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CYTOLYTIC ENZYMES IN BARLEY AND MALT

by

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ABSTRACT

The cytolytic enzymes, particularly their endo-enzyme components, play an important role in the conversion of barley to malt. In the first section of the present investigation a study of three endo-enzyme activities was carried out utilizing specific substrates to identify the various actitivies. The activities (barley- β-glucan endo-hydrolase, endo β 1-4 glucanase and endo β1-3 glucanase) were determined at sequential stages of germination for green malts prepared from six varieties of barley. The effect of adding gibberellic acid to the steep water was also assessed.

Of the three enzyme activities studied, barley- β-glucan endo-hydrolase activity exhibited the largest fold increase and was the only activity affected by gibberellic acid. The endo β 1-4 glucanase and endo β 1-3 glucanase activities also increased during germination but to a lesser degree than the barley- β-glucan endo-hydrolase activity. The possible role that each of these enzymes may play in malting is discussed.

In order to characterize the particular cytolytic endo-enzymes that hydrolyze β1-3 linked glucans and barley β-glucan, the enzymes (endo β 1-3 glucanase and barley- β-glucan endo-hydrolases M_I and M_{II}) were separated. The enzymes were separated and purified by ion-exchange column chromatography with DEAE- and CM-cellulose followed by molecular

sieving on Bio-Gel P-100. The endo β 1-3 glucanase had a pH optimum of 5.0 with laminarin and 5.9 with carboxymethyl pachyman as substrates. The enzyme was found to be heat-stable up to 60°C, was stimulated by NaCl and was inhibited by $CaCl_2$ and thioglycerol. The enzyme also had an unusually high isoelectric point at pH 9.8.

The barley- β -glucan endo-hydrolases M_I and M_{II} had the same pH optimum value of 4.8 but differed in their chromatographic properties on CM-cellulose. The endo-hydrolase M_I was unaffected by NaCl, EDTA and thioglycerol while endo-hydrolase M_{II} was stimulated by NaCl and was inhibited by $CaCl_2$. The two enzymes also differed in their heat stabilities, with endo-hydrolase M_I being heat-stable up to 35°C and endo-hydrolase M_{II} being stable up to 40°C. The limit products from barley- β -glucan produced by endo-hydrolases M_I and M_{II} appeared to be the same because in each case the products were eluted from a borate ion-exchange column at the same relative positions. The major products appeared to be tri- and tetrasaccharides by their relative chromatographic mobilities on paper. The significance of several of the enzyme characteristics is discussed with regard to the enzymes' possible roles in malting and brewing.

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OBJECTIVES

A problem sometimes encountered in brewing is the development of a viscous wort. This fault is known to be related to poor modification during malting. Slow filtration caused by viscous worts may result in back-up problems during production. Such worts may also have reduced extract yields and fermentation difficulties.

The viscous principle was first isolated from wort in 1949 (50). It was eventually shown to be primarily β -glucan and referred to as barley gum. The gum is considered to be a solubilized form of cell wall-like materials or matrix materials from the endosperm. The degradation of these materials is essential to expose starch and protein in the endosperm to amylases and proteases and is mediated by several endo-enzyme systems in barley and malt. As the β -glucan component makes up the major proportion of the gum, investigations were made in the areas of identification and characterization of the components of the β -glucanase systems in barley and malt.

The objectives of this study were two-fold. The first was to determine the changes during germination of three types of endo- β -glucanase activities in green malt. These were barley- β -glucan endohydrolase, endo β 1-4 glucanase and endo β 1-3 glucanase activities. Six varieties of barley of different row types were used in this study. Samples of mature barley were steeped and then germinated for from one to six days. Separate samples, from each of the varieties, were steeped

in a final steep with 4 p.p.m. gibberellic acid and then germinated two, three, and four days under the same condition as the untreated samples. Analyses of the endo-cytolytic activities were made using Ostwald-type viscometers with barley β -glucan, carboxymethyl cellulose and carboxymethyl pachyman as substrates.

The second objective was to separate, purify and characterize the endo-cytolytic enzymes. Three endo- β -glucanases were separated and partially purified using ion-exchange column chromatography and molecular sieving. Carboxymethyl pachyman was used as a viscous material for the measurement of endo β 1-3 glucanase activity. Although it is not a naturally-occurring substrate, carboxymethyl pachyman, which is composed entirely of glucose units linked β 1-3 (16), is useful for assaying β 1-3 glucanase activity. Barley β -glucan was used as a substrate for two β-glucan endo-hydrolase enzymes.

LITERATURE REVIEW

In considering the previous work relating to cytolytic enzyme systems in barley, three stages of development are apparent. These involve (i) the early work and the initial assessment of the viscous problem; (ii) the early attempts to characterize and separate substrate and enzyme systems and their practical relationships in malting and brewing; and (iii) the extensive characterization of both substrate and enzyme systems involved, using modern methods.

