

SOME ASPECTS OF ALPHA-AMYLASE CHEMISTRY

by

MARTA SUSANA MORO

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Major Professor

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INTRODUCTION

The use of amylase supplement is an old practice. The past century and a quarter has witnessed much research on the cereal amylases and many reports have appeared dealing with the presence of these enzymes in cereals and their mode of action in breadmaking.

Sound, ungerminated wheat has only very small amounts of alpha-amylase but substantial levels of beta-amylase. Germination of cereal grains is accompanied by a large increase in the activity of hydrolytic enzymes such as: amylases, proteinases, and cytases.

These hydrolytic enzymes are instrumental in mobilization of the endosperm reserves for the growth and development of seedlings. Little is known about how carbohydrate metabolism is controlled enzymatically, but it is difficult to conceive starch hydrolysis in the endosperm taking place in the absence of amylase activity, and any indication of the release of sugar coincident with, and possibly even dependent upon, amylase activation would be useful in estimating the endogenous role of this enzyme (Paley, 1960a).

A recurring suggestion in the literature is that enzymatic activity of the endosperm is derived by secretion of enzymes into that tissue by the embryo.

Early observations (Hayashi, 1940) showed that gibberellic acid stimulated the germination of cereal grains. Subsequent work has been conducted to determine the influence of gibberellic acid in the activation of the enzyme system in cereal grains.

The present study has been undertaken to test the effect of gibberellic acid on formation of alpha-amylase in separated wheat endosperm, as compared

with levels of enzyme formed in intact seeds. Use was made of measurement of starch liquefaction resulting from the action of alpha-amylase, since changes in viscosity are essentially unaffected by the presence of beta-amylase.

Sound wheats and the flours milled from them contain relatively large quantities of beta-amylase and very small amounts of alpha-amylase. This lack of alpha-amylase is corrected by the addition of alpha-amylase supplements.

Properties of amylases from commercial preparation are known to differ from those of purified amylases. While a large amount of work has been reported on the dextrinogenic and saccharifying properties of pure crystalline amylases, little work has been reported on the liquefying properties.

The second part of this report deals with the effects of crystalline amylases on liquefaction of pregelatinized starch and the inactivation of amylases from different sources.

REVIEW OF LITERATURE

Substrate

Next to cellulose, starch is the most abundant and widely distributed substance of vegetable life. It usually occurs as minute rounded spherules or granules in the root, tuber, seed or pith of the plant.

Starch granules are normally formed in plastids in the higher plants, chloroplasts in green tissues, and leucoplasts in the storage tissues, and they are almost invariably found in the proplastids of meristematic cells (Buttrose, 1960).

Duvick (1955) found starch granules in the maize endosperm developing in filaments resembling mitochondria. A further exception are the small granules of barley, rye and wheat endosperms. Badenhuizen and Ruhland (1958) suggested that these granules are formed in mitochondria.

From the biochemical standpoint, starch must be considered as a polymer of glucose units since on complete hydrolysis starch yields only glucose.

Native starch contains in general two types of moieties. The name "amylose" is given to the moiety which is believed to be a long unbranched chain. The second moiety, amylopectin, has been shown to be a branched-chain polysaccharide with a terminal glucose unit occurring for every 24 to 30 glucose residues. The glucose residue which is situated at each point of branching is substituted not only at carbon-4 but also at carbon-6 (White *et al.*, 1954).

Amylose may have a molecular weight of the order of 50,000 (300 glucose residues), whereas amylopectin has a considerably larger molecular weight of about 300,000 (1,800 glucose residues).

Bice *et al.* (1945) studied the starch deposition at different stages of maturity of the wheat grain. They observed that endosperm starch was abundant 8 days after pollination. From this time to maturity, the increase in amount of starch was approximately a linear function of time. Increase in starch granule size was marked up to 12 or 15 days after pollination. As the wheat matured there was an apparent increase in the amylose-amylopectin ratio as shown by the variation in the sorptive capacity of the starch for iodine. This variation may indicate a relatively more rapid synthesis of amylopectin in the earlier stages of deposition of starch in the endosperm than in the later stages.

Sandstedt (1946) studied the development of starch granules in the wheat grain. He found that starch granules were present in the unfertilized wheat ovary. Pericarp starch develops rapidly showing maximum diameter of granules by the 4th or 5th day. However, an enzyme was present in the pericarp which was capable of digesting starch granules. Pericarp starch practically disappears from the wheat kernel (due to enzymatic digestion) within a couple of days after the kernel attains its maximum length.

Minute endosperm starch granules may be found in the interior of the endosperm at about the time that cell walls form in this tissue, or approximately the 4th or 5th day after the flower has been fertilized. These granules develop into the large lenticular granules which are characteristic of wheat starch. Rye and barley starches also contain this type of granule.

Small spherical granules begin to develop in the cells containing lenticular granules at about the time the kernel attains full length.

Later on, Sandstedt and Beckord (1946) completed this study by studying the amylase system in the pericarp of immature wheat capable of digesting native starch granules. However, no active enzyme capable of attacking native granules could be found in the endosperm at any stage of its development. Chemical determinations of amylase action in extracts of pericarp and of endosperm tissue from wheat at several stages during its development substantiated the data obtained and indicated the presence of alpha-amylase in the pericarp with only traces in the endosperm. The concentration of alpha-amylase decreased during the growth of the kernel, whereas the total alpha-amylase content in the kernel increased during the first week of development (period of most rapid growth of pericarp tissue), and then decreased. An amylase inhibitor (which had no effect on the natural wheat

or malt amylase but which was effective in inhibiting salivary amylase) appeared in the endosperm at approximately the time that the immature kernel reached full length. This inhibitor was not found in the pericarp tissue.

Starch in the native state is seen as microscopically visible granules. Size and shape of the starch granules from different sources vary over a wide range. It seems well established that the shape is genetically controlled and depends to a large extent on environmental factors prevailing during the development of the starch granules (Kerr, 1950).

There is evidence that the starch granules grow over an extended period of time by apposition (May and Buttrose, 1959; Gaffin and Badenhuizen, 1959; Yoshida et al., 1958).

Numerous investigations have dealt with the distribution of amylose and amylopectin inside the granule (Meyer and Menzy, 1953; Cowie and Greenwood, 1957; Banks and Greenwood, 1959). Badenhuizen (1960) postulated that the structure of a layer in general would show long amylose molecules on the inside followed by shorter linear chains, and an increasing degree of branching toward the outside of the layer.

In the natural state starch is insoluble in cold water but appears to absorb about 25 to 30 per cent moisture and does not swell appreciably. At 60°C. according to Meyer and Bernfeld (1940), corn starch takes up about 300 per cent water, and at 70°C. about 1,000 per cent, based on its original weight. The granules lose some of their opacity and their polarization crosses disappear. This loss of polarization precedes swelling. The gelatinization temperature was defined by Schoch and Maywald (1956) as the point at which the starch granules lose their polarization crosses when heated in a swelling medium. All the granules in a given sample do not lose

their polarization simultaneously, but usually over a range of 8° to 10° C.

Schoch and Maywald (1956) studied the gelatinization temperatures of different starches. They found that gelatinization temperatures were fairly uniform within any given variety. The same authors found that the gelatinization range becomes an important consideration when starch is used in other than simple water systems. For example, increasing concentrations of ammonium nitrate or urea progressively lower the gelatinization temperature, while ammonium sulfate markedly raises it. Greenwood and Thomson (1962) found that no simple relation exists between gelatinization temperature and granule size or amylose content.

As a suspension of starch in water is heated several changes take place (MacMasters and Wolff, 1959). First birefringence is lost around the hilum and then there is a progressive loss of birefringence outward from the hilum. Finally, the granules become completely lacking in birefringence (isotropic). The range in temperature between that at which the first granules begin to show loss of birefringence around the hilum and that at which all granules have lost all birefringence is called the "gelatinization temperature range". The gelatinization temperature and the gelatinization temperature range are specific for a given starch and relatively specific for a type of starch, such as wheat, corn, or oat starch. The cereal starches cannot, however, be clearly distinguished from one another by their gelatinization temperatures or gelatinization temperature ranges.

The most important role of the starch in bread making is a substrate of the diastatic enzymes.

The Amylases

The enzymes which hydrolyze starch and its components (amylose and amylopectin), glycogen and certain degradation products originating from these polysaccharides are called amylases. The action of amylases is purely hydrolytic, the sole action of the amylases being a cleavage of the alpha-glucosidic 1,4 linkage, the "maltose linkage" (Myrbäck and Neumüller, 1950).

Today, one distinguishes mainly between endoamylases and exoamylases (Myrbäck and Neumüller, 1950).

Endoamylases act on the alpha-1,4 linkages; the alpha-1,6 bonds constituting the branching points of amylopectin and glycogen remain unattacked. There results a rapid diminution of viscosity and average molecular weight of the substrate; the primary products are oligosaccharides (dextrins), which are later broken down to yield maltose, some glucose, isomaltose and branched-chain products of relatively low molecular weight. A single type of endoamylase is known, namely alpha-amylase, the term being coined by Kuhn (1924; 1925), since the reducing hemiacetal group liberated by the hydrolysis is in the alpha optical configuration (Fischer and Stein, 1960).

Exoamylases are exclusively of vegetable or microbial origin. They are capable of attacking the polysaccharide only from the non-reducing outer chain ends and do so in a regular manner, breaking every glucosidic bond to produce solely alpha-glucose, or every alternate bond to produce maltose. The former enzymes are called glucamylases or gamma-amylases (they can hydrolyze alpha-1,6 bonds slowly), and the latter beta-amylase (their action stops at the alpha-1,6 bonds giving high molecular weight "limit dextrins") (Fischer and Stein, 1960).

Alpha-amylases are among the earliest known enzymes. Most of the more

common alpha-amylases have now been obtained in a highly purified form. Crystallization has been reported for the alpha-amylase of A. oryzae (Underkoffler and Roy, 1951; Fischer and Montmollin, 1951; Akabori et al., 1954); for the alpha-amylase of human saliva (Meyer et al., 1948); for human pancreas (Fischer et al., 1950; cited by Fischer and Stein, 1960); for hog pancreas (Meyer et al., 1947); for rat pancreas (Heatley, 1958); for barley malt (Schwimmer and Balls, 1949). Crystallization of alpha-amylase frequently becomes increasingly difficult on successive recrystallizations, and this has been attributed to a loss of various divalent metal ions resulting in a considerable increase in the apparent solubility of the protein (Fischer and Stein, 1960).

