ASSAY OF BETA-AMYLASE IN MALT

BY

OPTICAL ROTATION

by

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INTRODUCTION

Malt is prepared commercially by germinating cereal grains. Usually barley is used, but wheat, sorghum and other grains have limited use also. During germination alpha-amylase is produced and the beta-amylase content also increases. Methods are available for the analysis of alpha-amylase in the presence of beta-amylase, but the reverse, analysis of the beta-amylase in the presence of the alpha-amylase is not easily carried out.

In the present study an optical rotatory method is examined to analyze beta-amylase. The method is based on the principle that during alpha-amylolysis, the optical rotation drops an insignificant amount compared to beta-amylolysis. Barley, sorghum and wheat malts were analyzed and the method was used to test for the effectiveness of destroying beta-amylase in sorghum malt by heat treatment.

REVIEW OF THE LITERATURE

Malt is the product of germination of cereal grains controlled to limit sprout and rootlet development. The process known as malting (1) consists of three phases: steeping, germination, and kilning or drying. Grains are malted primarily to develop or activate enzyme systems, such as amylase (diastase), which are important in subsequent commercial uses of the malt.

The growing of barley and wheat is referred to in the earliest records of agriculture and the use of barley for the production of fermented beverages dates back to the ancients. It is not clear from the records just when the germination of grains to produce starch-splitting enzymes was discovered, but reference to the use of germinated barley or malt is recorded in the history of the Egyptians. The earliest uses of malt were primarily for the production of beer or other fermented beverages; barley was brought into North America with the first settlers, principally for the production of malt for the making of beer (2).

I. Malting

A. Raw Materials

Although barley, wheat, and rye all serve as raw materials for malting in the United States, barley is used in much larger amounts thanthe other two. Sorghum is used in India

and other Asian countries. Barley, wheat and rye when malted produce the two known starch-splitting enzymes, alpha-amylase and beta-amylase, in relatively large amounts. Oats, sorghum, corn, and rice produce essentially only alpha-amylase when germinated (3). Since the combination of the two enzymes results in more rapid and complete hydrolysis of starch to dextrins and fermentable sugars (4), malt from barley, wheat, and rye is more commonly used than malt from other grains.

In commerce, the term "malt" means barley malt, while malts from other grains are designated as wheat malt or rye malt. Wheat is malted in relatively small quantities and is used primarily as a flour supplement to increase the amylase activity of flour. The type, variety, growing conditions and harvesting conditions all influence the malting behavior, composition of the finished malt and, therefore, its subsequent value for specific uses.

B. Malting Process

In the present study a simplified malting process was used, and so the essential steps involved in the malting industry is discussed below. Since most of the malt produced goes into products for human consumption, it is important that the incoming barley be separated from dirt, weed seeds, and seeds of other grains before it goes to storage. During the cleaning process, the barley is separated into several sizes or grads. The size of the kernel influences the rate of water absorption during

steeping and germination, and the loss of moisture during kilning. Therefore, the several sizes are stored and malted separately. The cleaned barley is then stored under cool and dry conditions for from several weeks to several months. Freshly harvested barley is often dormant and shows low uneven germination. While in storage, the barley goes through an after-ripening process during which still obscure chemical and physical changes take place.

1. Steeping

Steeping temperatures usually fall in the range of 50-60°F. In the first step, water is allowed to run into a tank, and air under pressure is injected to mix the barley and water. After every 8-10 hours the steep water is changed. The rate of water absorption and time of steeping are influenced by temperature, size of kernel, type and variety of barley, and physical texture of the kernel (5). By steeping to an intermediate moisture content of 44-45%, uniform germination of desired type results.

2. Germination

The steeped barley is conveyed to drums or compartments for the germination step of the malting process. After the second or third day in the drum or compartment, the malt may be watered, with the moisture content kept at 45% or somewhat above. Relatively low germination temperature is required so as to reduce the loss of dry material through respiration and growth of rootlets or "sprouts" while still bringing about the desired physical and

chemical changes. The range of temperature employed is 60-70°F. The time of germination is influenced by moisture content, temperature, aeration, and vigor of the barley, varying from 5 to 7 days (6,7).

3. Kilning

Fundamentally, kilning terminates the growth and metabolic functions of the germinating grain at an appropriate time and introduces additional reactions catalyzed by heat. The simplest method, only for single-dect kilns, is to dry the green malt rapidly with high air flows and low temperatures, in the 110-130°F range, to a moisture content at which hydrolytic enzyme action does not take place. Kilning would then be completed either at low temperatures for maximum enzyme recovery or at selected high temperatures in the 175-195°F range for reduction of enzyme content and development of flavor.

C. Enzymes

Between the embryo and the endosperm lies a layer of enzymically active cells known as the scutellum. This whole embryonic area is important not only as a source of a variety of enzymes, both respiratory and hydrolytic, but also as a source of growth substances or stimulators which migrate to the aleurone cells and activate their enzyme-producing capabilities (8-13). While the two main portions of the kernel, germ and endosperm, can be made to perform somewhat independently, in practice their integrated actions result in the rapid production of enzymes and

subsequent functioning of these enzymes in the disaggregation of endosperm structure characteristic of the malting process.

