CYTOLYTIC ENZYMES IN GERMINATING BARLEY

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ABSTRACT

The malting quality of a new barley variety is judged by comparing important barley and malt properties with those of the variety O. A. C. 21, the standard of malting quality in Canada. As many important quality factors are as yet unknown, or cannot as yet be determined, intensive basic research on factors contributing to malting quality is required before satisfactory methods of assessing, and perhaps controlling, malting quality can be developed. The enzymatic dissolution of endosperm cell walls (cytolysis) appears to be an important quality characteristic that is not adequately measured by present procedures for determining malting quality. This thesis presents results of studies of cytolytic enzymes in barley and green malt, together with preliminary studies made with a bacterial enzyme preparation.

The chief difficulty in investigating cytolytic enzymes in the past has been the lack of adequate substrates. In the present studies, barley gums, which are believed to be derived from cell wall materials, are used as substrates for cytolytic enzymes.

A commercial bacterial alpha-amylase preparation served as the first source of cytolytic enzymes. A viscosimetric method of determining gum-degrading activity was developed. The enzyme, or enzyme system, involved in viscosity reduction, tentatively named X-enzyme, was separated from alpha-amylase in the crude bacterial preparation by column chromatography. Heating at 70°C. for

15 minutes in aqueous solution destroyed virtually all viscosity-reducing activity, but aqueous solutions of X-enzyme were remarkably stable in both crude and purified forms for long periods of time at room temperature or at 7°C.

Enzyme isolates were prepared from barley and green malt extracts by ammonium sulphate precipitation and by freeze-drying. The X-enzyme system contains no dialyzable component. X-activity appears to be inhibited by ammonium sulphate, but removal of ammonium sulphate by dialysis restores lost activity. Barley or green malt X-enzyme is heat labile, but green malt X-enzyme is stable on storage in the dry state at room temperature. Optimum X-activity occurs at pH 4.7 to pH 4.8. X-activity of green malt is about 90 times the activity of resting barley.

Oligopentosan, oligoglucosides, and glucose were identified, by paper chromatography, as reaction products of non-amylase gum-degrading enzymes of green malt. Oligosaccharides are thought to be reaction products of a non-specific endo-beta-poly-glycosidase, which is presumably the viscosity-reducing enzyme, X-enzyme. The presence of glucose among enzymic reaction products is attributed to the action of an exo-beta-glycosidase enzyme system, one portion of which operates by liberating cellobiose units from the ends of polyglucoside or oligoglucoside chains; cellobiose is then degraded by cellobiase to glucose. Evidence for the possible existence of an exo-beta-glycosidase system in green malt is presented.

The viscosity-reducing activities of green malts of nine barley varieties with widely different malting quality were determined. Large variations in X-activity were observed, suggesting that X-activity is an important factor in determining over-all malting quality.

A hypothesis of cytolysis in germinating barley is suggested.

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INTRODUCTION

One of the main difficulties in the development of new barley varieties, with improved agronomic characteristics and with desirable malting quality, is the lack of adequate tests by which malting quality may be defined. Analytical determinations of important properties of barley are made on small samples of barley. These determinations are used as prediction tests to eliminate obviously unsuitable material in early generations of the breeding program. Barley varieties considered promising, as judged by prediction tests, are then grown in larger quantities. When sufficient grain is available, samples of barley are malted, and malt extracts (worts) are prepared on a laboratory scale. Malt and wort properties are then determined, and malting quality is assessed with reference to malt and wort properties of O.A.C. 21, the standard of malting quality in Canada. Malting tests are time-consuming and expensive, and much time is wasted in testing varieties with unsatisfactory malt properties that are not predicted by results of barley analysis. Moreover, prediction tests and standard malt analyses do not always reveal faults that may develop in commercial malting and brewing practices. A recent example of this is the variety U. M. 1020, which appeared promising during three years of field and laboratory testing, but was rejected as a result of commercial malting and brewing trials.

Therefore there is a great need for more intensive studies of the factors contributing to malting quality, so that important quality characteristics may be recognized, methods for measuring and assessing these properties devised, and these methods applied to small samples of barley to aid in the early elimination of barley varieties of unsatisfactory malting quality. The present research is aimed at investigating what appears to be a significant quality factor in malting barley, the enzymatic dissolution of endosperm cell walls. The possible importance of this process, known as cytolysis, in malting arises from a consideration of the biochemical and structural changes occurring in the barley kernel during malting.

Malting is the process of germinating barley to provide a maximum yield of water soluble substances of satisfactory brewing quality. Barley is first steeped in cold water to about 44 per cent moisture content, then germinated. Temperature and aeration during germination are controlled by a current of cool moist air. Further growth and the accompanying consumption of food stored in the kernel are arrested by kilning. During steeping, barley undergoes chiefly physical changes due to water absorption. Respiration rate increases with moisture intake, but is limited, as is the growth of the embryo, by the limited supply of oxygen in the steep water. The embryo and endosperm, as well as the protective tissues, increase in size due to the physical intake of water into the cell walls, intercellular spaces, and cell contents. Barley dried after steeping is more mellow and friable in texture than before steeping. During the germination stage, rootlets and acrospire (or plumule) of the growing plant

are developed, and enzyme activity increases. It is generally assumed that enzymatic development is optimal when the acrospire has just reached the distal end of the kernel. The biochemical changes accompanying germination are largely enzymatic. Protein degradation and dissolution of endosperm cell walls are the chief processes, and the result is a general softening up or "modification" of the barley kernel. The purpose of malting is therefore modification of the barley grain by development of enzymes to the extent necessary for a maximum yield of water soluble substances of satisfactory brewing quality.

It is surprising that although extensive proteolysis occurs during germination there is no appreciable starch degradation. This cannot be attributed to a shortage of amylases in germinating barley, as ungerminated barley shows a relatively high level of free beta-amylase which, together with alpha-amylase, increases rapidly during germination. The reason for the resistance of starch to amylolysis must lie in the state of starch during germination. As starch is contained in cells in the endosperm, whereas the main enzyme-secreting sources are located in the scutellar epithelium and in the aleurone layer (11), it has long been suspected that the cell walls must be ruptured before the starch can become susceptible to enzymatic attack. The rupture of cell walls has been attributed to the action of cell wall degrading, or cytolytic, enzymes. This view is strengthened by the work of Dickson and Shands (11) whose photomicrographs of sections of germinating barley showed that dissolution of cell walls and starch-imbedding matrix precedes any

marked structural changes in the starch granules. Moreover, they showed that dissolution of cell walls progresses from the known enzyme-secreting source in the scutellum, and that disappearance of the cell walls is followed closely by dissolution of the matrix surrounding the starch granules. Finally, they noted few structural changes in the starch granules during germination except in regions immediately adjacent to enzyme-secreting tissues. These observations of Dickson and Shands suggest that a key biochemical process in the modification of the barley kernel during malting may be the enzymatic destruction of cell walls, which is generally known as cytolysis (37).

The preferred substrate for studies of cytolytic enzymes is cell wall material. Unfortunately, cell walls of barley endosperm have not yet been isolated. However, as is discussed later, there is reason to believe that the water-soluble barley gums are of cell wall origin (37). Thus, barley gums appear to be satisfactory substrates for cytolytic enzymes.

Although there is good reason to believe that some measure of the potential cytolytic activity of barley may be an important quality characteristic, there is little reason to believe that this measure alone will provide a complete index of malting quality. Malting quality is the sum of a variety of chemical and other processes, all of which must function at maximum efficiency at the appropriate time. Only through a thorough knowledge of the many factors contributing to quality will it be possible to assess accurately, and perhaps control, malting quality.

This thesis presents results of studies of cytolytic enzymes in resting and germinating barley. The first section reviews the literature on cytolytic enzymes up to the time this investigation was begun. The next three sections describe materials, equipment, and general methods used in the research. The fifth section summarizes preliminary studies that led to the search for a viscosimetric method of measuring cytolytic activity. The sixth section reviews studies of a bacterial alpha-amylase preparation. Included in this section are descriptions of a viscosimetric method for measuring cytolytic activity and of a method for separating cytolytic enzymes from alpha-amylase in the bacterial preparation. In experiments described in the seventh section, barley and green malt enzymes were isolated, certain enzymes that degrade barley gums were identified, and the existence of other enzymes was postulated. A short investigation of possible varietal differences in cytolytic activity was then undertaken. Finally, the results of this investigation are reviewed and compared with results of contemporary investigators; conclusions about the nature of cytolysis are drawn; and the possible value of the viscosimetric assay as an aid in evaluating malting quality is discussed.

REVIEW OF CYTOLYTIC ENZYMES

Although there are frequent references in the literature to the possible role of cytolytic enzymes in the malting process, there has been very little original work published on this subject. Interest in these enzymes was first aroused by Baker and Hulton (3) in 1917, and a significant contribution was made by Lüers and associates in 1928 (23, 24). However, little came of these investigations, probably due to lack of satisfactory substrates. Interest in the cytolytic enzymes was reawakened in 1948 when Preece, who had been investigating hemicelluloses of barley and malt for many years, published a review of cytolysis in germinating barley (37). About the same time Meredith isolated a viscous principle from malt extract (27, p.71) and later a similar material from barley (28). These materials were called barley and malt gums because of their similarity to plant gums, and to barley gums first isolated by O'Sullivan (32) and Brown (5). According to Brown, the solubilization of the highly colloidal gum of barley is one of the most significant changes which mark its conversion into malt. The literature thus suggested that barley gums might provide the means for studying cytolytic enzymes.

Studies of cytolytic enzymes were initiated by the author at the Grain Research Laboratory in 1949. In 1950, Preece, Ashworth, and Hunter, of the Heriot-Watt College, Edinburgh, Scotland, presented their preliminary results on barley and malt gums and cytolytic enzymes (38, 39).

Since then a number of papers on the gums and the enzymes that degrade them have been published by both laboratories, and recently similar investigations have been reported from the Central Laboratory, Stockholm Brewery, Stockholm, Sweden, by E. Sandegren and his associates (42, 13). These three groups have been the most active investigators of cytolytic enzymes, although related studies have been carried on in France under the direction of H. LeCorvaisier (41). This review of cytolytic enzymes will cover the period up to 1949, and contemporary investigations will be referred to in appropriate sections of the thesis and in the general discussion.

The first investigations of cytolytic enzymes arose from studies of pentosan modification during malting, and therefore involved pentosan degrading enzymes, pentosanases. Baker and Hulton (3), investigating the changes in furfurogenic substances during germination, observed that barley embryos, excised and grown in the dark on sand moistened with cane sugar solution, gave an increase in furfuraldehyde yield, accompanied by an increase in the weight of the embryo itself. Embryos grown normally with their own endosperms also gave an increase in furfuraldehyde, with a corresponding decrease in endosperm weight. The authors suggested that translocation of pentosans occurred due to the action of an enzyme that attacks insoluble pentosans, rendering these soluble and diffusible, and producing free pentose sugars. Later results appeared to confirm this conclusion. When ground barley and ground malt were separately digested with water, less furfuraldehyde-yielding material was extracted than when both were digested together. Also, when a suspension of the alcohol-precipitated enzyme was allowed

to act on malt husk, insoluble pentosan material was rendered soluble. Similar results were reported by Van Laer and Masschelein (43), in 1923. They found a 4 per cent loss during steeping of total pentosans occurring almost entirely in the husk. During germination they observed a general increase in pentosans, chiefly noticeable in the embryo, particularly in the rootlets, and to a lesser extent in the endosperm. Kiln drying also produced a general increase in pentosans, occurring chiefly in the endosperm and only to a very small extent in the rootlets, which are the first parts of the grain to dry up during kilning.

In 1928, Lüers and Volkamer (23), convinced that pentosan modification by pentosanase provided a key to barley modification, presented results of their fundamental investigations of a xylanase prepared from long-grown green malts. In their investigation, they assumed that hemicelluloses are cell wall material, and they therefore referred to their enzyme as "cytase". Their experimental work was very meticulous and merits detailed review, particularly as many of their methods and ideas were valuable guides to the present author in his studies.

Lüers and Volkamer used as substrates xylans prepared laboriously from elder pith and barley. Analysis showed that the pentosan content of these materials was about 77 per cent on a dry basis. Acid hydrolysis of the xylan yielded xylose which was identified by specific rotation of the purified hydrolysate, by appearance and by melting point of the osazone, and by a positive furfural reaction of the osazone.

Activity determinations were made by measuring the reducing power of an aliquot of a reaction mixture digested for 48 hours. Results were corrected for enzyme blanks, and net reducing power was converted to xylose content by referring to standard curves of reducing power vs xylose concentration. The potential xylose content of a similar quantity of xylan solution was determined by acid hydrolysis, and activity was reported as per cent degradation of xylan. At 45°C., the optimum pH was reported as 5.0, and the enzyme was completely inactivated when enzyme solutions were heated at 60°C. for 15 minutes. Some activity was reported when reaction mixtures were incubated at 60°C., and this was attributed to protection of enzyme by substrate.

Lüers and Volkamer next observed that activity could not be increased appreciably by increasing reaction time. Moreover, activity did not increase linearly with enzyme concentration. They reasoned that an equilibrium between reactants and products may exist, and that the reaction may therefore be retarded by products. This was confirmed by adding xylose to reaction mixtures and noting the resultant decrease in activity. The suggestion that an equilibrium exists between reactants and products (xylan and xylose) is probably not valid. Many enzymes are strongly inhibited by their products not through reversal of the reaction but by apparent competition with the substrate for the enzyme.

Identification of xylose as a product of enzymatic action was effected by fermenting 7-day reaction mixtures with brewer's yeast to remove fermentable sugars. Yeast and proteins were removed, and the reaction mixture was clarified by repeated treatment with

animal charcoal and by filtration. Xylose was identified in the clear filtrate as before. Xylose was similarly identified in kiln-dried malt.

Lüers and Volkamer next isolated their cytase by adsorption on and elution from alumina in water suspension. Enzymes were precipitated from a filtered malt extract with excess alcohol. The precipitate was dried with alcohol and absolute ether, then finely ground. Optimum conditions (concentration, temperature, pH, time) for adsorption and elution were determined. Adsorption proceeded most favorably in weakly acidic solution (pH 5.0), and the elution was most effective with phosphate buffer of pH 8.3. The investigators were able to obtain quantitative recoveries of cytase by a single adsorption and elution process, but higher purity and reduced yield were obtained by a double adsorption and elution process. The latter procedure increased specific diastatic activity six-fold, but increased specific cytase activity by a factor of 21. Lüers and Volkamer therefore concluded, as they expected, that amylase and cytase were two different enzymes.

In a later paper, Lüers and Malsch (24) reiterated the belief that cytase is responsible for the essential part of the solution of endosperm cell walls, and also admitted that they may have been investigating only a part of the complete cytase system, a xylanase. In this paper the authors obtained a direct proportionality between activity and enzyme concentration by reducing enzyme concentration and reaction time. They were thus able to determine the changes in xylanase activity during malting. They found that activity decreased slightly during steeping and early germination, but then increased markedly throughout the germination period. Kiln-drying then reduced xylanase activity to one-half the activity of resting barley.

The work of Lüers and associates would appear to open the way for studies of other components of the cytase system and their role in malting. Apparently, however, the lack of good substrates discouraged further investigation. Although several methods of preparing hemicelluloses had been developed (30), these materials were very complex and difficultly soluble, so that no serious attempt was made to use them as substrates for cytolytic enzymes. Instead, attention was again directed towards investigating changes in pentosan content during malting.

Fink and Hartmann (15) re-examined the pentosan yields (determined as furfuraldehyde) for malting barley, and concluded that the change from the hard barley kernel to the friable kernel of malt is probably due largely to changes in endosperm cell walls, and is accompanied by an increase in the soluble pentosans of the corn. This suggested that it might be possible to develop a method for assessing the degree of modification of a malt from the results of furfuraldehyde determinations. The merit of such a determination would be that its results should theoretically be closely related to the mechanical modification of the grain as envisaged by the practical maltster. Later, Fink (16) found that total pentosans increased slowly during germination, but soluble pentosans increased almost four-fold. However, after about half the germination period, the further production of soluble pentosans was balanced by synthetic processes in the developing embryo. Hence the determination of soluble pentosans as a measure of modification does not offer much promise, since the measured production stops before modification

is complete. Further objections were raised by Enders, Saji, and Schneebauer (14) and by Bishop and Marx (4), who found that both total and soluble pentosans (expressed as furfuraldehyde) appear to be varietal characteristics. Finally, Preece (36) warned that an increased furfuraldehyde yield at any point does not necessarily imply that there has been an increase in the actual amount of furfurogenic material; there may have been merely a change in its nature, such as decarboxylation of a uronic acid or anhydride.

Attempts to relate pentosan modification with barley modification were therefore not entirely successful, but studies of pentosan modification indicated that pentosanases, although an important sub-group of cytolytic enzymes, apparently do not reflect the complete cytolytic process. As cellulose is a major constituent of the cell wall it might be expected that cellulase plays at least an equally important part in cytolysis. Until recently very little attention was devoted to cellulase of barley and malt, but the importance of this and other cytolytic enzymes in the malting process is clearly demonstrated by a routine determination that is still regarded by some as the most reliable single test of malting quality. This test is the difference in extract yield between finely and coarsely ground malt.

The filtration of the wort (malt extract) in the mash tun requires a rather coarsely ground malt, and if the cell walls of the endosperm have not been adequately attacked the starch is not completely converted and the extract yield is low. With fine grinding this loss of extract does not arise, but clogging of the tun occurs. Moreover, a well modified malt may give a high extract yield in spite of coarser

grinding. Hence the difference between extracts yielded by finely and coarsely ground malt is an indication of the degree of modification. Degree of modification therefore appears to be related to the potential cytolytic activity of barley.

Another routine test that indicates the need for further research on cytolytic enzymes is the measurement of wort viscosity. Although information is limited concerning the effect of the individual constituents of wort on changes in viscosity, it is known that the viscosity of wort decreases as modification of the malt increases (35). Maltose contributes to wort viscosity, but the viscosity of wort is considerably higher than that of maltose solutions of the same density (27, p. 73). Piratzky and Wiecha (35) showed that neither protein nor starch at various stages in degradation appear to affect viscosity greatly, but that viscosity changes appear to be due to an enzyme system which hydrolyzes the viscous principle.

When Piratzky and Wiecha carried out viscosity measurements on worts from malts of four successive seasons (1933-1936), they found that maximum and average values for each year showed wide variations, but the minimum values agreed closely, suggesting that these values represented complete decomposition of the viscous principle. Piratzky and Wiecha stated that the minimum value probably indicates a condition in which all the convertible hemicelluloses in the worts have been converted. They further showed that incomplete malting, that is partial modification, produced highly viscous worts. From these worts they were able to isolate a polysaccharide

whose aqueous solution was very viscous. This material was degraded by enzymes of green malt which were extremely heat sensitive and had optimum activity between pH 5.0 and pH 5.4. These enzymes could not be detected in the finished malt, and were apparently inactivated by kilning.

The polysaccharide of Piratzky and Wiecha appeared to be the most suitable substrate available at that time for studying cytolytic enzymes. However, it had two serious disadvantages. First, it was susceptible to amylolytic action, thus it probably contained starch or degradation products of starch. Second, a polysaccharide from wort is probably structurally far removed from naturally-occurring barley polysaccharides due to the degradative action of enzymes developed during malting. Hence this polysaccharide of Piratzky and Wiecha aroused little interest.

In 1948, Preece (37) re-awakened interest in cytolysis with a review of cytolysis in germinating barley and its significance in the malting process. The best available evidence indicated that the plant cell wall is a complex association of cellulose, hemicellulose, and lignin, and that breaking of the cell wall probably involves enzymatic degradation of the cellulose and hemicellulose components. Thus cytolysis would appear to depend on the action of cellulase and hemicellulases. Preece describes hemicelluloses as cell wall materials of broadly carbohydrate nature, insoluble in water in their natural condition, not readily hydrolyzed by dilute acids, but comparatively readily soluble in dilute sodium hydroxide and easily hydrolyzed by boiling with dilute mineral acids. According to Preece, the essential difference between plant gums and hemicelluloses is that gums are soluble

in water. The fact that the gums agree in general characters with hemicellulose fractions suggests that hemicellulose is the precursor, although water solubility does not necessarily imply that gum molecules are smaller or less complex than the parent hemicellulose.

Clayson (9) states that hemicelluloses are made up of polymers of various sugars and uronic acids which may be linked in a variety of ways. Some hemicelluloses are short-chained hexosans and pentosans associated and oriented with cellulose, whereas others exist as amorphous encrusting substances associated with lignin. There is also considerable diversity of structure. For instance, the galactan associated with pectin in Lupinus alburs seeds is considered to consist of chains of beta-galactose units, linked in the 1:4-positions, whereas another galactan, the epsilon-galacto-araban of larchwood, has a branched chain structure with linkages in the 1:6-position and branches at the 3-position, a type of structure characteristic of plant gums and mucilages. The isolation and characterization of hemicelluloses is a relatively virgin field, and it is therefore premature to decide whether the enzymes that attack hemicelluloses are specific to certain types of linkage, or whether the specificity is of higher order. Further uncertainty arises from the fact that many hemicelluloses are not simply polymers containing just one repeating unit.

It is perhaps unfortunate from the standpoint of research on cytolytic enzymes, that Preece and others concentrated their efforts for a considerable time on studying the water-insoluble hemicelluloses, and completely neglected the water-soluble gums, such as Piratzky and Wiecha's polysaccharide. It was not until 1949 that Meredith (27, p. 71)

described the preparation of a wort gum and later a similarly prepared barley gum. The gums contained glucose, arabinose, and xylose residues, and about 3 per cent nitrogen. The gums were degraded by a bacterial alpha-amylase preparation which reduced viscosity and increased reducing power of aqueous gum solutions. Inactivation of alpha-amylase failed to alter viscosity-reducing activity, but heating at 70°C. for 15 minutes completely inactivated the viscosity-reducing enzyme without appreciably affecting alpha-amylase activity. It was therefore concluded that the bacterial preparation contained a cytolytic enzyme, and that barley gum was a promising substrate for studying cytolytic activity. Particularly encouraging was the fact that the gums apparently contain a variety of polysaccharides, both hexosan and pentosan. Thus barley gums may be sensitive to various cytolytic enzymes. This, then, was the basis for the studies of cytolytic enzymes described in this thesis.

MATERIALS

The materials discussed in this section include substances studied as potential substrates for cytolytic enzymes and materials investigated as possible sources of cytolytic enzymes. The source and preparation of all materials are given, and reference is made to the experiments in which the materials were used.

Substrates

Seven substrates for cytolytic enzymes were examined in this investigation. Four were early preparations of barley gum, described as whole barley gum, raw endosperm gum, alkali-digested endosperm gum, and boiled endosperm gum. Three substrates were more recent barley gum preparations, described as stabilized gum, papain gum, and "beta-glucosan". The preparation of these materials is outlined next.

Whole barley gum was prepared from Montcalm barley of the 1948 crop grown at the University of Manitoba. Barley was ground in a Wiley mill with a 1 mm. sieve to provide a whole barley meal. The meal was extracted at 20°C. for 2 hours with distilled water in the ratio of 1 g. meal to 8 ml. water. The mash was stirred continuously during extraction and was then centrifuged and filtered. The filtrate was poured into 3 volumes of 95 per cent ethyl alcohol with stirring. A stringy precipitate settled within an hour, and was recovered by centrifuging. The gum was dried by solvent exchange using ethyl alcohol, acetone, and ethyl ether, in that order. The final product was then air-dried.

Raw endosperm gum was prepared from the same sample of barley. The barley was milled, after tempering to 14 per cent moisture, in an Allis Chalmers mill to a short patent flour in approximately 40 per cent yield. The flour was extracted and the gum prepared as described in the previous paragraph.

Alkali-digested endosperm gum was prepared from raw endosperm gum by refluxing a 1 per cent solution of gum in normal sodium hydroxide for 2 hours in a boiling water bath. The liquor was cooled and insoluble material was removed by centrifuging. The supernatant was poured into 3 volumes of alcohol, and the precipitate was recovered by centrifuging and solvent exchange.

Boiled endosperm gum was similarly prepared by refluxing a 1 per cent solution of raw endosperm gum in water instead of sodium hydroxide solution.

Stabilized gum was prepared from the raw gum obtained by the procedure of Meredith, Watts, and Anderson (29). Barley grist was refluxed in boiling alcohol (specific gravity 0.850) for 30 minutes, and was then centrifuged, air-dried, and pulverized. The treated grist was mashed for 2 hours with distilled water at room temperature, using 50 g. grist with 400 ml. water. The mash was centrifuged and filtered, and the filtrate was poured into 3 volumes of 95 per cent ethyl alcohol. The raw gum settled out as a stringy white precipitate. The mixture was stored at room temperature overnight, and then centrifuged. The supernatant was discarded; the gum was washed with alcohol, acetone, and ether, then air-dried. Stabilized gum was prepared from this raw gum by alcoholic precipitation from hot aqueous

solution in the same way as boiled gum was prepared from raw endosperm gum.

Papain gum was also prepared from alcohol-treated grist according to the procedure of Meredith, Watts, and Anderson (29). Treated barley grist was mashed with a 0.25 per cent aqueous solution of papain for 2 hours at room temperature, using 50 g. of grist to 400 ml. papain solution. Papain was precipitated by adding a solution containing 27 g. trichloroacetic acid in 50 ml. distilled water, and storing for 30 minutes. The mixture was then centrifuged and filtered, and the filtrate was poured into 3 volumes of alcohol. After overnight storage, the precipitate was removed, washed and dried in the usual manner.