Alpha-amylase from different sources are capable of liquefying, dextrinizing and saccharifying starch which is not sufficiently damaged to be susceptible to beta-amylase. The specific action of alpha-amylase is essentially the same whether amylose or amylopectin is the substrate (Hopkins, 1946). The initial action is a very rapid liquefaction of starch paste caused by the rupture of the more centrally located alpha-1,4-glucosidic linkages. In contrast to the beta-amylases, the alpha-amylases are able to cleave branched substrate molecules between the branch points. In place of the high molecular weight limit dextrin produced by beta-amylase, the alpha-amylase "limit dextrins" are low molecular weight branched oligosaccharides containing only a few glucose units. Concomitant with dextrinization, the starch molecule gradually loses its capacity to form colors with iodine. Cleavage of only 0.1 per cent of the alpha-1,4-glucosidic links in the starch substrate reduces the viscosity of a paste to 50 per cent, liquefaction is complete when about 0.5 per cent of the

bonds are broken; dextrinization requires cleavage of about 7 per cent of the links (Hopkins, 1946). Even in the initial stages of amylolysis there is simultaneous production of high molecular weight dextrans and low molecular weight oligosaccharides (maltose, maltotriose and maltotetraose). At the end of the rapid phase of action, the entire structure has been converted to maltose and maltotriose. Production of reducing and fermentable sugars is relatively slow but may be eventually extensive (Franch, 1957). Pazur and Sandstedt (1954) found that after four hours action of malt-amyase on starch a series of sugars of low molecular weight including glucose, maltose, amylotriase, amylotetrose and amylopentaose were formed. This is in contrast to the action pattern of salivary amyase which produces only three reducing sugars of low molecular weight, maltose, amylotriase and amylotetraose (Bird and Hopkins, 1954). The smallest limit dextrin formed by malt alpha-amyase is 4-alpha-isomaltosylglucose (panose), by animal and fungal amyases 4-alpha-panosylglucose, and by Bacillus subtilis alpha-amyase it is a pentasaccharide (Whelan, 1961). Alfin and Caldwell (1948a) found that the extent of hydrolysis of soluble potato starch, whole potato starch, and linear fractions from corn starch by a highly purified pancreatic amyase, depended in each case within wide limits upon the concentration of amyase used. Maltose was present from the very early stages, both from linear fractions of corn starch and from whole potato starch, but in larger concentrations in the hydrolysates from the linear substrate (Alfin and Caldwell, 1948b). Glucose did not appear in the early stages of hydrolysis and was present in slightly larger concentrations in the hydrolysates of potato starch. Waxy maize starch was hydrolyzed more slowly by pancreatic amyase than unfractionated corn starch and much more slowly

than the linear fraction from corn starch (Mindell et al., 1949). From the very early stages of the hydrolysis of waxy maize starch by purified pancreatic amylase, maltose was present in significant concentrations; glucose also was liberated, but in smaller concentrations and not in the very early stages of the hydrolysis. Hanrahan and Caldwell (1950) reported that increases in the concentration of purified crystalline Taka-amylase effected an increase in the rate and extent of hydrolysis of a linear fraction from corn starch to end products maltose and glucose, although Taka-amylase did not hydrolyze maltose; it hydrolyzed trisaccharides and higher sugars composed of 1,4- α -D-glucosidic chains. The linear fraction from corn starch was hydrolyzed more extensively and rapidly than several branched chain substrates. Ulmann and Seidemann (1958) studied the behavior of amylases on potato and wheat starches. At 40°C. fungal amylase produced maltose and maltotriose in large quantities within 10 minutes, along with traces of maltotetraose and maltopentaose; after 20-30 minutes maltohexaose and maltoheptaose appeared. When compared with different malt preparations and with pancreatic amylase the fungal amylase was found to produce a greater number of higher saccharides and larger amounts of some of the intermediate sugars. Isomaltose has not been observed in fungal amylolysates of starch. With fungal preparations, glucose appeared at an earlier stage than was the case with malt, though the latter preparation yielded larger amounts of maltose. A temperature of 55°C. was optimal for formation of glucose by fungal amylase and least favorable for the production of higher sugars. Drapron (1962) reported that when starch was acted upon by bacterial amylases, the formation of glucose and maltose was at the beginning very slow and increased progressively. After 10 minutes maltoheptaose and

maltotetraose were formed, both of which disappeared after 15 minutes, concomitant with a rapid formation of maltotriose, maltohexose and maltoheptose. According to Hayden (1961) fungal-amylase produces primarily maltose; the main product of cereal amylases is a hexasaccharide.

Alpha-Amylase in Wheat Kernels

Germination of cereal grains is accompanied by a large increase in the activity of hydrolytic enzymes. These hydrolytic enzymes mobilize the endosperm reserves for the growing seedlings.

Hayashi (1940) showed that gibberellic acid stimulated the germination of barley, wheat and rice grains and the various processes associated with germination. Munekata and Kato (1957) and Sandegren and Beling (1958) showed that the effects of gibberellic acid on intact germinating barley were due to the gibberellic acid's effect on the embryo growth. While Pollack (1958) has reported that the effect of gibberellic acid is limited to the embryo, recent data (Dahlstrom and Sfat, 1961; Yomo, 1958, 1960a,b; MacLeod and Millar, 1962; Paleg, 1960b, 1961) show that the stimulation by gibberellic acid is not limited to the embryo alone.

Adding gibberellic acid during the early stages of germination has marked effects on the enzymes of barley (Dahlstrom and Sfat, 1961): increase in respiration, alpha-amylase, diastatic power, protease, cellulase, catalase and transaminase systems, and decrease in the dextrin-maltose ratio. The total carbon content of wheat seedlings is increased by adding gibberellic acid but increase in photosynthesis could not be demonstrated. Recent work (Dahlstrom and Sfat, 1961) on endogenous gibberellic acid in germinating barley indicates a distinct possibility that the biological form of

gibberellic acid may be a bound or complex form.

It has been believed for a long time that alpha-amylase is produced in the cells of the scutellar epithelium of the germinating cereal seed and is secreted into the endosperm (Myrbäck and Neumüller, 1950). The evidence for this secretion, as summarized by Dure (1960), is based on a) light microscope observations of the behavior of the scutellar epithelium and of the apparent migration of particles from it into the endosperm during germination; b) the isolation of particles from the scutellum that contain amylolytic activity; and c) excised cereal embryos exhibiting good germination growth on boiled endosperm and on starch paste. Dure (1960) studied the gross nutritional contributions of the scutellum and the endosperm to the germination growth of the maize axis. The results showed that the mature embryo depended on the endosperm only for a source of carbohydrates and inorganic ions but did not require hormones, vitamins or other factors originating in the endosperm for successful germination. The lag in growth in starch paste cultures was attributed to the time required for the scutellum to secrete amylases into the endosperm and absorb the sugars produced from the amylolysis of starch. These (Dure, 1960), and additional studies have led to the belief that alpha-amylase originates exclusively in the scutellum and is secreted during germination. An interaction between the scutellum and the endosperm was deemed essential before the scutellum secreted alpha-amylase into the endosperm.

Ikemiya (1956) suggested that possibly an unknown factor was produced in the germinating barley embryo and there activated the present, inactive amylase. Yomo (1958) dehulled barley seeds by a 3-hour immersion in 50 per cent sulfuric acid and sterilized the seeds without injuring their

germinating power by immersion for 3 minutes in 0.2 per cent bromine water. All the experiments conducted by Yomo lasted up to 7 days and were made on samples incubated aerobically in a Randolph-Cox culture medium and at a temperature of 17°C. Temperatures above 20°C. were found detrimental to beta-amylase and to some extent to alpha-amylase. The embryo and endosperm of the barley seed were separated under sterile conditions, and the amylase production in these tissues and their interaction under aerobic conditions was examined. The alpha-amylase produced in these organs was negligible when compared with that of ordinary malt endosperm. If, however, the separated embryo and endosperm were present together in a liquid medium a substantial increase of alpha-amylase in the barley endosperm took place. When the isolated barley endosperm was placed in a barley embryo culture medium or green malt extract, alpha-amylase in the endosperm increased remarkably (Yomo, 1960a). An ungerminated barley extract had no such effect. The activating substance was found to be dialyzable through a cellophane membrane, was stable at pH 5.5 against heat, but was destroyed by acid or alkali. The substance had an acidic character, could be adsorbed by an anion exchange resin, but not by a cation exchange resin, and could not be precipitated by either lead acetate or basic lead acetate. In a later publication, Yomo (1960b) reported that the barley endosperm, free from the embryo, could also produce or activate alpha and beta-amylase when in contact with a gibberellic acid solution under aerobic conditions for 2 days or 5 days. This gibberellin action was conspicuous at the concentration of 10^{-3} mcg. per ml. and reached maximum at the concentration of 0.1 mcg. per ml. The embryo-free endosperm was divided into two portions longitudinally or transversally. When the two portions were cultured

together in one vessel in the presence of gibberellic acid, the increase in alpha-amylase was almost the same as when non-injured endosperm was used. The purified amylase activating substance was shown (Yomo, 1960e) to have R_f values almost the same as gibberellin, and infra red spectra showed similarities with those of gibberellin. The amylase activator had a gibberellin-like, plant elongation activity. The substance had properties completely different from those of indole acetic acid or kinetin. Yomo concluded, therefore, that the amylase activating substance which is effective in the barley endosperm is a kind of gibberellin and that during germination this gibberellin-like substance is produced in the embryo and transported into the endosperm and there activates the alpha-amylase and beta-amylase in the endosperm.

The work of Yomo has been confirmed and amplified by Paleg (1960a) and Paleg et al. (1962). Barley seeds were surface sterilized by treatment with calcium hypochlorite solution and incubated (whole or cut transversally) in a solution containing streptomycin, to prevent bacterial development, and various levels of gibberellic acid. Amylase activity in the medium surrounding the endosperm was measured by incubating an aliquot of the medium with soluble starch, and assaying for maltose formed as a result of enzymatic action. Additionally, aliquots of the medium were assayed for reducing sugars after separation by means of chromatography. Concentrations from 2×10^{-4} mcg. to 2×10^2 mcg. of gibberellic acid were active in producing increasing amounts of maltose and glucose from the endosperm and in increasing the water soluble amylolytic activity of the endosperm. In addition to a substantial increase in beta-amylase activity and initiation of alpha-amylase activity, a third enzyme whose characteristics

closely resemble those of R-enzyme was also present in response to gibberellic acid. Since gibberellic acid application to the excised embryo resulted in the liberation of enzymes capable of affecting the carbohydrate metabolism, Paleg (1961) suggested that control of such a metabolism may be a function of an endogenous embryonic gibberellin. The presence of gibberellin-like substances in barley and malt has been reported by Radley (1959). As a response to increasing levels of gibberellic acid, the barley endosperm liberated increasing quantities of soluble protein (Paleg, 1961). This was accompanied by a proportional decrease in the resultant dry weight of the endosperm. The gibberellic acid-induced response could be prevented by preheating the intact grain before treatment with gibberellic acid or by incorporating one of several sulfhydryl-blocking compounds in the incubation solution. The response over the first 24 hours was also sensitive to temperature, indicating a maximum at about 30°C.

MacLeod and Miller (1962) reported that when slices of barley endosperm were incubated with gibberellic acid under aseptic conditions, the activities of salt soluble endo-beta-glucanase and endo-pentase were found greatly augmented. Gibberellic acid has been shown to act through the respiring aleurone cell layer; it has no effect on aleurone cell layer free endosperm or on endosperm slices in which the aleurone cell layer has lost the power of reducing tetrazolium salts. Gibberellic acid-induced sugar secretion from endosperm slices is maximal at 30°C., and completely inhibited at 37°C. Secretion, both of sugars and of endo-beta-glucanase, is unaffected under conditions that inhibit proteolysis. Excised barley embryos show no response to added gibberellic acid. MacLeod and Miller (1962) suggested, therefore, that when barley germinates without addition of gibberellic acid,

endogenous gibberellin present in the embryo is translocated to the aleurone cell layer where it induces secretion of hydrolytic enzymes from subcellular particles, causing a modification to proceed inward from the aleurone layer. Thus added gibberellic acid seems merely, or primarily, to enhance the effects of endogenous gibberellin.

Yomo (1961) incubated barley endosperm with and without the addition of gibberellic acid at 17°C. for 5 days. The incubated endosperm was dried and analyzed for protease activity. The addition of 1 mcg. per ml. of gibberellin I yielded a 4 fold increase in protease activity. It seemed that gibberellin I induced the formation or activation of protease in the endosperm. The protease level formed by the endosperm in the presence of 1 mcg. per ml. of gibberellin I was equivalent to that of ordinary malts.

Hydrogen-ion Concentration

The concept of alpha-amylase from different sources having distinct and characteristic pH is not new. It was Sørensen (1909) who first called attention to the effect of the hydrogen-ion concentration on enzyme activity. Caldwell and Adams (1946) and Kneen, Sandstedt and Hollenbeck (1943) showed that alpha-amylase from various sources were more sensitive to low pH levels than was beta-amylase.

Sherman et al. (1919) studied the behavior of three enzymes: pancreatic amylase, malt amylase and taka-amylase. They plotted the activity of the enzyme as a function of the hydrogen-ion concentration of the medium and the curve obtained showed a zone of optimum activity for each enzyme. Sherman (1923) found that pancreatic and malt amylases differ in the pH of optimum activity. He found the pH optimum to be 6.6 and pH 4.4 for

pancreatic and malt amylases, respectively; the first one loses its activity more rapidly than the latter when held in aqueous dispersion and is active over a narrower range of hydrogen-ion concentration. Redfern and Landis (1946) studied three alpha-amylases (barley malt, bacteria and mold) and found a broad pH optimum over the range 4.75 to 6.0.