Probably the first systems activated in the normal malting process are the oxidative and reductive systems associated with the use of oxygen for respiration and growth. Almost simultaneously another group of enzymes appears and evidences itself in the breakdown of large molecular structures to those of smaller size. Early in this evolution a group of enzymes variously known as cytases, hemicellulases, gumases, or gluconases are formed and begin the progressive breakdown of the cellular structure of the endosperm. These cellular structures are of a complex hexosan-pentosan nature, giving highly viscous solutions when suspended in water. The modification of the cell wall material is primarily responsible for the physical changes during malting which result in the conversion of hard, vitreous barley to friable, mellow malt.

Earley in the process proteolytic enzymes are elaborated and become distributed throughout the kernel during germination (14). These enzymes are important in the release of one of the amylase from a bound, inactive state. Their attack on the protein material in the germ, endosperm, and aleurone layer, renders about 40% of the total protein soluble in water or dilute salt solutions upon mashing. The rigid structure of the endosperm is, in part, due to the proteins present; breakdown of these high-molecular weight constituents into smaller, dispersible, products contributes to the physical modifications of the kernel.

Amino acids are produced within the kernel by proteolysis and are used for nutrition of the young plant and formation of high-molecular-weight materials in the developing rootlets and acrospire. Since rootlets are removed before ultimate processing of the malt itself, this portion of the nitrogenous materials is lost to the malt. The amino acids and soluble higher-molecular weight nitrogenous materials retained in the malt are important in the formation of malt flavor either on the kiln or in subsequent processing. They play an additional dual role in brewing - the small molecules for yeast nutrition and the large molecules for beer foam.

D. Malt Amylase

The starch-splitting enzymes known as amylases are very important enzymic constituents of malt. During germination a high percentage of the bound storage amylase (beta-amylase) is freed and alpha-amylase is synthesized (3,15). The alpha-amylase, synthesized primarily in the aleurone cells, diffuses into the endosperm and may, under certain conditions, convert a portion of the starch to dextrins and fermentable sugars. However, this is restricted to not over 10% of the starch; most of the starch granules carry through the malting process essentially intact. Some of the sugars used in metabolism of the germinating barley may come from the action of amylases on starch but the major source appears to be the sugar resulting from the hydrolysis of barley gums.

The amylases of malt play their primary role after the malt is made and introduced into its terminal use, whether this is for brewing, distilling, or food. Here they serve to convert gelatinized starch to fermentable sugars, syrup sugars, and dextrins. In some applications the amylases (primarily alpha-amylase) may be used only to thin the viscous pastes formed by concentrated gelatinized starch.

II. Alpha- and beta-amylases and their determination

Since Kuhn (16) reported the existence of alpha- and beta-amylase in 1924, much work has been done on alpha- and beta-amylase. The term alpha-amylase now connotes an alpha-1,4-glucan 4-glucanohydrolase and the term beta-amylases an alpha-1,4-glucan maltohydrolase. The alpha-amylases and beta-amylases are found in a variety of living matter. The alpha-amylase splits the alpha-1,4-glucosidic bonds of a polysaccharide throughout the molecule, while the beta-amylase attacks polysaccharide chains such as starch, glycogen or their degradation products effecting successive removal of maltose units from the nonreducing ends.

Native starches are mixture of two types of compounds which are separable from each other. Amylose is the component that is believed to be a long unbranched chain. Amylopectin has been shown on the basis of methylation studies to be a branched-chain polysaccharide, one terminal glucose occurring for every 24 to 30 glucose unit.

When amylose, which contains exclusively alpha-1,4-glucosidic bonds is attacked by alpha-amylase, cleavage of these bonds gives rise to a mixture of glucose and maltose, and higher linear oligosaccharides, whereas attack by beta-amylase gives pure maltose in almost quantitative yield. Amylopectin, when treated with alpha-amylase, undergoes rupture of alpha-1,4 bonds and yield, as ultimate products, a mixture of branched and unbranched oligosaccharides. When amylopectin is hydrolyzed by the action of beta-amylase, successive maltose units are liberated, commencing at the non-reducing ends of the polysaccharide molecule. This process continues until a branch point is approached. Since the enzyme has no capacity to hydrolyze the alpha-1,6 bond that it here encounters, all reaction stops. The polysaccharide fragment that remains after such incomplete hydrolysis is called dextrin, and specially, since this dextrin is the limit of attack of beta-amylase upon amylopectin, it is called limit dextrin. Limit dextrin produces viscous solution and is colored purple by iodine.

Salivary alpha-amylase produces mainly maltose, maltotriose, and maltotetraose up to the achroic point (17,18). The alpha-amylase of Bacillus subtilis (19) produces mainly maltopentaose, maltohexaose, and maltoheptaose, and the alpha-amylase of Bacillus polomyxa produces maltose. It has been noted that sorghum and barley alpha-amylase produces oligosaccharides of six to eight glucose units (20,21).

Beta-amylases are found in higher plants such as barley,

sorghum, wheat, potato and soybeans. During hydrolysis, beta-amylase splits alpha-1,4-glucosidic bonds forming maltose in the beta-configuration(22) from the non-reducing end of the polysaccharide. The reaction proceeds continuously until the branching point is approached. Generally only 70-85% conversion of amylose into maltose by beta-amylase is attained (23).

Oligosaccharide of even numbers of glucose units is hydrolyzed completely to maltose (24). That with odd numbers of glucose units produces maltose and maltotriose, the latter of which is hydrolyzed further to maltose and glucose at a much slower rate (25).