Preece and Mackenzie's method of preparing "beta-glucosan" (40) was considerably modified to conform with the general methods of preparing gums at the Grain Research Laboratory, so that the only similarity between the two methods is the use of ammonium sulphate as precipitating agent. A barley extract was prepared as for papain gum, and the extract volume was measured. Solid, pulverized ammonium sulphate was added in the ratio of 30 g. salt per 100 ml. extract. The mixture was stirred until all the salt had dissolved. It was then stored overnight at room temperature, and the precipitate was removed by centrifugation.

The excess ammonium sulphate was removed from the gum by one of two methods. For purifying small amounts of gum (one gram or less), the gum was redissolved in distilled water using 100 ml. of water per gram of gum. A calculated excess of a saturated solution of barium acetate was then added, and the voluminous precipitate of barium sulphate

was removed by centrifugation and filtration by suction through Celite. The filtrate was poured into three volumes of 95 per cent ethyl alcohol, which does not precipitate ammonium acetate or the small excess of barium acetate. The precipitated "beta-glucosan" was washed twice with alcohol to remove the last traces of these two salts, which are slightly soluble in alcohol. Finally the gum was washed with acetone and ether, and was then air-dried. For larger amounts of gum this procedure was somewhat laborious and yields of recovered gum were low. Preece and Mackenzie (40) purified their product by dialysing a solution of impure gum against distilled water for 24 hours, but this may be a drastic and equally laborious procedure. An alternative and simpler procedure for purifying larger amounts of beta-glucosan was to wash the crude precipitate repeatedly with a cold aqueous solution of alcohol sufficiently concentrated to prevent dissolution of gum but sufficiently dilute to permit solution of ammonium sulphate. A 50 per cent solution of alcohol (specific gravity 0.934) was suitable for these purposes, and four washings removed all traces of ammonium sulphate. The purified "beta-glucosan" was then dried by solvent exchange as before.

Barley gum solutions were prepared by mechanically mixing gum and water for 30 minutes in a water bath maintained at a temperature just below boiling. The hot mixture was filtered by suction through Whatman 41 H filter paper, and the filtrate was cooled to 30°C. before use. Gum concentration varied from 0.5 per cent to about 1.5 per cent ("as is" basis), depending on the substrate used and the initial viscosity required.

The first four substrates, whole barley gum, raw endosperm gum, alkali-digested endosperm gum, and boiled endosperm gum, were prepared from Montcalm barley of the 1948 crop, grown at the University of Manitoba. These substrates were used in preliminary investigations. Boiled endosperm gum was the most satisfactory of the early substrates for studies of cytolytic enzymes, and it was the chief substrate for investigations of cytolytic enzymes in a bacterial alpha-amylase preparation. Later substrates, stabilized gum, papain gum, and "beta-glucosan", proved to be more satisfactory than boiled endosperm gum. These materials were prepared from Montcalm barley of the 1949 crop, grown at the University of Manitoba, and were used in studies of barley and green malt.

Enzyme Sources

Raw barleys, green malts, and two commercial enzyme preparations were the sources of cytolytic and amylolytic enzymes used in these studies. Because of the diversity of source materials, it is perhaps most convenient to list enzyme sources as they occur in the experiments described in each section of the thesis.

Wallerstein's alpha-amylase, a preparation from a culture of B-subtilis, was the source of amylolytic and cytolytic enzymes in the preliminary investigations. This material, and Wallerstein's beta-amylase, a preparation from a special strain of barley, were the enzyme sources in experiments described in the section on bacterial alpha-amylase. In experiments described in the section on barley and green malt enzymes, source materials for cytolytic and amylolytic enzymes were the two Wallerstein preparations, Montcalm barley of the 1949 crop,

grown at the University of Manitoba, green malt of this barley malted by the Barley Improvement Institute, Winnipeg, and a commercial green malt obtained from the Canada Malting Co. For preliminary varietal studies, enzyme sources were Montcalm barley of the 1952 crop, grown at the University of Manitoba, Newal of the 1952 crop, grown at the Brandon Experimental Station, and the following varieties of the 1953 crop from the Tisdale Experimental Station: Montcalm, O. A. C. 21, U. M. 1020, U. M. 1623, U. M. 570, Lac. 5120, Br. 4298-3833, and Scott 151-62. Twenty-five gram samples of these varieties were malted by the Barley Improvement Institute. All samples of green malt were air-dried and stored in sealed cans in a refrigerator. Portions were removed as required, and coarse-ground in a Miag cone type mill before use.

EQUIPMENT

The equipment used in this research was standard cereal laboratory equipment, but two items merit further description: a freeze-drier for purifying barley and green malt enzymes, and the viscosimeters used in the research.

A Simple Freeze-Drier

A simple freeze-drier was constructed to aid in purifying barley and green malt enzymes. The assembled apparatus is shown in Fig. 1. It consists of four 250 ml. pyrex centrifuge bottles containing the material to be dried, connected to four vapor traps, which in turn are connected to a manifold. Suction is supplied by a high vacuum pump. Stopcocks are attached between each bottle and trap to permit independent evacuation of each container. Two stopcocks, one between the manifold and pump and the other between the manifold and atmosphere, permit the pump to be shut off without decreasing the vacuum, and prevent excessive moisture from entering the pump when the apparatus is dismantled. The centrifuge bottles are sealed with single-holed rubber stoppers coated with vacuum sealing compound to prevent leaks. All glass to glass connections are made with heavy walled rubber tubing lubricated with glycerol to give an air-tight seal. The vapor traps dip into an insulated wooden box containing a stainless steel box measuring 12 x 4 x $7\frac{1}{2}$ inches.

The procedure for freeze-drying was as follows. The lid of the insulated box was removed, and a mixture of dry ice and acetone was added to the freezing compartment. The lid was replaced, and the

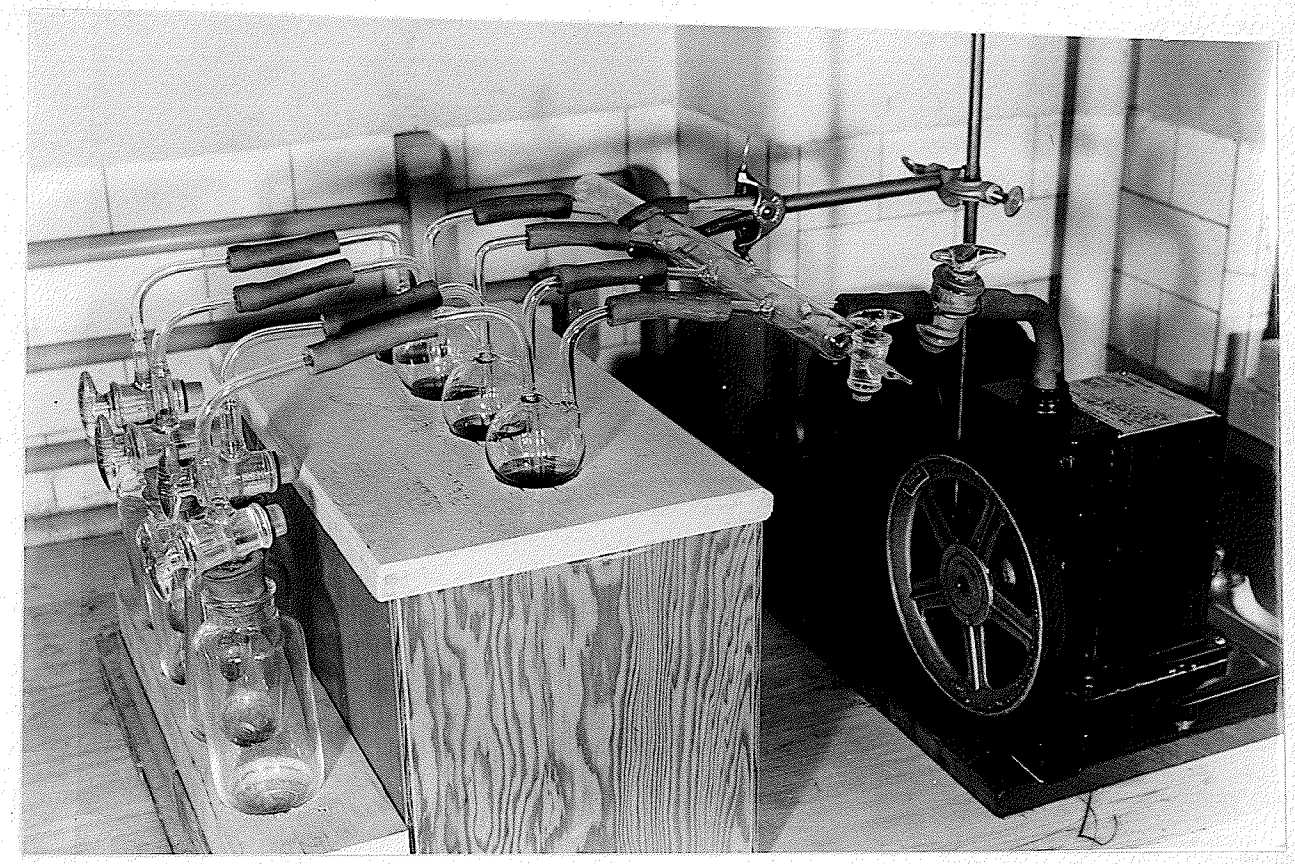

Figure 1.

Assembled freeze-drier.

apparatus assembled as in Fig. 1, except that stopcocks leading to the centrifuge bottles were off and the centrifuge bottles were not attached. The pump was then switched on. Fifty ml. of the material to be dried were added to each centrifuge bottle, which was swirled in a bath of dry ice and acetone to freeze the contents in a uniform film on the inside of the bottle. The bottles were then attached to the apparatus, and the suction line to each was turned on in turn. By this procedure, 200 ml. of an aqueous solution were freeze-dried in about 15 hours.

Viscosimeters

Solution viscosity is conveniently measured in Ostwald viscosimeters. The accuracy of such measurements depends on the validity of assumptions made in simplifying the Poisseuille equation governing the flow of liquids through capillaries, and on the errors inherent in the design of the viscosimeter. Accordingly, the fundamental equation for viscosity measurement was derived, and viscosimeters were designed to minimize instrument error. Viscosimeters were then calibrated with water and aqueous glycerol solutions to determine the range of viscosities that could be measured accurately. The derivation of the equation used to determine specific viscosity follows.

The absolute viscosity, in poises, of a liquid flowing through a capillary is given by Poisseuille's equation,

$$\eta = \frac{\pi P r^4 t}{8vl} \qquad (1)$$

where v = volume, in ml., of liquid flowing with uniform velocity in t seconds.

r = radius of capillary tube, in cm.

l = length of capillary tube, in cm.

P = pressure difference between the two ends of the capillary, in dynes/cm.^{2}

As the pressure, P, is directly proportional to density, d, of liquid in the tube, and as only d and t are variable for any one instrument, equation 1 may be rewritten as :

$$\eta = C.d.t. \qquad (2)$$

where C is a constant, characteristic of the viscosimeter.

According to Bull (7, p. 281), the absolute viscosity is given by:

$$\eta = C.d.t. - f\left(\frac{d.v}{l.t}\right) \qquad (3)$$

where $\left(\frac{d.v}{l.t}\right)$ is a function termed the "kinetic correction". This term may be neglected if t and l are sufficiently large, and equation 2 may then be used to calculate absolute viscosity.

The specific viscosity (η_{sp}) of a solution is defined (18, p. 500) as:

$$\eta_{sp} = \frac{\eta - \eta_s}{\eta_s} \qquad (4)$$

where η = absolute viscosity of the solution

η_s = absolute viscosity of the pure solvent

If the solvent is water, equation 4 reduces to the familiar form, $\eta_{sp} = \eta_r - 1$, where $\eta_r (= \frac{\eta}{\eta_s})$ is the relative viscosity, or viscosity ratio. From equation 2, $\eta_s = C.d_s.t_s$, where d_s and t_s are density and efflux time for pure solvent. Hence equation 4 becomes:

$$\eta_{sp} = \frac{C.d.t - C.d_s t_s}{C.d_s.t_s}$$

Or,

$$\eta_{sp} = \frac{d.t}{d_s.t_s} - 1 \qquad (5)$$

Finally, as the maximum gum concentration in enzyme studies was less than 1 per cent (dry basis), $d = d_s$. Hence,

$$\eta_{sp} = t/t_s - 1 \qquad (6)$$

Viscosimeters were designed to combine minimum outflow time with maximum accuracy. Rapid flow was desirable as initial enzymatic reaction rates were to be determined, and in most experiments six reaction mixtures were examined simultaneously. Hence viscosimeters were constructed to give efflux times of 10 to 13 seconds for water at 30°C. In addition, length and diameter of capillaries were selected so that the ratio l/d exceeded 100, as advised by Calderwood,

Douglas, and Mardles (8). These experimenters concluded that "In microviscometry, the Poisseuille equation needs modification to allow for the reversal of flow at the menisci; unless the length of liquid is at least a hundred times the diameter of the capillary, abnormal values are obtained, especially at low rates of movement". Other errors common to Ostwald viscosimeters, such as drainage error, working volume correction, and surface tension effects, were not considered, as these errors cannot be corrected by instrument design, but are usually negligible if viscosimeters and pipettes are scrupulously clean.

Twenty Ostwald type viscosimeters were made by R. J. Cheale, Grain Research Laboratory, to the author's specifications. These viscosimeters were cleaned, and calibrated with freshly prepared boiled glass-distilled water and with five aqueous glycerol solutions, as described next.

Before calibration, the viscosimeters were rinsed with chromic acid cleaning solution, tap water, distilled water, and drying liquid (a mixture of equal volumes of alcohol and ether). They were then dried overnight in a 70°C. oven. Viscosimeters were calibrated with distilled water by taking the mean of 10 consecutive efflux times. Two viscosimeters were discarded because of poor performance. The remainder were rinsed with drying liquid, oven-dried, and calibrated as before. Viscosimeters were then washed by overnight soaking in detergent, rinsed with tap water, distilled water, and drying liquid, then oven-dried. They were calibrated with distilled water, then washed, rinsed, dried, and calibrated again. Detergent washing and

oven drying had no effect on efflux time, and the highest deviation from a mean calibration was 0.7%. Mean efflux times are shown in Table I.

TABLE I

Calibration of Viscosimeters

Viscosimeter No.	Efflux Time for H_2O [1] seconds	Specific Viscosities of Glycerol Solutions				
		80%	77%	73%	64%	49%
2	12.00	32.1	23.5	14.5	7.26	2.94
3	11.02	32.4	24.0	14.7	7.38	2.96
4	10.20	32.4	23.9	14.7	7.37	2.99
6	11.18	32.1	23.5	14.5	7.22	2.94
7	11.45	32.4	23.8	14.7	7.25	2.98
8	10.77	32.2	23.7	14.6	7.38	2.98
9	12.92	-	23.5	14.4	7.38	2.90
10	11.48	32.8	24.0	15.0	7.24	3.02
11	11.22	32.9	24.2	14.8	7.47	3.03
12	11.18	32.9	23.9	14.8	7.51	3.00
13	11.07	32.7	24.0	14.7	7.42	3.00
14	10.91	32.8	24.1	14.9	7.39	3.01
15	10.31	32.8	24.2	15.0	7.48	3.02
16	10.40	32.3	23.8	14.6	7.52	2.94
17	10.58	32.7	23.8	14.7	7.36	3.02
18	10.51	32.1	23.5	14.5	7.45	2.91
19	10.88	32.1	23.5	14.4	7.26	2.95
20	11.15	32.1	23.7	14.6	7.29	2.94
Average		32.4	23.8	14.7	7.37	2.97
Theoretical		34.3	24.7	15.0	7.30	2.84
Max. deviation from mean, %		1.5	1.7	2.0	2.0	2.4
Mean deviation from mean, %		0.83	0.84	0.95	1.0	1.2

[1] Mean of four trials, each a mean of ten readings.

Five aqueous glycerol solutions, with approximate concentrations of 49 per cent, 64 per cent, 73 per cent, 77 per cent, and 80 per cent (volume per cent) were prepared next, and their specific gravities (25°/25°) were determined to be 1.1080, 1.1502, 1.1781, 1.1939, 1.2034. The theoretical absolute viscosities of these solutions were calculated by interpolation from a table of specific gravity vs absolute viscosity (19, p.1672) and specific viscosities were calculated from equation 4. Efflux times of all solutions were then measured in the 18 viscosimeters, and apparent specific viscosities were calculated.

Table I shows that all viscosimeters gave consistent results. The mean deviation from average specific viscosity varied from 0.83 to 1.2 per cent, and the maximum deviation from the mean varied from 1.5 to 2.4 per cent. For the 49 and 64 per cent solutions, mean viscosities were 4.6 and 0.96 per cent above theoretical, whereas for the 80, 77, and 73 per cent solutions, mean viscosities were 5.5, 3.6, and 2.0 per cent below the theoretical values. It appears, then, that viscosimeters were accurate to within about 2 per cent for specific viscosities ranging from 7 to 15. Below this range, specific viscosities were lower than theoretical. Observed specific viscosities above 30 were not reliable. It is very likely that the range 7 to 15 is actually a minimum one, as no estimate of the accuracy of theoretical viscosities was made. Hence for maximum accuracy in an enzyme reaction it would be desirable if the initial specific viscosity were no higher than 20, and the final viscosity no less than 5. There should be at least five readings in this range, and the time elapsed

between first and last readings should be about 1 hour. If the reaction is slow, then initial specific viscosity should be no higher than 15. Thus, most points will fall within the range of maximum accuracy, and points outside this range will be compensating.

It is possible to determine, from the data of Table I, whether the kinetic correction to the Poisseuille equation may be neglected. According to Bull (7, p. 281), "The most reliable method to determine whether the kinetic correction can be safely neglected is to measure the viscosity of two liquids whose viscosity is known. If the two measured viscosities bear the correct ratio to each other, and if the unknown viscosity has approximately the same value as that of the two known solutions, the kinetic correction is unnecessary". Table II shows that most ratios of observed specific viscosities are less than 5 per cent below theoretical ratios, and only three ratios are more than 5 per cent below theoretical ratios. Again, as the error in estimating theoretical viscosities was not calculated, these deviations probably represent maximum deviations. It therefore appeared safe to neglect the kinetic correction for all eighteen viscosimeters.

TABLE II

Specific Viscosity Ratios of Aqueous Glycerol Solutions

Viscosimeter No.	Ratios of Specific Viscosities			
	$80/77$	$77/73$	$73/64$	$64/49$
2	1.36	1.62	2.00	2.47
3	1.35	1.63	1.99	2.49
4	1.36	1.62	1.99	2.46
6	1.36	1.62	2.01	2.46
7	1.36	1.62	2.03	2.43
8	1.36	1.62	1.98	2.48
9	-	1.63	1.95	2.54
10	1.37	1.60	2.07	2.40
11	1.36	1.64	1.98	2.46
12	1.38	1.61	1.97	2.50
13	1.36	1.63	1.98	2.47
14	1.36	1.62	2.02	2.46
15	1.36	1.61	2.00	2.48
16	1.36	1.63	1.94	2.56
17	1.37	1.62	2.00	2.44
18	1.36	1.62	1.95	2.56
19	1.36	1.63	1.98	2.46
20	1.35	1.62	2.00	2.48
Theoretical	1.39	1.65	2.05	2.57
Max. deviation from theoretical, %	3.6	3.0	5.4	5.4
Mean deviation from theoretical, %	2.1	1.6	3.0	3.6

GENERAL METHODS

Much of the research presented in this thesis involved the development of methods that could be used in evaluating potential substrates, determining enzymatic activity, isolating and identifying enzymes, and estimating the potential viscosity-reducing activity of barley from a small sample. Many of these methods were developed for a specific purpose, and it is more convenient to discuss such methods in the section to which they belong. Other methods, however, had more general applications, and these methods are described next. They include analysis of barley gums, determination of alpha-amylase activity, determination of viscosity-reducing activity, filter paper chromatography, and column chromatography.

Analysis of Gums

All barley gums were regarded as potential substrates for cytolytic enzymes, hence all barley gum preparations were screened for desirable characteristics such as purity, low degree of degradation during preparation, and susceptibility to attack by cytolytic enzymes.

The degree of purity of barley gums were interpreted in terms of freedom from starch and contaminating nitrogenous material, including gum-degrading enzymes. The nitrogen content of gums was quantitatively determined by the A.A.C.C. procedure recommended for flour (1, p. 26). The presence of small amounts of gum-degrading enzymes in early gum preparations was suggested by a decrease in solution viscosity when aqueous solutions of nitrogen-containing gums were stored for 24 hours.

Stability of solution viscosity of all gum preparations was determined by calculating the per cent decrease in viscosity of concentrated aqueous solutions of gum after a 24-hour storage period at 30°C. Thus a low per cent decrease indicates a relatively stable gum and, presumably, a low degree of enzymic contamination. Absence of starch was checked qualitatively with a standard iodine-potassium iodide solution.

The degree of degradation of barley gum is an important characteristic, as it is desirable to have substrates as similar as possible in constitution, structure, and molecular size, to materials occurring naturally in barley. The extent to which barley gums were degraded by enzymes during extraction from barley grist could not be estimated quantitatively because the constitutions of cell wall materials and barley gums have not yet been determined. However, it seemed reasonable to assume that solution viscosity may be a measure of molecular size and complexity. Thus high solution viscosities probably indicate relatively undegraded gums, whereas low solution viscosity suggests that extensive enzymatic degradation has occurred during the process of extracting the barley gum. High solution viscosity is therefore a desirable property of potential substrates.

Susceptibility to attack by cytolytic enzymes is obviously the chief characteristic required of potential substrates. Hence all gums were tested for sensitivity to various enzyme preparations by one of two procedures. The first procedure was used in preliminary studies and involved determination of the effect of the bacterial alpha-amylase preparation on viscosity and reducing power of aqueous solutions of early barley gums. The second procedure was used in

testing more recent substrates, and involved determination of the viscosity-reducing activity of various enzyme preparations. A more detailed description of the two methods follows.

In preliminary studies, reaction mixtures containing 90 ml. of 1 per cent aqueous gum solution at 30°C., 10 ml. of a 5 mg. per cent aqueous solution of Wallerstein's alpha-amylase, and 4 drops of toluene to inhibit bacteria, were incubated at 30°C. Five-milliliter aliquots were transferred to viscosimeters in a 30°C. ($\pm$ 0.02°C.) water bath, and viscosity measurements were taken at regular intervals over a period of 2.5 hours. Five-milliliter aliquots were then removed for reducing power determinations at intervals over a period of 6 days. No growth of microorganisms was observed during the 6 day incubation period. Reducing powers were determined by the standard ferricyanide procedure for reducing substances (2, p. 103), which is described next. Blanks containing 10 ml. of distilled water instead of 10 ml. of enzyme solution were also measured for viscosity and reducing power, and data were calculated as net decrease in viscosity and net increase in reducing power.

The method for determining reducing power was as follows. Five-milliliter aliquots of reaction mixtures were pipetted into 125 ml. Erlenmeyer flasks, 10 ml. of 0.05 N alkaline ferricyanide solution (2, p. 103) were added, the contents were mixed, and the flasks were immersed in a vigorously boiling water bath for exactly 20 minutes. The flasks were cooled under running water, 25 ml. of acetic acid-salt solution (2, p. 103) and 1 ml. of potassium iodide solution (2, p. 103) were added, and the contents were mixed well. The solutions were then titrated with 0.05 N sodium thiosulphate (2, p. 103),

and results were calculated as volume of ferricyanide reduced per gram of gum.

In testing more recent barley gums, only viscosity-reduction was taken as a measure of susceptibility to cytolytic enzymes. Reaction mixtures containing 7 ml. of a 0.7 per cent aqueous gum solution, 2 ml. of McIlvaine buffer (pH 5.0) and 1 ml. of enzyme solution were prepared, and 5 ml. aliquots were pipetted into viscosimeters dipping into a water bath thermostatically controlled at 30°C. ($\pm$ 0.02°C.). The subsequent procedure is described later in the section under "Determination of Viscosity-Reducing Activity".

Acid hydrolysates of barley gums were also analyzed qualitatively for sugars by filter paper chromatography, and acid hydrolysates of stabilized gum and papain gum were analyzed qualitatively for amino acids by paper chromatography. These procedures are described in a later part of this section, as paper chromatography was also used to identify enzymic hydrolysates of some gum preparations.

Determination of Alpha-Amylase Activity

Alpha-amylase activity was determined by a method similar to the standard starch-iodine colorimetric method (2, p. 110a). Enzymic reaction mixtures containing 20 ml. starch solution, 10 ml. enzyme solution and 1 drop of toluene to inhibit bacteria were stored at room temperature. At intervals, 1 ml. of reaction mixture was pipetted into 5 ml. of dilute iodine solution (2, p. 110a), and the resultant color was recorded. As the degradation of starch by alpha-amylase proceeds, iodine colors change from the characteristic deep blue of starch through violet, red, brown, and eventually to the unchanged

yellow color of the iodine solution. Alpha-amylase activity is inversely proportional to the time required to develop a standard red color, and was assumed to be zero if there was no change in the blue starch-iodine color after an incubation period of at least 48 hours.