For cereal alpha-amylases, Creighton and Naylor (1933) reported that wheat alpha-amylase exerted its optimal dextrinogenic activity on potato starch at pH 4.6 to 6.3. For malt-amylase Fischer and Haselbach (1951) and Bernfeld and Studer-Pecha (1947) found a pH between 4.75 to 5.4. Recently, Dube and Nordin (1961) studying sorghum alpha-amylase, found that the enzyme was inactivated below pH 3.5 and above pH 9.0. Sorghum alpha-amylase was stable between pH 4.0 and 7.5 with an optimum pH at 4.6 quite similar to a reported value of barley alpha-amylase. Ito and Obata (1962) purified an alpha-amylase preparation from germinated rye seeds. The enzyme was found to have an optimal pH at 5.4-5.8.

For alpha-amylases from microbiological origin, Wallerstein (1939) found that alpha-amylase of B. subtilis showed its optimal activity at pH 6.5 to 8.0, but Menzy et al. (1947) reported the optimum pH for B. subtilis between 5.85 to 6.0. Redfern (1950) found that the optimum pH for bacterial amylases was species-dependent and varied over the range 4.8 to 6.8. Isemura and Imanishi (1960) found that the optimum pH for B. subtilis was around 5.7; the enzyme was stable over a wide pH range. Endo (1959) studied the alpha-amylase of mesophilic bacteria and found a pH optimum at 5.8. Manning and Campbell (1961) and Campbell and Cleveland (1961) working with a thermostable crystalline alpha-amylase found the pH range of maximum enzyme activity to be between 4.6 to 5.1, which is lower than the optimal range reported for most

microbial alpha-amylases. Cheng (1962) isolated an acid-stable alpha-amylase from a strain of A. niger. The optimum pH of this amylase was 3.5-4.5 and the enzyme was quite stable at pH 2.5 (at 40°C.) for a number of hours even in the absence of Ca-ions.

Pomeranz (1962) found that the optimum starch liquefying action of commercial alpha-amylases was about 4.0, 4.5, 6.5 and 7.0 for the fungal, cereal, bacterial and pancreatic enzymes respectively. The pH in each case was determined at the end of the enzymatic action. The thermostable bacterial amylase exhibited also high stability toward high acidity and was followed in this respect by the enzyme of fungal origin.

A comparison of the liquefying action of enzymes at a constant level of dextrinogenic activity showed the pancreatic enzyme to be most active, followed by cereal, and the fungal least active. The relative differences between the activity of the bacterial and fungal enzymes, at their optimum pH and at 37°C., depended on the levels of enzyme added.

Temperature

Temperature has a marked effect on the rate of enzymic activity. The enhancing effect of increased temperature is offset by denaturation of the enzyme at elevated temperatures. The rate of inactivation of an enzyme is a function of time as well as of temperature.

Wallerstein (1939) showed that the amylase from B. subtilis differed from others in its extreme thermostability and exerted marked liquefying activity at temperatures as high as 95°C. Conn et al. (1950) found, that amylases from different bacterial strains may differ in their thermostability. Bernfeld (1951) studied crude preparations of bacterial amylase

of B. subtilis. They were very resistant to high temperatures, and preserved their activity at 80°C.; the pure bacterial amylase, however, lost its activity even at 35°C. The high temperature resistance of the crude amylase is, therefore, not a property of the enzyme itself, but is result of a protection effect due to other substances which accompany the amylase in the culture medium as impurities.

Hartman et al. (1942) and Stark and Tetrault (1951) reported that a partially purified amylase obtained from B. stearothermophilus was active after 12 hours at 90°C. Campbell and Cleveland (1961) studying the thermostable alpha-amylase of B. stearothermophilus found that the optimum temperature for this enzyme was between 55° to 70°C.

Campbell (1954, 1955) purified and crystallized the alpha-amylase produced by the facultative thermophile B. coagulans. The enzyme lost only 10 per cent of its activity after 1 hour at 90°C. Endo (1959) studied the effect of temperature on the stability of an amylase from a thermophilic bacterium. There was a very small loss of activity during 180 minutes at 80°C. but at 90°C. enzyme inactivation proceeded very rapidly. Myrback and Neumüller (1950) found in a study of bacterial amylase, that the enzyme was stable at pH 5.8 to 10.8. The activity -pH curves at three different temperatures (30°, 40° and 50°) showed a rather broad optimum zone between pH 5 and 7. On the alkaline side of pH 6, the three curves were similar but on the acid side the curves differed widely. The acid branch of the pH curve may be attributed to a decrease in stability of the enzyme in acid solution.

Johnson and Miller (1948) studied the thermostability of amylases by means of the amylograph. Barley alpha-amylase was most resistant to thermal

inactivation, being closely followed by malted wheat alpha-amylase. Fungal alpha-amylase was the most susceptible to inactivation by heat. Inactivation was a gradual process, as shown by the fact that activity decreased over a relatively wide range of temperature.

Johnson et al. (1949) studied the thermostability of fungal, bacterial and malt alpha-amylase. They concluded that the inefficiency of the alpha-amylase from the fungal preparation in causing starch liquefaction was due mainly to the lack of thermostability. Alpha-amylase from fungal preparations was inactivated before a substantial portion of the starch became gelatinized and susceptible to enzymatic action. In contrast to the fungal preparations, the bacterial alpha-amylase had a high degree of thermostability. Consequently the effectiveness of starch liquefaction by amylases from different sources during the baking process would be related to their inactivation temperature.

With regard to the residual enzyme activity during baking, wheat and barley amylases lose a substantial part of their activity between 70-75° C. and 75-80° C., respectively. Fungal amylases are practically inactivated at 75° C., but have a greater gas-development rate than malt-amylases at fermentation temperatures. Bacterial amylases retain a 100 per cent efficiency even at 85° C. (Grep, 1953).

Inorganic Compounds

The participation of metal-binding agents in the proteolytic degradation of amylases strongly suggested that metal ions were present in the enzyme molecule (Fischer et al., 1958).

The stabilization of alpha-amylase by calcium ions is a well-established

phenomenon (Schwimmer and Balls, 1949; Hanafusa et al., 1955; Caldwell and Tung Kung, 1953). At least one equivalent of calcium per mole was present in all the enzymes tested by Fischer et al., (1958); zinc was also present in small amounts. Yamamoto and Fukomoto (1959) have reported that calcium in amylase can be exchanged with other cations such as Sr^{+++} , Mg^{++} and even Na^+ and the resulting enzyme retains part of its activity.

Bernfeld (1951) reported that the alpha-amylase of malt requires the presence of Ca-ions for its activity and stability. Crude malt extracts can be heated to 70°C. for 20 minutes without any loss of alpha-amylase activity. Like pure bacterial alpha-amylase, pure alpha-amylase of malt was much less stable at high temperatures than the crude enzyme.

Fischer and Stein (1960) summarize the action of Ca-ions in alpha-amylase as follows: "There is no indication that calcium participates directly in the formation of the enzyme-substrate complex. It appears that calcium performs a dual function. On one hand, it maintains the protein in the proper configuration for biological activity; on the other hand it stabilizes the secondary and tertiary structure, thus conferring to the amylase molecule its compact architecture."

All the alpha-amylases examined by Fischer et al. (1958) required Ca-ions for enzymatic activity. Generally, this metal is so strongly bound to the enzyme molecule that its addition to the assay system can be dispensed with, and hence, its indispensability for activity has remained unnoticed. Only when the binding of calcium is weak, as in the case of malt alpha-amylase, is the addition of calcium necessary, and this explains why the calcium requirement of this enzyme was first discovered.

Sherman et al. (1928) compared the activity of pancreatic amylase in

the presence of various salts. The data showed that anions exerted a specific influence on the activity of pancreatic amylase and that the chloride ion was the most effective activator. If in addition to the chloride ion another anion, such as the nitrate ion, was also present, the activity of the amylase was reduced to an extent which depended upon the relative concentrations of the two.

Aspergillus amylase was not influenced by sodium chloride (Ballou and Luck, 1940, 1941) but its optimum pH was altered to pH 5.1 by the presence of salts of fatty acids, at 30° C.

Whitaker et al. (1961, 1962) found that in relatively high concentrations cation-inhibited alpha-amylase catalyzed starch hydrolysis in the decreasing order: Li^+ , Na^+ , NH_4^+ , Rb^+ , Cs^+ and K^+ , and anions in the decreasing order: I^- , F^- , NO_3^- , SO_4^{2-} , Br^- and Cl^- . Inhibition was directly proportional to the square root of ionic strength.

Pomeranz (1962) studied the inactivating action of cobalt complexes on crystalline alpha-amylases of fungal, bacterial and pancreatic origin. The three alpha-amylases were irreversibly inactivated by incubation with a number of cobalt complexes. The inactivation was most effective at pH 9.0 and at room temperature after incubation for 18 hours. No equimolar stoichiometric relation seemed to exist between the cobalt complexes and the anionic groups of the amylases.

Ikemiya (1957) studied the effect of synthetic detergents on malt amylase. He found that sodium dodecyl sulfate, dodecylaminocarboxylic acid-sodium salt and alkyltrimethyl benzyl-ammonium chloride inhibited the amylase activity in decreasing order. Non-ionic detergents had no inhibiting action. Alpha-amylase seemed to be more resistant than beta-amylase to these

detergents. Calcium ions protected the alpha-amylase from inhibition by alkyl dimethyl benzyl-ammonium chloride at pH 8.5, but calcium ions could not remove completely the inhibition already established by this compound. In contrast beta-amylase was not protected by calcium ions.

Ikeda (1960) studied the effect of antibiotics on the activity of bacterial alpha-amylase. Crystalline alpha-amylase from *Pseudomonas* was markedly inhibited by tetracycline, chlortetracycline and oxytetracycline. Penicillin, kanamycin, novaliocin and carzinophilin also showed a slight inhibitory action on the enzyme, while sarcomycin, erythromycin and dehydrostreptomycin produced little or no inhibition. Simultaneous addition of calcium or sodium glutamate completely protected against the inhibition produced by tetracycline derivatives. Leucine, valine, glycine, alanine, and lysine also gave protection but to a lesser degree.

Sandstedt and Gates (1954) studied the effect of a cationic detergent (Roccal) on digestion of raw starch. The response of enzymes from different sources was different. In the case of the malt enzymes, little response was observed whereas the activities of enzymes from other sources on raw starch were materially augmented. They suggested that this could be an indication that the malt enzymes attacked the starch granule in a different manner. It seems, however, that the action of Roccal was not on the enzyme but on the substrate.

Radichevich et al. (1959) reacted crystalline porcine pancreatic amylase and crystalline taka-amylase with acetic anhydride under conditions that did not cause inactivation of the amylase. Free primary amino groups of porcine pancreatic amylase were essential to its enzymic action, and were also essential to the activity of taka-amylase. Evidences revealed that

only approximately 50 per cent of the free primary amino groups of the protein were essential to the activity of either enzyme and in both cases the alpha-amino or N-terminal amino groups were more important in that respect than the gamma-amino groups of lysine. Thus, although taka-amylase and porcine pancreatic amylase differ markedly in their action on the same substrate, these two alpha-amylases required primary free amino groups of the protein for their catalytic activity and both differed in this respect from beta-amylase.

Dedonder (1961) inactivated hog pancreatic and taka-amylase by acetylation of one-half of the amino groups of each of the alpha-amylases.

Onoue et al. (1962) reported that B. subtilis alpha-amylase lost its activity upon treatment with N-bromosuccinimide, presumably due to oxidation of the tryptophan residue, without changing the molecular configuration as indicated by immunochemical analysis.