Original Demonstration of Complex Carbohydrate and its Relation to Malting

Some of the first work related to this area goes back to 1880 with the isolation from barley and a few other cereals of a material referred to as β-amylan (52). O'Sullivan isolated this material from barley by ethanol precipitation of a 40°C aqueous extract. By hydrolysis, the precipitate was found to be very rich in glucose. When redissolved in hot water at two percent concentration, the ethanol precipitate formed a thick "jelly-like fluid." The solution was laevorotatory. Lacking current methods, it would have been very difficult to characterize this material further.

In 1890 a remarkably extensive microscopic examination of changes in the barley kernel during steeping and germination was made by Brown and Morris (13). The first observations of the true significance of "cyto-hydrolytic" enzymes were reported. Following steeping, signifi-

cant changes were observed in the endosperm tissue of the kernel. These changes involved the breakdown of the cell wall material and subsequent dissolution of starch granules. To quote from their paper with respect to endosperm cells, "The action upon starch granules never takes place as long as the walls of the cells containing them are intact". This sums up rather nicely the importance of this enzyme system. Without these enzymes, solubilization of starch does not occur and, consequently, the embryo will not grow. In malting, this would result in a lack of starch degradation to fermentable sugars. Thus, this enzyme system is responsible for cell-wall degradation and acts as the key to the further metabolism of the contents of endosperm cells. It should be noted that Brown and Morris made the unfounded assumption that the endosperm cell wall material was cellulose.

Involvement of Complex Carbohydrate in Viscosity of Wort

Between 1890 and 1935 little work was done in the area related to cell wall materials. Two types of materials were generally thought to be present. These were hemicellulosic and cellulosic materials. The cellulosic material was called cellulosan, and was considered to be cellulosic in structure but retained properties of hemicelluloses during its preparation. The method of separation probably produced a mixture of carbohydrate materials.

A glucosan that could be removed in varying quantities by hemicellulose solvents was classified as cellulosan (59). Cellulosan was thought to differ from true cellulose in having a considerably shorter chain length. That the glucosan linkage may have been anything but β 1-4 was

not suggested. This may seem logical since cellulose was the most widely known plant structural β-glucan. The hemicellulosic materials were described as being cell wall materials of broad carbohydrate nature (59). These were insoluble in water under natural conditions but soluble in dilute sodium hydroxide.

Within the hemicellulose classification was a subgroup called pentosans. Quantitative measurement of this subgroup was made by conversion of the pentose sugars to furfuraldehyde and subsequent estimation of this product. This method did not give a true measure of pentosan content as araban and xylan yield furfuraldehyde to different extents (58). However, using this method, it was estimated that during malting the pentosan content increased by twelve to twenty percent (58). This estimation was based on extractable materials suggesting increased solubilization of the native pentosan materials.

In 1936 Piratzky (55) described a problem encountered in producing worts from malts that had been germinated for different lengths of time. Those malts that were considered undermodified produced a considerably more viscous wort than that from a fully modified malt. Initially this was thought to be due to the presence of gelatinized, but undigested starch. However an iodine reaction gave negative results. As the degree of modification of the malt increased, the viscosity of the resulting wort decreased.

In a more comprehensive test, Piratzky and Wiecka (56) examined malts germinated for various times and prepared from four successive barley crops. Although the maximum values for wort viscosity varied

widely, the minimum values were quite close. This suggested that the conversion of all convertible "hemicellulose" materials had occurred. Whether the direct reference to hemicellulosic materials was made because previous workers (23, 76) had shown a four to twenty percent increase in soluble pentosans is not known. Regardless of the reason, the results of this study indicated that wort viscosity might be a useful way of measuring the degree of malt modification during germination.

Pursuing the problem of high wort viscosity from undermodified malts, Piratzky and Wiecha (57) isolated from a three-day germinated green malt a substance that would produce a highly viscous solution. A similar material could not be isolated from a well modified malt. This substance had similar properties to O'Sullivan's β-amylan (52) and, after acid hydrolysis, only glucose was detected. No further work was reported by these workers on this subject.

One final piece of research must be mentioned in connection with this early work. In 1941, Dickson and Shands (20) showed photographically what Brown and Morris (13) had described with respect to changes in the kernel during germination. Photomicrographs were obtained that showed the endosperm cell walls were completely disrupted before the matrix imbedding the granules, or the granules themselves, were dissolved. This again indicated the importance of the cytolytic enzyme systems.

Relations Between Viscosity and Other Malt Properties and Gum

The second stage of development relating to cytolytic enzymes involved the practical aspects of the cytolytic systems with regard to

malting and brewing. The change in wort viscosity with degree of malt modification, shown by Piratzky and Wiecha (56, 57), was of considerable interest. Work on possible practical applications was begun by Meredith and Sallans in 1943 (47).

During the early stages of germination, the high viscosity of the worts could be due to any type of soluble high molecular weight material including starch degradation products or nitrogenous compounds. This possibility was pointed out by Meredith and Sallans (47) in 1943 who showed a significant inverse correlation between wort viscosity and the degree of attenuation, and a direct correlation between wort viscosity and final turbidity. The degree of attenuation is a measure of fermentable materials and results primarily from starch degradation.