Alpha-amylase in aqueous solution was inactivated at low temperature and low pH. The solution pH was first adjusted to pH 3.3, and the solution was then stored at 0°C. for 48 hours. The solution was warmed to room temperature and its pH adjusted to pH 6.0. After this treatment alpha-amylase had no effect on soluble starch, as determined by the starch-iodine color.

Determination of Viscosity-Reducing Activity

A description of the method for determining the viscosity-reducing activity of various enzyme preparations on aqueous barley gum solutions is included in this section, as the method was used, with some modifications, in most of the investigations. The original procedure was developed early in the research, and revisions to the method were made as the need for greater precision arose. Improved substrates became available as studies of barley and green malt progressed, and the procedure was then standardized to control pH and composition of reaction mixtures.

In the original method, developed during studies of the bacterial alpha-amylase preparation, reaction mixtures contained 9 ml. of a 1 per cent solution of boiled endosperm gum and 1 ml. of a 10 mg. per cent solution of Wallerstein's alpha-amylase. The substrate, prepared by heating gum and water (page 20), was cooled to 30°C. before addition of enzyme, and the assay temperature was 30°C. Zero reaction time

was taken from the time of addition of enzyme to substrate. Five-milliliter aliquots were pipetted into viscosimeters dipping into a 30°C. (± 0.02°C.) water bath. Efflux times were taken with a stopwatch at regular intervals over a period of 45 to 90 minutes, and the initial time of each reading was recorded to the nearest 15 seconds. Initial times were corrected for efflux time by adding half of the total efflux time to the observed time. Specific viscosities were then calculated from efflux times by equation 6 (page 27). Initial specific viscosities were calculated from curves of specific viscosity vs corrected time for blanks containing distilled water instead of enzyme solution. These graphs were linear and could be extrapolated accurately to zero reaction time. Ratios of initial specific viscosity to specific viscosity at time, t, were then plotted against reaction time, and viscosity-reducing activity was arbitrarily determined as 40 times the slope of initially linear portions of reaction curves. That is, viscosity-reducing activity was arbitrarily defined as the difference between viscosity ratios at t = 40 minutes and t = 0, during which time the slope of the plot did not change. The development of this method is described in the section on bacterial alpha-amylase.

Modifications to the viscosimetric method were of two types: changes in composition of reaction mixtures, and changes in the method of plotting data. The need for accurate control of pH resulted in a change in composition of reaction mixtures to permit addition of buffer. This, in turn, was facilitated by the availability of improved substrates that yielded higher solution viscosities than boiled endosperm gum at lower gum concentrations. Thus, in studies

of barley and green malt the composition of reaction mixtures was revised to 7 ml. of 0.7 per cent gum (as is), 2 ml. of McIlvaine buffer (19, p. 1348), and 1 ml. of enzyme solution. The substrate, first cooled to 30°C., was added last, and time of addition of substrate was taken as zero reaction time. The assay temperature was 30°C. Initial specific viscosities of reaction mixtures were about 20 when the substrate was stabilized gum, papain gum, or beta-glucosan.

The method of plotting reaction data was also modified when studies of barley and green malt were initiated. The data were plotted as the reciprocal of specific viscosity vs corrected time. This is essentially the same as the earlier method of plotting, but it eliminates the need for carrying a substrate blank to determine initial specific viscosity, as initial specific viscosity can be calculated, if desired, by extrapolating reaction curves to zero time. The similarity between the two methods of plotting is best illustrated by comparing the equations derived from initially linear portions of reaction curves obtained by plotting reaction data by both methods:

$$\eta_o/\eta_t = b.t + 1 \qquad (7) \qquad \text{slope} = b \qquad \text{intercept} = 1$$

$$1/\eta_t = {}^{b}/_{\eta_o}.t + {}^{1}/_{\eta_o} \qquad (8) \qquad \text{slope} = {}^{b}/_{\eta_o} \qquad \text{intercept} = {}^{1}/_{\eta_o}$$

is to be created . . . responsible to the German people and not to any High Commissioners".[1]

On October 7, 1949, the Volksrat proclaimed itself Provisional Volkskammer (People's Chamber, the lower chamber of the Parliament of the GDR) and issued a manifesto establishing the German Democratic Republic.

> "We call on the German people to take salvation of the Nation into their own hands and, by supporting the struggle of the National Front of Democratic Germany, to pave the way for peace, reconstruction, and the national freedom of the united Germany Democratic Republic".[2]

The manifesto outlined the aims of the "National Front" that was to be an all-German extension of the "democratic block", called, among other things, for the abolition of the German Federal Republic and withdrawal of the occupation troops after the peace treaty, pledged loyalty to the Potsdam agreement and appealed for

> "an irreconcilable struggle against the instigators of a new war in Germany; prohibition of the war propaganda in the press and radio and at the meetings . . . irreconcilable struggle against drawing Germany into aggressive military blocks, into European Union and NATO . . , against traitors of the German nation . . . agents of American Imperialism".

The membership of the self-appointed Provisional Volkskammer was distributed according to a prearranged key, giving unchallengeable majority to the SED that combined its 90 seats with the 150 of the puppet parties and "mass organizations" against 45 seats allotted to each CDU and LDP. The general elections to the Parliament and to the Landtaege that were to take place in the fall of 1949, were postponed "due to the economic reasons" until October 1950. Meantime, the upper chamber of the East German

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[1] "Taegliche Rundschau", October 5, 1945, quoted in Keesing, Ibid., p. 1028.

[2] Keesing, Ibid.

mixture was placed at the bottom of the tank, and the papers were allowed to equilibrate for about 1 hour. The solvent phase was then poured into the troughs, and chromatograms were developed for a predetermined time. The solvent system used in these studies was ethyl acetate/pyridine/water in the ratio 5:2:5 (21, 22). This solvent system gave a clear separation of maltose, galactose, glucose, arabinose and xylose in 24 hours.

Dried hydrolysates were made up to about 5 per cent by the addition of 0.5 ml. distilled water, and approximately 3 to 6 µl. of these solutions were spotted along the base line of the chromatogram. Hydrolysates were spotted 1 inch apart, and standard solutions of known sugars were placed at each end of the base line, 1 inch from the edge of the paper. After developing, paper chromatograms were dried at room temperature, sprayed with aniline phosphate (6), and finally dried in a 100°C. oven for 5 minutes. Aldo-hexoses or oligo-hexosans appeared as brown spots, pentoses or oligo-pentosans appeared as pink spots. Unknowns were then identified by comparison with the positions of known sugars.

It was estimated that about 7.5 γ of monosaccharide or 15γ of disaccharide could be detected visually. Even smaller amounts could be detected by fluorescence in ultra-violet light. Thus the absence of free sugars on chromatograms of acid hydrolysates indicated that the dry hydrolysate contained considerably less than 5 per cent of these sugars.

Enzymic reaction products were similarly detected by incubating enzyme-substrate reaction mixtures and enzyme blanks for 24 hours at room temperature, then analyzing hydrolysates by paper chromatography. Reaction mixtures contained 10 ml. of 1 per cent aqueous gum solution, 2 ml. of McIlvaine buffer solution (pH 4.5), 1 drop of toluene to inhibit bacteria, and 10 ml. of a solution of barley or malt enzymes or 10 ml. of a 0.1 per cent solution of Wallerstein's alpha- or beta-amylase. Enzyme blanks contained 10 ml. of distilled water instead of gum solution. After incubation, reaction mixtures and blanks were heated in a boiling water bath for 20 minutes, cooled, passed through a mixed anion-cation exchange resin (Duolite A 7 and Amberlite IR-120 A.G.) then evaporated to dryness at reduced pressure. One-half to 1.0 ml. of distilled water was added to the residue, and the mixture was stirred, heated to boiling, and filtered through glass wool. About 6 to 9 µl. of concentrate were chromatographed with three different solvent mixtures: ethyl acetate/acetic acid/water (3:1:3), ethyl acetate/pyridine/water (5:2:5) and butanol/ethanol/water (5:1:4). Aniline phosphate was the spray reagent (6).

Acid hydrolysates of stabilized gum and of papain gum were analyzed for amino acids by filter paper chromatography to determine whether the low nitrogen content of these gums could be attributed to experimental error or whether the nitrogen content was due to proteinaceous material. Accordingly, these gums were hydrolyzed by digesting with normal hydrochloric acid in sealed tubes for about 24 hours at 95°C., using 30 mg. of gum and 1 ml. of acid. The acid was removed and the hydrolysate recovered by repeated dilution and vacuum distillation.

The dried hydrolysate was then suspended in 0.2 ml. of distilled water, and about 6 µl. were chromatographed for 24 hours. The solvent was butanol/acetic acid/water in the ratio of 4:1:5 (26) and the spray was 0.1 per cent ninhydrin in butanol (10). Amino acids appeared as blue or purple spots, and were identified by comparison with positions occupied by known amino acids.

Column Chromatography

Although column chromatography was used only for separating viscosity-reducing enzymes from alpha-amylase in the bacterial preparation, the method is outlined in this section because of its possible applications in purifying enzyme preparations from germinating barley. The materials and general procedure are described next.

Two columns were used. The first was a glass tube measuring 18 inches by 1 5/16 inches (inside diameter). A coarse sintered glass filter funnel was fused to the bottom end of the tube, and two inlet tubes were fused into opposite sides of the column near the top end. Compressed air, first passed through a mercury trap to eliminate large variations in pressure, was led into the column through a single-holed rubber stopper inserted into the top of the column. The funnel tip at the bottom of the column was fitted with a rubber tube and screw clamp to regulate the flow of liquid from the column. This column was used for preliminary investigations of different adsorbents. A second, smaller, column was used with alumina as adsorbent. This column measured 9 inches by 3/4 inch (inside diameter), and it was equipped with a sintered glass funnel but no side tubes.

Three adsorbents were examined for ability to adsorb alpha-amylase and viscosity-reducing enzymes from aqueous solution. These were Merck's insoluble potato starch, macerated Whatman No. 1 filter paper (cellulose), and Brickman's "alumina for chromatography". The starch adsorbent was prepared by making a slurry of starch in distilled water. The cellulose adsorbent was prepared by macerating filter paper strips with water to form a thick suspension. The alumina adsorbent was first activated by the method of Dupont, Dulou, and Vilkas (12) then stored in glass-stoppered bottles until required. An outline of the activation process follows.

The chromatographic alumina was activated before use to increase its adsorptive capacity and remove fine powders which retard flow. The alumina was first heated in a muffle furnace at 500 to 600°C. for one hour. It was cooled to room temperature then transferred to a 2 l. beaker. Concentrated hydrochloric acid was added with stirring until a supernatant layer of liquid was obtained. The mixture was stored for 2 to 3 hours with occasional stirring, the acid was decanted, and the beaker was filled with cold water. The alumina was then stirred into suspension, and allowed to settle for about 5 minutes, after which the supernatant was decanted. Washing was repeated until the supernatant cleared in 2 to 3 minutes. Five or six washings were usually necessary. The alumina was washed on a Buchner funnel, until wash waters were neutral and dried in a 105°C. oven for 2 to 4 hours. It was then stored in glass-stoppered bottles until required.

The general procedure for chromatography was as follows. The adsorbent, in slurry form or in aqueous suspension, was poured

into the column and allowed to settle to a depth of 10 inches in the large column or 3 inches in the small column. A plug of glass wool was inserted to keep the surface of the adsorbent intact, and the column was washed with 1 to 2 l. of distilled water. An appropriate weight of bacterial preparation in aqueous solution was then placed on the column and washed into the adsorbent with small amounts of water. Developers were passed through the column, and effluents were collected in equal portions. The ultra-violet absorption of each effluent was determined in a Beckman spectrophotometer at 265 mμ. wave length, and those portions containing the same fraction, as indicated by the chromatograph, were combined and tested for alpha-amylase and viscosity-reducing activities.

PRELIMINARY STUDIES

The first two requirements for the proposed investigations were a satisfactory substrate for studying cytolytic enzymes and an accurate measure of cytolytic activity. Preliminary studies dealt with these two points.

Four early barley gum preparations were screened for properties considered desirable in a satisfactory substrate. Gums were examined for nitrogen content, sugar content, starch content, initial solution viscosity, stability of viscosity, and susceptibility to enzymatic attack. A commercial bacterial alpha-amylase preparation was used in this phase of the investigation as this was a partially purified, concentrated, and readily available material.

Table III shows the analytical data for the four gum preparations. Yield of gum was about 3 per cent of the barley. Nitrogen content was high, but most of it was lost when the gum was reprecipitated from hot aqueous solution, and all the nitrogen disappeared when the gum was reprecipitated from hot normal sodium hydroxide solution. All gums contained the same sugar residues, although the relative proportions of these varied. Principal sugars were glucose, arabinose, and xylose, but traces of galacturonic acid, galactose, mannose, and ribose were also detected. All gums gave a negative test for starch.

TABLE III

Analytical Data on Four Barley Gum Preparations

Gum	Yield	Nitrogen Content	Sugar Content		Stability of Viscosity
			Glucose	Pentoses	
	% of barley	% of gum			% viscosity decrease in 24 hours
Whole barley	3.1	3.52	+	+	57
Raw endosperm	3.2	3.55	+	+	69
Alkali-digested	1.9	0	+	+	1
Boiled endosperm	1.8	0.72	+	+	28

Viscosities of aqueous solutions of all gums were determined next. Fig. 2 and Fig. 3 show the results of duplicate determinations of the relation between viscosity and gum concentration for whole barley gum, raw endosperm gum, and alkali-digested endosperm gum, and the results of a single determination for boiled endosperm gum. Viscosities of whole barley gum and alkali-digested gum were generally low, whereas viscosities of raw endosperm gum and boiled gum were higher and increased markedly with increasing gum concentration. The last column of data in Table III shows that solution viscosities of whole barley gum and raw endosperm gum were very unstable, the solution viscosity of alkali-digested gum was stable, and the solution viscosity of boiled gum was moderately stable.

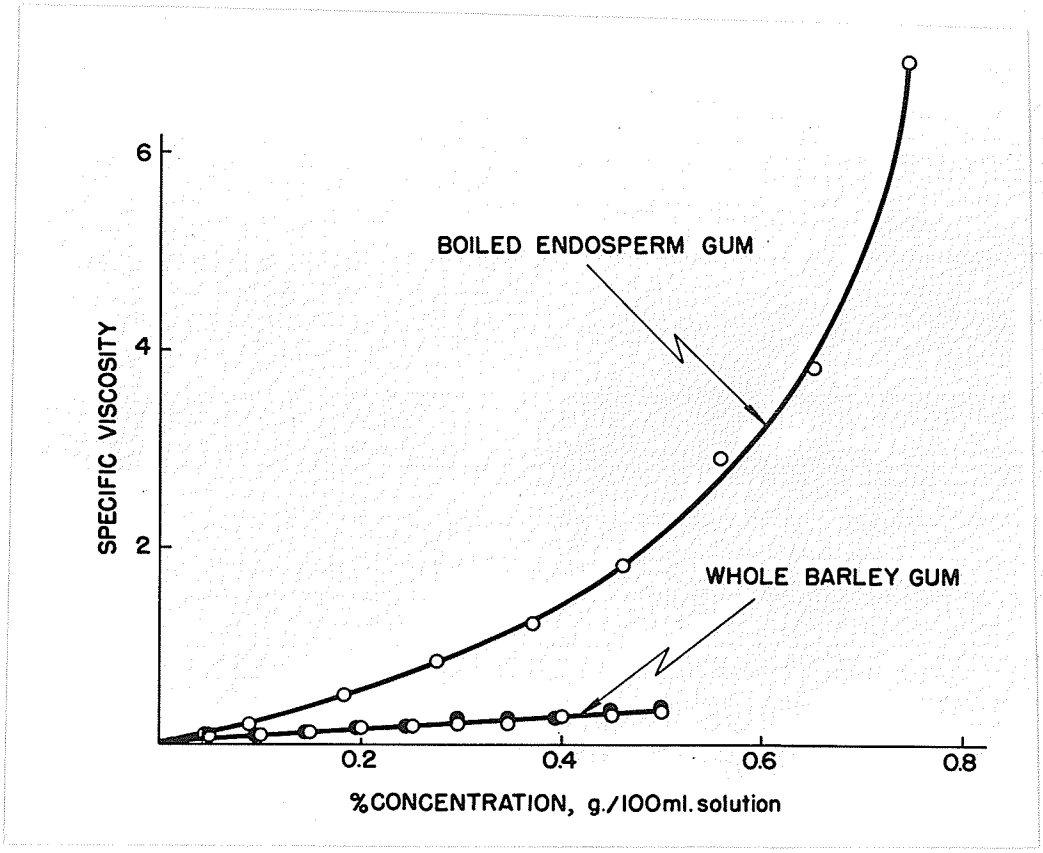

Figure 2.

Effect of concentration on viscosity of aqueous solutions of whole barley gum and boiled endosperm gum.

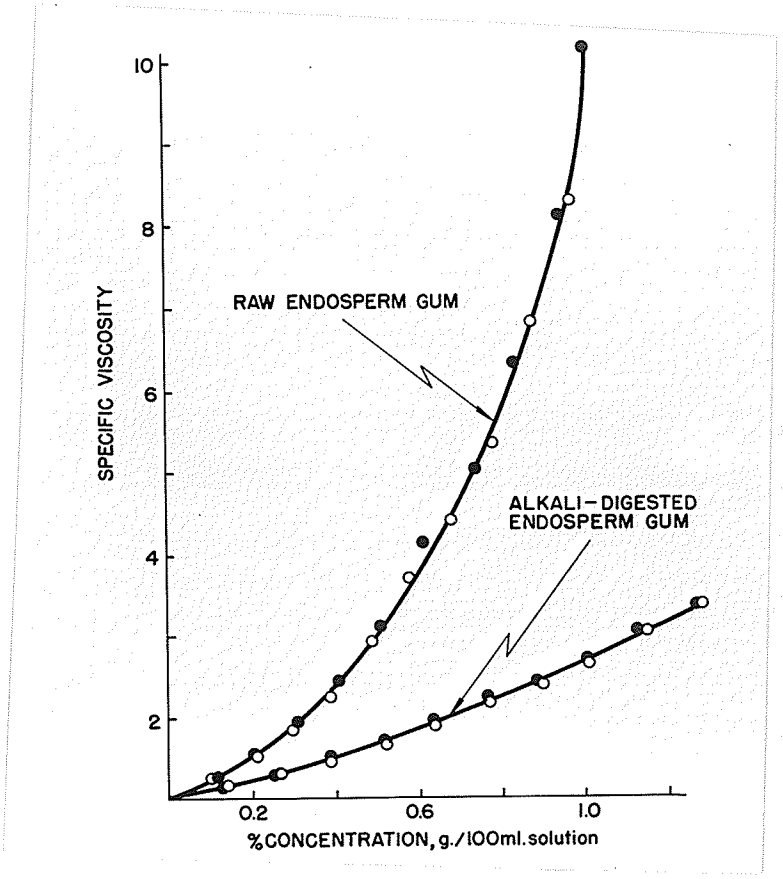

Figure 3.

Effect of concentration on viscosity of aqueous solutions of raw endosperm gum and alkali-digested endosperm gum.

Susceptibility of the gums to enzymatic attack was determined by recording changes in viscosity and reducing power of enzymic digests, as early work with the first barley gum preparation of Meredith indicated that enzymatic degradation of the gum was accompanied by a decrease in solution viscosity and by an increase in solution reducing power. Fig. 4 and Fig. 5 show typical changes in viscosity and reducing power due to the action of a 5 mg. per cent solution of Wallerstein bacterial enzyme preparation on a 1 per cent aqueous solution of boiled gum. It should be observed that the time scales in these figures differ by a factor of 60, as units of time in Fig. 4 are minutes and units of time in Fig. 5 are hours.

Paper chromatograms of enzymic digests showed no trace of free sugars, but slow-moving components, presumably oligosaccharides, were detected. One of these produced a pink spot after spraying and therefore contained pentosan material.

Results of preliminary enzymatic studies show that viscosity reduction appears to be more characteristic of the initial gum-degrading reaction than increase in reducing power, as viscosity decreased to a minimum value in about 2 hours, whereas with the same enzyme concentration maximum reducing power occurred after 5 days. Reducing power development can probably be accelerated by increasing the enzyme concentration, but this would involve relatively large corrections when extracts of green malt are used as enzyme sources. Hence it appears that a viscosimetric method of measuring cytolytic activity may be more desirable than a reducing power method.

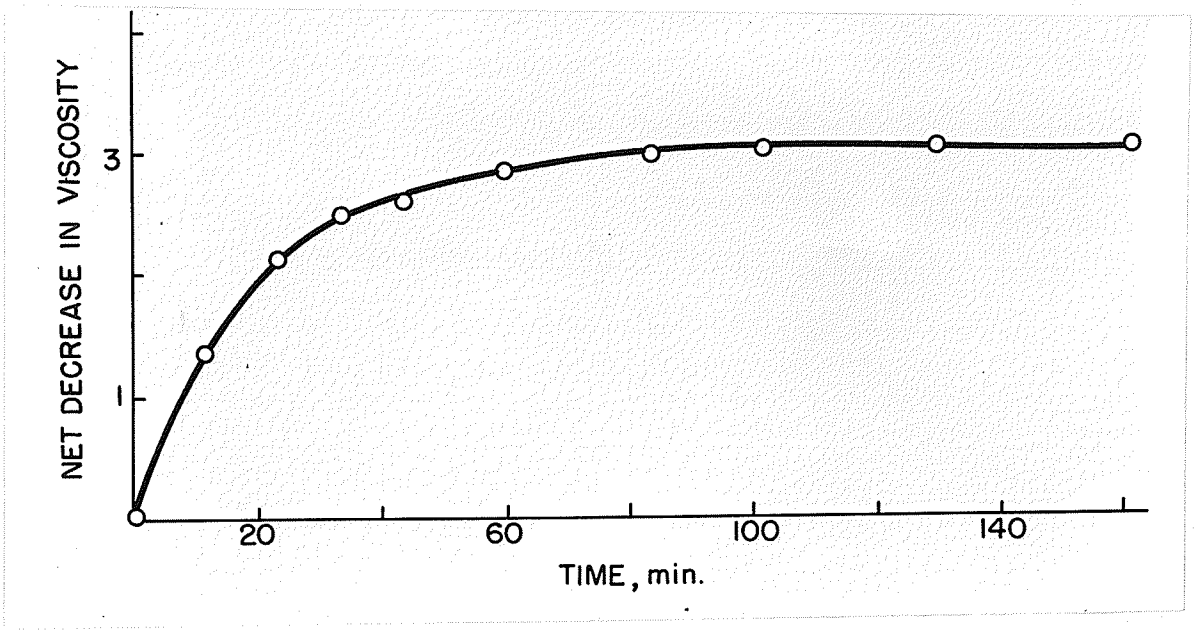

Figure 4.

Graph showing the effect of the Wallerstein bacterial enzyme preparation on viscosity of an aqueous solution of boiled endosperm gum.

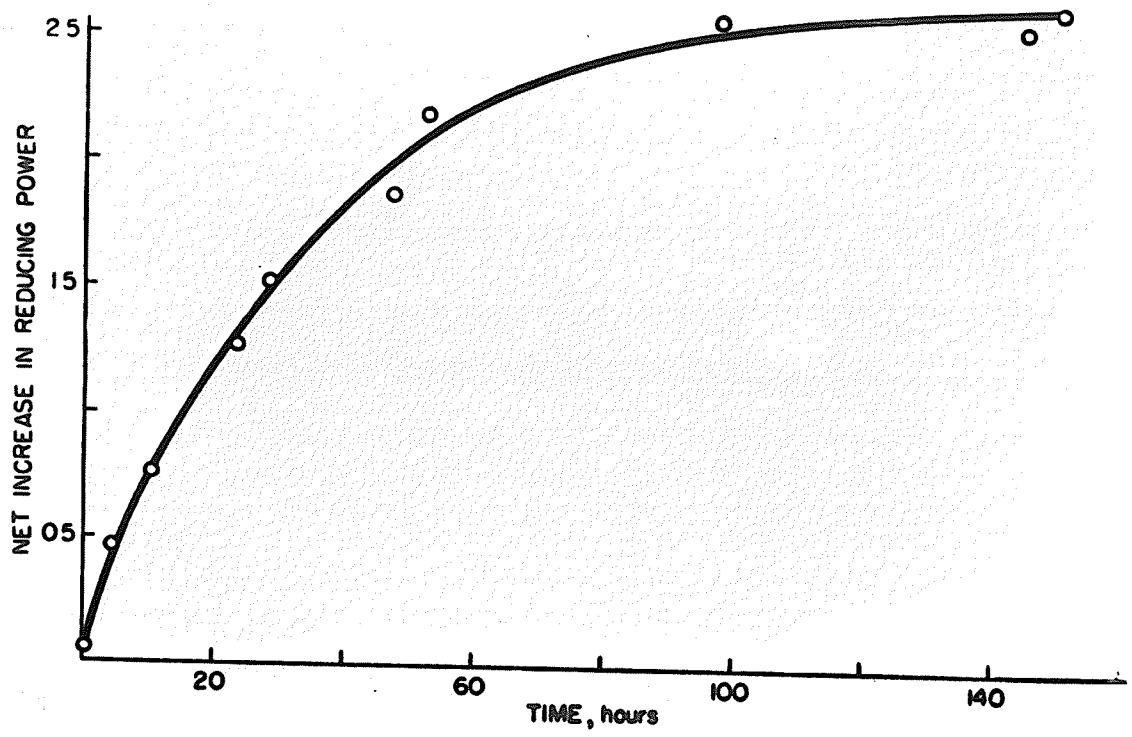

Figure 5.