The methods for quantitative estimation of the amylases have been developed in numerous ways. All of them are based on changes in physical or chemical properties of the solution undergoing enzymic reaction. With starch as substrate the changes in properties may be classified into five categories; change of iodine staining property, decrease in viscosity, change of turbidity, increase in reducing power and the change of optical rotation of the solution.

A. Iodine Staining

On adding iodine solution to a very dilute solutions of starch an intense blue color is produced, due to the formation of an iodine-starch complex. This action of iodine on starch was discovered by Claubry (26). The iodine test serves as a most useful control of hydrolysis of starch by alpha-amylase,

since the deep blue color characteristic of starch and soluble starch changes through violet or purple to reddish brown, and finally to pale yellowish red as hydrolysis progresses. The time for the disappearance of the blue color (achroic point) with potassium triiodide is measured (27).

B. Viscosity Method

If an aqueous suspension of starch is heated or if starch is mixed with hot water, the granules swell enormously and finally burst, the mixture forming the viscid, cloudy liquid known as starch paste. A rough idea of the stiffness or viscosity of the paste from a sample of starch is afforded by the sense of touch, but Erman (28) found that direct determinations of viscosity do not give constant results and he proposed to use solutions of starch prepared in the cold with NaOH. The activity of alpha-amylase on starch paste results in decrease of viscosity. Viscosity is determined at some standard temperature in viscosimeter.

C. Reducing Sugar Method

The basis for the determination of beta-amylase is upon the regular increase in reducing power due to maltose production. The progress of hydrolysis can be observed by measuring the increase in reducing sugar as assayed by 3,5-dinitrosalicylate reduction. The extent or percentage of hydrolysis is determined by comparing the amount of reducing sugar formed at any time with the amount of sugar present after total acid hydrolysis.

Kneen and Sanstedt (29) defined their unit of beta-amylase as the number of grams of starch converted to maltose by beta-amylase in one hour at 30°C. Meyer et al. (30) chose 1 unit of activity of beta-amylase as the number of mg of maltose produced when 1 ml enzyme was mixed with 1 ml of 1% starch in acetate buffer of pH 4.8 at 20° C for 3 minutes. Balls, Walden and Thompson (31) defined 1 unit activity as the amount of enzyme which liberated reducing sugar equivalent to one milliequivalent of $Fe(CN)_3$ in 10 minutes at 30°C in 30 ml of 1% soluble starch in pH 4.8 buffer. Thoma and Koshland (32) defined 1 unit of activity of beta-amylase as the amount of enzyme which produced 1 mg of maltose from 0.5% soluble starch in 30 minutes at pH 4.8 in 0.1 M acetate buffer at 25°C.

All of these units are not different from each other in any strict sense. They all were based upon the amount of maltose produced (1 mg ml^{-1} min^{-1} in nearly all methods) by the enzyme at a certain time period and temperature in acetate buffer of pH 4.8 which is near the optimum pH for most beta-amylase (33). The extent of the hydrolysis of starch by beta-amylse is not greatly influenced by relatively wide differences in the concentration of the substrate or on the conditions of the reaction (34). This method is also applicable to alpha-amylase provided that Beta-amylase is not present (41).

D. Optical Rotation Method

An optical rotation method developed by Chang (23) allows a quantitative evaluation of beta-amylase activity. The

method makes use of the change in optical rotation during enzymic reaction, and the activity unit of beta-amylase is defined in terms of change of optical rotation versus time. The method can be used for mixtures of alpha- and beta-amylase.

The magnitude and direction of rotation of the plane of polarized light by an asymmetric compound is a specific physical property of the compound that may be used in its characterization. When plane polarized light is passed through a solution the rotation of the light in degrees is found to be directly proportional to the number of asymmetric molecules of the compound through which the light passes. Therefore the rotation observed will be depended on the nature of the asymmetric compound, the concentration of the compound in solution, and the length of the light path through the solution. The variables are related as in the following formula:

$$[\alpha]_D^T = \frac{[\alpha]_{obs}}{l \times c}$$

Here $[\alpha]$ is the observed rotation, c is the concentration in g/ml, and l is the length of the light path in decimeters. The quantity is called the specific rotation at the temperature T when the D line of the sodium spectrum is used as the light source. Frequently this formula is written

$$[\alpha]_D^T = \frac{[\alpha]_{obs} \times 100}{l \times c}$$

where the units of c are g/100 ml instead of g/ml (35).

Mutarotation was first discovered by Dubrunfaut (36) in 1846. He observed that when glucose was dissolved in water, the optical rotation decreased until a final value was reached. During mutarotation only alpha and beta forms exist and the reaction follows first order kinetics (23). Maltose like glucose, mannose and lactose undergoes first order kinetics during mutarotation (37). The rate constant is governed by temperature, pH, solvent and salt in solution.

MATERIALS AND METHODS

I. Preparations of Barley, Sorghum and Wheat Malt

Barley (Will), sorghum (Plainsman), and wheat (Shawnee) were obtained from experimental station sources. The seeds were soaked in water overnight. They were treated with 0.2% formaldehyde solution for 40 minutes to insure a mold-free preparation, then washed and germinated at room temperature for three days - method adapted from Fleming et al. - (38). All equipment used in germination was sterilized, and aseptic conditions were maintained as far as poffible. After germination, the seeds were air-dried at room temperature and ground in Model 118 (Lab. Const. Co.)

