack native starch. The data in Table VI show the initial attack ($\leq$ 2 hr.) by either enzyme is approximately the same, but at longer digestion times, attack by Takamine Diazyme is more vigorous than attack by R. niveus glucoamylase. After 64 hr., Takamine Diazyme affected 84% conversion of starch to D-glucose, whereas R. niveus glucoamylase gave only 32% conversion.

---

[a]Evers (82) treated 5 g wheat starch with 3000 "Glaxo units" of Agidex for 5 days at 50°C. Assuming a doubling of the rate of reaction for every 10° increase, one can estimate Evers used approximately 0.09 International Units/mg of starch. In this work we used 5 IU/mg of starch at 37°C.

TABLE VI

CONVERSION (%) OF WHEAT STARCH[a] TO D-GLUCOSE BY GLUCOAMYLASE

Glucoamylase Source	Incubation period, hours							
	0.5	1	2	4	8	16	32	64
Aspergillus niger[b]	2.6	4.2	7.1	13.2	22.0	38.5	53.2	83.9
Rhizopus niveus	3.5	4.7	7.1	9.5	11.0	17.8	26.3	31.9

[a]Starch (100 mg) with glucoamylase (500 IU) in 50 ml of 0.05M citrate buffer (pH 4.8) at 37°C.

[b]Commercial preparation--Takamine Diazyme

Examination of the partially-digested, large, wheat starch granules by scanning electron microscopy revealed different morphological modes of attack by the two enzymes (Fig. 2). During the first 30 min. of digestion, both enzymes exposed the equatorial groove on many starch granules: (Fig. 2,Band E);the equatorial groove was not seen in the control starch (Fig. 2,A). At longer digestion times, R. niveus glucoamylase attacked principally on the surface of the large granules, giving their exterior a spongy appearance, which was occasionally pierced by a sharply defined, cylindrical hole of small diameter (Fig. 2, C and D).

On the other hand, digestion by Takamine Diazyme appeared to be less confined to the surface of the granules than digestion by R. niveus glucoamylase. Attack by the former enzyme was characterized by deeper and wider penetration into a granule's interior, which created scoop-shaped depressions on its surface (Fig. 2, F and G). In addition, tunnels, beginning chiefly at the equatorial groove and growing wider as they penetrated deeper into a granule, were often observed. These tunnels were not seen in granules exposed to R. niveus glucoamylase. The tunneling and higher degree of digestion by Takamine Diazyme could be due to the presence of α-amylase in this commercial enzyme. Other investigators (86, 87) previously demonstrated tunneling and extensive internal digestion of large wheat starch granules by α-amylase.

It is apparent then that in determining extent of gelatinization using glucoamylase, a correction is needed because of digestion of granular starch by the glucoamylase. This factor can be determined only after the glucoamylase concentration for digestion of partially-gelatinized starch is chosen.

THIS BOOK CONTAINS SEVERAL DOCUMENTS THAT ARE OF POOR QUALITY DUE TO BEING A PHOTOCOPY OF A PHOTO.

THIS IS AS RECEIVED FROM CUSTOMER.