Graph showing the effect of the Wallerstein bacterial enzyme preparation on reducing power of an aqueous solution of boiled endosperm gum.

Because products of enzymatic hydrolysis were oligosaccharides, it appears that the cytolytic enzymes of the bacterial preparation are endo-glycosidases, operating by rupturing internal glycosidic linkages. Endo-glycosidic ruptures satisfactorily account for a rapid decrease in viscosity accompanied by a slower increase in reducing power because viscosity may be profoundly affected by, say, a 50 per cent reduction in molecular size, whereas the accompanying reducing power increase may be a relatively small fraction of the total potential reducing power. Therefore the major drop in viscosity occurs rapidly, but reducing power continues to rise for a considerably longer time. Moreover, the products of endo-glycosidase action are presumably oligosaccharides and large polysaccharide fragments, with very little production of mono- or di-saccharides, at least in early stages of the reaction. This too supports the choice of viscosity as a good measure of cytolytic activity.

Of the four barley gums examined, three were considered to be unsatisfactory substrates for studying cytolytic enzymes. Whole barley gum was unsatisfactory because of low solution viscosity and instability of viscosity. Raw endosperm gum was satisfactory in initial solution viscosity, but viscosity was unstable. Finally, alkali-digested endosperm gum produced stable solutions, but this gum was probably extensively depolymerized by alkali treatment, as indicated by its low solution viscosity.

Boiled endosperm gum appeared to be the most satisfactory substrate for studying cytolytic enzymes as its nitrogen content was fairly low, its solution viscosity was relatively high, and its

solutions were moderately stable. However, these properties were still far from ideal. The preparation of barley gums with improved characteristics formed another field of study undertaken by Mr. T. A. Watts under the direction of Dr. W.O.S. Meredith. In the meantime, boiled gum was used as substrate in developing a method for measuring cytolytic activity and in separating cytolytic enzymes from alpha-amylase in the bacterial preparation.

STUDIES OF THE BACTERIAL ALPHA-AMYLASE PREPARATION

The Wallerstein alpha-amylase preparation was originally intended to be used solely as an aid in developing a viscosimetric method of measuring cytolytic activity. However, as this phase of the research progressed, it seemed desirable to extend the study to include an attempt to separate cytolytic enzymes from alpha-amylase in the bacterial preparation. As it was recognized that viscosity reduction may not be a measure of the activity of all cytolytic enzymes, it was considered advisable to replace the term "cytolytic activity" with "viscosity-reducing activity", and to refer to viscosity-reducing enzymes as "X-enzyme".

Determination of Viscosity-Reducing Activity

A reliable method of determining viscosity-reducing activity was sought by plotting viscosimetric reaction data in a number of ways. Reaction curves of specific viscosity *vs* time and of decrease in viscosity *vs* time indicated that the initial specific viscosity of reaction mixtures was a factor in determining viscosity-reducing activity. Hence it seemed worth while to plot change-time curves in which the change incorporates both initial specific viscosity (η_o) and specific viscosity at time t (η_t) in a function increasing with time. The ratio η_o/η_t is such a function. Therefore reaction curves were plotted as η_o/η_t *vs* time.

Fig. 6 shows reaction curves obtained when two solutions of boiled endosperm gum were incubated with a solution of the Wallerstein bacterial enzyme preparation (a + X), and with the same solution treated to inactivate alpha-amylase (X). Reaction mixtures consisted of 9 ml. of a 1 per cent solution of boiled endosperm gum and 1 ml. of a 10 mg. per cent solution of the bacterial enzyme preparation. Initial specific viscosities of reaction mixtures were 4.77 and 5.58. Reaction curves are essentially linear in initial stages of the reaction, and reaction rates diminish gradually in later stages. In addition, the two reaction curves for each enzyme preparation are superimposed, indicating that this method of plotting viscosimetric data may provide a satisfactory measure of viscosity-reducing activity.

Subsequent experiments showed that reaction curves were initially linear for appreciable periods of time. Fig. 7 shows typical curves obtained when boiled endosperm gum was infused with a 5 mg. per cent solution of bacterial alpha-amylase, and with the same solution first heated at 70°C. for 15 minutes. Reaction curves were linear for at least the first 50 minutes of the reaction.

Fig. 7 shows that heat-stable enzymes had negligible viscosity-reducing activity. Because alpha-amylase survived the heat treatment, as determined by the colorimetric starch-iodine procedure, it was concluded that X-enzyme is inactivated by heating at 70°C. for 15 minutes and that alpha-amylase has little or no viscosity-reducing activity on non-starch polysaccharides.

The slope of linear portions of reaction curves, i.e. $\frac{d}{dt} \eta_o / \eta_t$, is presumably a direct measure of X-activity. Fig. 8 shows that the slope is directly proportional to enzyme concentration for enzyme

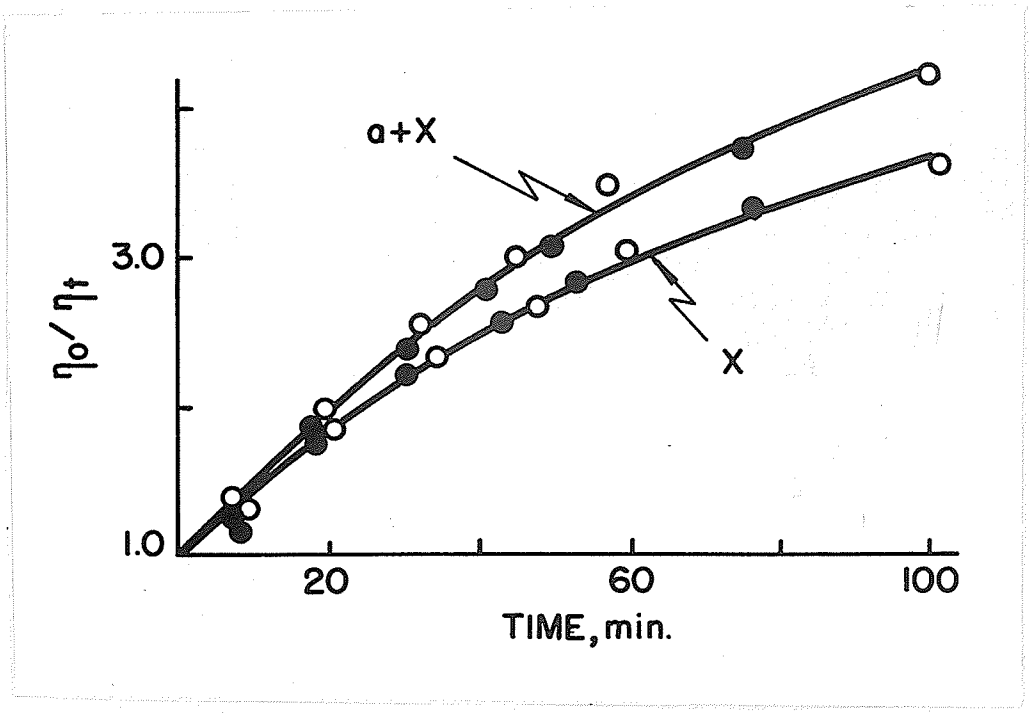

Figure 6.

Comparison of the actions of two Wallerstein bacterial enzyme preparations, with and without alpha-amylase inactivation, in reaction mixtures of different initial specific viscosities.

o——o Reaction mixtures having an initial specific viscosity of 5.58

●——● Reaction mixtures having an initial specific viscosity of 4.77

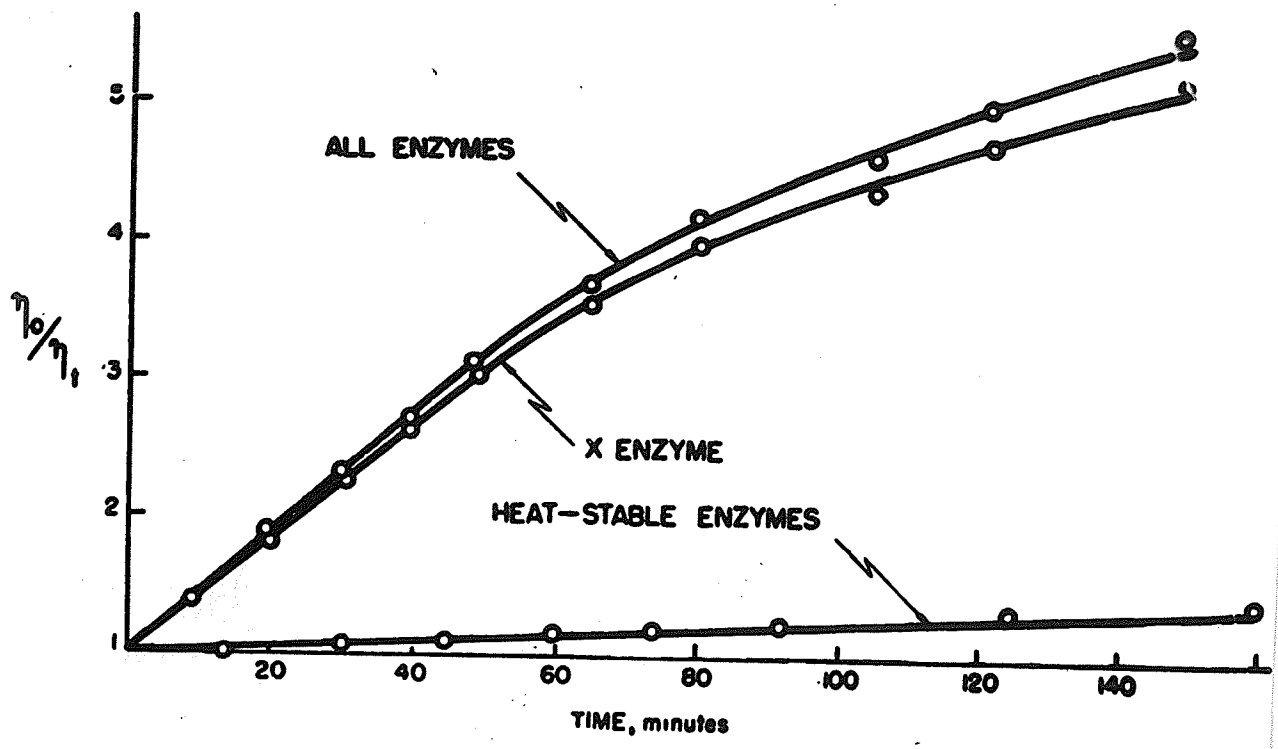

Figure 7.

Comparison of the actions of Wallerstein bacterial enzyme preparations with different pre-treatments.

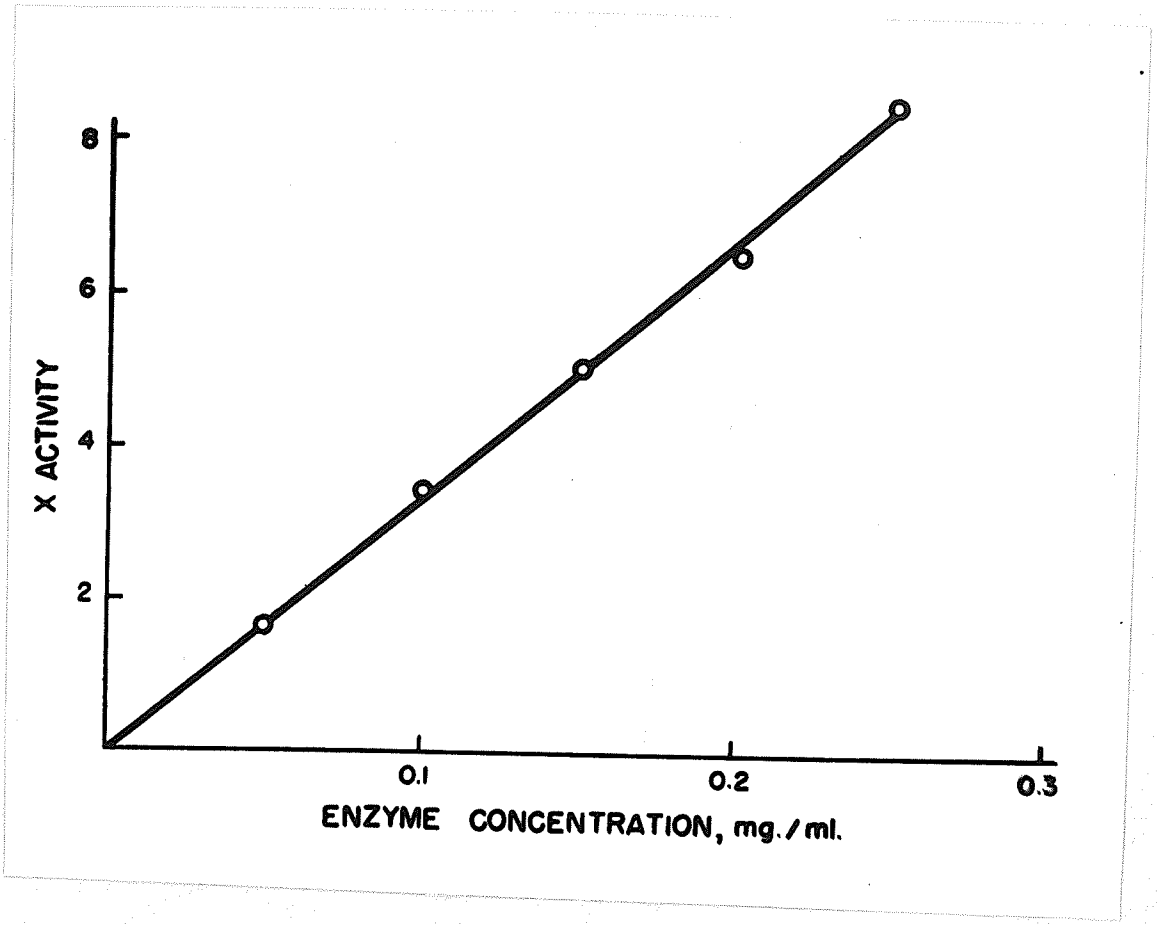

Figure 8.

Effect of enzyme concentration on viscosity-reducing activity.

concentrations of 5 mg. per cent to 25 mg. per cent. When enzyme concentration was higher than 25 mg. per cent, the reaction proceeded too rapidly for accurate measurement, and when enzyme concentration was less than 5 mg. per cent the reaction was too slow. Nevertheless, the initial slope of reaction curves appears to be a suitable measure of X-activity over the working range of enzyme concentration.

In all reaction curves the activity of X-enzyme appeared to be less than the activity of the whole enzyme preparation. This was probably due to partial inactivation of X-enzyme by the acid-cold treatment for inactivating alpha-amylase, as alpha-amylase itself showed little or no viscosity-reducing activity. However, the possibility that alpha-amylase, or some other constituent of the bacterial preparation, may inhibit X-enzyme should not be overlooked. It was therefore decided to isolate X-enzyme while studies on the bacterial preparation were in progress, so that a similar procedure could be developed and applied to isolation of viscosity-reducing enzymes in barley and green malt, if it should become necessary to study these enzymes in the absence of amylases. The necessity for isolating viscosity-reducing enzymes of barley and green malt did not arise during the studies reported in this thesis, but the isolation of bacterial X-enzyme did prove useful in identifying some gum-degrading enzymes of barley and green malt, and it may prove even more useful if kinetic studies of cytolytic enzymes are undertaken in the future.

Before isolation procedures could be investigated, it was first necessary to determine the stability of viscosity-reducing enzymes in the bacterial preparation to see if special procedures

were required to stabilize these enzymes during the fractionation process.

The stability of a 50 mg. per cent solution of Wallerstein's alpha-amylase was therefore determined for a storage period of one month at room temperature and at 7°C. The enzyme solution was divided into two portions, one of which was treated to inactivate alpha-amylase. Half of each portion was stored in a refrigerator at 7°C., and half at room temperature. At intervals over a period of one month a small portion of each solution was removed, warmed to room temperature, diluted 10-fold, and the viscosity-reducing activity of the diluted enzyme solution was determined. Reaction mixtures contained 1 ml. of enzyme and 9 ml. of a 1 per cent aqueous solution of boiled gum. Table IV shows that the intact enzyme solution was stable for more than a month under both storage conditions. The enzyme solution treated to inactivate alpha-amylase was stable for about 3 weeks at room temperature and for more than a month at 7°C. Such stability in a heat labile enzyme is surprising. Apparently, then, no special precautions are necessary to ensure the stability of X-enzyme in a 50 mg. per cent solution of Wallerstein alpha-amylase.

TABLE IV

Stability of Bacterial X-Enzyme

Time (days)	Activity of Intact Enzyme		Activity of Acid-Cold Treated Enzyme	
	Storage at Room Temperature	Storage at 7°C.	Storage at Room Temperature	Storage at 7°C.
1	1.60	1.60	-	-
5	-	-	1.17	1.17
7	-	1.47	-	1.07
10	1.49	-	0.98	-
11	-	1.68	-	1.47
14	1.47	1.45	1.17	1.27
17	1.49	-	1.17	-
18	-	1.52	-	1.29
24	1.46	1.45	1.09	1.25
33	1.55	-	1.01	-
34	-	1.57	-	1.34

Isolation of Bacterial X-Enzyme

Column chromatography appeared to be the most promising method for the proposed isolation of X-enzyme. Accordingly, studies were made of various adsorbents and eluting agents commonly used in this type of chromatography. Activated alumina proved to be a suitable adsorbent for both alpha-amylase and X-enzyme, and phosphate buffer appeared to elute X-enzyme preferentially. Hence optimum conditions for the preferential elution of X-enzyme were determined, and the stability of purified X-enzyme was investigated. A detailed account of these investigations is presented next.

The first requirement in column chromatography is a satisfactory material to form the stationary medium. This material should adsorb from solution all, or all but one, of the components to be separated,

while permitting the solvent to pass through the column. Three factors influenced the choice of adsorbents. First, as alpha-amylase is one of the components of the bacterial enzyme, it was thought that insoluble starch, which in solution is a substrate for alpha-amylase, might adsorb this enzyme. Similarly, as cellulose is a substrate for cellulase, which may be a component of the cytolytic enzyme system, macerated filter paper might adsorb cellulase. Finally, as Lüers and Volkamer (23) used alumina to adsorb their xylanase, alumina might also be a suitable adsorbent for viscosity-reducing enzymes. Accordingly, each of these materials was tested for adsorptive capacity by adding 50 ml. of a 50 mg. per cent aqueous solution of Wallerstein bacterial enzyme preparation to the large column containing the adsorbent, developing the column with distilled water, collecting 25 ml. fractions then testing the effluents for alpha-amylase and viscosity-reducing activities. As it was laborious to test all effluents for enzymatic activity, a method was first developed for detecting potentially active components in effluents by measuring their absorption in a Beckman spectrophotometer against a blank containing pure solvent.

The absorption spectrum of a 50 mg. per cent aqueous solution of the bacterial enzyme is shown in Fig. 9. There was only one maximum, at 265 mμ. Experience proved that enzymatically active components always exhibited high absorption at this wave length. This is not surprising, as strong absorption in the ultra violet is characteristic of most proteins. This phenomenon made it possible to construct chromatograms showing the optical density of each fraction collected.

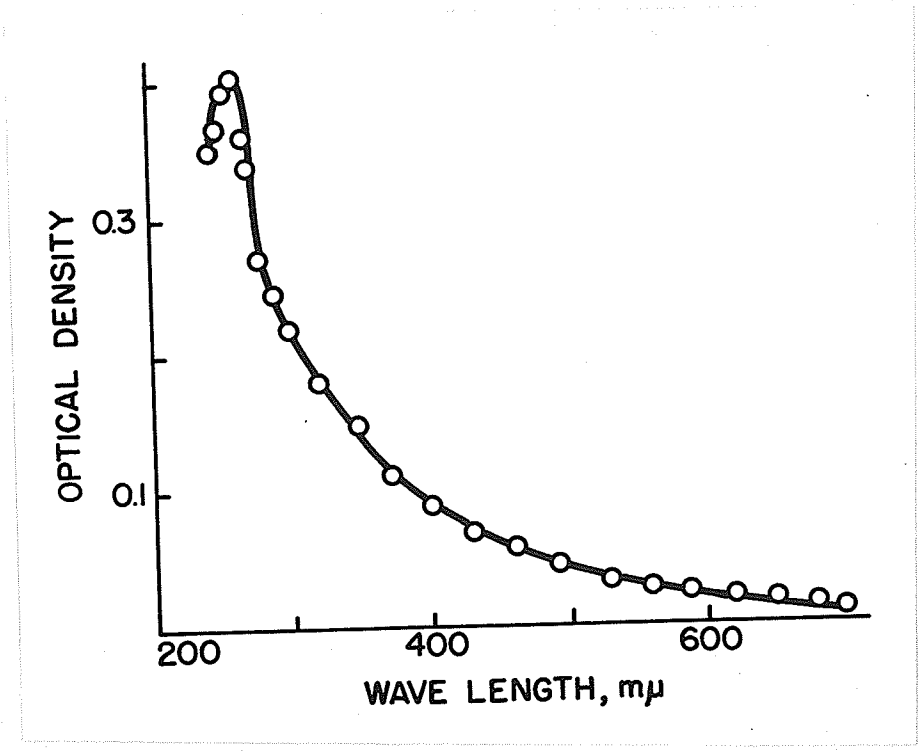

Figure 9.

Absorption spectrum of a 50 mg. per cent solution of Wallerstein alpha-amylase.

Those fractions having optical densities of 0.1 or higher were considered to contain potentially active components. Adjacent fractions showing optical densities greater than 0.1 were then combined, and the combined effluents were tested for enzymatic activity. All effluents were adjusted to pH 6.0 by the addition of a minimum amount of hydrochloric acid or sodium hydroxide before testing for enzymatic activity.

Both starch and cellulose failed to adsorb alpha-amylase and X-enzyme. Fig. 10 shows that, with starch, potentially active material appeared in the effluent immediately, and the largest part of it was removed by about 125 ml. of water. There was considerable tailing, suggesting weak retention, but all fractions showed both alpha-amylase and X-activity. Fig. 11 shows that macerated filter paper apparently retarded the enzymes, but again water alone eluted both enzymes together. The total enzymatic activities eluted from both adsorbents were approximately equal to the total enzymatic activities added to the column. Possibly some pre-treatment of these two adsorbents may render them satisfactory for chromatography, but this proved to be unnecessary.

When alumina was used as adsorbent the enzymes were not eluted by water. Fig. 12 shows that water removed no potentially active fraction. Later batches of alumina surrendered one such fraction, but it proved to be enzymatically inactive. Alumina was therefore selected as the adsorbent for chromatography.

The next requirement in column chromatography is a developer or eluting agent that will elute the components differentially. Eluting agents are frequently aqueous solutions of electrolytes, and their eluting power apparently depends on ionic strength and pH.

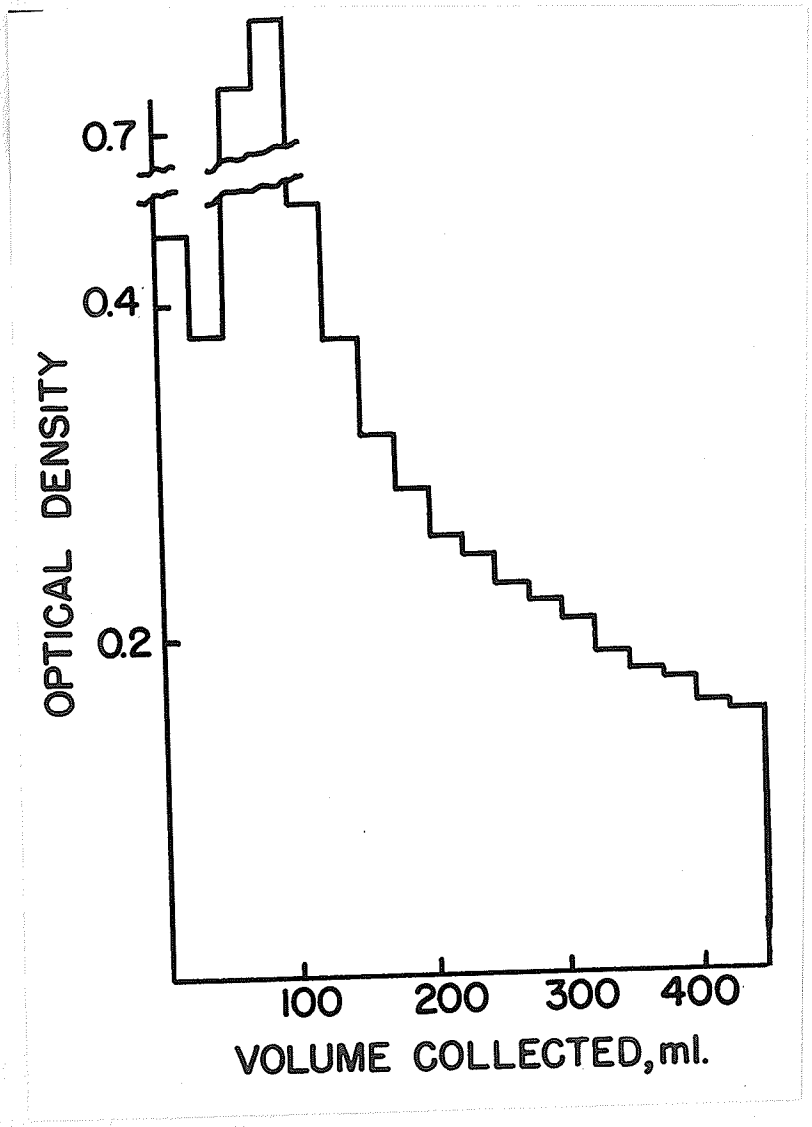

Figure 10.