Although Piratzky and Wiecha (57) had isolated a gummy material from germinated barley, there was no evidence that this was the same material as was causing the viscosity problem in the wort. In 1949, Meredith (50) isolated the viscous principle from the wort by precipitation with alcohol and showed it to be primarily carbohydrate in nature. Although some nitrogenous material was also present, it was not thought to be of major importance because its presence varied considerably from one preparation to the next.

Identification of Gum Components

Preece, Ashworth and Hunter (60) in 1950 described several hemicellulose fractions that they had prepared. A malt polysaccharide, B_2,

that was extracted at room temperature and precipitated with Fehling solution and acetone, yielded primarily glucose when hydrolyzed. The fact that it gave a high positive rotation suggested it was largely starch dextrin. Later work (61), using α-amylase, confirmed this.

A second fraction, C_2, from the same grains was obtained by extraction at 40°C followed by precipitation using the same reagents. This material yielded arabinose, xylose and glucose upon hydrolysis. Because the proportions of these three major components varied depending upon the methods of extraction and precipitation, it seemed likely that a mixture of products was present.

Meredith, Bass and Anderson (48) prepared hemicellulose gums from raw barley, malt and the resulting wort. The water-extracted material was precipitated with ethanol to yield a crude gum fraction. To remove nitrogenous materials, the crude gum fractions were treated with normal sodium hydroxide. This treatment accomplished its purpose. Even after removal of nitrogenous materials, a viscous solution could still be prepared, confirming an earlier suggestion of Meredith's (50) that nitrogenous materials did not play a major role as the viscous principle.

Of the four raw gums prepared, that from the endosperm produced the highest viscosity with one of the lowest pentose (arabinose plus xylose) to glucose ratios of sugars from hydrolysis. It was also noted that after alkali treatment the ratio of pentose to glucose increased markedly while the arabinose to xylose ratio remained unchanged. The loss of glucose suggested hydrolysis of a glucan component to a less

precipitable form. Also following this treatment, the high viscosity of the endosperm raw gum was reduced by almost one half to a level comparable to all the other alkali-treated gums.

Their results also indicated a decrease in the ratio of glucose to pentosan when going from barley to malt. To attribute this change to a single factor would be difficult as several possibilities exist. Previous workers (23, 76) had shown that an increase in soluble pentosans occurred during germination. Whether this pentosan material was of sufficiently high molecular weight to be precipitable, and thereby account partially or solely for this change, is not known. A second possibility was enzymic degradation of the glucan component reducing the amount of precipitable glucan relative to the pentosan material. A third possibility is a combination of the first two.

Preece and Mackenzie (62) in 1952 successfully fractionated a glucosan free of arabinose and xylose by ammonium sulfate precipitation of a barley extract. The pentosan-rich fractions each contained glucose to some extent and this was thought to be in the form of more soluble glucosan components. The components of the gum fractions were determined by paper chromatography of hydrolysis products. Hydrolysis was carried out with normal sulfuric by refluxing for three hours. It is possible that if very minor xylose and arabinose components were present, they would have been destroyed by the long reflux time. The pentose sugars have since been shown to be more susceptible to degradation than glucose during acid hydrolysis (27).

Preece and Mackenzie's method of gum separation was certainly the best available and was a major breakthrough in this field. Their glucosan, G_{20}, was thought to be degraded during the malting process since none could be found in the malt. The glucosan gave a moderately high viscosity as a 0.5% solution and was laevorotatory (α_D -11^{o}). This suggested the molecule was essentially linear with β-glucosidic linkages. Two important results of this work (62) were noted: (i) The glucosan which produces a viscous solution is degraded during malting and is degraded in the extracted state by malt enzymes; (ii) A pentosan-rich fraction, G_{40}, also yielded a highly viscous solution at the 0.5% level. However, this fraction contained less than a tenth of the amount of material present in the G_{20} fraction. The conclusion drawn was that because the malt gums were still moderately viscous even though the glucosan had been removed, these pentosan-rich fractions must make a major contribution to normal wort viscosity.

Preparation of Stable Gums

A seond major advance in the field of the preparation of barley gums was made by Meredith, Watts and Anderson (49). They developed a method whereby larger amounts of a largely undegraded β-glucosan could be prepared. All the researchers in this field at that time were preparing gums by using a half-hour reflux with 85% ethyl alcohol to inactivate the enzymes present. The gums were then extracted from this alcohol-treated grist into water. The alcohol treatment helped to give

a much more viscous gum product. However, solutions prepared from these gums were shown to be quite unstable with an appreciable loss in viscosity over a twenty-four hour period. Meredith's group found by using this alcohol treatment, followed by extraction of the dried grist with a papain solution, that the gum preparations were quite stable as viscous solutions.

In a previous paper by Meredith, Bass and Anderson (48), alkali treatment had been used to prepare nitrogen-free gums. The effect of the alkali on the gum was not assessed at the time. However, when the papain gum (49) was dissolved in N NaOH and then recovered, a considerable change in viscosity had occurred (750 cp to 4 cp). Thus, the glucosan linkages seemed to be exceedingly labile to alkali treatment.