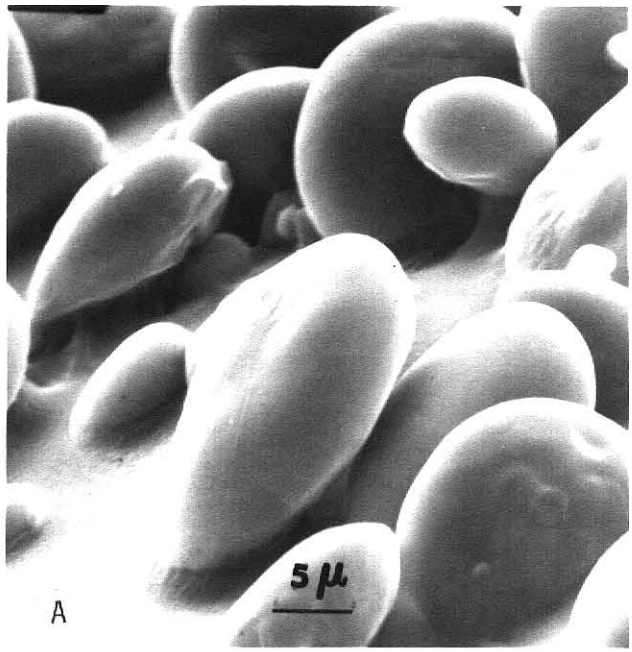
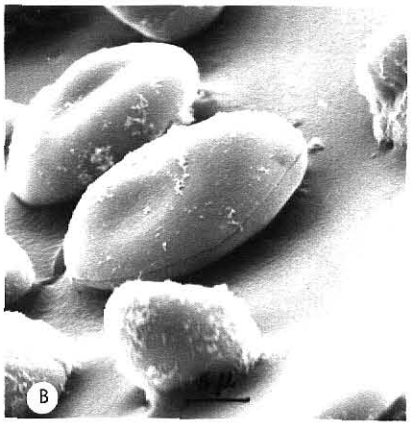
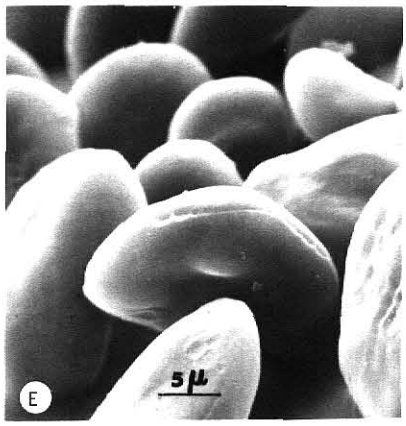
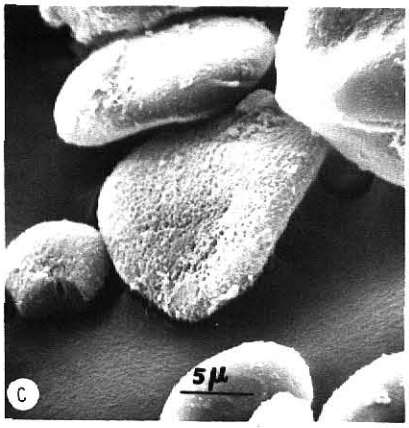


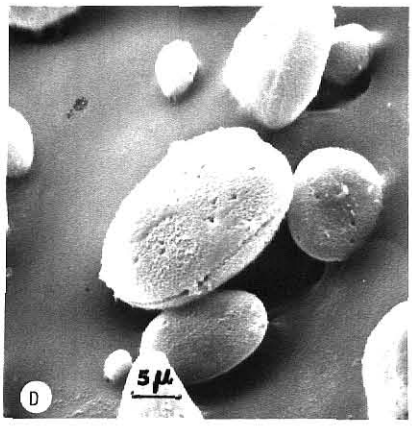
Fig. 2. Attack of glucoamylases on wheat starch (see also Table IV): A. control: B through D, Rhizopus niveus; and E through G, Takamine Diazyme. Digestion periods: B and E, 0.5 hr.; C and F, 8 hr., and D and G, 32 hr.

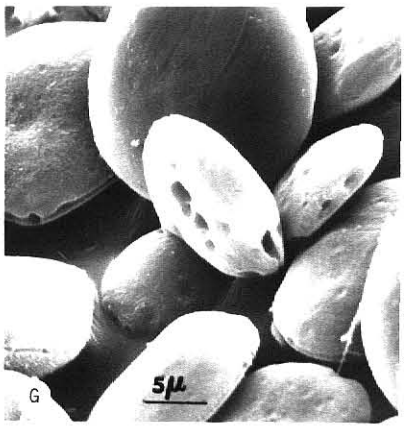

B
5μ


E
5μ


C
5μ


F
5μ


D
5μ


G
5μ

The amount of glucoamylase used to digest partially-gelatinized starch samples (to obtain the value of X in equation I) was determined empirically. We wished to convert a 100 mg sample of starch in an aqueous medium completely to D-glucose in 30 min., even when the sample contained 100% gelatinized starch. We found approximately 500 IU of Takamine Diazyme was needed to accomplish this conversion under our experimental conditions.