Chromatogram of elution by water through a starch column.

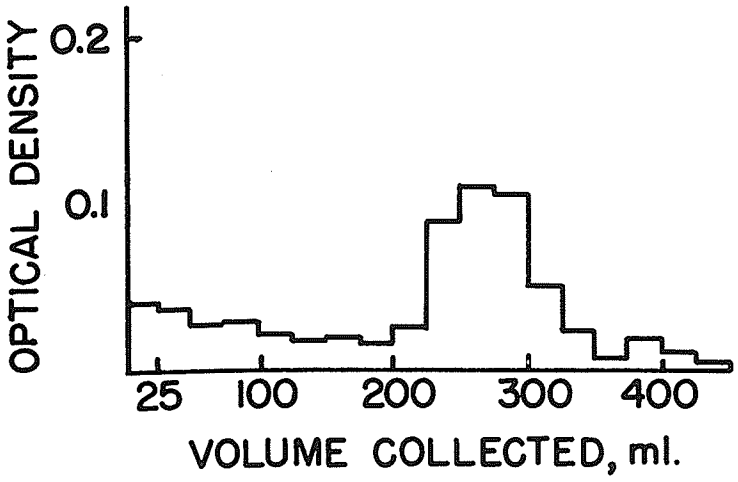

Figure 11.

Chromatogram of elution by water through a cellulose column.

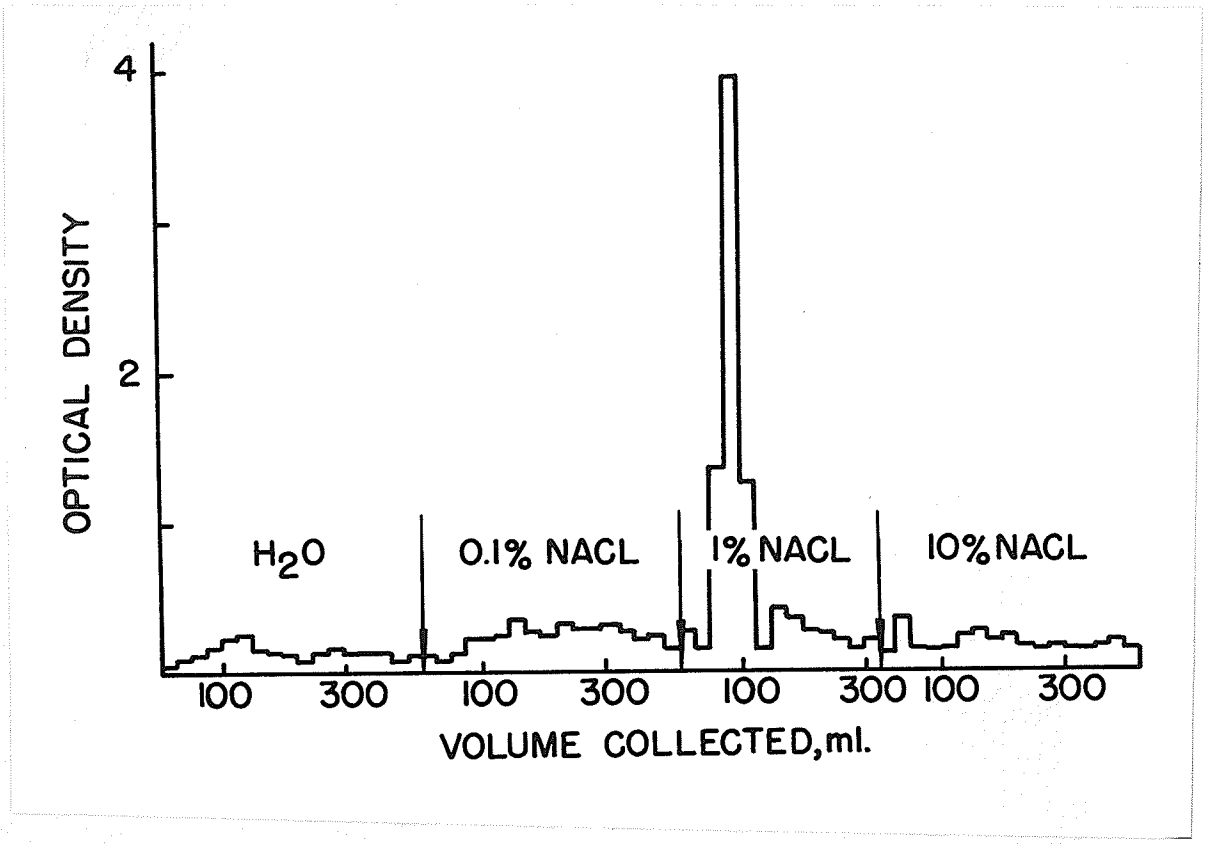

Figure 12.

Chromatogram of elution by water and aqueous sodium chloride solutions through an alumina column.

The simplest type of eluting agent is a salt such as sodium chloride, as only ionic strength of salt solutions can be varied. Accordingly, elution of enzymes was attempted with salt solutions of increasing concentration. Fig. 12 shows that only one potentially active fraction was eluted by sodium chloride of 1 per cent concentration. This fraction was enzymatically inactive, and no other fractions were eluted even by 10 per cent sodium chloride. It must be concluded, then, that sodium chloride fails to elute the enzymes.

Phosphate buffer was used next, so that both pH and ionic strength could be varied. Lüers and Volkamer (23) found that elution of their xylanase from alumina was most effective with phosphate buffer of pH 8.3. When 0.1 M phosphate buffer of pH 8.3 was used as eluting agent, three fractions were obtained, as shown in Fig. 13. The first two fractions were enzymatically inactive, but the third fraction showed both alpha-amylase activity and X-activity. Thus 0.1 M phosphate buffer elutes both enzymes. Higher concentrations of phosphate buffer fail to elute any additional fractions.

Normally, the next step would be to test lower concentrations of phosphate buffer, but it was decided to see first if a starch solution would elute alpha-amylase. At this stage the smaller column was used to reduce experimental time. One ml. of a 1 per cent enzyme solution (10 mg.) was passed into the column, followed by 25 ml. of a 2 per cent buffered starch solution prepared as for determination of alpha-amylase activity. The column was then washed with distilled water until effluents were free of starch, as indicated by the iodine reaction.

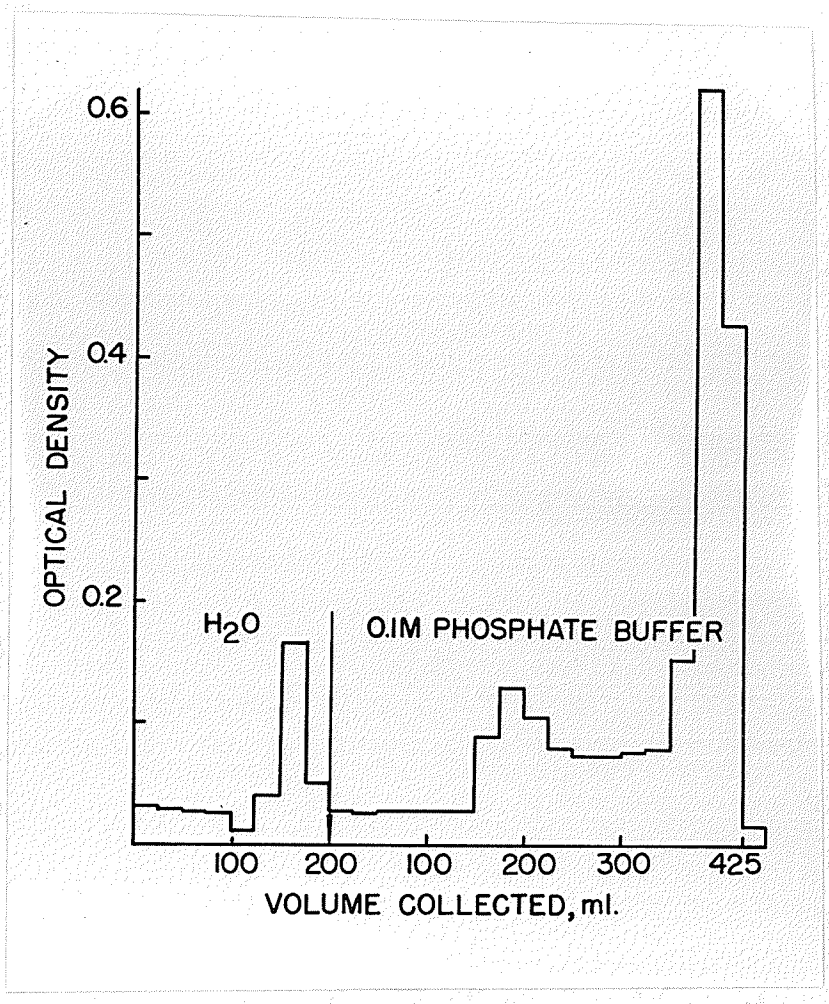

Figure 13.

Chromatogram of elution by water and phosphate buffer through an alumina column.

About 1100 ml. of water were required to free the column of starch. Enzymes were then eluted with 0.1 M phosphate buffer of pH 8.3, and total enzymatic activities of the effluents were determined after effluents had been adjusted to pH 6.0. As the total activities of the enzymes originally present in the column were known, it was possible to calculate per cent recoveries. The claculated recovery of X-enzyme was 45 per cent, whereas recovery of alpha-amylase was 7 per cent. It thus appears that the starch removed about 90 per cent of alpha-amylase and about 50 per cent of X-enzyme from the column, although it is also possible that washing with starch made the alpha-amylase non-extractable by the phosphate buffer, and that the 50 per cent of X-activity not recoverable by elution with phosphate buffer is lost through causes other than elution by starch solution. Later work showed that approximately 50 per cent of X-activity appears to be the maximum recovery by chromatography, so that a preparatory elution with starch is not nearly as wasteful as might appear at first glance. This experiment also indicated the interesting possibility that a preliminary fractionation of enzymes by chromatography can be achieved by developing a column with successive solutions of pure substrates.

The need for a preliminary washing with starch solution disappeared when lower concentrations of phosphate buffer were used for development. Fig. 14 shows that as buffer concentration decreased X-enzyme was eluted preferentially to alpha-amylase. With 0.005 M phosphate buffer no detectable amount of alpha-amylase was eluted, and recovery of X-enzyme was about 32 per cent. Phosphate buffer, of 0.005 M concentration, is therefore a satisfactory eluting agent.

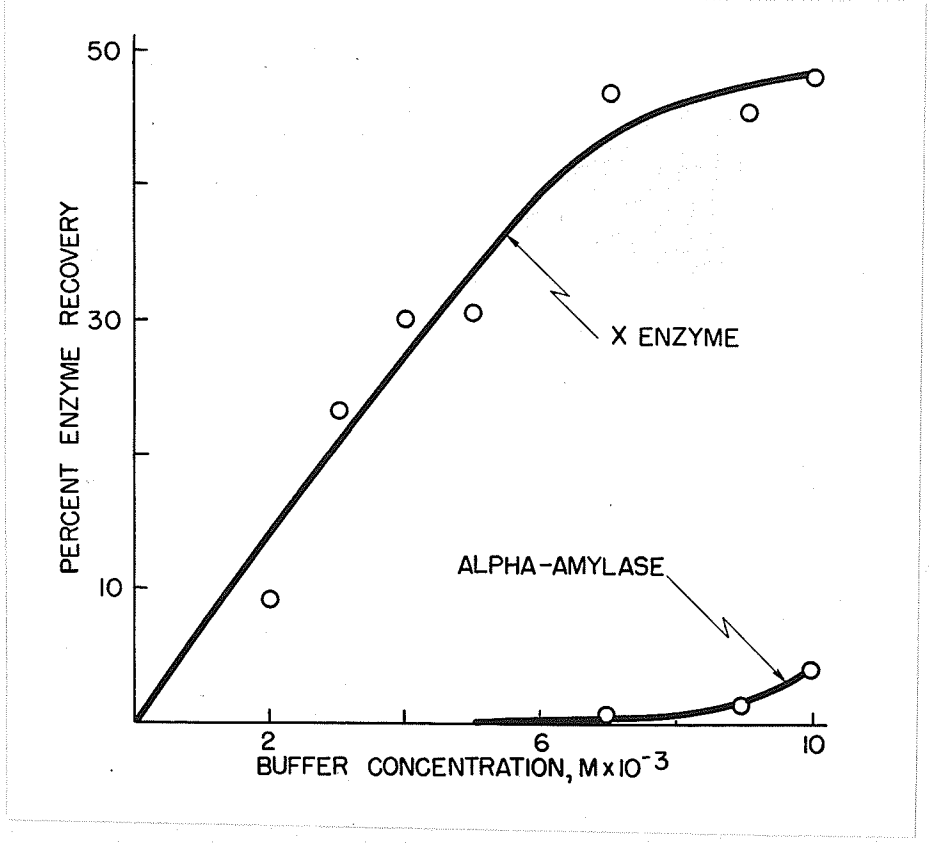

Figure 14.

Effect of phosphate buffer concentration on elution of alpha-amylase and X-enzyme from an alumina column.

The effect of the pH of 0.005 M phosphate buffer on recovery of X-enzyme was investigated next. Ten milligrams of the bacterial preparation were added to the small column, which was washed with water and developed with 0.005 M phosphate buffer. Effluents were collected in 10 ml. portions, and effluents having optical densities of 0.05 or higher were combined, adjusted to pH 6.0, and tested for alpha-amylase and X-activities. Enzymes were eluted by 40 to 80 ml. of phosphate buffer, and subsequent effluents showed little or no enzymatic activity. Fig. 15 shows that recovery of X-enzyme was very low at extreme pH's and high between pH 6.5 and pH 8.0. A maximum recovery of about 40 per cent was obtained at pH 6.5.

The capacity of the small column was determined by chromatographing 100 mg. and 400 mg. of the bacterial preparation with 0.005 M phosphate buffer of pH 6.5. Alpha-amylase recoveries were zero and 0.05 per cent. Hence the column can accommodate between 100 mg. and 400 mg. of Wallerstein's alpha-amylase preparation. One hundred milligrams appear to be a safe maximum.

The viscosity-reducing activities of all chromatographically purified X-enzyme preparations were almost completely destroyed by heating at 70°C. for 15 minutes. Hence X-enzyme is heat labile, as was concluded earlier (page 56).

Finally, a chromatographically purified X-enzyme preparation, having viscosity-reducing activity equal to that of a 7.6 mg. per cent solution of the intact bacterial preparation, was tested for stability at room temperature and at 7°C. Table V shows that purified X-enzyme is fairly stable for about two weeks at room temperature and is considerably less stable at 7°C. Purified X-enzyme is therefore less stable than the crude preparation, as might be expected. Nevertheless its stability at room temperature is still remarkable.

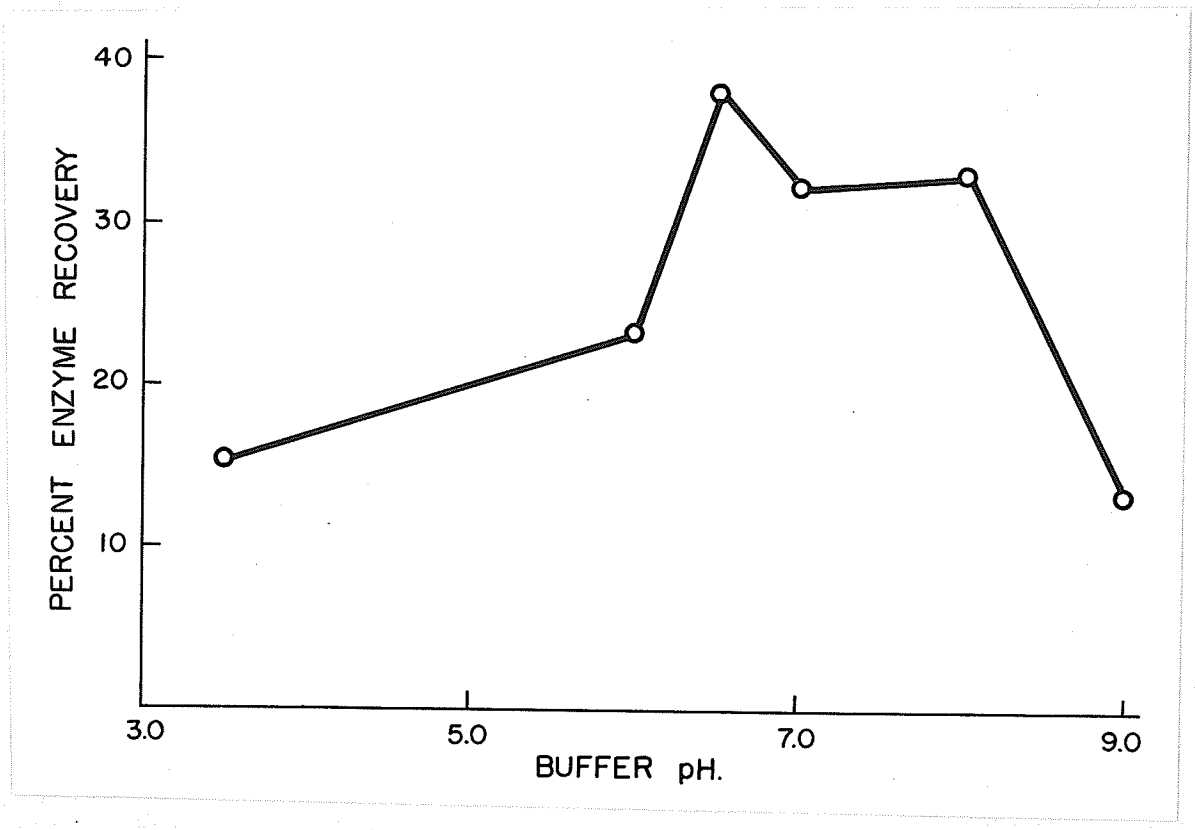

Figure 15.

Effect of the pH of a 0.005 M phosphate buffer on elution of X-enzyme from an alumina column.

TABLE V

Stability of Purified X-Enzyme

Time (days)	Activity	
	Storage at Room Temperature	Storage at 7°C.
0	1.16	1.16
3	1.13	-
7	1.19	0.94
11	1.22	0.88
14	1.22	0.84
19	0.97	-
20	-	0.83
48	1.01	0.78

STUDIES OF BARLEY AND GREEN MALT

In experiments described in this section, cytolytic enzymes of barley and green malt were isolated and studied after three new barley gum preparations were screened as potential substrates for viscosity-reducing enzymes. Enzymes of Montcalm barley and green malt were extracted and precipitated by procedures that were developed with the use of a commercial green malt. Activity-pH relations of extracts and precipitates were determined, and partially purified enzyme concentrates were prepared. Finally, some gum-degrading enzymes of barley and green malt were identified, and the existence of others was postulated.

Enzyme Preparations from Barley and Green Malt

Methods of preparing barley and green malt extracts, of precipitating barley and green malt enzymes, of preparing enzyme solutions, and of preparing reaction mixtures for determinations of viscosity-reducing activities are described next.

Extracts of green malt were prepared by two methods, by maceration in a Waring blendor or by mashing at room temperature, and barley extracts were prepared only by maceration. In the first method, 50 g. of coarse-ground green malt (or barley grist) were suspended in 100 ml. of distilled water and macerated in a Waring blendor for 2 minutes. The mixture was centrifuged and the supernatant filtered by suction through celite. The spent grains were then resuspended in 100 ml. of water, and again macerated for 2 minutes. The mixture

was centrifuged, the supernatant filtered as before, and the two filtrates were combined. In the second method, 50 g. of course-ground green malt were mashed with 200 ml. of distilled water for 2 hours at room temperature. The mash was filtered through E & D 509 x 32 cm. fluted filter paper. The first 100 ml. of filtrate were returned to the filter, and filtration was then continued for 2 hours.

Various protein precipitants were investigated to determine the most satisfactory enzyme precipitant, and a standard procedure for precipitating enzymes of barley and green malt was developed. Three volumes of alcohol, or graded amounts of ammonium sulphate, magnesium sulphate, or trichloroacetic acid were added to extracts of commercial green malt, and precipitates were removed by centrifugation then washed by solvent exchange with alcohol, acetone, and ether. All fractionations and washings were performed at room temperature. Ammonium sulphate appeared to be the most satisfactory precipitant of X-enzyme, and organic solvents inactivated this enzyme. Hence the precipitation procedure was standardized as follows. An extract of barley or green malt was made half saturated with ammonium sulphate by adding one volume of saturated aqueous ammonium sulphate solution. The precipitate was removed by centrifugation, washed once with half saturated ammonium sulphate solution, then dried <u>in vacuo</u> over phosphorous pentoxide. The residual liquor was saturated with ammonium sulphate by adding the solid salt, and the precipitate was collected, washed once with saturated ammonium sulphate solution, and dried as before. For convenience, the half saturated ammonium sulphate precipitate is referred to as P_1, and the saturated ammonium sulphate precipitate, P_2.

Solutions of ammonium sulphate precipitates were first prepared by mixing precipitate and water in a Burrell Wrist Action Shaker for 20 minutes at room temperature. As there was some doubt whether X-enzyme was completely dissolved by this procedure, later enzyme solutions were prepared in 20 ml. glass homogenizers. Solutions of enzyme concentrates were also prepared in 20 ml. glass homogenizers.

Three substrates were used in experiments described in this section, and the composition of reaction mixtures used in determining viscosity-reducing activity was varied as mentioned earlier in the section on General Methods. For evaluation of substrates, reaction mixtures consisted of 7 ml. of 0.7 per cent gum, 2 ml. of McIlvaine buffer (pH 5.0), and 1 ml. of enzyme solution. In determining viscosity-reducing activities during preliminary experiments on isolation of green malt enzymes, reaction mixtures contained 9 ml. of 0.6 per cent stabilized gum and 1 ml. of enzyme solution. In determining activity-pH relations, reaction mixtures contained 7 ml. of 0.7 per cent papain gum, 2 ml. of McIlvaine buffer, and 1 ml. of enzyme solution. For subsequent experiments on isolation of barley and green malt enzymes, reaction mixtures contained 7 ml. of 0.7 per cent stabilized gum, 2 ml. of McIlvaine buffer (pH 4.5), and 1 ml. of enzyme solution. Finally, viscosity-reducing activities of enzyme concentrates were determined using 7 ml. of 0.7 per cent papain gum or "beta-glucosan", 2 ml. of McIlvaine buffer (pH 4.5), and 1 ml. of enzyme solution.

Evaluation of New Substrates

One new barley gum preparation became available before studies of barley and green malt were initiated, and two more barley gums were made available as these studies progressed. The three gums, stabilized

gum, papain gum, and "beta-glucosan", were screened for use as potential substrates for viscosity-reducing enzymes. The barley gums were screened, as outlined previously in the section on General Methods, by analysis for nitrogen, sugars, starch, viscosity, and stability of viscosity, and by determination of susceptibility to enzymatic attack. In addition, stabilized gum and papain gum were analyzed for amino acid content.

Analysis. Table VI shows the analytical data for stabilized gum, papain gum, and beta-glucosan". Yields were considerably lower than in earlier gum preparations, but the quality appeared higher. Thus, nitrogen content was very low in stabilized gum and papain gum, and there was no nitrogen in the "beta-glucosan". The low nitrogen content of papain gum and stabilized gum could not be attributed to experimental error as these gums yielded detectable amounts of amino acids by chromatography after acid hydrolysis. It has been suggested (28) that this small amount of nitrogenous material may be an integral part of the whole gum - a cement that holds the polysaccharide together. Convincing evidence of a protein-carbohydrate linkage is lacking, however, and will be difficult to provide.