Following these two major advances in barley gum preparation, only a few changes have been made in the procedure commonly used. Bass and Meredith (4) compared the products produced from papain extracts by using ammonium sulfate (30%) and ethyl alcohol (75%) as fractionating agents. The ammonium sulfate method proved to be better as glucose was the only detectable hydrolysis product, while the ethanol-precipitated gum produced arabinose and xylose as minor constituents. Thus, the best method for β-glucan preparation is a combination of the methods of Preece et al. (62) and Meredith et al. (49) and is similar to that described in the Methods section.

Structure of Gums

Aspinall and Telfer (3) were the first to do a structural analysis of barley β-glucan after it had been prepared in a pure form by Preece and Mackenzie (62). Previously, Gilles, Meredith and Smith (25) had analyzed the mixed gums. The β-glucan components appeared to be linked β1-4 in a manner analagous to cellulose.

Using methylation followed by acid hydrolysis, Aspinall and Telfer found that the β-glucosan was linked about 50 percent β1-3 and 50 percent β 1-4. Using periodate, later workers (15, 17, 36, 54) found the ratio of β 1-4 to β1-3 to be closer to 7:3 than the 1:1 ratio reported by Aspinall and Telfer. Thus, it is generally accepted that a 7 to 3 ratio exists. Although several different ideas on the fine structure of β-glucan from barley were proposed on the basis of acid hydrolysis prior to 1960, it was only when better methods of detection and purification became available that significant results were produced.

So far, only a consideration of the viscous wort material, its isolation and partial characterization has been considered. This has been done in order to better appreciate the changes in ideas that occurred in the period 1940-1960.

From the early observations (13, 20), work on cell wall hydrolyzing enzymes had proceeded on the assumption that the cell walls were made up of cellulose and hemicelluloses. The work on gums just discussed, prior to the work of Aspinall and Telfer in 1954 (3), supposedly supported

this theory, as the gums were thought to be breakdown products from the endosperm cell walls. Hydrolysis of barley gums indicated xylose, arabinose and glucose were the main products (i.e., hemicellulosic and cellulosic materials).

Enzymatic Degradation of Gums

Some early work (1940) on cellulases by Enders and Saji (21) demonstrated that cellobiase was present in both barley and malt. During germination, this enzyme increased two and a half times above the level in barley. The measurement of endo-cellulase activity was reported shortly after by several Swedish workers using hydroxymethyl cellulose as a soluble derivative of cellulose (39). This material yielded a viscous solution upon which viscosity reducing activity could be measured. One of the studies carried out involved a comparison of cellulase activity of two-row and six-row barley (39). The six-row barley was found to have about seven times as much activity as the two-row barley.

From Lunden's concluding remarks on cytase enzymes, in an address given in 1959 (39), it was apparent that he considered cellulose to be a major cell wall material. He felt that in the absence of adequate cellulase activity, poor germination would result because of inadequate endosperm cell wall breakdown.

Sandegren and Enebo (73), two of the Swedish workers referred to above, did further work on cellulases in barley using hydroxymethyl cellulose as a substrate. The effect of pH was determined, and the

cellulase had an optimum of about 5.0. The enzyme began to lose activity at a temperature of 45°C.

Degradation of the hemicellulosic components had been studied much earlier by Luers and Volkamer (37) in 1928. A xylanase from green malt was reported, as the enzyme hydrolyzed preparations of "xylan" from elder pith and from barley. It seems doubtful that a pure xylan preparation was available from barley, since later researchers working with barley gums were unable to separate xylan and arabinan components.

Luers and Malsch (38) followed the change in activity of this xylanase enzyme during steeping and germination. The enzyme activity increased rapidly during germination to a level two and one half times that found in the barley. During kilning, the activity decreased to a level lower than that of the original barley, suggesting a high degree of heat lability.

Distinction Between Types of Enzyme Degradation of Gums

Preece and Ashworth concluded from their work (61) relating to enzymolysis of mixed gums, that two separate types of enzymes were involved in hydrolysis. These two types of enzymes were referred to as cytoclastic and cytolytic enzymes. The cytoclastic enzymes were responsible for random chain cleavage and produced a rapid loss in viscosity. The cytolytic enzymes cleaved sequentially from the chain end and caused an increase in reducing power without significantly affecting the viscosity.

Preece and Ashworth theorized that the use of artificial substrates was to be avoided as much as possible in characterization of an enzyme system. The reason suggested was that an artificial substrate would not necessarily parallel changes that would occur when the enzyme degraded its natural substrate. The disadvantage of this suggestion was that, at that time, 1950, a mixed product of pentosan and glucosan was the only substrate available for characterization of cytolytic enzymes.

After 1952, with the preparation of a β -glucosan polysaccharide that was free of pentosan material, the work on gum enzymolysis shifted to the β-glucosan hydrolases. This occurred for two reasons. First, a natural substrate had finally been obtained in a pure form. Secondly, the glucosan was the largest proportion of the precipitable gum from barley (62). During malting, this material was almost completely degraded. This suggested that degradation of the β-glucosan was necessary before other changes could occur during germination. Thus, the β-glucosan hydrolases would be of considerable importance in producing the necessary degradation.