II. Extraction of Barley, Sorghum and Wheat Amylase

Crude amylase was prepared by extracting barley, sorghum and wheat malt overnight with water at 2°C, in the ratio of 400 g malt to 1 liter of water. The above preparations were used directly as the malt extracts. For the concentrated extracts, the suspension was further precipitated with 70% $(NH_4)_2SO_4$ saturation, and the precipitate was dialyzed in water overnight. Ammonium sulfate as well as malt extracts were used for enzyme studies. They were lyophilized and collected in the powder form and kept out of contact with air (23).

III. Inactivation of the Beta-Amylase by Heat (39)

150 mg of crude amylase was dissolved in 15 ml 0.1 M acetate buffer, pH 5.9, containing 0.2% calcium acetate. The solution was heated at 70° C for 15 minutes with stirring (1% solution of concentrateed extracts). The suspension was cooled in an ice-bath and the coagulated protein removed by centrifugation (5000 R.P.M., 3020 x G). For malt extracts, the same heat-treatment was made on the 40% malt solution (400 g/L) directly.

IV. Substrate

Amylase substrates of 0.4% Merk's soluble starch, 0.1 M sodium acetate buffer, pH 4.8 were used in all assays.

V. Determination of Beta-Amylase Activity by Polarimetry

Polarimetry measurements were made with an ETL-NPL Automatic Polarimenter Type 143A. The cell was on all glass unit described by Chang (23). Its effective length was determined with standard solutions of vacuum dried sucrose 1% and 1.17%. The concentrations were checked with a Rudolf Model 80. Polarimeter. Observed optical rotations on the Bendix Model 143 A at 30°C were .400 and .460 for the solutions 1% and 1.17% respectably. When these values were put into the formula $[\alpha]_D^T = \frac{\alpha \times 100}{\ell \times C}$ together with corres-

ponding specific rotations, +66.5 for sucrose, the effective lengths calculated were .6157 and .6181 respectably. The average value .62 was adopted in this work.

The concentration of enzyme was chosen so that when 1 ml enzyme was mixed with 9 ml substrate at 30°C, the achroic point would be observed within 15-20 minutes. An aliquot of enzyme and substrate was withdrawn and transferred to the polarimeter cell and the optical rotation measured at 30°C. At the same time, aliquots from the digest in the water bath were withdrawn at intervals to determine the achroic point. The optical rotation was observed over a time span past the achroic point. The beta-amylase activity was calculated from the slop of the curve. When starch was hydrolyzed it yields beta-maltose and limit dextrin. Starch and limit dextrin have specific rotations of +200 and of +111.7 respectably (40). With these numbers one can relate the change of rotation to the beta-amylase unit as follows:

$$\underset{+200}{\text{Starch}} \longrightarrow \underset{+111.7}{\text{Beta-maltose}} + \underset{+200}{\text{Limit dextrin}}$$

The observed average optical rotation of starch substrate (.4%) was .413.

$$.413 \times \frac{111.7}{200} = .231$$ (observed rotation of solution if completely hydrolyzed to maltose)

$$\therefore \quad .413 - .231 = .192$$ (change in rotation corresponding to 100% conversion or 4 mg/ml of maltose)

$\therefore$ 1 unit enzyme = 1 mg ml^{-1} min^{-1} maltose

$$= \frac{.192}{4} = .048 \ \Delta^{\circ}$$

The change in optical rotation of .048 represents 1 unit beta-amylase activity. Beta-amylase activity was obtained by dividing the value of the slope of a graph plotting optical rotation during hydrolysis vs. time.

VI. Determination of Alpha-Amylase Activity - Reducing Sugar Method (41)

1 g of Merk's soluble starch was dissolved in 100 ml of 0.02 M phosphate buffer, pH 6.9. 1 g of 3,5-dinitrosalicylic acid in 20 ml of 2N NaOH and 50 ml of H_2O, and 30 g of Rochelle salt were added and the solution made up to 100 ml with distilled H_2O. The solution was kept sealed to protect from CO_2.

1 ml of properly diluted beta-amylase inactivated enzyme (heated for 15 minutes at 70° C at Ph 4.8, then cooled rapidly and centrifuged) was incubated for 3 minutes at 20°C with 1ml of the substrate solution. The enzyme reaction was interrupted by the addition of 2 ml of dinitrosalicylic acid reagent. The tube containing this mixture was heated for 4 minutes in boiling water and then cooled in running tap water. After addition of 20 ml of H_2O, the optical density of the solution containing the brown reduction product was measured at 540 n.m. in a Spectronic 20 colorimeter. A blank was prepared in the same manner without enzyme. A calibration curve established with maltose (0.2 yo 2 mg in 2 ml of H_2O) was used to convert the colorimeter readings into mgs of maltose.

VII. Determination of Aminoid Nitrogen

In order to express, alpha-amylase activity in terms of mg maltose produced per mg nitrogen, micro-kjeldahl method was applied to determine nitrogen contents (42). The procedure was as follows:

A. 1. 1 ml of dialyzed enzyme was diluted to 10 ml with acetate buffer pH 5.9 in a 10 ml volumetric flask.

2. 1 ml of this solution was put into a 30 ml kjeldahl flask and to this was added 1 ml of concentrated H_2SO_4 and a few mgs of catalyst composed of one part K_2SO_4 and three parts $CuSO_4$. The flask was heated on a microelectric heater until the contents were digested and the fuming from the oxides of sulfur subsided.