The sugar compositions of stabilized gum and papain gum appeared similar, and the presence of galactose, not observed in previous preparations, may indicate a higher state of molecular aggregation. The "beta-glucosan" showed a complete absence of nitrogen and of sugars other than glucose. This gum appears to be a pure poly-glucoside, and the absence of a characteristic starch-iodine color suggests that the glucose residues may be linked in the beta positions. Later evidence

TABLE VI

Analytical Data on New Barley Gum Preparations

Gum	Yield	Nitrogen Content	Amino Acid Composition	Sugar Composition	Stability of Viscosity	Starch Test
	% of barley	% of gum			%	
Stabilized gum	0.85	0.29	alanine glutamic acid glycine serine aspartic acid arginine lysine	galactose glucose arabinose xylose	13.7	+
Papain gum	1.38	0.08	glutamic acid glycine serine lysine	galactose glucose arabinose xylose	0	+
"Beta-glucosan"	0.98	0	-	glucose	0	-

confirmed this conclusion. Stabilized gum produced the blue color with iodine characteristic of starch, and this preparation may therefore be contaminated with a small amount of starch. Papain gum produced a plum color with iodine that is quite different from the colors given by starch or its degradation products. It is suggested that this color is due to some material resembling, but not identical to, starch. The available evidence appears to favor this view. Aqueous solutions of all three gums had high viscosities, indicating a high degree of polymerization, as might be expected for naturally occurring materials extracted in essentially undegraded form. Fig. 16 shows the relation between viscosity and concentration for the three gums. Finally, the most significant factor illustrating the superiority of these gums over earlier preparations is the remarkable degree of stability (Table VI) of the more recent barley gums. Stabilized gum had a solution viscosity considerably more stable than that of boiled gum, whereas papain gum and the "beta-glucosan" were stable after standing for 24 hours. This is a most desirable feature, as with stable substrates viscosity decrease is a precise and accurate measure of enzymatic activity. Thus papain gum and "beta-glucosan" appear to be satisfactory substrates for studies of X-enzyme.

Susceptibility to Enzymatic Attack. The viscosity-reducing activities of various enzymes on stabilized gum, papain gum, and "beta-glucosan" are shown in Fig. 17 to Fig. 19. Enzyme solutions in all experiments were: 4 mg. per cent aqueous solutions of Wallerstein alpha-amylase, the same solutions first treated to inactivate alpha-amylase, the same solutions first treated to inactivate X-enzyme, and 0.1 per cent aqueous solutions of Wallerstein beta-amylase preparation.

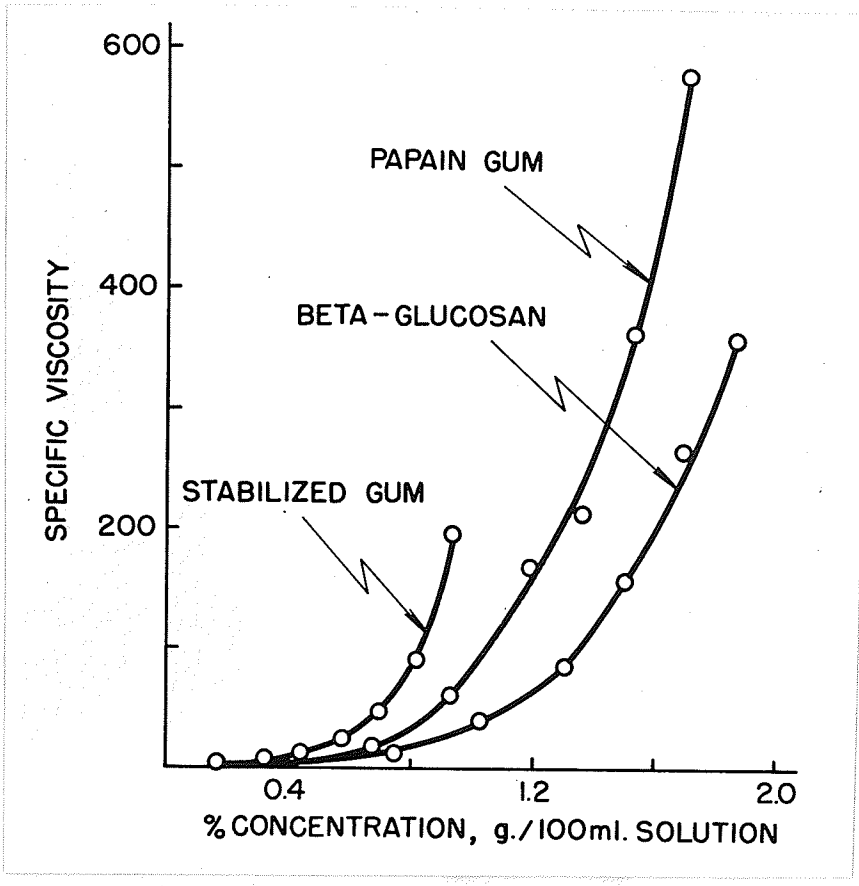

Figure 16.

Effect of concentration on viscosity of aqueous solutions of stabilized gum, papain gum, and "beta-glucosan".

All curves were linear for periods exceeding 1 hour, and the viscosity-reducing activities of alpha- and beta-amylase were negligibly small. On stabilized gum and "beta-glucosan", the activities of the intact alpha-amylase preparation were considerably higher than the activities of the preparation treated to inactivate alpha-amylase, as was observed earlier in experiments involving the use of boiled endosperm gum (page 60). However, the activities of the two enzyme solutions on papain gum were approximately equal, hence the difference in activity on stabilized gum and "beta-glucosan" appeared to be due to partial inactivation of X-enzyme by the acid-cold treatment, and not to inhibition of X-enzyme by alpha-amylase. This conclusion is supported by the data of Table IV, which show that the initial activity of an acid-cold treated enzyme solution was lower than the activity of an untreated enzyme solution of the same concentration. The fact that little or no inactivation of X-enzyme was observed in the experiment on papain gum may be explained by assuming that slight differences in the procedure for inactivating alpha-amylase may affect the degree of inactivation of X-enzyme.

All three potential substrates reacted similarly with extracts of green malt. Solution viscosities of the barley gums were markedly reduced by green malt extracts diluted 10-fold, and reaction curves were linear for periods exceeding 1 hour. The initial slope of reaction curves was a measure of viscosity-reducing activity, and viscosity-reducing activity was directly proportional to enzyme concentration over a wide range of enzyme concentration. Extracts of green malt (containing calcium ions to protect malt alpha-amylase from heat inactivation), heated at 70°C. for 15 minutes, had little or no effect on the viscosity of aqueous barley gum solutions.

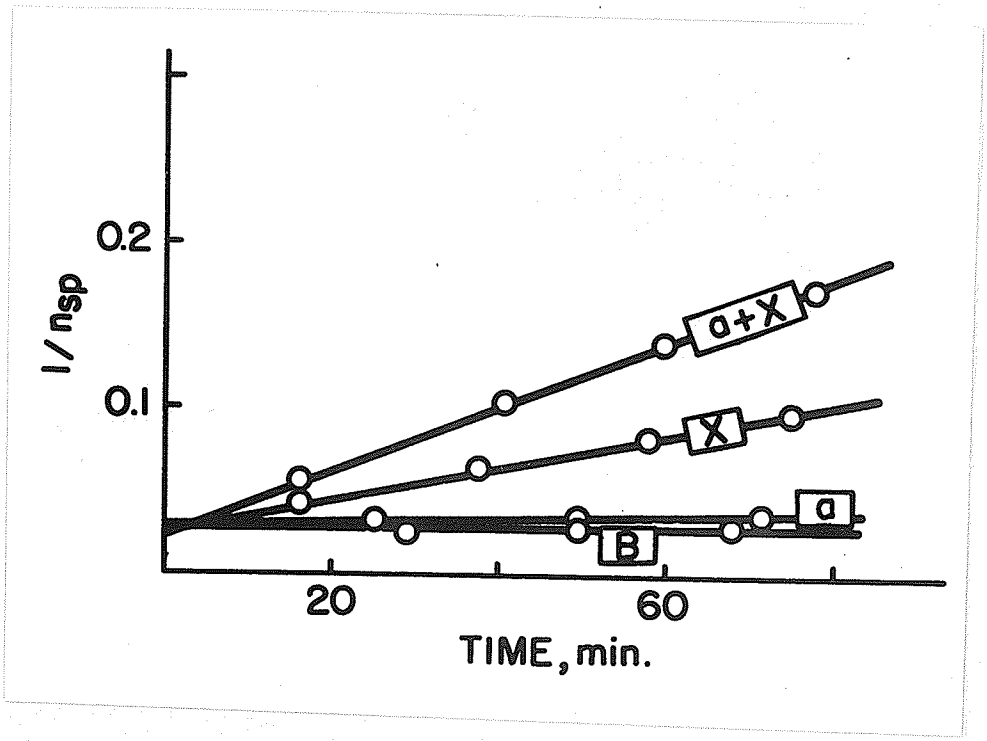

Figure 17.

Comparison of the activities on stabilized gum of the Wallerstein alpha-amylase preparation, with different pre-treatments, and of the Wallerstein beta-amylase preparation.

a = alpha-amylase; B = beta-amylase; X = X-enzyme.

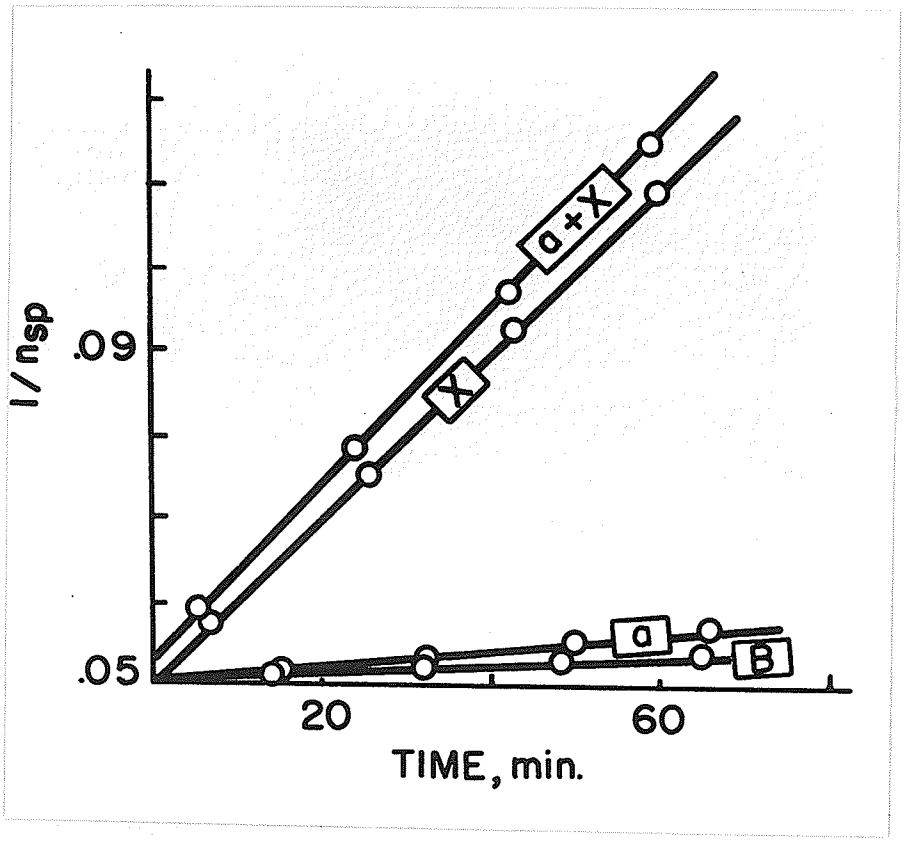

Figure 18.

Comparison of the activities on papain gum of the Wallerstein alpha-amylase preparation, with different pre-treatments, and of the Wallerstein beta-amylase preparation.

a = alpha-amylase; B = beta-amylase; X = X-enzyme.

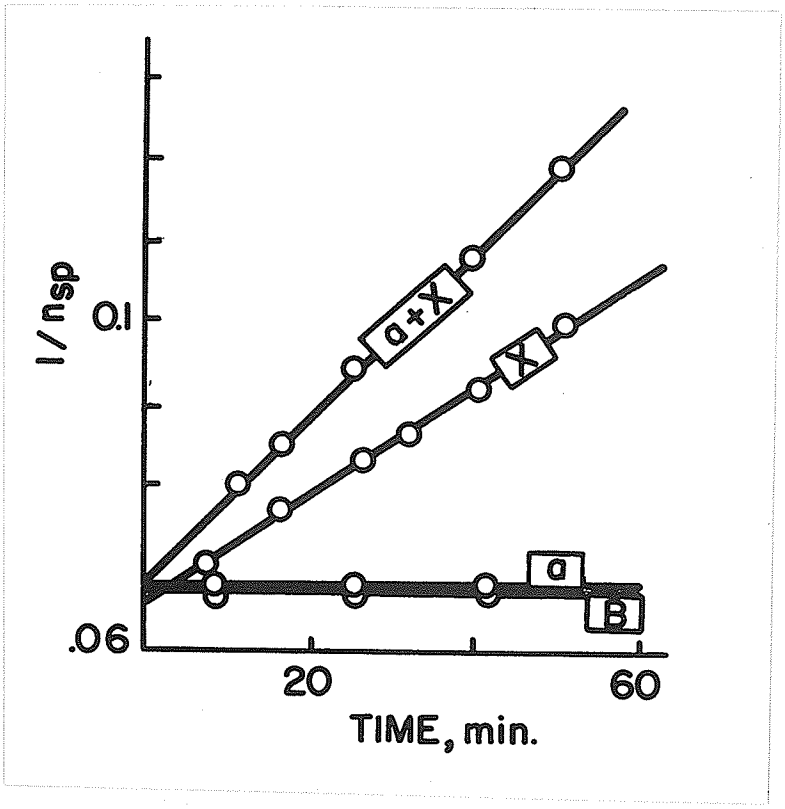

Figure 19.

Comparison of the activities on "beta-glucosan" of the Wallerstein alpha-amylase preparation, with different pre-treatments, and of the Wallerstein beta-amylase preparation.

a = alpha-amylase; B = beta-amylase; X = X-enzyme.

Fig. 20 shows the relation between activity and enzyme concentration, with stabilized gum as substrate. In this experiment, a portion of a commercial green malt extract was diluted with distilled water to give solutions containing 20, 40, 60, and 80 per cent of the extract by volume, and viscosity-reducing activities of each solution and of a water blank were determined. The remainder of the extract was stored overnight in a refrigerator, diluted to yield solutions containing 5, 10, 20 and 25 per cent of extract, and activities of the solutions and of a water blank were determined. Fig. 20 shows that activity-concentration curves are linear for both concentration ranges, and both curves can be extrapolated to pass through the origin. The curve for the stored enzyme solution is slightly below the curve for the freshly prepared enzyme solution, but the difference between them (less than 8 per cent) can probably be explained by a slight loss in activity during storage. Hence it is reasonable to assume that there is a direct proportionality between activity and enzyme concentration over a much wider range of enzyme concentration than is likely to be realized if conditions for maximum accuracy in determining enzymatic activities viscosimetrically (page 30) are observed.

These experiments show that stabilized gum, papain gum, and "beta-glucosan" are superior to earlier gum preparations as substrates for viscosity-reducing enzymes. The nitrogen content of stabilized gum and papain gum is low, and "beta-glucosan" contains no nitrogen. Stabilized gum solutions were more stable in viscosity than boiled endosperm gum, and the other two preparations produced aqueous solutions

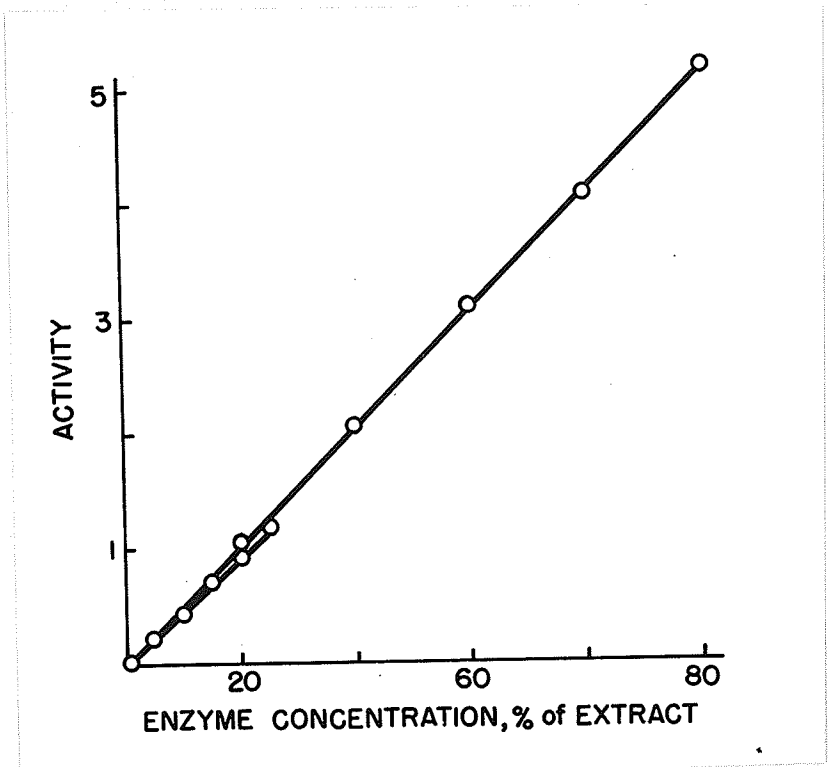

Figure 20.

Effect of enzyme concentration on viscosity-reducing activity.

whose viscosity remained unchanged after 24 hours storage. The viscosity of aqueous solutions of the three gums was high, suggesting that relatively little enzymatic degradation of gum occurred during extraction of the alcohol-treated barley grist. All three gums were extensively degraded by X-enzyme of the bacterial alpha-amylase preparation and of green malt in low concentration. In only one respect were stabilized gum and papain gum apparently inferior to boiled endosperm gum: aqueous solutions of these two gums produced a characteristic color with iodine, suggesting the presence of starch or a starch-like polysaccharide.

It is of interest, at this point, to speculate about the possible significance of this iodine color. It was suggested earlier in this section that iodine color may be due to a polysaccharide resembling, but not identical to, starch. Evidence for the existence of such a polysaccharide in barley gum was provided by the investigations of Gilles, Meredith, and Smith (17), who reported that the gum isolated by aqueous extraction of alcohol-treated barley (i.e., the precursor of stabilized gum) appeared to contain three components: a pentosan, a "poly-beta-glucosan", and a "poly-alpha-glucosan". The last component resembled, but was not identical with, amylopectin. This polysaccharide is the only component of the gum likely to produce a characteristic iodine color and to be susceptible to amylolysis. However, as amylases have little or no viscosity-reducing activities on barley gums, it appears that the "poly-alpha-glucosan" may be only a minor constituent of barley gum contributing little to viscosity of aqueous gum solutions. Moreover, this component may not be of cell wall origin, by analogy with a similar material (31) found in association with yeast cell wall materials.

Isolation and Some Properties of Barley and Green Malt Enzymes

Procedures for extraction and precipitation of X-enzyme from barley and green malt were developed with the use of commercial green malt. These procedures were then applied to isolate enzymes of Montcalm barley and green malt. Activity-pH relations for extracts and enzyme precipitates were determined next. Finally, enzyme concentrates were prepared from extracts and precipitates of Montcalm green malt.

Extracts of commercial green malt, prepared by maceration and by mashing at room temperature, were compared for total viscosity-reducing activity. Stabilized gum was the substrate for activity determinations, and extracts were diluted 10-fold before use. Table VII shows that the efficiency of enzyme extraction was approximately the same by both procedures, but the maceration procedure was much more rapid. This technique was therefore used in subsequent investigations.

TABLE VII

Comparison of Viscosity-Reducing Activities of Green Malt Extracts Prepared by Two Methods

	Trial I		Trial II	
	Mashing	Maceration	Mashing	Maceration
Activity of aliquot	0.91	0.73	0.97	0.77
Activity/ml. extract	9.1	7.3	9.7	7.7
Volume of extract, ml.	100	127	106	142
Total extract activity	910	927	1030	1090
Time of preparation, hours.	5	0.5	5	0.5

Various protein precipitants were compared next for ability to precipitate viscosity-reducing enzymes from extracts of commercial green malt. With magnesium sulphate or ammonium sulphate, one precipitate was obtained at 35 to 50 per cent saturation, and a second precipitate at 100 per cent saturation. With a saturated solution of trichloroacetic acid, one precipitate was obtained after addition of 5 ml. precipitant to 35 ml. extract, and no further precipitation occurred on subsequent additions of trichloroacetic acid. Alcohol produced a stringy brownish precipitate. All precipitates were washed by solvent exchange with alcohol, acetone, and ether. Viscosity-reducing activities were 5 to 12 per cent of initial extract activity, and ammonium sulphate precipitates were the most active.

Low enzymatic recoveries were attributed either to incomplete precipitation of enzymes or to inactivation of viscosity-reducing enzymes by the organic solvents. The latter possibility was investigated first. A fresh extract of commercial green malt was prepared, a small aliquot was removed for activity determinations, and the remainder was divided into four equal portions. The first portion was made 95 per cent of saturated with ammonium sulphate, and the precipitate was washed with a saturated solution of ammonium sulphate. An aliquot of the residual liquor was removed for activity determinations. The next two portions were treated in the same way, but the precipitate from one was washed with alcohol and the precipitate from the other was washed with acetone. The fourth portion was precipitated with alcohol, and the precipitate was washed with alcohol. Viscosity-reducing activities of the two liquors and four precipitates were then determined, using stabilized gum as substrate. The original extract

was high in viscosity-reducing activity, but the liquor remaining after precipitation with ammonium sulphate had negligible activity. Activity of the first ammonium sulphate precipitate was 85 per cent of theoretical, whereas activities of the second (alcohol-washed) and third (acetone-washed) precipitates were only 4 per cent and 12 per cent of theoretical. Activity of the alcohol precipitate was only 5 per cent of theoretical activity. There is little doubt, then, that alcohol inactivates viscosity-reducing enzymes at room temperature, and acetone has a similar effect. Hence washing precipitates with organic solvents is inadvisable. Ammonium sulphate appears to precipitate viscosity-reducing enzymes completely.

The ammonium sulphate precipitation of viscosity-reducing enzymes of Montcalm green malt was investigated next. As was observed in preliminary experiments on commercial green malt extracts, very small amounts of precipitate were obtained at low concentrations of ammonium sulphate. Hence only two fractions were collected, one precipitated from half saturated ammonium sulphate solution, and one precipitated from saturated ammonium sulphate solution. The first precipitate was washed once with a half saturated aqueous solution of ammonium sulphate, and the second precipitate was washed once with a saturated solution of ammonium sulphate. Both precipitates were dried <u>in vacuo</u> over phosphorous pentoxide. For convenience in the following and subsequent discussions, these precipitates are referred to as P_1 and P_2.

Viscosity-reducing activities of the original extract, precipitates, and liquors were determined with stabilized gum as substrate. Solutions of precipitates were prepared by mixing precipitate and distilled water for 20 minutes in a Burrell Wrist Action Shaker.

Table VIII shows that yields of precipitates were approximately the same, but later preparations showed greater variability. Recoveries of viscosity-reducing activity also appeared to be constant, but here too greater variability was observed in later experiments. In general, enzyme recoveries from saturated precipitates were satisfactory -- 70 per cent or higher -- but recoveries from half-saturated precipitates were low -- 15 to 35 per cent. Variability in yield is easily understood, as yield is probably affected by the amount of ammonium sulphate adhering to precipitates. But recoveries of viscosity-reducing activity should be high, as activities of residual liquors after removal of P_2 were usually less than one per cent of the original extract activity.

TABLE VIII

Ammonium Sulphate Precipitation of X-Enzyme from Extracts of Montcalm Green Malt

	Trial 1	Trial 2
Total extract activity	1100	1010
Yield of $\frac{1}{2}$ saturated precipitate, P_1, g.	0.138	0.144
Activity of $\frac{1}{2}$ saturated liquor	845	737
Theoretical activity of $\frac{1}{2}$ saturated precipitate	255	273
Observed activity of $\frac{1}{2}$ saturated precipitate	57.9	73.2
Recovery, %	23	27
Yield of saturated precipitate, P_2, g.	0.775	0.804
Activity of saturated liquor	2	2
Theoretical activity of saturated precipitate	843	735
Observed activity of saturated precipitate	641	534
Recovery, %	76	73
Total recovery, %	64	60

Poor enzyme recoveries were attributed to two possible causes: first, incomplete solubility of green malt precipitates, and second, differences in the pH of reaction mixtures. Accordingly, solutions of ammonium sulphate precipitates were prepared in 20 ml. glass homogenizers, and the effect of pH on viscosity-reducing activities of green malt extract and of ammonium sulphate precipitates was determined. As arbitrary units of activity were used, the activities of ammonium sulphate precipitates were multiplied by an appropriate constant so that their activities at pH 4.4 were equal to the activity of the green malt extract at pH 4.4. Thus the shapes of pH-activity curves could be compared without affecting the position of the pH optimum.

Figs. 21 to 23 show that the pH-activity curves for all three enzyme sources were strikingly similar, suggesting that the same viscosity-reducing enzyme was active in all enzyme solutions. The pH optimum was pH 4.7 to pH 4.8, and activity decreased markedly at lower or higher pH. Hence subsequent reaction mixtures were buffered at pH 4.7. This was accomplished when reaction mixtures contained 7 ml. of 0.7 per cent aqueous gum solution, 2 ml. of McIlvaine buffer solution (pH 4.5), and 1 ml. of enzyme solution.