Bass, Meredith and Anderson (5) indicated that two or possibly three enzymes were required for the complete degradation of β-glucan to glucose. It was proposed that an endo-enzyme would degrade the high molecular weight material to a dextrin, followed by cleavage of the dextrin by an exo-enzyme to cellobiose or glucose. If cellobiose was produced, the third enzyme, cellobiase, would cleave cellobiose to form glucose.

At the same time, Enebo, Sandegren and Ljungdahl (22) suggested that their cellulase system was actually comprised of two enzyme systems. The first was a hydrolytic poly- β-glucosidase, and the second was a non-hydrolytic transglucosidase. Evidence for the presence of the second enzyme was not very substantial.

Preece, Aitken and Dick (63) sought to re-examine the activity of Sandegren's "hydroxymethyl" cellulase (73), using the natural β-glucan substrate (63). During the course of this investigation, the results of the structural analyses on their β-glucan was completed by Aspinall and Telfer (3). These results indicated that the β-glucan was not a pure β 1-4 linked polymer, but rather, it contained approximately equal proportions of β 1-3 and β 1-4 linkages. Ultra-centrifugal analysis indicated the presence of one component.

For the first time, Preece, Aitken and Dick (62) could postulate the presence of an endo- β -glucanase enzyme as a distinct entity to cellulase. Attempts to purify the endo-glucanase had limited success. Analysis of enzymatic hydrolysis products by paper chromatography revealed mono- and disaccharides. The disaccharide was identified as cellobiose by its comparative mobility to standard samples of cellobiose. It was proposed that as cellobiose was detected and not laminaribiose, the β 1-3 linkages were selectively cleaved by the endo- β -glucanase enzyme.

Bass and Meredith (6) reaffirmed the multi-enzyme nature of enzymes responsible for β-glucan degradation proposed earlier (5). Evidence was presented in the form of a hydrolysis time study of β -glucosan using a

green malt enzyme preparation. Glucose was the first detectable product, followed by some higher molecular weight components of low chromatographic mobility. This suggested the presence of both endo- and exo-enzymes.

Preece and Hoggan (64) discussed the probable existence of the multi-enzyme system for β-glucosan degradation and came to the same conclusion as Bass and Meredith (6). Preece and Hoggan showed that the exo-activity could be inhibited completely by phenylmercuric nitrate with little effect to the endo-activity. Cellobiase activity could not be selectively inhibited without inhibiting the endo-activity.

These same workers studied the development of these three enzymes during steeping, germination and kilning. Again, the lack of parallel development of enzyme activities further emphasized the independent nature of the enzymes. The activity of all three enzymes increased during germination and was lost to some extent during kilning. The exo-enzyme proved to be the most heat-labile, and the endo-enzyme was the most heat-stable.

Relations Between Cytolytic Activity and Malting Quality

Since cytolytic enzymes play a controlling role during malting and were shown to increase during germination, a proposal was made that the cytolytic activity in mature barley might be used as a criterion for measuring malting quality. Bass and Meredith (17) discussed the results reported by two Swedish workers, Thunaeus and Sandegren, who had tried

to find a correlation but had failed. Preece, Aitken and Potter (66) had also tested this criterion without success. Bass and Meredith (7) believed a better measure would be between malting quality and cytolytic activity in green malt rather than in barley. Their results indicated a correlation did exist but not without exceptions. In general, quite significant varietal differences had been shown between good and poor malting varieties.

Continuing this work, Bass, Bendelow and Meredith (8) measured the relationships between cytolytic activity, as assessed by enzymic degradation of β-glucan, and other barley and malt properties. In general, cytolytic activity in green malt was related to saccharifying activity, alpha-amylase activity, cold water extract, malt extract and the index of protein modification. All the latter properties were measured in kilned malt. Since no direct relationship seemed to exist for the general correlation between cytolytic activity and malting quality, the relationship was assumed to be indirect.

Another problem, studied by Preece and MacDougall (65), was the autolysis of barley pentosans. Release of soluble pentosan was found to be particularly dependent upon solvent penetration. Also, it was theorized that enzymolysis of non-pentosan materials, particularly β-glucan, and also possibly protein, would have significant effects on the release of pentosan.

A more complete analysis of the enzyme systems responsible for pentosan degradation was carried out by the same researchers (67). In a

manner analogous to the work on β-glucan, it was shown that there were four enzymes in the pentosanase system. The substrate used was an arabinoxylan from rye. By degradation time studies and inhibition studies using a barley enzyme preparation, evidence was presented for the presence of four enzymes. These were an arabinosidase, an exoxylanase, a xylobiase and an endoxylanase.