3. The flask was removed and 2 or 3 drops of 30% H_2O_2 were added.

4. The digestion was recontinued until the solution was clear and colorless. If the solution was not colorless after the first addition H_2O_2, a few more drops were added and the solution reheated, until a colorless solution was obtained.

5. The flasks were colled and the digest was transferred quantitatively with 5 ml of distilled water to a standard micro-kjeldahl distillation apparatus and to it was added 7.0 ml of 30% NaOH. Any solution adhering to the transferring funnel was washed into the flask with 3 ml of distilled water, and the

solution was steam-distilled.

6. The distillate was collected in 5 ml of 4% boric acid solution during 4 minutes and this solution was titrated with 0.01 N H_2SO_4. The end point of titration was indicated by using a mixed indicator (43). A standard nitrogen solution (0.60024 mg/ml) was prepared from $(NH_4)_2SO_4$ and treated in the same as the unknown sample.

7. A blank was always run simultaneously with the samples. A conversion factor of 6.25 was used to convert the obtained nitrogen value to protein contents of the samples.

Calculations:

The amount of nitrogen per 100 ml is calculated as follows:

g of nitrogen/100 ml =

$$\frac{\text{ml of std. acid used for sample} \times 0.60024}{\text{ml of std. acid used for standard}}$$

if protein is required that is multiplied by 6.25 value.

Indicator:

Methylene blue-methyl red mixed indicator
This solution consists of 1 part of 0.2% methyl red in alcohol and 1 part of 0.1% methylene blue in alcohol. The pH changes is at 5.4. The color is green in alkaline solution (pH 5.6), colorless at pH 5.4 (end point), and reddish-pink in acid solution (pH 5.2).

VIII. Chromatography (20)

A chromatographic analysis of the hydrolysates was done as follows:

A. 1. To 9 ml of 0.4% starch solution was added 1 ml of

heat-treated enzyme. The hydrolysis was carried out in a water bath at 30°C.

2. Portions of the hydrolyzing mixture were withdrawn at 0', 5' and beyond achroic point, and boiled for 3 minutes to stop the enzyme reaction.

3. The products were then identified by paper chromatography using a solvent mixture of butanol: pyridine: water (6:4:3 v/v). Six to eight ascends were found satisfactory. Reducing sugars were revealed as follows. Chromatograms were dipped first with 0.4% silver-nitrate in acetone. After drying, this was followed with a dip in methanol containing 0.4% NaOH. When spots were well formed, chromatograms were stabilized by dipping in 5% $Na_2S_2O_3$ followed by washing and drying.

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RESULTS AND DISCUSSION

Some special precautions are required before valid results can be obtained by the optical rotatory method. There is first of all the fact that starch solutions become turbid on standing. While amylopectin is reasonably stable in solution, amylose retrogrades readily causing turbidity. To avoid these problems one should use a soluble starch prepared by a Lintner Process. Fresh substrate should be used and it should prefereably be clarified by centrifugation at low speed. The enzyme also should be centrifuged to ensure clarity. It has been observed that when the enzyme is added to the substrate a physical reaction appears to take place (independent of enzyme action) which results in a slight lowering of the optical rotation. Consequently it may not be possible to extrapolate optical rotation values to zero time. Generally from 2-3 minutes are required before the cell can be filled and a reliable reading taken. A third requirement is therefore introduced; the enzyme should be dilute enough so that a reasonably linear section of optical rotatory change with time is observed at the beginning. The initial slope under these conditions give the maltose production. Alternately one can use the zero time value for the heat inactivated sample. This will be less accurate because the change may not be linear. Calculations reported in this thesis for sorghum are based on initial slope.

EXPLANATION OF FIGURE 1

Fig. 1 Beta-amylase activities of Barley, Sorghum and Wheat in their treated and untreated states (Concentrated - Crude Amylase).

			Achroic Point
○	B	Barley	12.05'
○	B^h	Barley, heat-treated	48.89'
□	S	Sorghum	15.41'
□	S^h	Sorghum, heat-treated	11.08'
△	W	Wheat	27.34'
△	W^h	Wheat, heat-treated	26.30'