To find factor B in equation I, needed to correct for attack of glucoamylase on granular (ungelatinized) starch, we digested various amounts (25-100 mg) of prime wheat starch with 500 IU glucoamylase for 0.5 hr. at 37°C and measured the amount of D-glucose released (Table VII). The data in Table VII shows a linear correlation between the D-glucose released during digestion and the amount of granular starch. The linear correlation is important in using equation I to determine the percentage gelatinization in an unknown sample, since it can be shown that a correction for the digestion of intact starch can be applied without prior knowledge of the sample's degree of gelatinization.

To determine the accuracy of our proposed glucoamylase method of determining the degree of gelatinization, we prepared a series of starch standards containing varying amounts of (0-100%) gelatinized starch (Table VIII). Total starch determination, using dissolution in 90% DMSO, on three of the samples gave an average of 98.0% theoretical conversion to D-glucose; thus T in equation I was 98.0%. To determine the value of X, each standard mixture was digested in 0.05M citrate buffer (pH 4.8) at 37° for 0.5 hr. using 500 IU of Takamine Diazyme. A linear correction was then applied to the experimentally determined digestibility (X) by using equation I (Appendix) to eliminate the error incurred by the partial digestion of intact granular starch. The corrected digestibility represents the experimentally

TABLE VII

HYDROLYSIS OF GRANULAR STARCH DURING 0.5 HOUR DIGESTION[a]

Sample weight mg	Glucose released mg	% hydrolysis of starch
100	2.63	2.52
100	2.70	2.60
100	2.73	2.62
100	2.78	2.66
100	2.67	2.56
75	2.04	2.61
50	1.37	2.62
25	0.69	2.64

[a]Wheat starch (25-100 mg) treated with Takamine Diazyme (500 IU) in 50 ml of 0.05M citrate buffer at pH 4.8 at 37°C.

TABLE VIII

COMPARISON OF KNOWN AND EXPERIMENTALLY DETERMINED DEGREES OF GELATINIATION

Standard Sample[a] % Gelatinization	% Digestibility[b] (X)	% Gelatinization[c] (Y)
0	2.65	0
5	6.84	4.3
10	11.5	9.1
25	26.8	24.8
50	49.9	48.5
75	75.3	74.6
100	98.4	98.4

[a]Starch standards with different degree of gelatinization prepared by mixing prime starch and pre-gelatinized starch (see p. 17).

[b]Experimentally determined digestibility of a standard sample (sample weight 100 mg) in 50 ml of 0.05M citrate buffer for 30 min. at 37° with Takamine Diazyme (500 IU). Percentages based on total starch content (see page 22).

[c]Values of $Y = \frac{100\ (X-B)}{100-B}$ (see Appendix) where B = 2.6%, the digestibility of granular starch (0% gelatinization) in 0.5 hours digestion time.

determined degree of gelatinization (Y). The latter values were found to be in excellent agreement with the theoretical values (Table VIII). The largest error observed was at the 5.0% gelatinization level, where our analytical procedure gave 4.3% gelatinization.

This method can probably be used to determine the degree of gelatinization of starch in foods and feeds by incorporating simple procedures listed below:

(1) The sample should be dried at room temperature to eliminate excess moisture. Freeze drying shall be done whenever possible.

(2) Glucose and oligosaccharides made up of D-glucose units, if present, should be removed prior to analysis (by extraction with 80% ethanol).

(3) Excess fat should be removed from fatty foods by extraction with a solvent such as petroleum ether.