In 1959, MacLeod and Napier (42) published a significant paper that clarified certain problems relating to cell-wall structure. The term "cellulase" had been misused repeatedly, almost from the earliest work on cell wall degradation. "Cellulase" had been used to describe cytolytic enzymes in many instances when no evidence was presented to substantiate that cellulose was actually the substrate. MacLeod and Napier measured the cellulose content of barley that had been subjected to various degrees of pearling. They found only 1/25 of the total cellulose in the final pearl, and estimated that this fraction was in the furrow. The data indicated that there was essentially no cellulose in the endosperm. It seemed unlikely that a cellulase enzyme would be required for degradation of the cell wall material of endosperm tissue and would, therefore, be of small consequence for changes observed during malting.

Following their earlier work, Bass and Meredith (9) determined the correlation between the quantity of barley gums, cytolytic activity, and malting quality. A high correlation was found between the reciprocal of cytolytic activity and cold water extract, a parameter that is used to determine malting quality. The quantity of gums in each sample was

determined but did not correlate with cytolytic activity or malting quality. It was concluded that, within the limits of the investigation, the endo- β -glucanase activity was the principal biochemical factor for evaluating malting quality.

Glucan Structure and Enzymic Action

As both β 1-3 and β 1-4 linkages were present in barley β -glucan, studies were initiated to determine whether cytolytic enzymes had different types of bond specificities. The enzymes might preferentially attack either β 1-3 or β 1-4 linkages or attack both types of linkages.

Bass and Meredith (10) attempted to resolve this problem, using laminarin, a β 1-3 linked polyglucose substrate. The enzyme extract was fractionated by absorption on an alumina column. A separation of exo- and endo-enzymes was accomplished with regard to enzymes that degrade laminarin. The pH optima, using β -glucan and laminarin for the endo-enzyme fractions, were 4.5 and 4.9, respectively, suggesting that different enzymes attacked each substrate. Heat stability studies confirmed this conclusion. The study also revealed that two different enzymes were responsible for the hydrolysis of barley β -glucan. These β -glucan hydrolase enzymes may have been the complementary exo- and endo-glucanase enzymes demonstrated in previous studies (5). The results (10) supported the possibility of linkage-specific enzymes for hydrolyzing barley β -glucan.

Along a similar line, Luchsinger, Cochrane and Kneen (29) differen-

tiated between the endo- β-glucan enzymes in terms of heat stability. The enzymes were separated by ammonium sulfate fractionation, and the pH optima of the enzymes were 4.4 and 4.7. Neither enzyme fraction attacked carboxymethyl cellulose, suggesting the β 1-4 linkages of barley β-glucan might also be impervious to attack by these enzymes.

Preece, Garg and Hoggan (68) attempted to characterize an endo-enzyme responsible for β-glucan degradation by analysis of oligosaccharide products. The results indicated that a separation of endo- and exo-enzymes was not accomplished. However, the products produced, and the method used to separate these products, were significant. All of the possible oligosaccharides, up to the tetrasaccharide level, were found except one, that being cellotriose. This indicated a very random mixture of linkage existed. Similar results were found, using a β-glucan substrate prepared from oats.

Further study using both oat and barley β-glucan (69) demonstrated that the mode of attack by the endo- β-glucanase from barley differed for the two substrates. The oat glucan was degraded smoothly to low molecular weight materials, but the barley β-glucan appeared to equilibrate with a "dextrin" material that was degraded further by an exo β-glucanase system. The enzyme mixture was prepared from barley and contained endo-laminarinase and endo-cellulase activities. An exo β 1-4 glucanase, laminaribiase, and a general β-glucosidase were postulated as being present. The exceptional activity of the extract with laminarin (a largely β 1-3 linked polymer) suggested that the β1-3 glucan linkages

of barley β-glucan might be particularly susceptible to enzyme attack. However, this would suggest a relative increase in β 1-4 linkages of the hydrolysis products, which by previous analysis of oligosaccharide products (68) was not found. Of three possible structures postulated for barley glucan, a structure with an alternating series of β 1-3 and β 1-4 units seemed most likely, based on the available evidence.

This completes the second developmental stage of work relating to separation and characterization of cytolytic enzymes and substrates. During this period, there was a rapid expansion of knowledge relating to the nature of the material in the endosperm cell wall of barley. An insight was gained regarding the complex nature of the hydrolase enzymes that attacked this cell wall material. And finally, the involvement of these systems and their importance during malting and brewing were recognized. The accumulated knowledge of the period established a firm basis for work that was to follow.

Attempts to Isolate and Characterize Cytolytic Enzymes

The third developmental stage is separated from the second stage primarily because of the improvement of separational and detection techniques. These techniques have resulted in the preparation of highly purified cytolytic enzymes.

A possible problem in analyzing products of an enzyme degradation study, arising primarily when a limit degradation is sought, is the possibility of transglucosylation. As enzymes are only catalysts, the

theoretical possibility of a reverse reaction exists. In an equilibrium system, as the concentration of products increases, there is also an increase in the rate of the reverse reaction.

Anderson and Manners (1) first demonstrated the existence of a transglucosylation system in barley. Although transglucosylation did not occur using glucose alone, transglucosylation did occur using cellobiose, producing several different oligosaccharides. The rate of such reactions was very low, and was below the one percent concentration level. Transglucosylation reactions are not unusual. MacLeod and Sandie (93) found that similar reactions occurred with extracts of Bromus seed.