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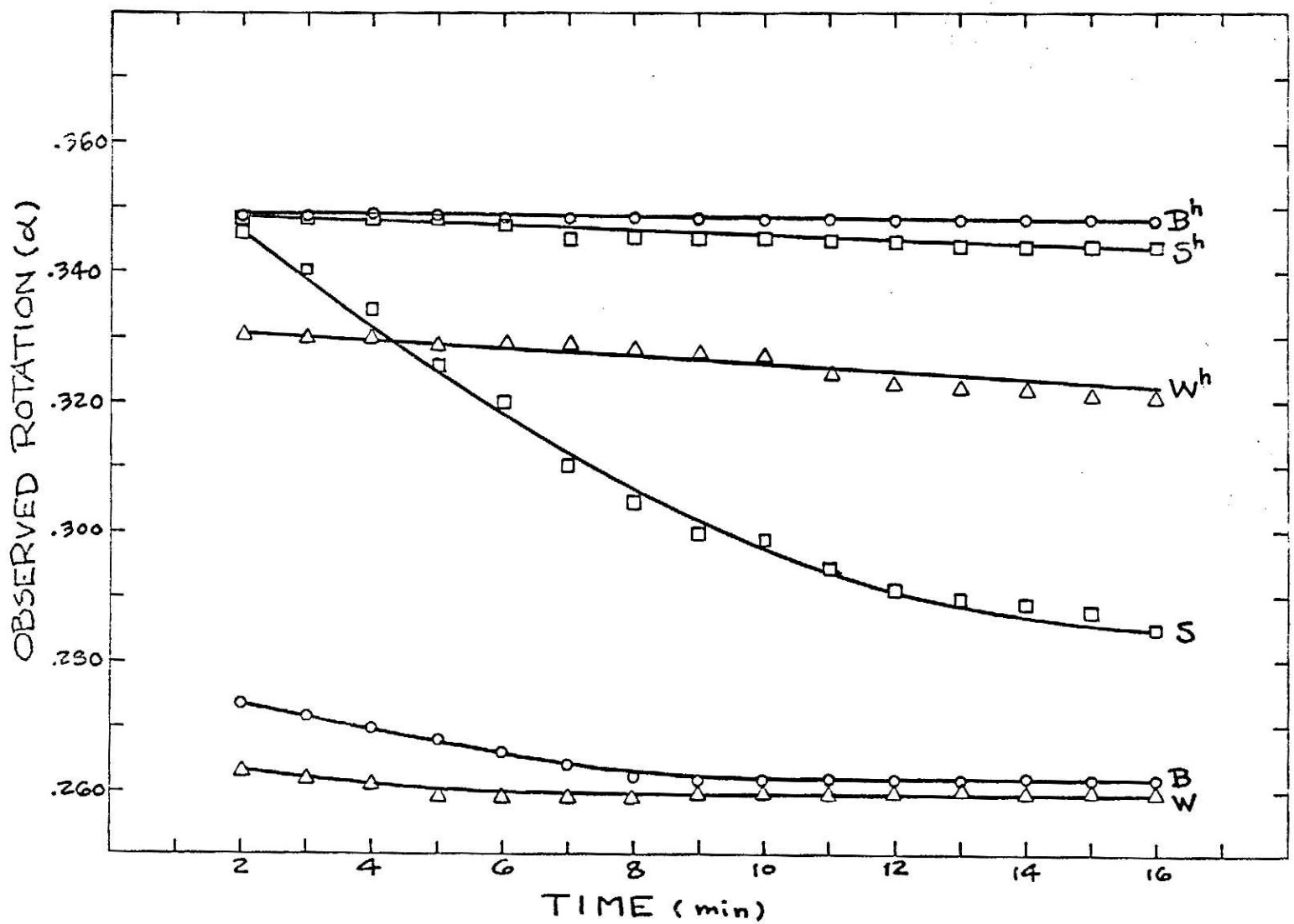
OBSERVED ROTATION (α)
.360
.340
.320
.300
.280
.260
2
4
6
8
10
12
14
16
TIME (min)
B^h
S^h
W^h
S
B
W

TABLE 1

MALT EXTRACT (WATER EXTRACT)*

Sample	N(mg/ml)	Alpha-amylase u/ml	Beta-amylase u/ml	Alpha-amylase u/g malt	Beta-amylase u/g malt
Sorghum	1.122	.546	.456	1.365	1.140
Wheat	.636	.900	-	2.250	
Barley	.990	.894	-	2.235	

* 400 g malt was dissolved in 1 L of H_2O.

TABLE 2

CONCENTRATED EXTRACT (CRUDE AMYLASE)*

Sample	N(mg/ml)	Alpha-amylase u/ml	Beta-amylase u/ml	Alpha-amylase u/mg N	Beta-amylase u/mg N
Sorghum	.156	.243	.165	1.543	1.058
Wheat	.147	.240	-	1.616	-
Barley	.090	.336	-	3.732	-

* .1g crude amylase was dissolved in 10ml H_2O.

EXPLANATION OF FIGURE 2

Fig 2 Hydrolysis products of 0.4% soluble starch by crude alpha-amylases to the achroic stages in 0.1 M sodium acetate buffer of pH 4.8 at 30°C.

1	Barley	0' time
2	Barley	5'
3	Barley	Achroic Point
4	Sorghum	0' time
5	Sorghum	5'
6	Sorghum	Achroic Point
7	Wheat	0'
8	Wheat	5'
9	Wheat	Achroic Point
R	Mixture of G_1 - G_{10}	