All the above mentioned procedures should be carried out at room temperature as far as possible or at temperatures much below the gelatinization temperature of the particular starch sample.

APPENDIX

Derivation of Equation I

$$Y = X - \left(B \frac{100-Y}{100}\right)$$

Where Y = Percent gelatinization of starch in a sample.

X = Percent of starch converted to glucose from a sample by glucoamylase digestion. See page 22.

B = Percent granular starch digested by glucoamylase in 0.5 hour digestion period.

100-> represents the total starch content since all the variables Y, X and B are calculated based on total starch content.

$B \frac{100-Y}{100}$ = Percent digestion contributed by the portion of intact granular starch present in the sample. (The variable T_1 - page 23 is not required here because the intact starch is a part of the given sample having the same proximate analysis.)

$$X - Y = \frac{B\ 100 - BY}{100}$$

$$100X - 100Y = B100 - BY$$

$$100X - B100 = 100Y - BY$$

$$100\ (X-B) = Y\ (100-B)$$

$$Y = \frac{100\ (X-B)}{100-B}$$

SUMMARY AND CONCLUSIONS

The steps involved in the determination of the degree of gelatinization of starch by the enzymic procedure developed in this investigation are as follows:

(1) A proximate analysis of the starch sample is obtained and an "expected" starch content is calculated. This step is optional and can be skipped in routine analysis. (Time required is variable).

(2) Total starch content (T) is determined in the given sample after DMSO solubilization. (Time required is 2 hours).

(3) Digestible starch (X) is determined by glucoamylase hydrolysis (Time required is 1 3/4 hours).

(4) Digestibility of starch (B) by glucoamylase is determined. In routine analysis this step needs to be done only once for each botanical source of starch). (Time required is 1 3/4 hours).

(5) The degree of gelatinization (Y) is calculated by using the equation

$$Y = \frac{100\ (X-B)}{100-B}$$

The time indicated for each stage is its duration in a routine analysis if all facilities are ready. Stages 2, 3 and 4 can be done simultaneously if adequate facilities are available. Therefore, a single sample can be analyzed in two hours in a routine analytical procedure with adequate facilities.

We conclude that this dual enzymic method is the most accurate, precise and sensitive method developed so far to determine the degree of gelatinization of starch.

LITERATURE CITED

1. POWELL, E. L. Production and use of pregelatinized starch. In: Starch chemistry and technology, ed. by R. L. Whistler and E. F. Paschall, Vol. II, pp. 523-536. Academic Press: New York (1967).

2. SCHOCH, T. J., and MAYWALD, E. C. Microscopic examination of modified starches. Anal. Chem. 28(3):383 (1956).

3. MYHRE, D. V. The function of carbohydrates in baking. Baker's Dig. 44(3):32 (1970).

4. WILLIAMS, P. C., and LESEELLEUR, G. C. Determination of damaged starch in flour. A comparative study of present-day procedures. Cereal Sci. Today 15(1):4 (1970).

5. HAENLEIN, C. F. W., BURTON, D. W., HOYT, H. C., MITCHELL, W. H., and RICHARDS, C. R. Effects of expanding or pelleting upon feed digestibility and heifer growth. J. Dairy Sci. 45:754 (1962).

6. LEACH, H. W. Gelatinization of starch. In: Starch Chemistry and Technology, ed. by R. L. Whistler and E. F. Paschall, Vol. I, pp. 289-307. Academic Press: New York (1965).

7. STERLING, C. The structure of the starch grain. In: Starch and its derivatives. ed. by J. A. Radley, pp. 139-167. Chapman and Hall Ltd: London (1968).

8. MEYER, K. H. The past and present of starch chemistry. Experientia 8:405 (1952).

9. KENT-JONES, D. W., and AMOS, A. J. eds. Modern Cereal Chemistry. pp. 12-17. Food Trade Press Ltd: London (1967).

10. LEACH, H. W., and SCHOCH, T. J. Structure of the starch granule. II. Action of various amylases on granular starches. Cereal Chem. 38: 34 (1961).