Pomeranz (1962) studied the effects of prolonged action of a number of synthetic detergents on inactivation of pure, crystalline amylase, from fungi, bacteria and pancreas. Only cetylquarternary ammonium compounds were effective in inactivating the amylases. The anion of the quarternary ammonium salts seemed to be unimportant but a long chain, asymmetric compound was essential for effective inactivation. The enzyme of bacterial origin was more resistant than the fungal amylase to inactivation by cetyl quarternary ammonium compounds. The amylase inactivation was pH dependent. The extent of inactivation depended on the length of incubation of the enzyme with the quarternary ammonium compound.

Concentrations above 0.1 per cent of detergent were necessary to attain a substantial extent of enzyme inactivation. Mixtures of cationic detergents

with sodium lauryl sulfate or of the anionic detergent with calcium chloride resulted in an inactivation which was proportional to the amount of free synthetic detergent. Amylase inactivated by the anionic detergent could be partly reactivated by adding calcium chloride but the inactivation was irreversible with the quaternary ammonium salts.

Assay of Alpha-Amylase Activity

Amylase activity can be described as saccharifying, liquefying and dextrinizing.

Dextrinization. It can be considered as the production from the starch of sugar polymers of lesser complexity than the starch. Dextrinogenic activity is usually determined by the Wohlgemuth procedure (1908) modified by Sandstedt, Kneen and Blish (1939). The Wohlgemuth procedure involves the hydrolysis of a buffered starch solution at a constant reaction temperature until a definite stage is reached.

The modifications of Sandstedt, Kneen and Blish are:

I. Since the Wohlgemuth values are indicative only of the combined dextrinizing activity of alpha and beta-amylase, these workers render the method specific for alpha-amylase by adding an excess of beta-amylase to the reaction mixture. This procedure is based on their finding that increases in the ratio of beta to alpha-amylase in the reaction mixture increase the rate of dextrinization up to a point beyond which further increments of beta-amylase had no effect.

II. They used a commercial dextrin which gives a red-brown color with iodine as a color standard.

Redfern (1947) modified the Sandstedt, Kneen and Blish assay. He found

that No. 17 Hellige glass varnish color standard when used in a Hellige comparator could replace the dextrin-iodine color standard with considerable advantage. Briggs (1961) modified the assay so that a photoelectric colorimeter could be employed in conjunction with a standard graph. The precision of the method was thereby improved and the work needed for each assay reduced.

Saccharification. The action of amylases, particularly of the beta-type, on starch causes a production of compounds which have reducing properties. When this hydrolysis of the starch results in the production of sugars, the process involved is termed saccharification. In addition to their reducing properties, these sugars are fermentable by yeast. The saccharogenic activity is determined by the method of Kneen and Sandstedt (1941) based on the reduction of ferricyanide.

Liquefaction. A number of methods have been proposed to determine the liquefying properties of alpha-amylase.

Liquefying activity of alpha-amylase is determined by measuring the decrease in viscosity of reaction mixtures. Viscosimetric methods are influenced by a number of factors. However, if starches rendered soluble by chemical or mechanical means are used, and care is taken to standardize other conditions, reproducible measurements may be made (Geddes, 1946).

Jossa and Johnson (1935) proposed a method to determine the liquefaction activity of alpha-amylase. The method has not met with widespread use because of the meticulous care required to prepare the starch substrate with a specific initial viscosity, and because it requires a large quantity of substrate for each determination.

Blom and Bak (1938) proposed a method which depended upon observing the time required for the viscosity of a starch paste to change from a

viscosity equal to twice the viscosity of a reference solution of sucrose to the same viscosity as the sucrose solution. Since it was impossible to determine these two times by only two measurements, the results were obtained by interpolation from several measurements.

Landis and Redfern (1947) modified the method of Jozsa and Gore (1930) to measure liquefaction activity. They prepared a standard starch substrate from potato starch and used a modified Ubbelohde viscometer for the measurement of viscosity. By careful gelatinization and beating, the viscosity was adjusted to that stipulated. The enzyme was then added and after a definite reaction time the viscosity was again measured. Enzyme activity was calculated from a calibration table. The unit values proposed for amylase content were designated "liquefons".

Hagberg (1960) suggested a simple and rapid method for determination of alpha-amylase activity. The test may be completed in one or two minutes, depending on the amount of alpha-amylase present. The principle of the method depends on heating a flour-water suspension very rapidly to determine the time required to gelatinize, and subsequently, to liquefy the flour suspension. Hagberg (1961) modified his previous method, using as a measure of alpha-amylase activity "the falling number". The "falling number" is expressed as the total time in seconds needed by a stirrer to fall by its own weight through a distance of 70 mm. This time varies from 60 or more in samples of high amylase activity (i.e. flour from sprout-damaged grain) to 400 or more in samples of low activity.

The Brabender amylograph is a torsion viscosimeter (Anker and Geddes, 1944) which provides a continuous automatic record of changes in the viscosity of a starch paste.

According to Katz and Hanson (1939) gelatinization of wheat starch begins at 65°C. Amylase action is slight until the gelatinization point is reached; differences in the thermostabilities of amylases from various sources produce differences in the extent of enzymatic action and are reflected in the amylograph curve. It is well established that bacterial alpha-amylase is most thermostable, cereal alpha-amylase possesses intermediate thermostability and fungal alpha-amylase is least thermostable. As half of the fungal alpha-amylase activity is destroyed prior to starch gelatinization and the remaining half is inactivated at about 75°C. the amylograph cannot be employed to evaluate the level of alpha-amylase of fungal origin added to wheat flour or native starch.

Pomeranz and Shellenberger (1962a, b) found, however, that if the fungal enzyme is allowed to act on available (pregelatinized) starch, it is possible to employ the amylograph to determine levels of alpha-amylase of fungal origin.

According to Kneen (1950) the kinetics of the liquefaction of starch by amylases apparently differ somewhat from those operative in starch dextrinization or saccharification. Over no range was there a linear relationship between the quantity of enzyme and decrease in viscosity.

Pomeranz (1962) found a linear relation between enzyme activity and viscosity reduction for very low levels of enzyme concentration. Higher levels of enzyme resulted in a curvilinear relation. The linear relation was exhibited for the range up to 0.02 SKB units in case of pancreatic alpha-amylase, up to 0.04 SKB for cereal amylase, and up to 0.2 SKB in case of bacterial and fungal amylase. It is possible, however, that the results at low concentration of enzyme are influenced by partial inactivation of the

amylase.

Importance of Alpha-Amylase in Breadmaking

Enzyme supplements are useful in baking primarily as sources of alpha-amylase and proteinase. An adequate level of alpha-amylase activity is essential for the maintenance of a sufficiently high rate of gas production to leaven the dough and to provide a residual sugar to yield bread with a desirable taste and crust color (Geddes, 1950).

The effect of amylase on bread quality was pointed out by Jago as early as 1885. Baker and Hulton described the use of malt supplement as already "common" in 1908.

Kozmin (1933) recognized the effect of amylases on bread quality and postulated that excessive dextrinization was responsible for sticky crumb found when flours milled from highly germinated wheats were baked into bread.

Different studies (Sandstedt, et al., 1939; Kneen and Sandstedt, 1942) have established definitely that alpha-amylase is the diastatic component responsible for the increase in dough mobility, the increase in gas production of doughs and the increase in loaf volume caused by malt supplementation.

Kneen and Sandstedt (1942) showed clearly that the gas production of sugar-deficient doughs is increased by supplementing with alpha-amylase but not by raising the level of beta-amylase.

Freeman and Ford (1941) summarized the reasons for using supplement in baking as: (1) to increase gas production; (2) to improve the crust color; (3) to increase the moistness of the crumb and the keeping quality, and (4) to impart additional flavor. Kneen and Sandstedt (1946) added a fifth

reason, to increase the gas-retaining ability of the dough.

On the other hand, Kozmin (1933) pointed out that an excessive amount of supplement produced an undesirable, sticky, moist and inelastic crumb.

Stamberg and Bailey (1939) found that small amounts of malt alpha-amylase improved the bread considerably, but larger amounts of alpha-amylase resulted in bread having an inferior crumb, grain and texture.

Kneen and Sandstedt (1942) suggested a beneficial role for alpha-amylase in breadmaking, apart from producing sugar for fermentation.

Selman and Sumner (1947) found that the final effect of the amylases in the oven is the most important function of malt. It is clear, however, that not all the activity of the amylases takes place in the oven. The fermentation of the dough piece before entering the oven allows amylolytic activity properly to condition the starch so that when a gel is formed in the oven it will be of optimum viscosity provided the correct level of alpha-amylase activity is added. They suggested that a large portion of the total effect of the amylase on the starch granules takes place in the oven during the first stage of baking. An important function of alpha-amylase is to prepare the starch so that it will gelatinize at the right temperature and to the correct viscosity. It would be expected that flour deficient in alpha-amylase activity would produce a gel of high viscosity in the oven and that as the starch in the exterior of the loaf, which was first exposed to the heat, gelled, the expansion of the loaf would be reduced and the resultant loaf would be small in volume and characterized by a coarse texture.

On the other hand, if the flour is overtreated, the low viscosity of the starch gel will not securely establish the structure of the loaf and

here again there will be some diminution of volume, coalescence of the cellular structure of the crumb and a dark crumb color.

If the flour has been treated to the optimum range, the starch viscosity will be low enough to allow for the expansion of the loaf and yet will be high enough so that the established cell structure is of fine and desirable grain. When the temperature reaches 75° to 85°C. during baking, the gelling starch assumes the load which to this point has been carried by the gluten. The importance of the gluten as a structural unit is greatly reduced as the starch gel takes over this function.

Difficulties in distinguishing between the effects of amylase and proteases, both present in malt supplements, were overcome by preparation of amylases free from proteinases. Baking improvement was shown to result from the addition of moderate levels of alpha-amylase to the baking formula, whereas high levels produced sticky crumbs.

The action of alpha-amylase and proteinase can be considered during two phases of the baking process. During fermentation, the small fraction of the wheat starch that has been severely damaged by milling is characterized by a high degree of availability for degradation by amylases and by high water-holding capacity. Liquefaction of this fraction by alpha-amylase in addition to influencing its capacity for water retention, renders it more susceptible to attack by beta-amylase. Dextrinization and saccharification follow, and the net observable result, especially in flours containing large quantities of damaged starch, is a decrease in water absorption capacity, a slackening of the dough, and the development of stickiness (Kneen and Sandstedt, 1946).

The extent of starch modification during the baking process differs

with cereal, fungal and bacterial alpha-amylases.

Walden (1955) studied the action of wheat amylases on starch under conditions of time and temperature as they exist in baking. He found that starch was rapidly attacked over the approximate temperature range of 58° - 78°C., while above 78°C. the rate of starch conversion declined, owing to enzyme denaturation. Starch conversion was small for 0.28 alpha-amylase units per gram, the level normally employed in baking practice. For 6.1 units of alpha-amylase per gram, the final amount of conversion was 6.1 per cent, and the average dextrin chain was composed of 60 D-glucose units.

Walden (1955) found that levels of alpha-amylase corresponding to those in flours yield loaves with sticky crumbs produced considerable quantities of maltose and materially reduced the chain length of starch when tested under time-temperature conditions of baking. At much higher levels of alpha-amylase, starch conversion may be considered to be complete, under the time conditions prevailing. He considered that sticky crumbs are caused by an unbalance between beta and alpha-amylase. However, studies made by Stamberg and Bailey (1939) showed that beta-amylase, even when added in large amounts, did not improve the bread. Mamaril *et al.* (1963) showed that purified wheat and sorghum alpha-amylases exhibited a beta-amylase response, apparently due to their low beta-amylase content, but commercial malts high in beta-amylase showed no increase in formation of fermentable sugars as a result of supplementation with additional beta-amylase.