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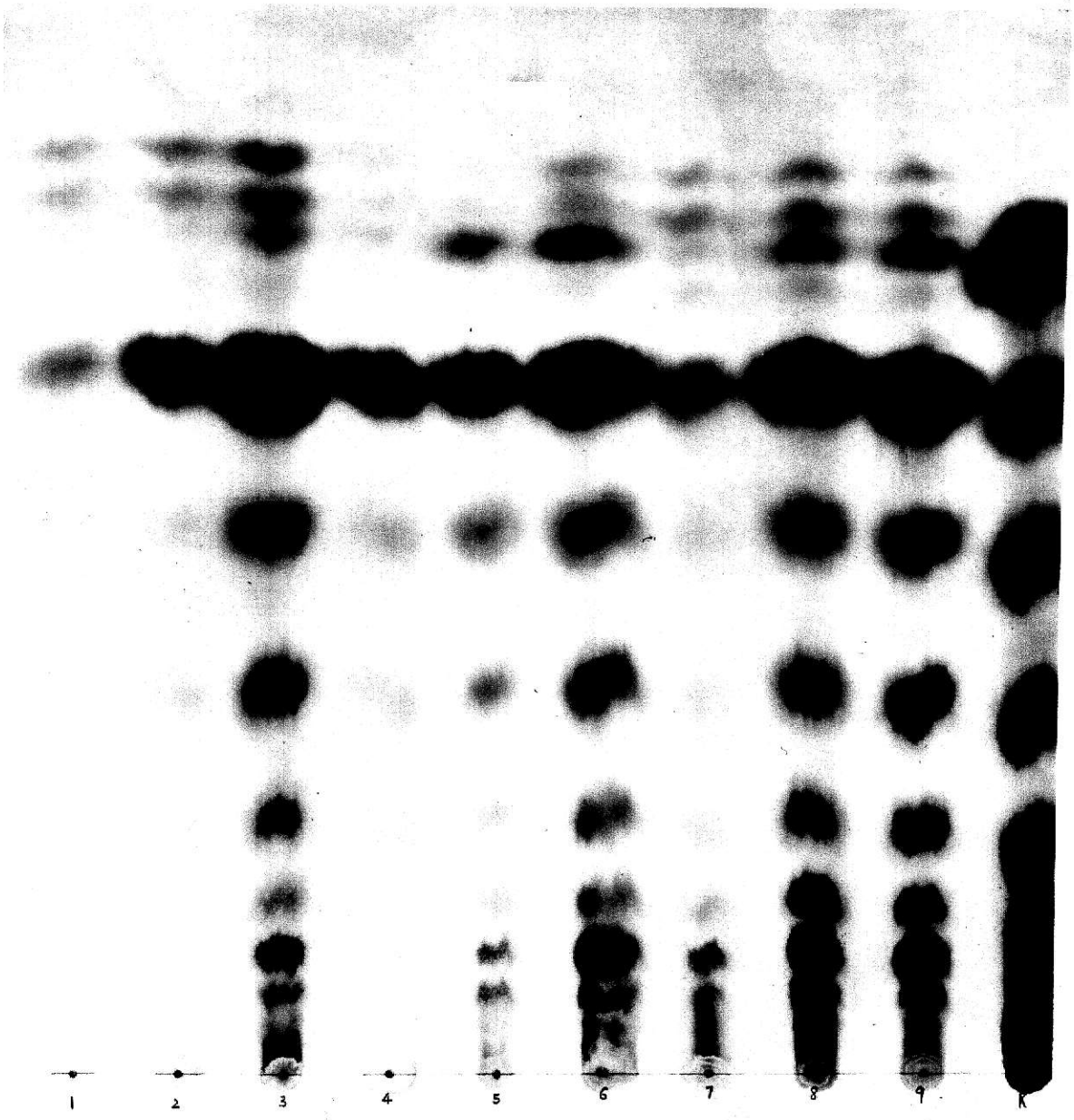

8-4-70 W.H.

The actual optical rotation readings observed for sorghum, wheat and barley, concentrated extracts are shown in Fig. 1. The achroic points are recorded also for these samples as an approximate indication of alpha-amylase activity. The alpha-amylase and nitrogen contents for these enzyme extracts are recorded in Table 1.

A similar set of date were obtained for the partially purified enzyme. Both alpha- and beta-amylase were precipitated with 70% ammonium sulfate saturation. They were collected by centrifugation, dialyzed to remove the salts and then freeze-dried (39). Not only was the enzyme obtained in a more concentrated form but it also eliminated much extraneous matter, such as sugars, proteins and polysaccharides extracted from the malt. Results for these concentrated extracts are shown in Table 2. The oligosaccharides produced by the three enzymes are shown in Fig. 2.

In the present work on sorghum beta-amylase, the studies showed that malt extract (water extract) and concentrated extract (crude amylase) had activities of .406 u/mg N and 1.058 u/mg N respectably.

When unit activities, i.e. mg maltose ml^{-1} min^{-1} are expressed in terms of u moles of maltose ml^{-1} min^{-1} at 30°C, the values can be compared with those of Novellie's (47).

Malt Extracts (Water Extracts)

.456 units of beta-amylase activity of sorghum

.456 mg ml^{-1} min^{-1}

$$\frac{456 \text{ g} \times 10^{-6}}{336 \text{ g} \times 10^{-6}} = 1.357 \text{ u mole of maltose}$$

Specific activity = u mole / mg N

= 1.357 u mole / 1.122 mg N

= 1.209 u mole / mg N

Concentrated Extract (Crude Amylase)

.165 units of beta-amylase activity of sorghum

.165 mg ml^{-1} min^{-1}

$$\frac{165 \text{ g} \times 10^{-6}}{336 \text{ g} \times 10^{-6}} = .491 \text{ u mole of maltose}$$

Specific activity = u mole / mg N

= .491 u mole / .156 mg N

=3.147 u mole / mg N

Novellie determined beta-amylase activity by the saccharifying method (39). In determining beta-amylase, he employed 2% gelatinized soluble starch solution in 0.05 M acetate buffer of pH 5.0. The saccharifying unit was arbitrarily defined as that amount of beta-amylase which would liberate 1 u mole maltose

per min at 30°C by Novellie. Specific activities were also expressed as saccharifying units per mg protein nitrogen (47). Novellie found values of 2.4 u mole/mg N for a water extract of malt and 21.6 u mole/mg N for ammonium sulfate precipitate similar to the one studied here. The results compare favorably in case of the water extracts.