11. BADENHUIZEN, N. P. Chemistry and biology of the starch granule. Protoplasmologia Band II (1959).

12. ULMAN, M. The outer membrane of starch granule. Die Starke, 8:109 (1956). Chem. Abstr. 51:1632 (1957).

13. ULMAN, M. Determination of the chemical nature of the hull of a swollen starch granule. Kolloid. Z. 150:128 (1957). Chem. Abstr. 51:14301a (1957).

14. BUTTROSE, M. S. The influence of environment on the shell structure of starch granules. J. Cell. Biol. 14:159 (1962).

15. GREENWOOD, C. T. Starch and Glycogen In: The Carbohydrates: Chemistry and biochemistry. ed. by W. Pigman and D. Horton, Vol II B, pp. 471-513. Academic Press: New York (1970).

16. KERR, R. W., ed. Chemistry and industry of starch. Chap. I. pp. 3-25. Academic Press: New York (1950).

17. COLLISON, R. Swelling and gelation of starch In: Starch and its derivatives. ed. by J. A. Radley, pp. 168-193. Chapman and Hall Ltd.: London (1968).

18. SEIB, P. A. Starch gelatinization: Chemical and physical effects. FEEDSTUFFS 43: pp. 44-45, 50. (March 13, 1971).

19. HELLMAN, N. N., FAIRCHILD, B., and SENTI, F. R. The bread staling problem. Molecular organization of starch upon aging of concentrated starch gels at various moisture levels. Cereal Chem. 31:495 (1954).

20. LEACH, H. W., and SCHOCH, T. J. Structure of the starch granule III. Solubilities of granular starches in dimethyl sulfoxide. Cereal Chem. 39:318 (1962).

21. MULLEN, J. W., and PACSU, E. Starch Studies. Gelatinization of Starches in Water and in aqueous pyridine. Ind. Eng. Chem. 34:807 (1942).

22. TAFT, R. Liquid ammonia as a lyophilic dispersion medium, J. Phys. Chem. 34:2792 (1930).

23. ULMANN, M., and SCHIERBAUM, F. The determination of various types of boundwater in potato starch. Ernaehrungsforschung 3:65 (1958). Chem. Abstr. 52:19197 (1958).

24. KATZ, J. R., and DERKSEN, J. C. Physical chemistry of starch and bread making. VI. Z. Phys. Chem. 150A:100 (1930). Chem. Abst. 25:16 (1931).

25. SCHIERBAUM, F., TAUFEL, K., and ULMANN, H. Die Hydration der Starke Die Starke 14:161 (1962).

26. COLLISON, R., and ELTON, G. A. H. Some factors which influence the rheological properties of starch gels. Die Starke 13:104 (1961).

27. BOURNE, E. J., TIFFIN, A. I., and WEIGEL, H. Interaction of starch with sucrose stearates and other antistaling agents. J. Sci. Food Agric. 11:101 (1960).

28. JONES, C. R. The production of mechanically damaged starch in milling as a governing factor in the diastatic activity of flour. Cereal Chem. 17:133 (1940).

29. SANDSTEDT, R. M., and ABBOTT, R. C. A comparison of methods for studying the course of starch gelatinization. Cereal Sci. Today 9:13 (1964).

30. JASKA, E. Starch gelatinization as detected by proton magnetic resonance. Cereal Chem. 48:437 (1971).

31. STEVENS, D. J., and ELTON, G. A. H. Thermal properties of the starch/water system. Part I. Measurement of heat of gelatinization by differential scanning calorimetry. Die Starke 23:8 (1971).

32. COULTER, P. R., and POTTER, O. E. Measurement of the susceptibility of starch to hydrolysis by malt enzymes. J. Inst. Brew. 78:445 (1972).

33. SULLIVAN, J. W., and JOHNSON, J. A. Measurement of starch gelatinization by enzyme susceptibility. Cereal Chem. 41:73 (1964).