When the dough reaches the oven, further alpha-amylase action depends on the heat stability of the enzyme. If the enzyme is heat stable, as in the case of bacterial amylases, and to a somewhat lesser extent with that from malt, enzyme activity reaches a maximum just after the time when the

dough reaches the temperature of starch gelatinization. If there is an excess of heat stable alpha-amylase still active at this stage, a large amount of starch will be converted to low molecular weight dextrans. This will give rise to greater fragility of the loaf and may result in a large number of crippled loaves and poor performance at the slicer, as well as a caved-in appearance of the loaf. If there is too much active enzyme remaining after baking, the crumb will become gummy and sticky and eventually "ropy" (Silberstein, 1961).

Beck et al. (1957) studied the soluble dextrin fraction and sugar content of bread baked with alpha-amylases from different sources. The amount of soluble dextrans extracted from bread crumb 1 hour after baking increased with each increment of malted wheat flour and bacterial alpha-amylase. Fungal alpha-amylase did not affect the amount of soluble dextrans obtained. The residual glucose in bread crumb increased rapidly with increasing concentrations of bacterial amylase, but less rapidly with increasing concentrations of cereal and fungal alpha-amylase. The residual maltose in bread crumb increased with cereal or bacterial and, to a lesser extent, with fungal amylases.

Johnson and Miller (1948) compared the thermostability of alpha-amylases from wheat, barley and fungi. Barley alpha-amylase was the most resistant to thermal inactivation, being closely followed by malted-wheat alpha-amylase. Fungal alpha-amylase was the most susceptible to inactivation by heat.

In a study of the effects of malted wheat flour and fungal extracts (Johnson and Miller, 1949), the use of high amylase concentrations increased the loaf volume. The grain and texture were improved by the use of a limited amount of either supplement. As the concentration of malted wheat

flour extract was increased, the grain of the crumb became more open and the texture became increasingly soft. The texture became particularly gummy and undesirable at the highest concentrations. The crumb did not become as gummy with the fungal as with the malted wheat flour extract; use of fungal amylase gave a harsh and coarse crumb.

Johnson and Miller (1949), studying the effect of supplements from which the proteinase was removed, concluded that increasing the alpha-amylase in the absence of the proteinase was less effective in grain and texture improvement than if proteinase was also present in small amounts. At low concentrations of supplement alpha-amylase from either the malted wheat flour or a fungal preparation was responsible for a sharp decrease in dough consistency. Further increase in the concentration of alpha-amylase appeared to produce no further change in dough mobility. This suggested that the quantity of the starch substrate which is susceptible to attack was limited. It would appear that the protein substrate was not limiting for the proteinase, as was the substrate for alpha-amylase. The presence of both enzyme components is more effective than that of either component alone in decreasing dough consistency.

Miller et al. (1953) compared cereal, fungal and bacterial preparations as supplements for breadmaking. All three types of enzymes decreased the rate at which bread became firm with the age. The bacterial enzyme, however, caused the bread crumb to be sticky and gummy when sufficient quantities were used to maintain a soft crumb for several days. The fungal amylase was the least, and the bacterial amylase the most thermostable of the three preparations. The bacterial and malted wheat flour preparations produced larger quantities of sugar than was produced by the fungal preparations.

Schultz et al. (1952) studied the effect of bacterial alpha-amylase in relation to the staling process. Adding 3 to 6 SKB units of bacterial alpha-amylase per pound of bread baked on a commercial scale, resulted in an improved product which staled at an appreciably decreased rate, with no evidence of sticky or gummy crumbs.

Among the molds, certain strains of Aspergillus oryzae are particularly adapted to the production of alpha-amylase. The fungi amylase has starch degrading properties similar to cereal alpha-amylase; its thermostability is, however, low (Kneen and Sandstedt, 1946). The economy of the production, studied by Green (1934) and by Read and Haas (1936), indicated that mold amylase could be used in baking.

There is an extensive commercial production of alpha-amylase from selected strains of B. subtilis and B. mesentericus. These enzymes, however, have a much higher degree of thermostability than malt alpha-amylase (Kneen and Sandstedt, 1946). Not only is the starch breakdown in the oven excessive, but apparently some of the amylase may remain active throughout the baking period and cause liquefaction of the gelatinized starch after the bread is removed from the oven. The use of amylases of such high thermostability produces undesirable bread-crumbs characteristics similar to those associated with "ropy" bread.

Pancreatic amylase has a thermostability of about the same order as mold amylase or as cereal beta-amylase. Its optimum pH is above the hydrogen ion concentration likely to occur in the dough. Green (1934), however, showed satisfactory results in breadmaking by use of pancreatic alpha-amylase.

MATERIALS

The starch used as the substrate for measuring alpha-amylase activity was Amaizo 721A-pregelatinized waxy maize starch, from American Maize Products Company, New York.

The samples of wheat used were soft red winter, durum, hard red spring and hard red winter. The sample of mold-free hard red winter wheat of the Turkey variety from the irrigated region of Idaho was obtained through the courtesy of Prof. C. M. Christensen, University of Minnesota.

The pure, crystalline Taka-amylase A was a gift from Shoji Matsubara, Osaka University, Japan. The pancreatic, crystalline amylase was purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio and the crystalline bacterial enzyme from Worthington Biochemical Corporation, Freehold, New York.

Additionally, crude amylases from cereal, bacterial and fungal origin were used. Among the cereal amylases, two samples of wheat, two samples of barley and a sample of sorghum amylases were tested. Of the amylases of microbiological origin, fungal and bacterial enzymes were tested. One of the wheat malt preparations and also the sorghum malt were partly purified. The other enzymes were concentrates furnished by three different commercial firms. The dextrinogenic activities of the non-crystalline preparations (expressed as the S.K.B. units per gram) were:

Wheat	19 and 414
Barley	52 and 284
Sorghum	840
Fungal	908;4,590;5,000;7,900 and 8,140
Bacterial	5,480;7,950 and 11,880

The quarternary ammonium compounds were analytically pure, and were purchased from Bios Laboratories Inc., N. Y.

The gibberellin used was potassium gibberellate from Merck and Company, Rahway, N. J.

For studies on the effect of crystalline bacterial amylase on dough consistency a commercial unmalted hard red winter, straight-grade flour was used, (protein content, 11.9 per cent, ash content 0.43 per cent; and water absorption as determined by the farinograph, 64.9 per cent).

METHODS

Gibberellic Acid Induction

Seeds were rinsed three times with water and placed in 50-seed-(or embryo free endosperm) lots in sterilized 4-inch Petri dishes containing one piece of filter paper and 2 ml. of water. Unless stated otherwise, 10 ml. of a solution containing various amounts of gibberellic acid and 1500 mcg. of streptomycin were added and the samples incubated for 24 hours at 24°C. in the dark. Control tests were carried out on the mold-free wheat, employing no streptomycin in the incubation mixture or using wheat endosperm which had not been surface sterilized with the calcium hypochlorite solution.

At the end of the incubation period, the solution was decanted, the filter paper removed, and the wheat seeds or wheat endosperms were dried in the Petri dishes in a forced-draught oven at 43°C. overnight. After cooling, the samples were ground in a Wiley micropulverizer, through mesh No. 40. Homogenates were prepared by grinding sliced endosperm halves

with water or a gibberellic acid solution for 15 minutes in a micro-attachment of a Servall Omni-Mixer at 16,000 r.p.m.

Throughout the incubation procedure, sterilized water and sterilized glassware was used. As far as possible the incubation has been carried out under sterile conditions. To eliminate the influence of microorganisms on the formation of alpha-amylase, seeds were soaked for 45 minutes in a 5 per cent calcium hypochlorite solution. The solution was freshly prepared before each experiment by shaking the calcium hypochlorite with water for 10 minutes in a glass-stoppered flask and passing the resultant mixture through one thickness of Whatman No. 1 filter paper. All the determinations were made in duplicate.

Alpha-Amylase Activity

Unless stated otherwise, alpha-amylase activity was measured by following the liquefying action of the enzyme. The amount of pregelatinized starch to obtain a reading of 1000 B.U. on the amylograph chart was 33.5 g. The starch was added slowly to 320 ml. of a phosphate buffer solution 0.02 M and mixed by a laboratory stirrer at 1450 r.p.m. in a beaker. The starch suspension was mixed for 3 minutes. The apparently homogenous dispersion was transferred with the aid of 120 ml. of additional buffer solution to an amylograph bowl.

The Brabender amylograph, a torsion viscosimeter which provides a continuous automatic record of changes in viscosity of the starch, was employed to measure changes in starch viscosity as a result of the action of alpha-amylase. The instrument bowl was operated at 75 r.p.m.; the enzymatic action was measured at a temperature of 37° C. After the transfer

of the starch to the amylograph bowl, the instrument was operated for 10 minutes. After this period of temperature equilibration, the enzyme preparations were added.

Enzyme Preparations

I. Gibberellic acid induction: The samples were ground in a Wiley micro-pulverizer and 400-mcg. lots were extracted during 1 hour with 16 ml. 0.2 per cent calcium chloride, filtered through Whatman No. 4 filter paper, and added to the starch suspension in the amylograph bowl. The total volume of liquid in the bowl was adjusted to 450 ml. and the viscosity recorded during 20 minutes.

II. Starch liquefying properties of crystalline alpha-amylases: Ten ml. of a 0.2 per cent calcium chloride or 10 ml. of a 0.02 M sodium chloride solution containing the enzyme were added and the viscosity recorded by the instrument during 40 minutes.

III. Effect of pH or inactivation on enzyme stability: Microgram quantities of pure enzyme (or 10 S.K.B. units of a commercial preparation) suspended in 10 ml. of 0.2 per cent calcium chloride or 0.02 M sodium chloride in case of pancreatic amylase, and 10 ml. of 0.2 M phosphate buffer were held in a stoppered Erlenmeyer flask for 18 hours before being placed in the instrument bowl. The enzyme was then added to a starch suspension buffered to give optimum pH at the end of the starch-hydrolysis measurement.

Dough Consistency

The flour was mixed in a Hobart mixer to optimum consistency and portions corresponding to 50 g. of flour were mixed in a small farinograph bowl for 10 minutes immediately, and after holding at 30°C. and 85 per cent R. H. for 2 and 4 hours, respectively.

Baking Tests

The baking experiments were on a laboratory scale employing the straight-dough procedure. The basic formula was:

<u>Ingredients</u>	<u>Parts</u>
Flour (unmalted)	100
Water	variable
Yeast	2
Sucrose	variable
Milk solids	3
Shortening	3
Sodium chloride	2
Arkady	0.5

The following variables were introduced:

Dough No.	Sugar %	Pancreatic amylase mg.	Bacterial amylase mg.
1	2	-	-
2	5	-	-
3	2	0.1	-
4	2	0.2	-
5	2	0.4	-
6	2	0.8	-
7	2	1.6	-
8	2	-	0.1
9	2	-	0.2
10	2	-	0.4
11	2	-	0.8
12	2	-	1.6

After mixing to optimum consistency, the doughs were fermented at 30°C. After 110 minutes the doughs were punched, and 50 minutes later were punched again, divided, rested for 20 minutes, and moulded. The doughs were proofed at 30°C. for 45 minutes and baked for 25 minutes at 210°C.

RESULTS AND DISCUSSION

Gibberellic Acid Induction of Alpha-Amylase in Wheat Endosperms

Preliminary experiments were made to determine the range of gibberellic acid that effectively induces the liberation of alpha-amylase.

Paleg (1960) reported that an increase in reducing sugars and amylase activity in the liquid surrounding the endosperms was induced over a range of 2×10^{-4} mcg. to 2×10^2 mcg. or a factor of 10^6 , but induction of measurable alpha-amylase by gibberellic acid by the proposed method was much smaller. Only concentrations between 0.1 to 100 mcg. of gibberellic acid per ml. of solution increased alpha-amylase in the calcium chloride extracts of endosperm halves of seeds. A similar range has been reported by Fleming and Johnson (1961) in studies on effectiveness of potassium gibberellate on amylase and protease activities during steeping of wheat. Using extracts from wheat seeds or wheat endosperm halves heated for 1 hour at 100°C., in either the absence or presence of gibberellic acid, resulted in no measurable drop in starch viscosity.