In 1960, Parrish, Perlin and Reese (58) studied the structure of β -glucan polymers from oats and barley, using specific enzymes. Their results indicated that the glucan was composed almost entirely of two types of structural sequences. These were: (i) units of four glucose residues in which a single β 1-3 linkage alternates with two β 1-4 linkages, and (ii) units of five glucose residues in which one β 1-3 alternated with three β 1-4 linkages. The enzymes used were not from barley but the results suggested an interesting possibility for barley and malt enzymes responsible for β -glucan degradation.

The enzymes used were a cellulase (β 1-4) and laminarinase (β 1-3). The reaction conditions were chosen to minimize any possible transglucosylation side reactions. Cellulase cleaved β 1-4 linkages, provided that another β 1-4 linkage occurs adjacent to it towards the non-reducing end of the chain. When the linkage adjacent to the β 1-4 linkage was

β 1-3, no cleavage occurred. The laminarinase rapidly degraded laminarin, a β 1-3 linked polymer. However, its action on barley β -glucan was to cleave a β 1-4 linkage and not a β 1-3 linkage. The β 1-4 linkage was cleaved, provided that the non-reducing glucosyl unit was substituted at the 3-position. Thus, both of these enzymes demonstrate a degree of stereospecificity with β -glucan as substrate. Such stereospecificity from non-barley enzymes suggest the possibility of a similar specificity from the barley and malt enzymes.

Luchsinger, Hou, and Schneberger (30), working with green malt, separated three fractions by means of ammonium sulfate precipitation. Two endo-barley β -glucanase enzymes were thought to be present because of different heat stabilities. Although a viscous substrate was not used, they also proposed to have found an endo β 1-3 glucanase. The fraction that degraded laminarin rapidly had little effect on β -glucan. A suggestion that linkage specificity was related to types of adjacent bonds was put forward.

The uncertainty of working with crude extracts and being unable to distinguish between a specific hydrolase and a non-specific hydrolase, necessitates greater purifications being made before characterizations are attempted. Most of the following studies were performed with partially-purified or purified enzyme preparations.

Luchsinger (31) fractionated, by successive ammonium sulfate precipitation, three fractions containing endo- β -glucanase activity. He showed different degrees of heat stability for these fractions. However,

his concept of differential stability upon dilution with water, and subsequent stabilization by addition of a variety of material, does not appear too sound, and the results were inconclusive.

In 1963, Luchsinger, Ferrell and Schneberger (32) described the preparation and purification of a laminarinase using ion-exchange column chromatography on phosphorylated (P) cellulose, diethylaminoethyl-cellulose (DEAE) and carboxymethyl-cellulose (CMC). A 50-60 fold increase in laminarinase specific activity was obtained with a 36 percent recovery. The method of ion-exchange chromatography is far preferred over a separation in terms of solubility, as differences in chemical properties of the protein will be more selective than physical properties. The purified laminarinase exhibited no detectable action (endo- nor exo-) on barley β-glucan, CMC, cellobiose, or gentiobiose. Its pH optimum curve was atypical, peaking at 4.59. The enzyme was relatively heat-stable at 50°C for two hours, and appeared to be activated by NaCl. The hydrolysis products of laminarin yielded glucose and a whole series of oligosaccharides. The oligosaccharides appeared prior to glucose, indicating that random hydrolysis was occurring. The degradation to glucose suggested that some exo-laminarinase activity was also present.

Anderson, Cunningham and Manners (2) demonstrated the presence of separate β1-3 and β 1-4 glucanases in extracts of barley, using laminarin and cellodextrins as substrate. The crude extract was fractionated by ammonium sulfate precipitation. No clear-cut separations of enzyme activities were obtained. Several inhibitors were examined, and glucono-1,

4-lactone was found to be the most selective, causing greater inhibition to cellodextrinase activity than to laminarinase activity. These two activities were further differentiated in terms of heat stability, with the cellodextrinase being the more heat stable.

A slightly different approach to the study of endo- β-glucanase specificity in barley was taken by Preece and Bhuiyan (71). The enzymolysis of barley β-glucan by enzyme preparations from barley and fungus was compared. One of the fungal enzyme preparations was thought to be specific in attacking only β1-3 linkages because it was found to attack both laminarin and β-glucan while having little effect on cellodextrin. The barley enzyme preparation readily attacked laminarin, cellodextrin, and β-glucan. The barley and fungal enzyme preparations were then adjusted to have the same initial endo activities upon β-glucan. When analyses of the hydrolysis products of the β-glucan by these two enzyme preparations were compared, it was found that less β-glucan dextrin was recoverable, by precipitation, from the sample treated with barley enzymes. It was known that the β-glucan contained both β1-3 and β1-4 linkages, and that the fungal enzyme preparation appeared to have a β1-3 linkage preference. Therefore, it was suggested that the difference in the degree of degradation by these two enzyme preparations may be due to the presence of a β1-4 linkage cleaving enzyme in the barley extract, not present in the fungal preparation. This suggestion seems to be based on the fact that the crude barley preparation cleaved all three types of substrates.