34. NEWTON, J. M., FARLEY, F. F., and NAYLOR, N. M. The use of soybean beta-amylase to follow the modification of starch. Cereal Chem. 17:342 (1940).

35. MARTIN, V. D., and NEWTON, J. M. Comparitive rates of amylase action on starches. Cereal Chem. 15:456 (1938).

36. WATSON, C. A., and JOHNSON, J. A. Studies on gelatinization of starch I. Competition for water by protein and cellulose derivatives. J. Food Sci. 30:450 (1965).

37. TOYAMA, T., HIZUKURI, S., and NIKUNI, Z. Estimation of starch gelatinization by means of glucoamylase. J. Soc. Starch Technol. (Japan) 13:69 (1966) and references therein.

38. ANSTAETT, F., and PFOST, H. B. Effects of temperature, pressure, moisture content and time on starch gelatinization in cooking grain. Trans. Am. Soc. Agr. Eng., 12:493 (1969).

39. LIANG, Y. T., MORRIL, J. L., ANSTAETT, F. R., DAYTON, A. D., and PFOST, H. B. Effect of pressure, moisture and cooking time on susceptibility of corn or sorghum grain starch to enzymatic attack. J. Dairy Sci. 53:336 (1970).

40. NOVELLE, L., and SCHUTTE, R. J. Kaffircorn malting and brewing studies. X. The susceptibility of sorghum starch to amylolysis. J. Sci. Food. Agr. 12:552 (1961).

41. FLEMING, I. D. Amyloglucosidase. In: Starch and its derivatives, ed. by J. A. Radley, pp. 498-508. Academic Press: New York (1968).

42. ROBYT, J. F., and WHELAN, W. J. The α-amylases, In: Starch and its derivatives, ed. by J. A. Radley, pp. 430-476. Academic Press: New York (1968).

43. ROBYT, J. F., and WHELAN, W. J. The β-amylases, In: Starch and its derivatives, ed. by J. A. Radley, pp. 477-497. Academic Press: New York (1968).

44. SHILDNECK, P., and SMITH, C. E. Production and uses of acid-modified starch. In: Starch Chemistry and Technology, ed. by R. L. Whistler and E. F. Paschall, Vol. II, pp. 217-235. Academic Press: New York (1965).

45. SUNDERMAN, F. W., MACFATE, R. P., EVANS, G. T., and FULLER, J. B. Symposium on blood glucose. Am. J. Clin. Path. 21:901, (1951).

46. SAIFER, A., VALENSTEIN, F., and HUGHES, J. P. The photometric determination of sugar in biological fluids by ferricyanide reduction. J. Lab. Clin. Med. 26:1969 (1941).

47. NELSON, N. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153:375 (1944).

48. SCOTT, T. A., and MELVIN, E. H. Determination of dextran with anthrone. Anal. Chem. 25:1656 (1953).

49. DUBOIS, M., GILLES, K. A., HAMILTON, J. K., REBERS, P. H., and SMITH, F. Colorimetric method for determination of sugar and related substances. Anal. Chem. 28:350 (1956).

50. MULLER, D. Studies on the new enzyme glucose oxidase. BIOCHEM. Z 199:136 (1928). Chem. Abstr. 23:166 (1929).

51. FRANKE, W., and LORENZ, F. Glucose oxidase. Liebigs Ann. Chem. 532:1 (1937) Chem. Abstr. 32:1731 (1938).

52. KESTON, A. S. Specific colorimetric enzymatic analytical reagents for glucose. In: Abstracts of papers, 129th Meeting, Am. Chem. Soc., p. 31c (April 1956).

53. COMER, J. P. Semiquantitative specific test paper for glucose in urine. Anal. Chem. 28:1748 (1956).

54. REED, G. Enzymes in Food Processing. pp. 177-181. Academic Press Ltd: New York (1966).

55. BENTLEY, R. Glucose oxidase. In: The Enzymes. ed. by P. D. Boyer, H. Lardy, and K. Myrback, 2nd ed., Vol. 7, pp. 567-568. Academic Press: New York (1963).