The use of mold-free wheat, a short (24 hrs.) incubation time, and practically sterile conditions, made it possible to use surface sterilized wheat and to compare the formation-activation of alpha-amylase in the presence and absence of streptomycin.

The results, summarized in Table 1, show no significant effect from adding streptomycin, in agreement with results reported by Paleg (1960a).

Table 1. Effect of streptomycin on activation of alpha-amylase.

Treatment	Viscosity drop (Instrument units)	
	Without GA	With GA
Incubated with streptomycin	0	210
Surface sterilized	0	165
Surface sterilized and incubated with streptomycin	0	235

The short-termed contact of seeds with water during the surface-sterilization period has been assumed to have no effect on the grain. But as MacLeod and Millar (1962) pointed out, such an assumption may be wrong. Material such as endogenous gibberellin, may transfer from the germ to the aleurone and the endosperm and activate alpha-amylase during such a brief period. To test this hypothesis, endosperms from sound, essentially mold-free wheat were incubated both in the presence and absence of gibberellic acid. No significant difference could be detected between the samples. This result was in agreement with the results reported by Paleg (1960a).

The effect of adding various levels of gibberellic acid to the various classes of wheat endosperms is summarized in Fig. 1.

Two conclusions can be derived from these data. Over the effective range of gibberellic acid-induced alpha-amylase activation, enzyme activity

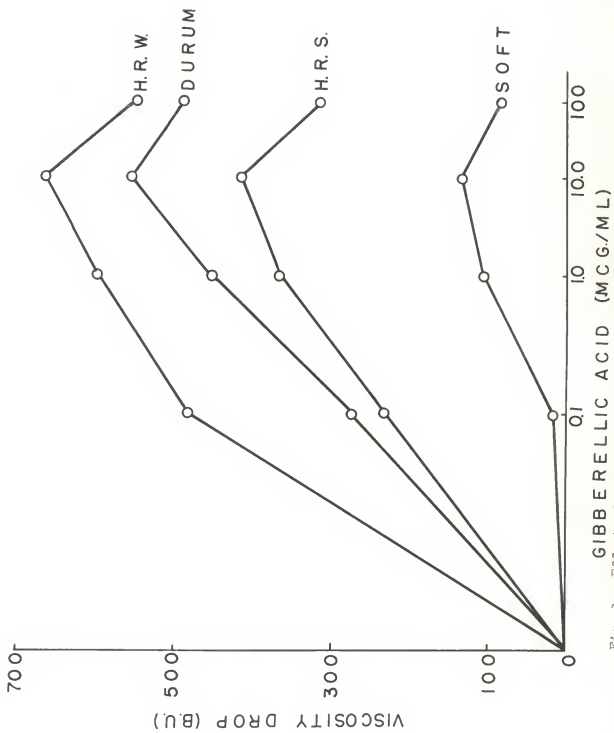


Fig. 1. Effect of various levels of gibberellic acid on the various classes of wheat endosperm.

gradually increased up to 10 mcg. per ml. of solution; levels of 100 mcg. per ml. decreased amylase activity. The amylase activity varied with the class of wheat tested. Despite this variation, the procedure might be employed to estimate the amount of gibberellic acid in a preparation, as no alpha-amylase activity was recorded in any of the samples incubated in the absence of gibberellic acid. The results in Table 2 show a comparison of alpha-amylase present in separated wheat endosperm halves and in whole seeds, in both the absence and presence of gibberellic acid.

These results are in line with the well established fact that gibberellic acid not only activates the alpha-amylase in the separated endosperm, but also increases substantially the levels of enzyme in the intact seed germinated for a short period. However, use of optimal concentrations of gibberellic acid resulted in release of higher concentrations of amylase in

Table 2. Effect of gibberellic acid (10 mcg./ml.) on starch liquefaction due to the action of alpha-amylase.

	Viscosity drop (Instrument units)			
	Whole Wheat		Separated Endosperm Halves	
	Without GA	With GA	Without GA	With GA
Soft red winter	40	120	0	135
Hard red winter	120	650	0	660
Durum	260	600	0	560
Hard red spring	130	560	0	420

intact grain than in separated endosperm. This seems to indicate that while, under the test conditions, the added gibberellic acid replaces a substantial part of the endogenous activators, the scutellum may play a supplementary role in augmenting the amylase activation.

The effect of incubation of whole seeds and of separated endosperms, at various temperatures in the presence or absence of gibberellic acid, is shown in Fig. 2.

The actual extract of amylase was prepared in each case from the endosperm portion only. In one case, however, this separation was carried out at the end of the incubation period; in the other case, the endosperm was separated from the embryo prior to incubation. The optimum temperature seems to be about 30°C., This is in agreement with the data obtained by MacLeod and Millar (1962). But whereas the activation due to the addition of gibberellic acid showed a sharp drop beyond 30°C., the formation of amylase in the whole seed without gibberellic acid showed a gradual decrease.

It has not been possible in the absence of exogenous gibberellic acid to induce, during the short period of 24 hours, the activation of alpha-amylase in the endosperm by keeping it in a Petri dish with the separated embryos, as was reported by Yomo (1958).

Cutting seeds longitudinally into two halves, or splitting the embryo without separating the adjacent endosperm resulted in a slight decrease in alpha amylase content of the calcium chloride extracts. The results are shown in Table 3.

When the separated endosperms were homogenized, the alpha-amylase activity was substantially decreased. This decrease might be, in part at least, due to the increased solubility of alpha-amylase in the finely-divided

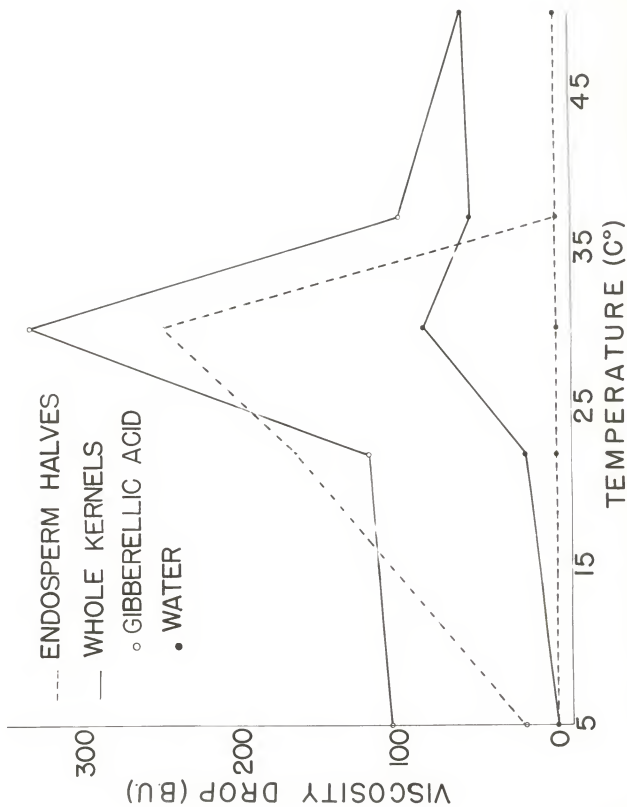


Fig. 2. Effect of temperature on activation of alpha-amylase, in wheat endosperm.

Table 3. Effect of various mechanical methods of seed treatment on alpha-amylase activity.

Treatment	: Viscosity drop (Instrument units)	
	: Without GA	: With GA
Heat treated endosperm halves ¹	0	0
Heat treated seed ¹	0	0
Endosperm halves	0	165
Endosperm halves cut after incubation	55	160
Whole seeds	90	515
Endosperm halves incubated with cut germs ²	0	220
Seeds cut longitudinally	35	415
Seeds cut longitudinally through germ only	45	380
Homogenates of endosperm	0	45

¹Heated for 1 hour at 100°C. prior to incubation.

²For CaCl₂ extract, the endosperm halves only were used.

homogenate and to retention of enzyme in the whole endosperm.

Starch Liquefying Properties of Crystalline Alpha-Amylase

Effect of pH on Enzyme Activity and Enzyme Stability. Optimum pH for amylase activity varies with the enzyme source (Figs. 3, 4 and 5). The three crystalline amylases exhibited maximum activity at (or about) pH 4.5, 6.8 and 6.0 for the fungal, pancreatic and bacterial amylases, respectively. These results are in agreement with the values given by Myrbäck and Neumüller (1950) and Pomeranz (1962).

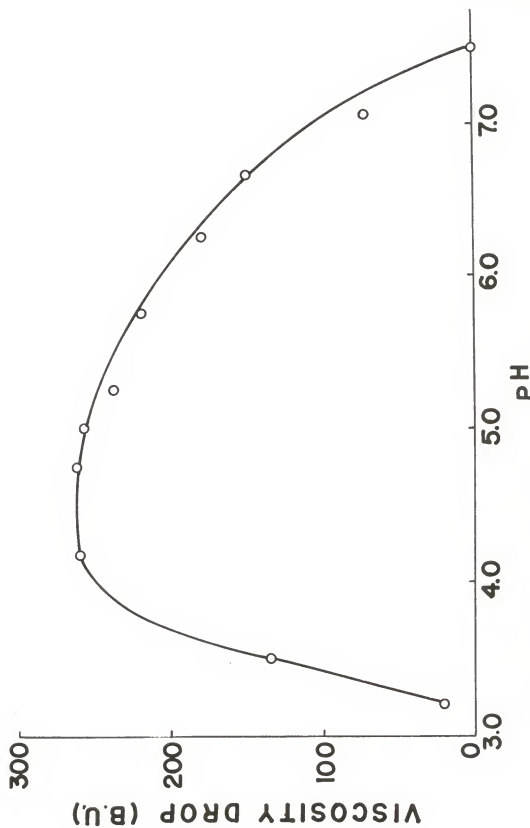


Fig. 3. Effect of pH on activity of Taka-amylase.

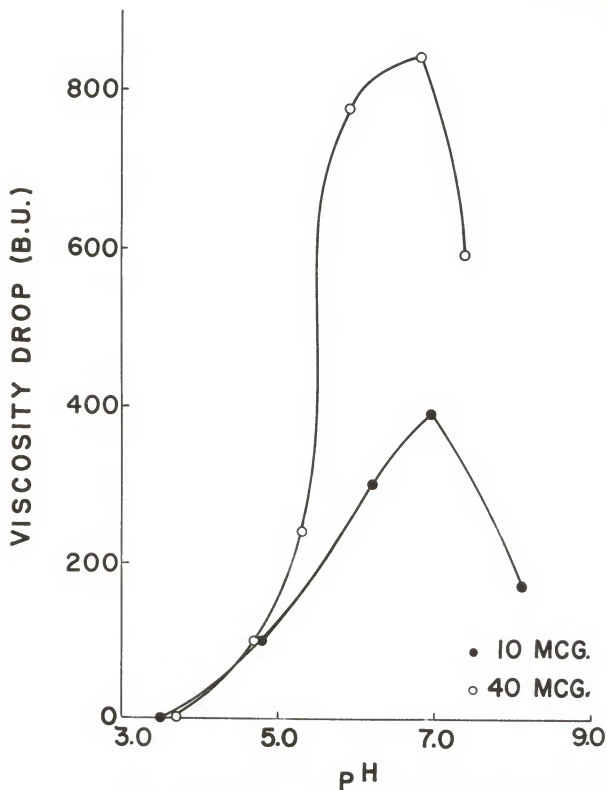


Fig. 4. Effect of pH on activity of pancreatic alpha-amylase.