Extraction and precipitation of Montcalm green malt enzymes were then repeated, and viscosity-reducing activities of the extract and precipitates were determined. Table IX shows that the new procedure apparently increased activities by a factor of about 26 over the values in the previous experiment, but percent enzymic recovery was the same. A recovery of approximately 60 per cent therefore appears to be the maximum obtainable under these conditions of

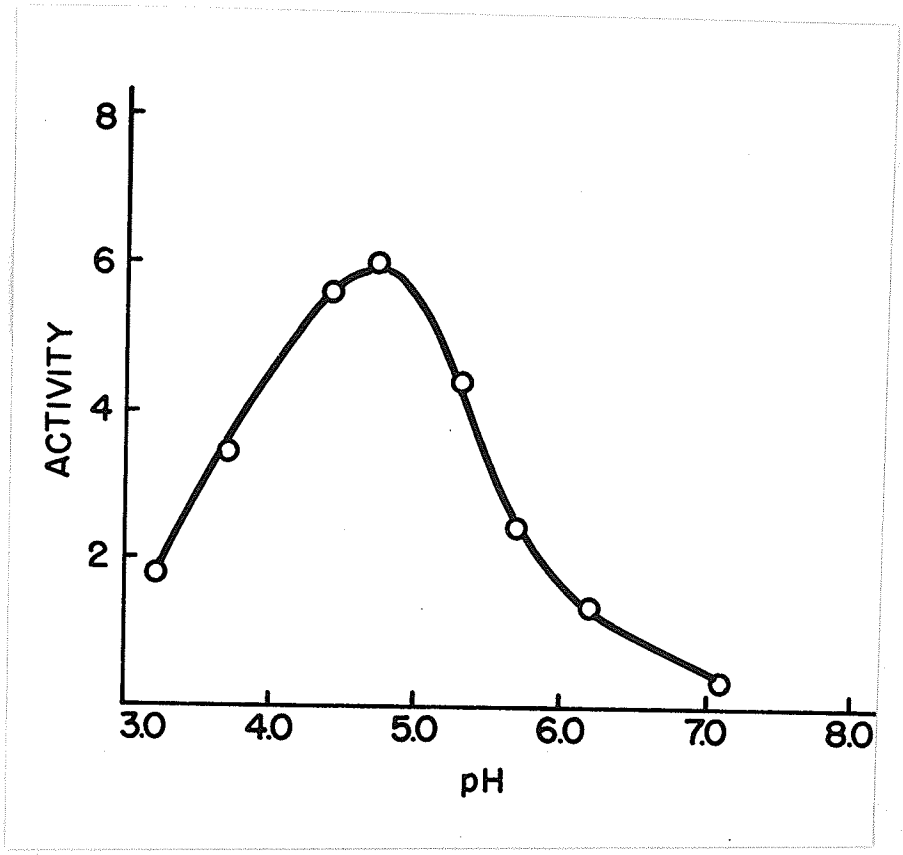

Figure 21.

Effect of pH on viscosity-reducing activity of an extract of Montcalm green malt.

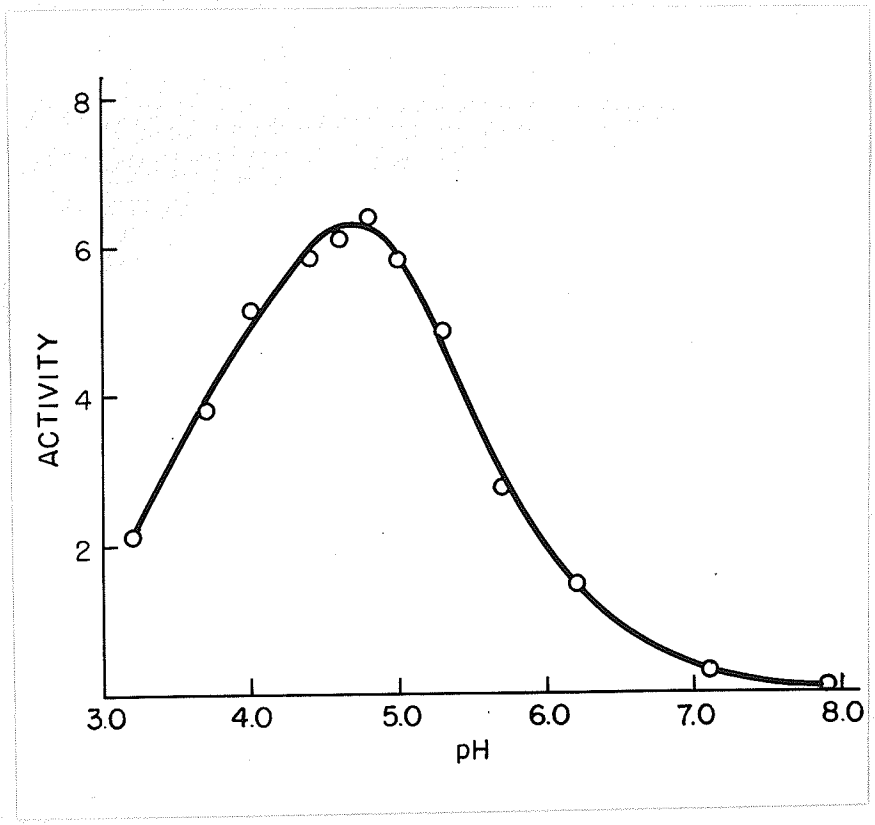

Figure 22.

Effect of pH on viscosity-reducing activity of the half saturated ammonium sulphate precipitate.

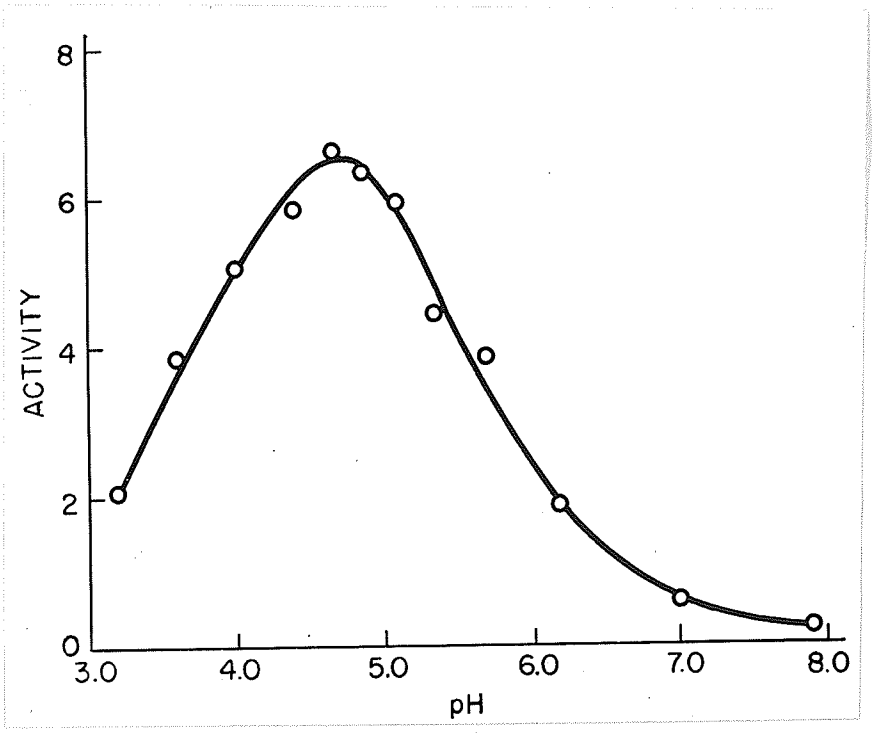

Figure 23.

Effect of pH on viscosity-reducing activity of the saturated ammonium sulphate precipitate.

extraction and precipitation. Ammonium sulphate precipitates suffered no appreciable loss of viscosity-reducing activity after storage for 6 months at room temperature.

TABLE IX

Recovery of Viscosity-Reducing Activity of Montcalm Green Malt by Ammonium Sulphate Precipitation

Total extract activity	28,600
Activity of $\frac{1}{2}$ saturated liquor	9,950
Theoretical activity of $\frac{1}{2}$ saturated precipitate	18,600
Observed activity of $\frac{1}{2}$ saturated precipitate	12,300
Recovery, %	66
Activity of saturated liquor	13
Theoretical activity of saturated precipitate	9,940
Observed activity of saturated precipitate	5,060
Recovery, %	51
Total recovery, %	61

Enzymes of Montcalm barley were then isolated by similar procedures. Barley extracts were prepared by maceration in a Waring blendor, and half saturated and saturated ammonium sulphate precipitates were collected, as before. Solutions of precipitates were prepared by homogenization, and reaction mixtures were buffered at pH 4.7. Table X shows the results of two experiments on isolation of barley enzymes. The first was unsatisfactory, as all viscosity-reducing activity was apparently removed by half saturation with ammonium sulphate, and recovery of this activity was only 27 per cent. The second determination resulted in 89 per cent recovery of viscosity-reducing activity.

TABLE X

Recovery of Viscosity-Reducing Activity of Montcalm Barley by Ammonium Sulphate Precipitation

	Trial 1	Trial 2
Total extract activity	350	270
Activity of $\frac{1}{2}$ saturated liquor	16	100
Theoretical activity of $\frac{1}{2}$ saturated precipitate	334	170
Observed activity of $\frac{1}{2}$ saturated precipitate	92	143
Recovery, %	28	84
Activity of saturated liquor	11	4
Theoretical activity of saturated precipitate	5	96
Observed activity of saturated precipitate	0	92
Recovery, %	0	96
Total recovery, %	27	89

The increase in viscosity-reducing activity from barley to green malt was very marked. If representative values of the viscosity-reducing activity of Montcalm barley and green malt are taken as 310 and 28,200, then the viscosity-reducing activity of green malt was about 90 times the activity of the barley.

Three enzyme concentrates were prepared and studied next. Two of these were prepared from extracts of Montcalm green malt, and one was prepared from green malt P_2. Viscosity-reducing activities of concentrates and source material were compared, and recoveries of activity were calculated.

The first concentrate was prepared by freeze-drying an extract of Montcalm green malt obtained from 50 g. green malt and 200 ml. water. Yield of freeze-dried product was about 5 g. The product was deliquescent, and dark brown in color. Its viscosity-reducing activity

on papain gum was 92 per cent of the activity of the extract. On storage in a sealed glass vial, this material hardened into a sticky, glossy mass and became unmanageable.

The second concentrate was prepared by freeze-drying an extract of Montcalm green malt that had first been dialyzed against tap water for 24 hours and filtered by suction through celite. The freeze-dried product was light tan colored and pulverulent. Its yield was 0.9 g. Activity of the dialyzed extract was about 64 per cent, and activity of the freeze-dried powder was about 37 per cent, of the activity of the extract. No further appreciable loss in activity of the freeze-dried powder could be detected after a storage period of 6 months. Loss of viscosity-reducing activity during dialysis was attributed to one, or to a combination of more than one, of three causes: first, partial enzymatic inactivation due to the 24-hour dialysis period; second, destruction of viscosity-reducing enzymes by other enzymes of green malt; third, removal of some dialyzable component of the viscosity-reducing enzyme system.

The third concentrate was prepared by freeze-drying a dialyzed solution of green malt P_2. This product was a light grey colored powder. Its yield was about 13 per cent, by weight, of the ammonium sulphate precipitate. The total viscosity-reducing activity of the freeze-dried product on either papain gum or "beta-glucosan" was about 50 per cent higher than the total activity of the ammonium sulphate precipitate, and no appreciable loss of activity could be detected after a storage period of 6 months. It thus appears that low recoveries of enzymatic activities from ammonium sulphate precipitates may be due largely to inhibition or reversible inactivation of X-enzyme by ammonium sulphate.

In addition, this experiment shows that there is little loss of viscosity-reducing activity during dialysis. Hence it is unlikely that the viscosity-reducing enzymes system contains a dialyzable component or that any appreciable inactivation of X-enzyme occurs during the 24-hour dialysis period. Therefore the chief factor in reducing activity in a stored extract of green malt appears to be enzymatic destruction of viscosity-reducing enzymes.

In brief summary of experiments described in this section, it appears that a concentrated, stable, and partially purified enzyme preparation from green malt may be prepared by freeze-drying a dialyzed aqueous solution of an ammonium sulphate precipitate of green malt extract. The yield of freeze-dried powder is about 0.3 per cent of the green malt, and the partially purified product contains most of the viscosity-reducing activity of the original extract. This product is therefore an excellent starting material for further purification procedures, such as chromatography. Dialysis, followed by freeze-drying, appears to be a convenient and safe method of preparing stable, solid isolates.

Identification of Some Gum-Degrading Enzymes of Barley and Green Malt

Two experiments were designed to provide information on the identity of enzymes that degrade barley gums. In the first, the effect of different enzyme preparations on the ability of stabilized gum and papain gum to produce characteristic colors with iodine was determined, and the enzymes responsible for destroying this ability were identified. In the second experiment, enzymic reaction products were determined by paper chromatography, and conclusions were drawn about the possible

identity of enzymes liberating these products. Some of the enzyme preparations described in the previous section, as well as the Wallerstein alpha- and beta-amylase preparations were used in comparisons designed to characterize some gum-degrading enzymes of barley and green malt.

The effect of various enzymes on the ability of stabilized gum and papain gum to form characteristic colors with iodine was determined first. As the iodine colors of these substrates can be attributed to their alpha-glucosan fraction[1], only those enzymes that degrade this fraction should destroy the ability of either gum to give characteristic iodine colors. Accordingly, solutions of stabilized gum and papain gum were incubated with various enzyme solutions for 24 hours at room temperature, and iodine colors were determined at intervals during the reaction period. Reaction mixtures contained 10 ml. of 1 per cent aqueous solution of gum, 10 ml. of enzyme solution, 2 ml. McIlvaine buffer (pH 4.5), and 1 drop of toluene to inhibit bacteria. Enzyme solutions were 2 per cent homogenates of ammonium sulphate precipitates from barley and green malt, 0.1 per cent Wallerstein alpha-amylase preparation, 0.1 per cent Wallerstein beta-amylase preparation, and a chromatographed bacterial X-enzyme solution with viscosity-reducing activity equal to that of a 0.1 per cent solution of Wallerstein alpha-amylase. Included also were the same solutions heated at 70°C. for 15 minutes and the Wallerstein alpha-amylase preparation treated to inactivate alpha-amylase.

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[1] Personal communication from Dr. F. Smith, Department of Agricultural Biochemistry, University of Minnesota, St. Paul, Minn.

Before green malt enzyme solutions were heated, calcium acetate was added in the proportion of 1 ml. of 2 per cent calcium acetate per 5 ml. of enzyme solution, to protect alpha-amylase from heat inactivation, and the amount of enzyme solution used was increased proportionately. All enzyme solutions were tested for alpha-amylase and viscosity-reducing activities before use. Table XI shows the active enzymes present in each enzyme preparation.

TABLE XI

Effect of Various Enzymes on the Ability of Stabilized Gum and Papain Gum to Yield Characteristic Iodine Colors

Enzyme Source	Enzyme Composition			Iodine Color	
				Stabilized Gum	Papain Gum
	α-amylase	β-amylase	X-enzyme		
Water	-	-	-	blue	plum
Barley, $\frac{1}{2}$ saturated precipitate, P_1	-	+	+	yellow	yellow
Barley P_1, heated	-	-	-	blue	plum
Green malt, $\frac{1}{2}$ saturated precipitate, P_1	+	+	+	yellow	yellow
Green malt P_1, heated	+	-	-	yellow	yellow
Green malt, saturated precipitate, P_2	+	+	+	yellow	yellow
Green malt P_2, heated	+	-	-	yellow	yellow
Wallerstein alpha-amylase	+	-	+	yellow	yellow
Wallerstein alpha, heated	+	-	-	yellow	yellow
Wallerstein alpha, acid-cold	-	-	+	blue	plum
Chromatographed X-enzyme	-	-	+	blue	plum
Wallerstein beta-amylase	-	+	-	yellow	yellow

Table XI shows that the only enzyme solutions that destroyed ability to give colors with iodine were those containing active amylases. All reaction mixtures containing active amylases, except barley P_1, produced the iodine colors reported in Table XI in less than 2 hours: barley P_1 required 15 hours, presumably due to the low concentration of beta-amylase; all other reaction mixtures were allowed a 24 hour reaction period. There is little doubt, then that the alpha-glucosan moiety of barley gums is attacked only by the amylase system.

Valuable clues to the identity of gum-degrading enzymes were obtained next from paper chromatograms of enzymic reaction products. The enzyme solutions and reaction mixtures used in iodine color determinations were also used for this study. In addition, a 0.2 per cent solution of the freeze-dried dialyzed solution of green malt P_2 was also used as enzyme source, and enzyme blanks were included. Substrates were stabilized gum and papain gum. Both substrates yielded identical reaction products, hence these are summarized in one table, Table XII. The hydrolysis products of ammonium sulphate precipitates of barley extracts are not included in this table, as these products were present in trace amounts only, presumably due to the low concentration of gum-degrading enzymes in barley extracts. All three solvent mixtures, ethyl acetate/acetic acid/water (3:1:3), ethyl acetate/pyridine/water (5:2:5), and butanol/ethanol/water (5:1:4), gave similar results, but the ethyl acetate/pyridine/water mixture gave the best separations and clearest chromatograms.

TABLE XII

Paper Chromatography of Enzymic Reaction Products

Enzyme Source	Enzyme Composition			Reaction Products
	α-amylase	β-amylase	X-enzyme	
Water	-	-	-	nil
Wallerstein alpha-amylase	+	-	+	oligopentosan oligoglucosides
Wallerstein alpha, heated	+	-	-	oligoglucosides
Wallerstein alpha, acid-cold	-	-	+	oligopentosan oligoglucosides
Chromatographed X-enzyme	-	-	+	oligopentosan (?) oligoglucosides
Wallerstein beta-amylase	-	+	-	trace maltose
Green malt $\frac{1}{2}$ saturated precipitate, P_1	+	+	+	oligopentosan oligoglucosides galactose (?) glucose
Green malt P_1, heated	+	-	-	nil
Green malt saturated precipitate, P_2	+	+	+	oligopentosan oligoglucosides galactose (?) glucose
Green malt P_2, heated	+	-	-	nil
Freeze-dried dialyzed green malt P_2	+	+	+	oligopentosan oligoglucosides galactose (?) glucose

Fig. 24 and Fig. 25 show typical chromatograms of reaction products. Fig. 24A shows reaction products obtained when papain gum was incubated with the Wallerstein bacterial preparation. Three oligosaccharides were released. The top, or slowest-moving, oligosaccharide produced a pink colored spot after spraying, and the other two oligosaccharides produced brown spots. All three oligosaccharides were hydrolyzed by Meredith, Watts, and Anderson (29), who found that the first oligosaccharide yielded arabinose, xylose, and some glucose, and the other two yielded only glucose. Fig. 24B shows reaction products obtained when papain gum was incubated with a solution of green malt P_1, and with the heated enzyme solution containing calcium ions. Green malt P_1 yielded oligosaccharides, glucose, and possibly another monosaccharide with R_F value slightly less than that of glucose. An enzyme blank and the heated enzyme solution yielded no detectable products.

Fig. 25 shows similar monosaccharide spots obtained when papain gum and "beta-glucosan" were incubated with a solution of green malt P_2 and with a solution of the freeze-dried product obtained from green malt P_2. The monosaccharide spots covered the region normally occupied by glucose and galactose. However, as no galactose was detected in acid hydrolysates of "beta-glucosan", and as the ethyl acetate/pyridine/water solvent usually gave a clear-cut separation of galactose and glucose from a mixture containing pure sugars, it appears that the elongated spot may be characteristic of glucose in these enzymatic reaction mixtures. Nevertheless, some galactose may be associated with enzymic digests of papain gum, because the elongated

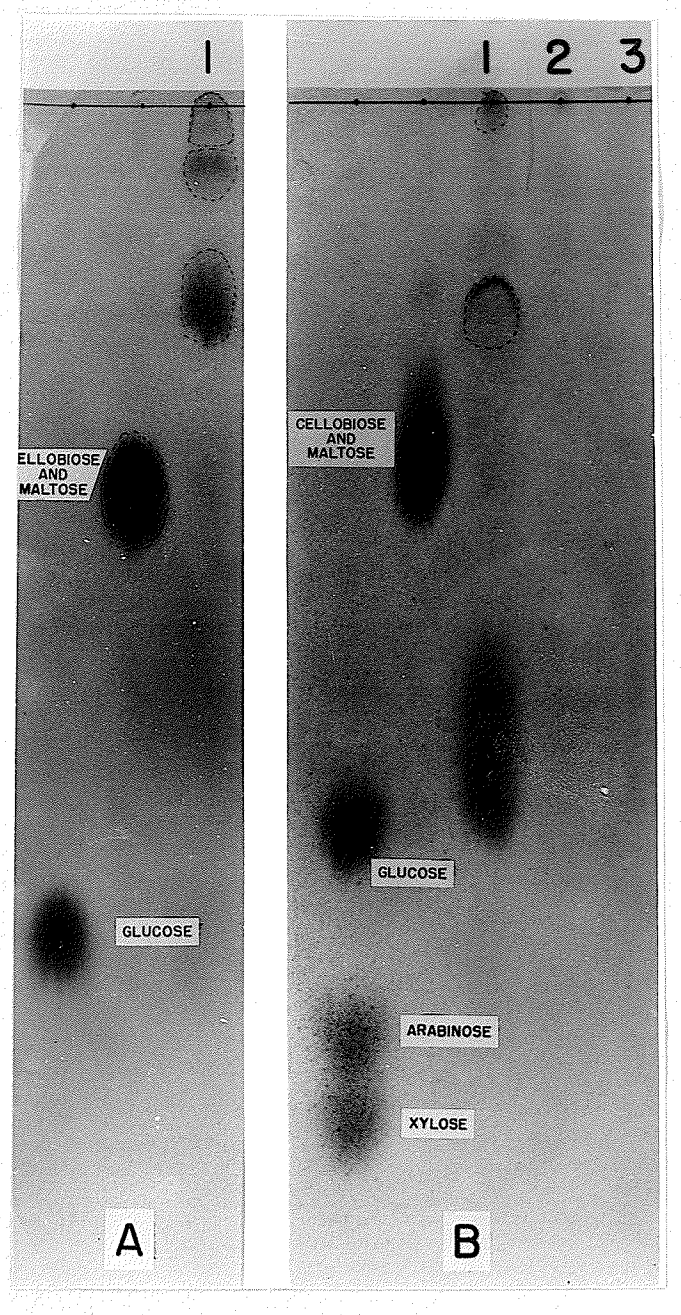


Figure 24.

Typical chromatograms showing products of enzymic action on papain gum.

A 1. Action of Wallerstein bacterial enzyme preparation.

B 1. Action of half-saturated ammonium sulphate precipitate from green malt extract.
2. Action of heat-treated enzyme solution.
3. Enzyme blank.

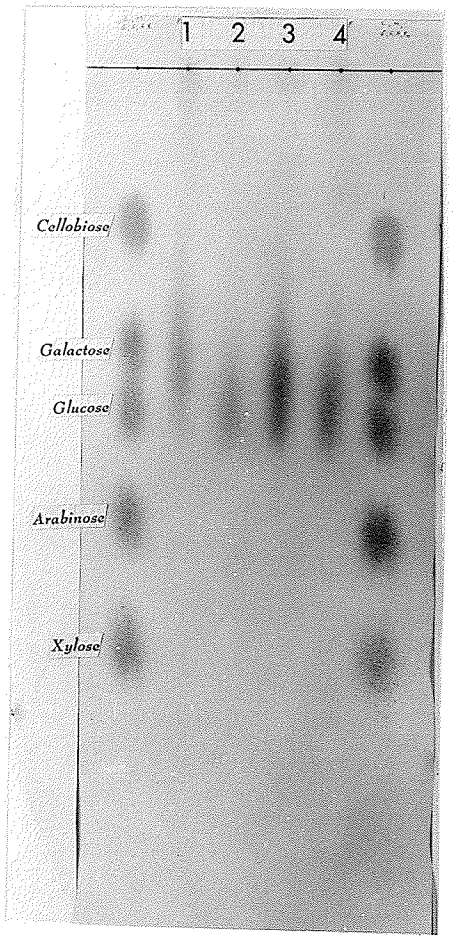


Figure 25.

Chromatogram showing products of enzymic action on papain gum and beta-glucosan.

1. Action of saturated ammonium sulphate precipitate (P_2) from green malt extract on papain gum.

2. Action of green malt P_2 on beta-glucosan.

3. Action of freeze-dried dialyzed solution of green malt P_2 on papain gum.

4. Action of freeze-dried product on beta-glucosan.

spots for papain gum appeared to be uniformly dense over the whole glucose to galactose region, whereas for "beta-glucosan" the smear was more dense in the glucose region and seemed to "tail" into the galactose position. However, the presence or absence of galactose is of little interest at present, as only trace amounts of galactose were observed in acid hydrolysates of papain gum.

Table XII shows that bacterial alpha-amylase yielded oligo-glucosides, whereas green malt alpha-amylase yielded no detectable products. Apparently, then, green malt alpha-amylase degraded the alpha-glucosan fraction of barley gum sufficiently to destroy its ability to produce a characteristic iodine color, but not sufficiently to yield products that can be separated chromatographically. Thus, reaction products liberated by green malt preparations may be attributed to the action of gum-degrading enzymes other than alpha-amylase.

Table XII also shows that both green malt X-enzyme and bacterial X-enzyme liberated oligosaccharides, which are presumably products of endo-glycosidase action, as suggested earlier (page 53). Hence both enzyme sources appear to contain the same endo-glycosidase. However, only green malt X-enzyme liberated glucose, suggesting that green malt may contain a second glycosidase, an exo-glycosidase that attacks the ends of polysaccharide or oligosaccharide chains, releasing glucose.