56. UNDERKOFLER, L. A. Glucose oxidase: Production properties, and present and potential applications. Soc. Chem. Ind. (London) Monograph II: pp. 48-70 (1961).

57. KEILIN, D., and HARTREE, E. F. Properties of glucose oxidase (Notatin), Biochem. J. 42:221 (1948).

58. KEILIN, D., and HARTREE, E. F. Specificity of glucose oxidase (Notatin), Biochem. J. 50:331 (1952).

59. TELLER, J. D. Direct, quantitative, colorimetric determination of serum or plasma glucose. Abstracts of Papers, 160th Meeting, Am. Chem. Soc., p. 69c (Sept. 1956).

60. McCOMB, R. B., YUSHOK, W. D., and BATT, D. B. Colorimetric estimation of D-glucose and 2-deoxy-D-glucose with glucose oxidase. J. Franklin Inst. 265:417 (1958).

61. KINGSLEY, G. R., and GETCHELL, G. Direct ultramicro glucose oxidase method for determination of glucose in biologic fluids. Clin. Chem. 6:466 (1960).

62. SAIFER, A., and GERSTENFELD, B. S. The photometric microdetermination of blood glucose with glucose oxidase. J. Lab. Clin. Med. 51:448 (1958).

63. HUGGETT, A. ST. G., and NIXON, D. A. Use of glucose oxidase, peroxidase, and o-dianisidine in determination of blood and urinary glucose. Lancett ii, 368 (1957).

64. WASHKO, M. E., and RICE, E. W. Determination of glucose by an improved enzymatic procedure. Clin. Chem. 7:542 (1961).

65. DAHLQVIST, A. Determination of maltase and isomaltase activities with a glucose oxidase reagent. Biochem. J. 80:547 (1961).

66. LLOYD, J. B., and WHELAN, W. J. An improved method for enzymic determination of glucose in the presence of maltose. Anal. Biochem. 30:467 (1969).

67. ANNONYMOUS, Glucostat for the enzymatic determination of glucose, Technical Brochure, Worthington Biochemical Corp. Freehold, New Jersey (December 1971).

68. FIELD, J. B., and WILLIAMS, H. E. Artifactual hypoglycemia associated with leukemia. New Eng. J. Med. 265:946 (1961).

69. BANDS, W., and GREENWOOD, C. T., The characterization of starch and its components Part IV. The specific estimation of glucose using glucose oxidase. Die Starke 23:222 (1971).

70. WOLF, M. J. Wheat Starch isolation. In: Methods in Carbohydrate Chemistry ed. by R. L. Whistler. Vol. IV, pp. 6-9, Academic Press: New York (1964).

71. FELLERS, D. A., PHILIP, H. J., SMITH, S., MOSSMAN, A. P., and SHEPHERD, A. B. Process for Protein-Starch Separation in Wheat Flour. Food Technol. 23:560 (1969).

72. LINEBACK, D. R., RUSSELL, I. J., and RASMUSSEN, C. Two forms of the glucoamylase of Aspergillus niger. Arch. Biochem. Biophys. 134:539 (1969).

72a. ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS, Official methods of analysis, 11th ed., The Association: Washington, D. C. (1970).

73. LIBBY, R. A. Direct starch analysis using DMSO solubilization and glucoamylase. Cereal Chem. 47:273 (1970).

74. MARSHALL, J. J., and WHELAN, W. J. Incomplete conversion of glycogen and starch by crystalline amyloglucosidase and its importance in the determination of amylaceous polymers. FEBS Letters 9(2):85 (1970).

75. GARCIA, W. J. and WOLF, M. J. Polarimetric determination of starch in corn with dimethyl sulfoxide as a solvent, Cereal Chem., 49:298 (1972).