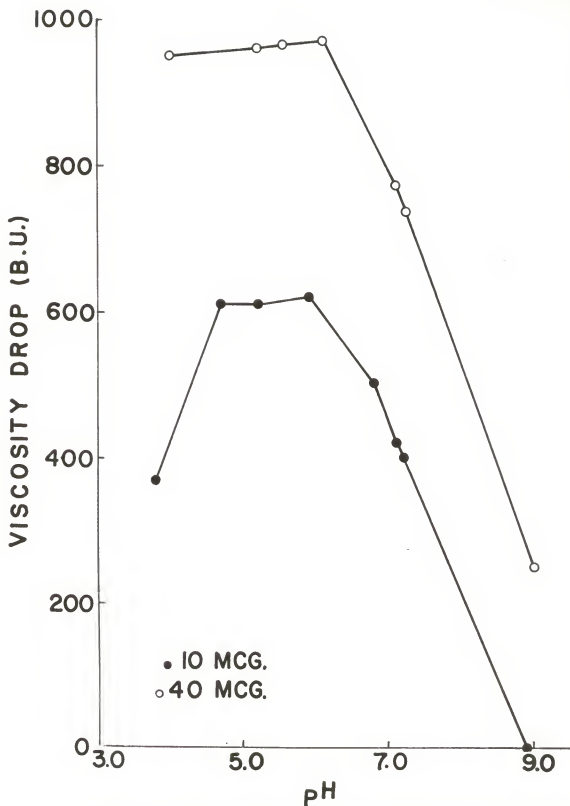


Fig. 5. Effect of pH on activity of bacterial alpha-amylase.

No appreciable irreversible inactivation took place after incubation between pH 4-8, 5-8 and 5-12 for the fungal, pancreatic and bacterial amylases, respectively (Figs. 6, 7 and 8). In addition to exhibiting the widest range of pH optimum, the amylase of bacterial origin was most stable at low pH values, despite its relatively high pH optimum. This is in agreement with previously reported results on the high stability of a number of commercial amylases of bacterial origin. The addition of calcium chloride seemed to have no effect on enzyme stability at extreme pH values (Figs. 6, 7 and 8). This apparently was due to the fact that the enzymes tested were purified in the presence of calcium ions. The amount of calcium necessary for amylase stabilization is bound very strongly and can be removed only by prolonged dialysis against ethylenediaminetetraacetic acid, (Fischer and Stein, 1960). Today calcium is routinely added during the purification of alpha-amylases to stabilize the enzyme and also to promote its crystallization. Recent spectrophotographic analysis (Vallee et al., 1959) of five crystalline alpha-amylases from widely different biological sources, and purified according to five different procedures showed that each had at least 1 gram-atom of calcium per mole.

Effect of Temperature. The effect of temperature on the rate of amylase activity was studied. Table 4 shows the relation between log of velocity of enzymatic action and temperature. Taka-amylase showed enhanced activity up to a temperature of 50°C.; the increase in activity in case of pancreatic-amylase was up to 45°C.; bacterial-amylase activity decreased above 60°C. Johnson et al. (1949), Miller et al. (1953) and Fleming et al. (1961) studied the thermostability of commercial fungal, bacterial and malt alpha-amylases. They concluded that alpha-amylases from fungal preparations are inactivated

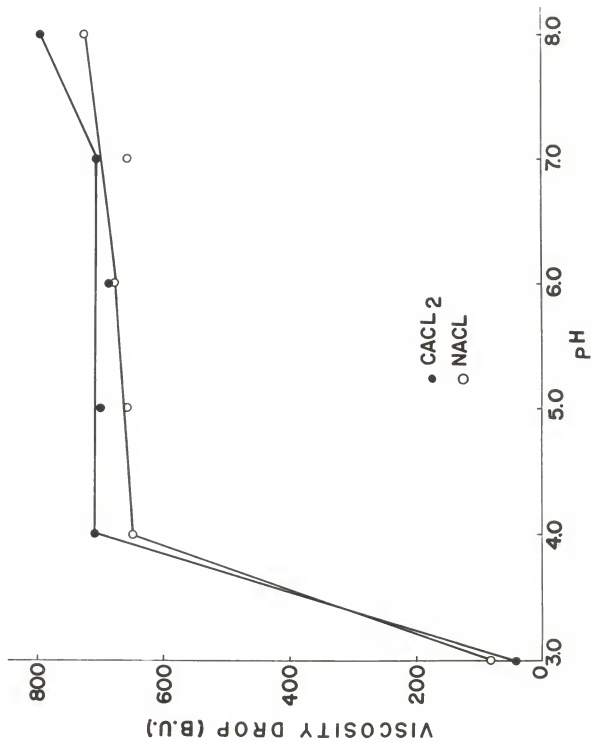


Fig. 6. Effect of pH on stability of Taka-amylase.

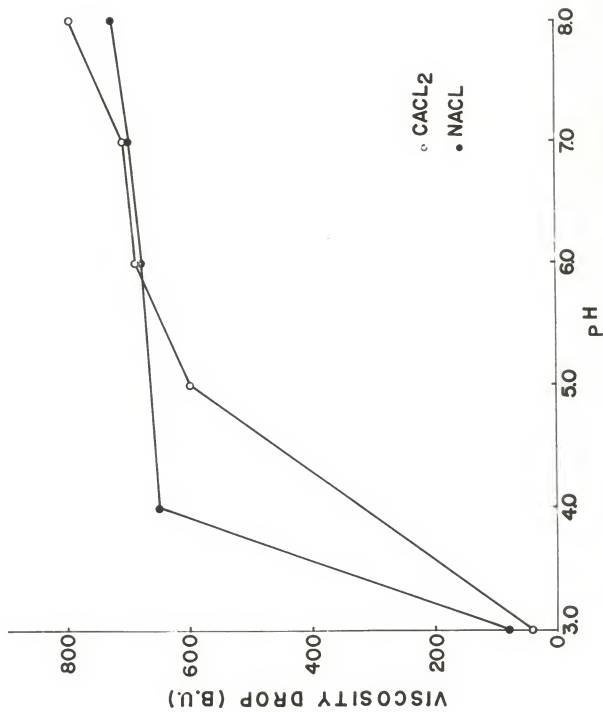


Fig. 7. Effect of pH on stability of pancreatic alpha-amylase.

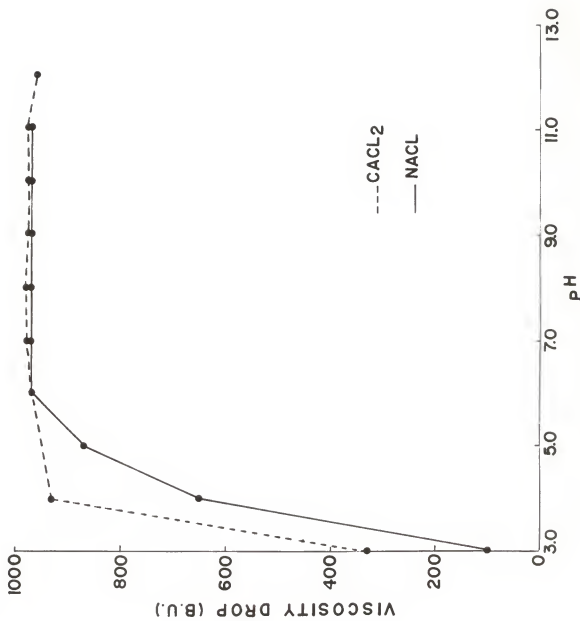


Fig. 8. Effect of pH on stability of bacterial alpha-amylase.

Table 4. Effect of temperature on amylase activity.

Temp. : (°C)	:1/T x 10 ⁴	:Taka- :amylase (100 mcg.)	Log V			
			:Pancreatic alpha-amylase (40 mcg.) :in presence :of NaCl	:Bacterial alpha-amylase (40 mcg.) :in presence :of CaCl ₂	:Pancreatic alpha-amylase (40 mcg.) :in presence :of NaCl	:Bacterial alpha-amylase (40 mcg.) :in presence :of CaCl ₂
25	33.5	1.954	2.556	2.556	2.462	2.447
30	33.0	2.114	2.613	2.699	2.544	2.532
35	32.4	2.146	2.699	2.716	2.562	2.586
40	31.9	2.204	2.935	2.748	2.695	2.686
45	31.4	2.260	2.929	2.763	2.724	2.708
50	30.9	2.301	2.929	2.748	2.744	2.712
55	30.4	1.605	2.623	2.690	2.763	2.720
60	30.0	--	--	--	2.690	2.724

before a substantial portion of the starch becomes gelatinized and susceptible to enzymatic action; the bacterial alpha-amylase, in contrast, has a high degree of thermostability.

Caldwell and Adams (1946) showed that in crude aqueous extracts malt alpha-amylase was usually more thermostable than malt-beta-amylase; upon purification, however, this difference between the two amylases became less marked.

The results in Table 4 show that the difference in thermostability for crystalline preparations are much smaller than for enzymes from commercial sources.

Effect of Enzyme Concentration. The mode of action of an amylase is essentially that of a hydrolytic enzyme. Two desirable considerations for the

relationship between the quantity of enzyme used and the changes resulting from enzymatic action are: 1) a linear relationship between the quantity of enzyme and the measured product, and 2) a linear time course. These considerations may be achieved when the enzyme is saturated by the substrate used, or when measurements are made at points where only small portion of the substrate has been converted.

In selecting the optimum substrate concentration, the amount of pregelatinized starch was chosen to the maximum reading (1000 B.U.), prior to the beginning of enzymatic action.

No linear relationship between the quantity of the enzyme and decrease in viscosity could be observed. It seems, therefore, that to satisfy the requirements regarding substrate concentration, the results might follow a linear course over the low range of enzyme concentration. This has been previously shown to be true for commercial preparations, and also for the three crystalline alpha-amylases studied (Fig. 9). It should be noted, however, that in employing very low levels of alpha-amylase the results may be affected by denaturation.

Effect of Crystalline Bacterial Alpha-Amylase on Dough Consistency.

The results summarized in Table 5 showed that adding high levels of enzyme (above 0.4 mcg. to 50 g. of flour) resulted in no additional consistency drop. Treatment of the crystalline preparation so as to inactivate its amylolytic activity eliminated the consistency drop, due to absence of action on the available starch. Treatment under conditions which would tend to inactivate any proteolytic impurity present in the crystalline bacterial amylase gave results equal to those in the untreated amylase. These results seem to confirm the findings of Johnson and Miller (1949),

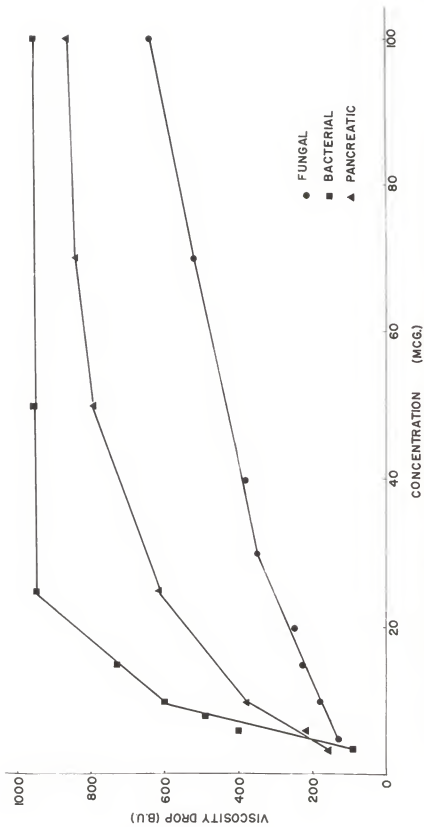


Fig. 9. Relation between concentration of Taka-amylase, pancreatic amylase and bacterial amylase and starch liquefaction.

Table 5. Effect of crystalline bacterial alpha-amylase on dough consistency.

Enzyme level (mg/50g. flour)	Treatment	Consistency (B.U.) after		
		0 hours	2 hours	4 hours
0	none	370	380	460
0.4	none	300	300	360
0.8	none	310	300	340
1.6	none	305	300	350
0.8	Kept at pH 3.0 overnight ^a	320	340	430
0.8	Incubated at pH 11.5 at 50°C for 1 hour ^a	300	310	360
0.8	Held in 0.1M NaCl overnight ^a	310	310	350

^aSamples were readjusted to pH 6.0 after incubation with various reagents.

who indicated that alpha-amylase was responsible for the sharp decrease in dough consistency up to a point at which further increase in the concentration of alpha-amylase appeared to produce no further change in dough mobility. This suggested that the quantity of starch substrate which is susceptible to attack is limited.