When calculated in terms of Novellie's unit of specific activity, values of 1.211 u mole/mg N and 3.147 u mole/mg N were obtained for malt extract (water extract) and concentrated extracts (crude amylase) respectably. Considering the effects of different malting conditions, variations in variety, season, maturity and age of grain, these differences are not surprising (47).

Meyer and coworkers (48,49,50) have crystallized wheat and malt beta-amylases, and Balls and coworkers (51) malt alpha-amylase. The wheat beta-amylase was found to be electrophoretically homogeneous but was not examined in the ultracentrifuge. The homogeneity of the beta-amylase from barley malt was not determined. Not until Novellie (47) isolated sorghum beta-amylase in a homogeneous state in 1967, had any of the cereal beta-amylase been obtained in a homogeneous state as defined by both electrophoretic and ultracentrifuge analysis.

The determination of the alpha-amylase content of malt is of importance not only in obtaining a better understanding of the mash behaviour of the malt, but also in estimating beta-amylase

which cannot be determined directly but only via the alpha-amylase determination (52). The determinations of beta-amylase in sorghum malts is of particular importance because it is essential, by selection of suitable varieties and malting procedure to raise the usually low content of this enzyme to as high a value as possible. The present study makes it possible to determine the beta-amylase activity directly in short time with a very simple procedure.

It was confirmed in the present study that when a crude sorghum malt extract was heated at 70°C for 15 min in the presence of 0.2% calcium acetate, the beta-amylase activity was destroyed but the alpha-amylase was resistant to heat denaturation. The preferential thermal inactivation of beta-amylase in the presence of the alpha-component has found wide application in the isolation of cereal amylase.

The action pattern of sorghum, wheat and barley amylase were studied with 0.4% soluble starch substrates. The products were examined by paper chromatography. High concentrations of maltohexaose, maltoheptaose, and maltooctaose were produced, in addition to lesser amounts of other oligosaccharides. The oligosaccharides of eight or less glucose units persisted after the achroic point. The production of high concentrations of oligosaccharides of six, seven and eight glucose units is a pecularity of sorghum alpha-amylase which has also been reported by Dube and Nordin (20).

Crude extracts of grain are rich in pentosans and gums

(53,54,55). The crude enzyme would also contain hydrolase which can split these polysaccharides producing D-xylose and arabinose (56,57). Thus during a digest these bi-products might appear. An examination of the chromatogram shows that these are in minute quantities and it is not likely that they influence the rotation significantly. The main interest is the production of oligosaccharides. All three enzymes are very similar in this respect. These enzymes have not been compared in this manner.

Since sorghum alpha-amylase exhibits many other properties which are identical to barley alpha-amylase (20), they might be expected have a similar action pattern also. It has been noted that barley alpha-amylase produces oligosaccharides of 6-8 glucose units (21). The two enzymes and wheat alpha-amylase were examined under similar conditions, they were indeed identical in pattern.

SUMMARY

1. Barley, sorghum and wheat malts were prepared through soaking, germination, drying and finally grinding. Alpha- and beta-amylase are found in a variety of living matter, and the enzyme found in barley, sorghum and wheat exhibit similar actions toward carbohydrate substances like starch.
2. Crude amylase was prepared by extracting malts with water. It was 70% saturated with solid ammonium sulfate which precipatated alpha- and beta-amylase and the solution containing these precipitates were lyophilized and collected in powder form.
3. Proper dilutions were made and optical rotation was observed. In the presence of alpha-amylase, due to beta-amylase activity in producing maltose, the decrease in optical rotation was observed. Inactivation of beta-amylase was achieved by heat-treatment at 70°C for 15 minutes. Enzyme solutions were prepared with acetate buffer pH 4.8.
4. Inactivation of beta-amylase by heat-treatment did not affect the activity of alpha-amylase greatly. Alpha-amylase activity was closely followed with iodine staining method. Heat-treated enzyme of alpha-amylase activity was determined and expressed in mg maltose per mg nitrogen, and also in mg maltose per g malt.

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ASSAY OF BETA-AMYLASE IN MALT

BY

OPTICAL ROTATION

by

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ABSTRACT

Malts of sorghum, wheat and barley were prepared from the germinated seeds. Amylase extracts of the malts and concentrates prepared by ammonium sulfate precipitation were analyzed for beta-amylase using an ETL-NPL Automatic Polarimeter. Sorghum malt extract contained 1.140 units beta-amylase per gram malt and sorghum crude amylase contained 1.058 units per mg nitrogen.

Inactivation of beta-amylase without affecting alpha-amylase activity was achieved by heat-treatment at 70°C for 15 minutes. Enzyme solution was prepared with acetate buffer pH 4.8.

Alpha-amylase activity was observed by the iodine staining method and quantitative measurements were obtained by a reducing sugar method. The results were expressed in mg maltose produced per mg of nitrogen by a micro-kjeldahl nitrogen determination method and also in mg maltose produced per g malt.