76. LINEBACK, D. R., and SAYEED, A. L. Effect of dimethylsulfoxide on amylase activity. Carbohyd. Res. 17:453 (1971).

77. MARSHALL, J. J., and WHELAN, W. J. Detection of endoacting carbohydrases, particularly in the presence of exoenzymes acting on the same substrate. Anal. Biochem. 43:316 (1971).

78. ANONYMOUS, Alpha Amylase (hog pancreas). Product Catalog, pp. 55. Nutritional Biochemical Corp. Cleaveland, Ohio. (Fall 1972).

79. PAZUR, J. H. and ANDO, T. The action of amyloglucosidase of Aspergillus niger on starch and malto-oligosaccharides. J. Biol. Chem. 234:1966 (1959).

80. MACRAE, J. C., and ARMSTRONG, D. G. Enzyme method for determination of α-linked glucose polymers in biological materials. J. Sci. Fd. Agri. 19:578.

81. MANNERS, D. J. Some industrial aspects of the enzymic degradations of starch. Biochem. J. 123:1p (1971).

82. EVERS, A. D., GONG, B. M., and PYBUS, J. N., Scanning electron microscopy of wheat starch. IV. Digestion of large granules by glucoamylase of fungal (Aspergillus niger) origin. Die Starke 23:16 (1971).

83. HATORI, Y., and TAKEUCHI, I. Studies of amylolytic enzymes produced by Endomyces Sp. Part II. Purification and general properties of amyloglucocsidase. Agri. Biol. Chem. 25:895 (1961).

84. LEACH, H. W., and SCHOCH, T. J. Structure of the starch granule. Action of various amylases on granular starches. Cereal Chem. 38:34 (1961).

85. MARSHALL, J. J. Action of amylolytic enzymes on a chromogenic substrate. Anal Biochem. 37:467 (1970).

86. EVERS, A. D., and McDERMOTT, E. E. Scanning electron microscopy of wheat starch. II. Structure of granules modified by alpha-amylolysis. Die Starke 22:24 (1970).

87. DRONZEK, B. L., PAULINA HWANG, and BUSHUK, W. Scanning electron microscopy of starch from sprouted wheat. Cereal Chem. 49:232 (1972).

ACKNOWLEDGEMENTS

It is a great pleasure to acknowledge my sincere gratitude to Dr. P. A. Seib, my major professor, for his suggestions and encouragement throughout the period of this research work and preparation of this manuscript. Dr. Seib's patience and sympathetic attitude at times when we ran into unexpected problems in our study, was commendable.

I wish to thank Dr. D. R. Lineback for his valuable suggestion and comments in the preparation of this manuscript.

My thanks are also due to Dr. P. Nordin for being on my advisory committee and reviewing the manuscript.

I appreciate very much the help of my friends S. F. Quadri, C. S. Rao and Varalakshmi Bhupal in preparing this manuscript.

Lastly I am grateful to Kansas Wheat Commission for financing this research project.

DETERMINATION OF THE DEGREE OF GELATINIZATION OF STARCH

by

RAVIPRASAD M. SHETTY

B.Sc., University of Agricultural Sciences, Bangalore, India, 1968

AN ABSTRACT OF A THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

in

Food Science

Department of Grain Science and Industry

KANSAS STATE UNIVERSITY

Manhattan, Kansas

1973

ABSTRACT

A method has been devised to measure the extent of gelatinization of starch. The procedure involves selective digestion of the gelatinized fraction of starch with glucoamylase followed by determination of the D-glucose released using D-glucose oxidase. Glucoamylase was also used to determine total starch in the sample. The method was tested on known mixtures of gelatinized and prime starch, and was found to give an accurate measure of gelatinization. A scanning electron microscopic study of the attack of two glucoamylases on intact starch granules was made and the extent of attack was measured. This enzymic method provides a precise, accurate and sensitive measure of degree of gelatinization.