The doughs to which only proteinase was added also decreased in consistency but not in the same relationship to concentration as the doughs containing alpha-amylase. Dough mobility continued to increase as the concentration of proteinase was increased. It would appear that the protein substrate was not limiting for the proteinase as was the substrate for alpha-amylase. The presence of both enzyme components was more effective than either component alone in decreasing the dough consistency.

Inactivation by Quarternary Ammonium Compounds. The effect of different

amounts of cetyl pyridinium chloride at three different levels of crystalline bacterial alpha-amylase is shown in Table 6.

Table 6. Effect of different amounts of cetyl pyridinium chloride on activity of bacterial alpha-amylase.

Cetyl pyridinium chloride (mg.)	: Viscosity drop (B.U.)		
	: Enzyme level		
	: 20	: 40	: 80
	(mcg.)		
0	600	870	960
2	550	840	940
5	540	740	940
10	400	640	940
20	340	550	910
40	250	490	840

The results summarized in Table 7 show that when a constant amount of cetyl pyridinium chloride was used (40 mg. per 20 ml. of solution) and the amount of enzyme was varied between 5-100 mcg. of amylase, there was no substantial difference in the extent of bacterial amylase inactivation.

The extent of inactivation was computed from a calibration graph correlating the effect of enzyme concentration on viscosity drop.

The extent of inactivation was essentially the same whether cetyl ammonium bromide, cetyl pyridinium chloride or cetyl trimethyl ammonium bromide was used, (Table 8).

Table 7. Effect of cetyl pyridinium chloride on crystalline bacterial alpha-amylase.

Enzyme (mcg.)	Inactivation (%)
5	87.0
10	86.0
15	83.3
20	85.0
25	84.0
40	86.3
50	76.0
100	83.0

Table 8. Effect of various quaternary ammonium salts (40 mg. per 20 ml.) on crystalline bacterial alpha-amylase.

Enzyme (mcg.)	Viscosity drop (B.U.)			
	: Control	: With Cetyl Ammonium Bromide	: With Cetyl Pyridinium Chloride	: With Cetyl Trimethyl Ammonium Bromide
25	860	640	740	760
50	960	925	940	960
100	975	975	970	970

Whereas bacterial amylase was partly inactivated by the action of quarternary ammonium compounds, the inactivation of pancreatic and fungal amylases by any of the tested quarternary ammonium compounds, was practically complete. Cetyl pyridinium salts were equally, or more effective than cetyl trimethyl ammonium salts (Pomeranz, 1962). The enzyme of bacterial origin was much more resistant than the fungal amylase to inactivation by cetyl quarternary ammonium compounds. By use of various amounts of bacterial amylase and various levels of cetyl pyridinium bromide, it was shown that a fairly constant amount (about 40 per cent) of the enzyme remained active, irrespective of wide variations in the ratio of enzyme and quarternary ammonium compounds. The complete inactivation of fungal amylase might be, therefore, due to an irreversible reaction of the abundant anionic groups with the cationic quarternary ammonium compounds. The reaction was dependent on pH, and incubation of the fungal enzyme with cetyl trimethyl ammonium bromide at pH 9 at 5°C. resulted in a very low degree of inactivation (5-10 per cent).

The results of testing commercial and partly purified amylases confirm those obtained with crystalline enzymes. Whereas the enzymes of fungal origin were completely inactivated, a substantial residual activity was recorded in the bacterial amylases, as shown in Table 9.

These results must, however, be interpreted with caution as the nature of impurities and their affinity for the quarternary ammonium compounds is unknown. That such impurities may exert a pronounced effect is shown by the fact that whereas the two partially purified cereal amylases were completely inactivated by the detergent, crude preparations of low activity were only partly inactivated.

Table 9. Effect of 40 mg. cetyl pyridinium chloride in 15 ml. solution containing 10 SKB units of amylase on enzyme activity.

Enzyme source	Viscosity drop (B.U.)	
	Control	With Cetyl Pyridinium Chloride
Fungal	430	0
Fungal	330	0
Fungal	300	0
Fungal	270	0
Bacterial	970	640
Bacterial	960	780
Bacterial	960	640
Wheat malt ^a	530	0
Sorghum malt ^a	420	0
Barley malt	740	470
Barley malt	890	610
Wheat malt	880	390

^aPartly purified.

Effect of Crystalline Bacterial Alpha-Amylase on Breadmaking. Crystalline bacterial and pancreatic alpha-amylases were used at five different levels. The results are summarized in Table 10. Description of the samples has been given under Materials and Methods. The volume, crust color, symmetry, break and shred were improved by the addition of bacterial alpha-amylase. Bacterial alpha-amylase, however, caused the bread crumb to become sticky and gummy. Pancreatic amylase had a light, overall improving effect.

Table 10. Effect of bacterial and pancreatic alpha-amylase supplements on baking characteristics of a commercial, unmalted hard red winter flour.

Sample No.	Loaf : volume	Crust : color	Symmetry	Break : Shred	Texture	Grain	Crumb Color
	(cc)						
1	650	4	5	5	15	16	10
2	800	9	8	7	17	17	10
3	670	4	6	5	15	16	10
4	695	5	6	5	16	16	10
5	700	5	6	5	15	16	10
6	710	4	7	5	16	16	10
7	675	5	8	5	16	16	10
8	760	9	8	7	12	12	8
9	825	8	8	8	12	10	7
10	820	8	8	8	12	10	7
11	875	9	10	9	12	9	6
12	880	9	10	9	12	8	5

Conn *et al.* (1950) suggested that the difficulties involved in using bacterial amylases in flour supplementation may be due not only to their thermostability but also to their lesser affinity for low molecular weight dextrans. Accordingly, bacterial alpha-amylase molecules may be free to split greater numbers of starch molecules, with a corresponding increase in dextrin formation and stickiness of bread crumb. Fungal amylase produced the smallest amounts of fermentable sugars; malted wheat flour preparations were slightly lower than bacterial supplements (Miller *et al.* 1953).

SUMMARY

Two aspects of alpha-amylase chemistry were studied. One phase dealt with the activation of alpha-amylase in wheat endosperm by adding exogenous gibberellic acid, and measuring the starch liquefying properties of calcium chloride extracts of the endosperm. Activation has been confirmed by experiments using fungi-free wheat and wheats from four different classes. The absence of measurable amounts of alpha-amylase in endosperms of any of the tested wheats, and the enzymatic activities of samples incubated with gibberellic acid at levels ranging between 0.1 to 100 mcg. per ml. of solution, point to the possibility of using the method for assaying gibberellic acid. The activation of amylases in endosperm by the exogenous gibberellic acid decreased sharply beyond 30°C., but only slowly in seeds germinated at higher temperatures. The alpha-amylase activities of either whole seeds or of endosperm halves incubated with gibberellic acid at the optimal level of 10 mcg. per ml. were substantially higher than those of similar preparations incubated without gibberellic acid. Endosperm from whole seeds incubated under similar conditions showed amylase activities slightly higher than those of cut endosperms incubated with gibberellic acid. Longitudinal sections of whole seeds had amylase activities slightly lower than whole seeds, and in endosperm-homogenates there was a slight activity in the presence of gibberellic acid.

The second phase of this report dealt with the starch liquefying properties of crystalline alpha-amylase. The pH optima for liquefying action of crystalline amylases were 4.5, 6.8 and 6.0 for the enzymes from fungal, pancreatic and bacterial sources, respectively. The enzymes exhibited a

stability over the range of 4-8, 5-8 and 5-12 respectively. The temperatures at optimum pH at which inactivation started were 50°C. for Taka-amylase, 45°C. for the enzyme of pancreatic origin, and above 60°C. for the enzyme of bacterial origin. An essentially linear relation was obtained between low levels of enzyme activity and viscosity decrease.

Decrease in dough consistency due to the action of bacterial amylase remained constant, even at levels of supplementation corresponding to a four fold increase above that employed in commercial practice. Doughs supplemented with crystalline bacterial alpha-amylase gave breads of increased volume but with sticky and gummy crumbs. Supplementation with pancreatic amylase had an overall slight improving effect.

Both purified and commercial preparations of fungal and pancreatic amylases were completely inactivated by incubation with a number of quaternary ammonium compounds; the inactivation in case of the enzyme of bacterial origin was partial, only. With the cereal amylases, the extent of inactivation varied with the concentration of the enzyme, the more purified enzymes being inactivated to a larger extent than the low-activity amylases.

SUGGESTIONS FOR FUTURE RESEARCH

The primary purpose of this work was to determine the starch liquefying properties of crystalline amylases from various sources, and the relation of these properties to the bread baking process. Additionally, the formation of alpha-amylases in embryo-free wheats, as induced by exogenous gibberellin has been studied. The results obtained have suggested additional areas for future investigations.

The pattern of action of different crystalline amylases should be

followed by study of the products of enzymatic hydrolysis of the starches. Such information could be obtained by available fractionation methods or by use of molecular sieves. The results would be valuable in evaluating the availability of hydrolytic products to panary fermentation.

The role of gibberellin in induction of amylase formation should be further studied with supplementation with anti-gibberellins.

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SOME ASPECTS OF ALPHA-AMYLASE CHEMISTRY

by

MARTA SUSANA MORO

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The present study has been undertaken to test the effect of exogenous gibberellic acid on formation of alpha-amylase in separated wheat endosperm, as compared with levels of the enzyme formed in intact seeds. The amylolytic activity was followed by measuring liquefaction resulting from the action of alpha-amylase. Changes in viscosity were measured in the amylograph with pregelatinized starch used as substrate. Amylase activation has been confirmed by carrying out experiments with fungi-free wheat and with wheats from four different classes. Concentrations between 0.1 and 100 mcg. of gibberellic acid per ml. of solution increased alpha-amylase activity in the calcium chloride extracts of endosperm halves or seeds. Extracts from wheat seeds or from wheat endosperm-halves heated for 1 hour at 100°C. in either the absence or presence of gibberellic acid, resulted in no measurable drop in starch viscosity. Activation of endosperms due to exogenous gibberellic acid decreased sharply beyond 30°C., whereas the formation of amylase in the whole seed without gibberellic acid showed a gradual decrease.

The alpha-amylase activities of either whole seeds or endosperm halves incubated with gibberellic acid at the optimal level of 10 mcg. per ml. were substantially higher than those incubated without gibberellic acid. On the other hand, whole seeds incubated with gibberellic acid showed amylase activities slightly higher than those of cut endosperms incubated with gibberellic acid. Longitudinal sections of whole seeds had amylase activities slightly lower than whole seeds and in endosperm-homogenates there was a slight activity in the presence of gibberellic acid.

The second part of this thesis deals with the study of some properties of purified amylases. The pH optima for liquefying action of crystalline amylases were 4.5, 6.8 and 6.0 for the enzymes from fungal, pancreatic and

bacterial sources, respectively. The enzymes exhibited a stability over the range of 4-8, 5-8 and 5-12 respectively. The pH temperatures at which inactivation started were 50°C. for Taka-amylase, 45°C. for the enzyme of pancreatic origin and above 60°C. for the enzyme of bacterial origin. An essentially linear relation was obtained between low levels of enzyme activity and viscosity decrease.

Dough consistency decrease due to the action of bacterial amylase remained constant, even at levels of supplementation corresponding to a four fold increase above that employed in commercial bread production. Supplementation with bacterial amylase substantially increased the loaf volume but affected adversely bread crumb and texture. Pancreatic amylase had a slight overall improving effect.

Both purified and commercial preparations of fungal and pancreatic amylases were completely inactivated by incubation with a number of quaternary ammonium compounds; the inactivation in case of the enzyme of bacterial origin was partial, only. With the cereal amylases, the extent of inactivation varied with the concentration of the enzyme, the more purified enzymes being inactivated to a larger extent than the low-activity amylases.