The specificity of X-enzyme (the viscosity-reducing enzyme) can be fairly well established by considering the available evidence on the structure of barley gum polysaccharides. The investigations

of Gilles et al. (17) provide the starting point for discussion of polysaccharide structure. These investigations have shown that methylated barley gum appears to contain three components: a methylated "arabo-xylan", a methylated "poly-beta-glucosan", and a methylated "poly-alpha-glucosan". As results presented earlier in this thesis (page 89) suggest that the alpha-glucosan may be a minor constituent of barley gum that contributes little to solution viscosity and that is degraded only by the amylase system, the structure of this polysaccharide need not be considered here. The methylated poly-beta-glucosan appeared to be structurally related to cellulose, as it had a negative specific rotation and yielded upon cleavage only 2, 3, 6-trimethyl-D-glucose. The methylated pentosan also had a negative rotation, which, together with the observed hydrolysis products (2, 3, 5-trimethyl-L-arabinose, 2, 3-dimethyl-D-xylose, 2-methyl-D-xylose, and D-xylose in the molecular ratio of 1:12:4:2) suggest that the principal linkages are beta-1:4-xylosidic. This is also considered the chief linkage in xylans from straw and other woody materials (33, p. 121), and recently Perlin (34) suggested that the pentosan of wheat flour is composed of straight chains of anhydro-D-xylose residues, linked beta-1:4- , to which are appended single units of anhydro-L-arabofuranose through 1:2- or 1:3-linkages. Hence it appears that both pentosan and beta-glucosan moities of barley gum may contain similar linkages that may be ruptured by the action of a single non specific beta-polyglycosidase. Thus the viscosity-reducing enzyme, X-enzyme, is probably an endo-beta-polyglycosidase.

The possibility that X-enzyme is a mixture of two enzymes, an endo-beta-polyglucosidase and a pentosanase should not be overlooked, however, in view of the known specificities of other polysaccharidases. Nevertheless, the linearity of enzymic reaction curves and the sharpness of pH-activity curves strongly suggest the action of a single enzyme. Moreover, the ratio of activity on "beta-glucosan" to activity on papain gum (e.g. Table XIII) is usually constant, which also strongly suggests that one viscosity-reducing enzyme operates on both substrates. Thus, presently available evidence indicates that X-enzyme is a single non-specific endo-beta-polyglycosidase, but additional information is required to substantiate this conclusion.

The appearance of glucose among hydrolysis products released by enzymes of green malt may be explained by three possible mechanisms. First, the endo-beta-polyglycosidase may degrade its beta-polyglucoside substrate directly to glucose, whereas the bacterial endo-beta-polyglycosidase may not cause such extensive hydrolysis. Second, glucose may be released from beta-polyglucoside or beta-oligoglucoside chain ends by an exo-beta-glycosidase. Finally, by analogy with the amylase system, cellobiose may be the final product of either endo- or exo-beta-glycosidase action, and this product may then be degraded by cellobiase to glucose.

The possible presence of an exo-beta-glycosidase in green malt was demonstrated by examining enzymic hydrolysis products in early stages of the enzymic reaction. If glucose is the product of endo-glycosidase action, oligosaccharides should be liberated before glucose, whereas if glucose is the product of exo-glycosidase action, both products should be released simultaneously. With either papain gum or beta-glucosan as substrate, and green malt P_2 as enzyme source, both glucose and oligosaccharides were detected on paper chromatograms after a 1-hour reaction period. Hence the existence of an exo-beta-glycosidase is strongly suggested, but the possibility should be considered that some glucose liberation from oligosaccharides occurs relatively rapidly, so that both glucose and oligosaccharides are present after 1 hour's digestion.

The possibility that cellobiose may be the product of exo-beta-glycosidase action was investigated next. Cellobiose is a possible final product if enzyme solutions show cellobiase activity, because any cellobiose formed would immediately be degraded to glucose. Hence green malt precipitates were next tested for cellobiase activity by incubating cellobiose solutions with green malt precipitates for

24 hours at room temperature, then identifying the products by paper chromatography. The Wallerstein bacterial preparation was similarly tested for cellobiase activity. Reaction mixtures were the same as before, except that 10 ml. portions of a 10 per cent aqueous solution of cellobiose were used instead of 10 ml. of gum solution. The bacterial preparation failed to degrade cellobiose, but the ammonium sulphate precipitates from green malt extract degraded cellobiose to glucose. Therefore cellobiose is a possible product of exo-beta-glycosidase action.

In summary, then, barley gums appear to be degraded by at least two enzyme systems, the amylase system and a beta-glycosidase system. The amylase system degrades the alpha-glucosan moiety of barley gum, and has little or no effect on the viscosity of aqueous barley gum solutions. It is postulated that the beta-glycosidase system is a two-component enzyme system. One component is a non specific endo-beta-polyglycosidase that ruptures internal beta-glycosidic linkages of beta-polyglucoside and pentosan fractions of barley gum, thereby reducing the viscosity of aqueous barley gum solutions. The second component is an exo-beta-glycosidase that attacks the ends of beta-polyglucoside chains, liberating cellobiose, which is then degraded by cellobiase to glucose.

Preliminary Varietal Studies of Green Malts

Varietal differences in many properties of Canadian barleys and malts, including certain enzyme activities, have been established previously (27a, and previous papers in the same series), hence a short investigation was next undertaken to examine the possibility of

varietal differences in viscosity-reducing activity of green malts. Eight barley varieties of widely different malting quality were obtained from the 1953 crop of one station, Tisdale, Saskatchewan. A sample of Newal from the preceding crop year of this station was also included, as Newal has the unusual malt property of high diastatic activity coupled with low extract.

The nine varieties were divided into two lots, each lot containing a sample of Montcalm barley as the reference standard. Twenty-five gram samples of each barley variety in each lot were then malted by Mr. V. M. Bendelow, Barley Improvement Institute, Winnipeg. The green malts were air-dried, at room temperature, and the viscosity-reducing activities of barleys in each lot were determined on successive days.

TABLE XIII

Viscosity-Reducing Activities of Green Malts

Variety	Activity per Gram of Dry Barley	
	Papain Gum as Substrate	Beta-glucosan as Substrate
U. M 570	19.4	51.4
Lac. 5120	18.9	51.0
Montcalm*	16.4	43.8
Montcalm	16.4	43.1
U. M. 1623*	14.9	40.6
O. A. C. 21	14.8	40.7
Scott 151-62	13.6	36.1
Br. 4298-3833*	11.8	30.7
U. M. 1020*	11.2	26.2
Newal*	10.6	26.0

* Barley varieties in lot 1.

Since agreement between activities of the reference standard in each lot was excellent, it was assumed that the two lots received identical malting treatments. Accordingly, the varieties were listed in one group, in order of decreasing activity, in Table XIII. The order is the same for both substrates. The data show that there are large variations in the viscosity-reducing activities of the nine barley varieties, particularly with beta-glucosan as substrate. The highest activity (U. M. 570) is almost twice the value of the lowest activity (Newal). It thus appears that viscosity-reducing activity is a varietal characteristic, and there is good reason to believe that this property plays a significant role in establishing differences between varieties in over-all malting quality.

GENERAL DISCUSSION

This investigation of cytolytic enzymes in germinating barley will be discussed in terms of its possible contribution to fundamental knowledge of cytolysis, a process that appears to be of considerable importance in the conversion of barley to malt. Results presented in this thesis are first compared with results obtained by Sandegren and his associates (42, 13); their investigations were conducted simultaneously with, and were similar to, the present research. Possible types of enzymic reactions involved in cytolysis are discussed next, and these reactions are then used to suggest a mechanism of cytolysis. Finally, practical applications of this investigation are discussed briefly, and paths for future research are indicated.

The view that barley gums are derived largely from cell wall material is widely accepted today (e.g. 37, 38). Hence enzymes that degrade barley gums may be classed as cytolytic enzymes. Alpha- and beta-amylases appear to degrade some small portion of recent barley gum preparations (pages 89, 104), but this appears to be an incidental function of amylases, and it is not thought that amylases function as cytolytic enzymes.

In this investigation, Wallerstein's bacterial alpha-amylase preparation was the first source of a gum-degrading enzyme whose activity could be demonstrated independently of alpha-amylase. Although a considerable portion of this investigation was devoted to studies

of the gum-degrading enzyme of Wallerstein's preparation, bacterial enzyme relations are not discussed here. The bacterial preparation was used principally as a convenient tool to develop methods that could be applied to studies of cytolytic enzymes in barley and green malt, hence bacterial enzyme relations per se were not considered relevant to this discussion.

Comparisons with the research of Sandegren and his associates (42, 13) show that there is general agreement between their results and results presented in this thesis. Thus, the "cellulase", which Sandegren describes as a "typical hydrolytic beta-polyglycosidase" has the same specificity, heat sensitivity, and approximately the same optimum pH as the endo-beta-polyglycosidase described in the present research. Sandegren's method of plotting reaction data as $1/\eta_{sp}$ vs time is essentially the same as the original method, used in the present investigation, of plotting η_o/η_t vs time (page 39). In fact, to eliminate the need for determining the initial specific viscosity (η_o) of reaction mixtures, Sandegren's method of plotting $1/\eta_{sp}$ vs time was adopted in the present research when studies of barley and green malt were initiated. Other similarities between the two investigations were: the linearity of reaction curves, the calculation of enzyme activity from the slope of reaction curves, and the use of ammonium sulphate to precipitate enzymes from barley and green malt.

There is disagreement between interpretations of the two investigations in only two respects. First, a non linear relation between cellulase activity and enzyme concentration was reported by

the Swedish group, whereas in experiments described in this thesis a direct proportionality was observed between viscosity-reducing activity and enzyme concentration (Fig. 8, Fig. 20). As there is no essential difference between the two methods of calculating enzyme activity, the mechanism of the reaction between cellulase and ethyl-hydroxyethyl-cellulose (Sandegren's substrate) must differ from the mechanism of the reaction between the endo-beta-polyglycosidase and barley gum. Hence the opinion expressed earlier (page 34), that substrates for cytolytic enzymes should be as similar as possible to materials occurring naturally in barley, appears justified if in vivo processes in germinating barley are to be deduced from results of in vitro studies of cytolytic enzymes.

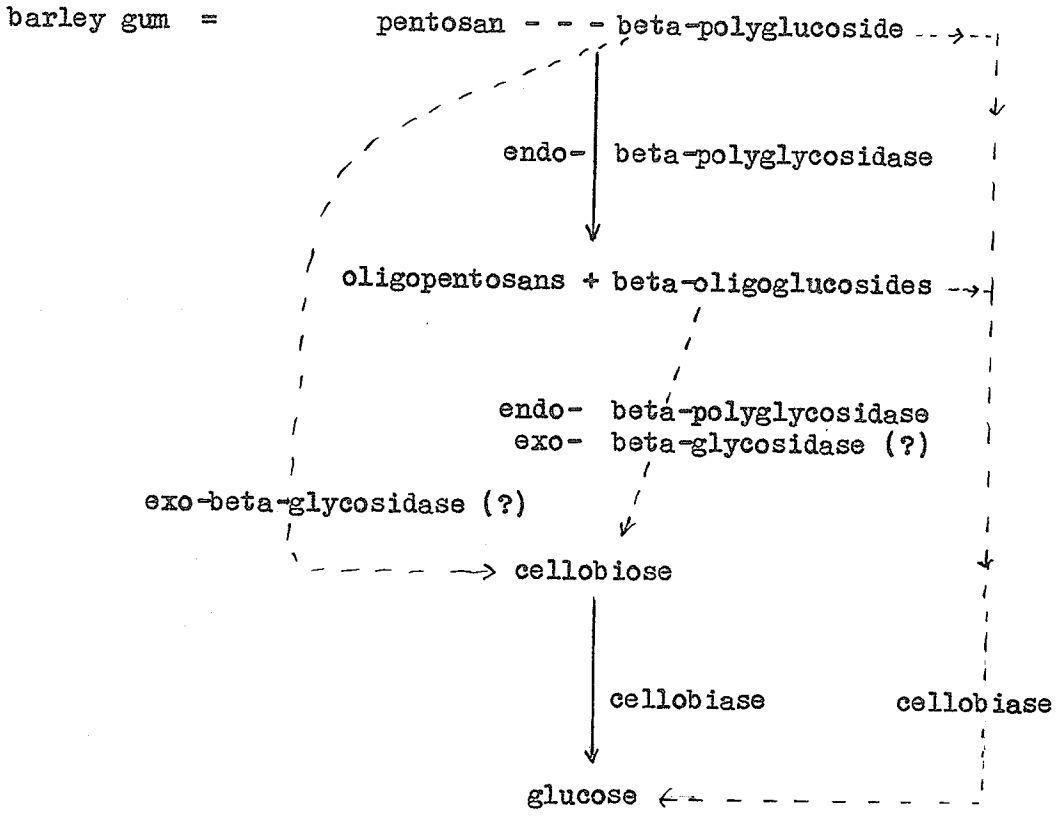
Secondly, Sandegren suggested the existence, in green malt, of a second beta-glycosidase, which he describes as a "nonhydrolytic enzyme of transglycosidase type". The existence of this enzyme was postulated to account for the observed stimulation of the cellulase activity of a purified cellulase preparation, by glucose, mannose, and xylose. Sandegren characterizes the proposed transglycosidase by the reaction:

$$A - O - B + R - OH = A - O - R + B - OH$$

where A - O - B is a beta-linked polysaccharide, and R - OH is a reducing sugar. Such a process represents essentially the reverse of exo-beta-glycosidase action postulated in this thesis to account for the presence of glucose in enzymic hydrolysates. That is,

a transglycosidase would tend to remove reducing sugars to form short-chained polysaccharides, whereas an exo-beta-glycosidase would tend to degrade short-chained polysaccharides to simple sugars. Hence, although exo-beta-glycosidase action does explain the stimulation of cellulase activity by certain sugars, transglycosidase action does not explain the accumulation of glucose in enzymic hydrolysates, unless only a small amount of glucose is consumed in transglycosidase action. Additional research is required to test these two different hypotheses.

Various possible reactions for the enzymatic degradation of barley gums are suggested by the results presented in this thesis. These reactions are discussed next, and a schematic diagram of the reactions is given below to clarify the discussion. In the diagram, solid lines indicate the paths of established reactions, and broken lines indicate the paths of unconfirmed reactions. Question marks indicate materials whose existence is not definitely established.



Barley gum, which, for this discussion, may be considered either as a combination of beta-linked polysaccharides (page 110), or as a mixture of beta-linked polysaccharides, is degraded by the endo-beta-polyglycosidase to a mixture of beta-linked oligopentosan and oligoglucoside material. This reaction has been fairly well established (page 43, Table XII). Cellobiose is considered to be the penultimate degradation product of the beta-polyglycoside, from which it is released by either or both, of two enzymes -- endo-beta-polyglycosidase and exo-beta-glycosidase. Although no trace of cellobiose has been observed on paper chromatograms of enzymic reaction products, this product is suggested by analogy with the amylase system, and by the observation

of Sandegren (13) that cellobiose is one of the reaction products of a bacterial cellulase preparation. Cellobiose is then immediately degraded, by cellobiase, to glucose (page 112), thus completing the degradation of beta-polyglucoside material.

The detection of cellobiase activity in green malt, and the postulation of an exo-beta-glycosidase (page 111) suggest additional side reactions in the enzymatic degradation of the beta-polyglucoside component of barley gum. As both enzymes operate by splitting external beta-glycosidic linkages, and as cellobiase is considered to be a non specific beta-glycosidase, the following reactions are also possible, but not confirmed: the liberation of cellobiose from beta-polyglucoside by the exo-beta-glycosidase; and the release of glucose from beta-polyglucoside and beta-oligoglucoside by cellobiase.

There is no direct evidence for the enzymatic degradation of oligopentosan material, as no free dipentoses or pentoses have as yet been detected in enzymic hydrolysates of pentosan-containing gums such as papain gum. However, free pentoses have been detected in worts of commercial and experimental malts[2]. If the free pentoses appear as a result of enzymatic degradation, there is no need to postulate the existence of an exo-pentosanase, because either exo-beta-glycosidase or cellobiase may be a non specific beta-glycosidase that can degrade a wide variety of beta-linked polysaccharides.

A hypothesis of the mechanism of cytolytic action may now be qualitatively developed. To simplify the discussion, reference will be made to an exo-beta-glycosidase enzyme system. This enzyme

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[2] Personal communication from Dr. W. O. S. Meredith, Grain Research Laboratory, Winnipeg.

system is assumed to include all exo-enzymes of germinating barley that degrade substrates for, or reaction products of, endo-beta glycosidase action. There may be only one such enzyme, with a low order of specificity, or there may be several enzymes, each highly specific; the total number does not affect the argument. Thus, for the present, cytolytic enzymes may be considered to include a single non specific endo-beta-polyglycosidase, and an exo-beta-glycosidase system.

Cytolysis may then be described by the following sequence of enzyme-catalyzed reactions. Beta-linked cell wall polysaccharides are attacked principally by the endo-beta-polyglycosidase. The exo-beta-glycosidase system probably has little effect on the composition of the cell wall, because no appreciable number of polysaccharide chain ends are likely to be exposed to the action of the exo-enzyme system. Even if a considerable number of chain ends were exposed, it is extremely unlikely that the relatively slow degradation produced by the successive removal of single di- or mono-saccharide units would have any profound and rapid effect on the over-all properties of the cell wall. However, the exo-enzyme system can act freely on the ends of chains of polysaccharides or oligosaccharides liberated by the endo-action.

In the absence of the exo-enzyme system, the endo-enzyme may show some affinity towards its reaction products. If so, the process of cell wall degradation would be retarded. In the presence of the exo-enzyme system, however, reaction products may be degraded to such an extent that the endo-enzyme no longer acts on products, and the

process of cell wall degradation is therefore accelerated. Thus, when the exo-enzyme system is in harmony with the endo-beta-polyglycosidase, there is a continuous conversion of cell wall material to low molecular weight products, until the cell walls have been completely broken down.

This qualitative picture of cytolysis in germinating barley suggests that the initial cytolytic action, the direct attack on the cell wall, is chiefly the result of the activity of a single enzyme -- the endo-beta-polyglycosidase. The principal role of the exo-beta-glycosidase system in cytolysis therefore appears to be to ensure an optimum level of cytolytic activity by removing products of cytolytic activity that might otherwise compete for the cytolytic enzyme with intact materials of the cell wall.

With the exception of the brief preliminary study of varietal differences in the viscosity-reducing activities of green malts, this study has been confined to basic research on enzymes involved in a fundamental aspect of the malting process -- cytolysis. However, the practical implications of this investigation are clear, and there is now good reason to apply the results of this research to applied research on the role of cytolysis in over-all malting quality.

There is now little doubt that viscosity-reducing activity is a measure of cytolytic activity, as both viscosity reduction and cytolysis appear to be due to the action of the same enzyme. Hence the viscosimetric assay provides a useful means of measuring cytolytic activity. Since preliminary studies have shown (page 114) that significant varietal differences can be expected in cytolytic activities

of green malts, these studies will be extended to include a number of barley varieties from several stations. Results of cytolytic activity determinations can then be subjected to statistical analysis to determine whether cytolytic activity of green malt is a significant varietal characteristic contributing to varietal differences in malting quality.

The results of these trials will largely determine the path of future research. Thus, if significant varietal differences, related to malting quality, are observed, attention will be directed next towards developing a method for predicting the cytolytic activity of green malt from a small sample of barley. If results are discouraging, basic studies will be resumed. The factor under investigation then will be the exo-beta-glycosidase system, as this enzyme system may well be equal in importance in the process of cytolysis to the endo-beta-polyglycosidase.

SUMMARY

(1) Cytolytic enzymes in a bacterial alpha-amylase preparation, in resting barley, and in green malts were isolated or partially purified, and some properties were determined.

(2) Seven barley gum preparations were screened as potential substrates for cytolytic enzymes. All were susceptible to attack by cytolytic enzymes, and all except the recently prepared "beta-glucosan" contained three principal sugar residues, glucose, arabinose, and xylose. Three of the gums, prepared most recently, proved sufficiently stable and viscous to serve as substrates for the enzyme study. The first, "beta-glucosan", was believed to contain only one major polysaccharide, a beta-polyglucoside, whereas the other two, papain gum and stabilized gum, appeared to contain three polysaccharides, a poly-alpha-glucosan, a beta-polyglucoside, and a pentosan.

(3) The bacterial enzyme preparation was studied first, as it was a partially purified, concentrated, and readily available material. Preliminary experiments showed that enzymatic gum degradation in aqueous solution was accompanied by an increase in solution reducing power and by a decrease in solution viscosity. As viscosity reduction appeared to be more characteristic of the initial enzymic reaction, a viscosimetric method of measuring gum-degrading activity was sought. Viscosity reduction

was attributed to the action of a system of endo-polyglycosidases. This enzyme system was tentatively named X-enzyme.

(4) A viscosimetric method of determining X-activity was developed. Data were then plotted as η_o/η_t vs reaction time (where η_o = initial specific viscosity, η_t = specific viscosity at time, t), or more simply, as $1/\eta_t$ vs reaction time. Reaction curves were linear in initial stages of the reaction, and the slope of linear portions of reaction curves was a measure of X-activity. Thus, X-activity was directly proportional to enzyme concentration for a wide range of enzyme concentrations.

(5) Bacterial X-enzyme was separated from alpha-amylase by column chromatography, with activated alumina as adsorbent and phosphate buffer as eluting agent. Separation of the two enzymes was optimal when buffer concentration was 0.005 M and buffer pH was 5.0. The maximum recovery of X-enzyme, free from alpha-amylase, was about 40 per cent.

(6) X-activity in the crude preparation or in purified form was almost completely destroyed by heating in aqueous solution at 70°C. for 15 minutes. Solutions of the crude preparation suffered little or no loss in X-activity after storage for more than 1 month at room temperature or at 7°C. Solutions of the purified preparation were stable for about 2 weeks at room temperature, but were unstable at 7°C.

(7) Solid enzyme isolates were prepared from barley and green malt extracts by precipitation with ammonium sulphate. Three additional enzymic isolates from green malt were prepared by freeze-drying a green malt extract, by freeze-drying a dialyzed green malt extract, and by freeze-drying a dialyzed solution of an ammonium sulphate precipitate of green malt extract. X-activity of all preparations was almost completely destroyed by heating in aqueous solution at 70°C. for 15 minutes. X-activity of ammonium sulphate precipitates was reduced markedly by alcohol- or acetone-washing at room temperature.

(8) X-activity of green malt, on a weight basis, was estimated to be about 90 times the activity of the barley from which the green malt was prepared. X-activities of barley and green malt precipitates were about 90 and 60 per cent of the activities of the extracts from which the precipitates were prepared. X-activity of the freeze-dried dialyzed green malt extract was only 37 per cent of the activity of the extract, whereas X-activity of a freeze-dried dialyzed extract of green malt and X-activity of a freeze-dried sialyzed solution of the ammonium sulphate precipitate from green malt were about 90 per cent of the activity of the parent green malt extracts. It was therefore concluded that the X-enzyme system contains no dialyzable component, and that ammonium sulphate appears to inhibit X-activity. The low activity of the freeze-dried dialyzed extract of green malt was attributed to destruction of X-enzyme by other enzymes of green malt.

(9) Alpha- and beta-amylases were the only enzymes found in the bacterial preparation or in barley and green malt that degraded the poly-alpha-glucosan fraction of barley gum; these amylases destroyed the ability of papain gum or stabilized gum to produce characteristic colors with iodine.

(10) Products of enzymatic action on stabilized gum, papain gum, and "beta-glucosan" were identified by paper chromatography. With stabilized gum or papain gum as substrate, bacterial X-enzyme yielded at least one oligopentosan and one or more oligoglucosides; green malt X-enzyme liberated glucose in addition to these products. With "beta-glucosan" as substrate, green malt X-enzyme liberated oligoglucosides and glucose. As oligosaccharides are probably products of endo-enzymic action, and as there is good reason to believe that the principal linkages in pentosan and beta-polyglucoside fractions of barley gum are beta-glycosidic, it was suggested that X-enzyme is a non-specific endo-beta-polyglycosidase, capable of rupturing internal beta-glycosidic linkages. However, the possibility that X-enzyme may be a mixture of an endo-beta-polyglucosidase and an endo-pentosanase should not be overlooked.

The presence of glucose among reaction products of green malt enzymes is attributed to the existence of an exo-beta-glycosidase system in green malt. It is suggested that cellobiose is a reaction product of this enzyme system, and that cellobiose is degraded by cellobiase to glucose. The results of two experiments provided evidence favoring such an enzyme system.

(12) Studies of X-activities of the green malts of nine barley varieties showed large variations in X-activities and thus suggest that X-activity of green malt is a varietal characteristic contributing to malting quality.

(13) A hypothesis of cytolysis in germinating barley is suggested. It is postulated that the rupture of cell wall materials is effected by the action of a single enzyme, a non-specific endo-beta-polyglycosidase. Products of endo-beta-polyglycosidase action are then degraded by an exo-beta-glycosidase system to simple sugars or other materials not attacked by the endo-beta-polyglycosidase. It is suggested that the principal role of the exo-beta-glycosidase system in cytolysis is to ensure an optimum level of cytolytic activity by removing products that might otherwise compete for the cytolytic enzyme with intact materials of the cell wall.

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