In comparing the work of Luchsinger, Ferrell and Schneberger (32) with that of Preece and Bhuiyan (71), a possible discrepancy appears to be present. Luchsinger et al. (32) isolated from germinated barley a β 1-3 glucanase which had no activity upon barley β-glucan. Preece and Bhuiyan (71) assumed that the crude enzyme preparation which degraded laminarin was also responsible for partial degradation of β-glucan. Although Luchsinger et al. (32) were using germinated barley and Preece et al. (71) were using ungerminated barley, this is not believed to explain the difference. In fact, no definite evidence is given for the assumption made by Preece et al. (71). The comparison of these two sets of results illustrates the uncertainty of working with crude extracts, and the possible discrepancies that may appear.

In 1964, Luchsinger and co-workers (14, 33) described the separation and characterization of β-glucanase enzymes from green malt. Two endo- β-glucanases, a laminarinase and carboxymethyl cellulase, were separated by chromatography on phosphorylated cellulose (33). The separation of two endo- β-glucanases that were specific for barley β-glucan was of particular interest. Neither of these enzymes would attack laminarin or CM-cellulose. Therefore, it was suggested that these enzymes might require a particular arrangement of linkages rather than a specific β1-3 or β1-4 bond.

The hydrolysis products produced by these four enzymes acting on barley β-glucan were investigated (14). The addition of the laminarinase and carboxymethyl cellulase to the endo- β-glucanases A_I and A_{II}

had no effect compared to hydrolysis products of A_I and A_{II} alone. Endo- β-glucanases A_I and A_{II} each produced four products, as determined by paper chromatography. The Rg values of the A_I products were identical to those of the A_{II} products. Two of the four products in each case had low chromatographic mobility, and, as a result, it was not certain that these two hydrolysis products were identical. The other two hydrolysis products were identified as tri- and tetrasaccharides by measurement of their degree of polymerization. Acid hydrolysis of these components and separation of the products showed that in both cases β 1-3 and β 1-4 linkages were present. It was suggested that, as β -glucan contained β 1-3 and β 1-4 linkages in the ratio of 3:7, one would not expect to see many β 1-3 linkages if these were being cleaved to any extent.

Luchsinger, Chen and Richards (36) characterized the tri- and tetrasaccharide produced by A_{II} hydrolysis of β -glucan as 3-O- β -D cellobiosyl-D-glucose and 3-O- β-D cellotriosyl-D-glucose, respectively. The minimum yield of these was 53% and 26.5%, respectively, of the total products. The two higher oligosaccharides were found to be primarily β 1-4 linked, terminating with a β 1-3 linkage at the reducing end. The determinations were made using periodate cleavage, acid hydrolysis, enzyme (emulsin) hydrolysis, degree of polymerization and melting points.

Further analysis of β -glucan structure (36) using periodate cleavage and the action of A_{II} on several tri- and tetrasaccharides of specifically arranged linkages, led Luchsinger and co-workers to conclude

the following: the β-glucan structure is 70% β 1-4, 30% β 1-3 linked glucose, as had been measured previously by several groups (15, 54). The A_{II} endo-glucanase cleaves a β 1-4 linkage with a β 1-3 linkage on the non-reducing side, and a β1-4 linkage on the reducing side; that is,

-G1-3G1-4G1-4G-

Parrish, Perlin and Reese (53) had suggested previously the possibility of stereospecificity in endo- β-glucan hydrolases. The evidence (36) seemed fairly sound except that oligosaccharides longer than tri- and tetrasaccharide should have been used for an endo-enzyme whose limit products are tri- and tetrasaccharides.

Of interest in an earlier paper by Cunningham and Manners (19) is the enzymic digestion of lichenin by malt enzymes. The lichenin polysaccharide has a β-glucan structure similar to barley β-glucan. The predominant trisaccharide produced was 4-O- β-glucosyl laminaribiose (same as 3-O- β-D-cellobiosyl-D-glucose). Evidence was also presented for the presence of two tetrasaccharides with β 1-3 linkages at the reducing ends, and acid hydrolysis of the tetrasaccharides led to production of some laminaribiose in the hydrolysate. These data tend to support the conclusions of Luchsinger et al. regarding the mode of attack by β-glucanase A_{II}. The two tetrasaccharides would be G1-4G1-4G1-3G and G1-4G1-3G1-3G, as both groups of researchers (19, 36) agree that β 1-4 linkages are being cleaved.

The problems of limit degradation of β-glucan using an insufficiently

purified enzyme were illustrated by Luchsinger and Richards (35). The problem of transglucosylation reactions was previously discussed by Anderson and Manners (1) with respect to cellobiose. Luchsinger and Richards (35) partially purified a transglucosylase enzyme. The di-, tri- and tetrasaccharides common in β-glucan hydrolysis products served as substrates for this enzyme. If the reaction was allowed to proceed long enough, the products were hydrolyzed to glucose. Whether this was a transferase reaction to water or hydrolysis by a separate enzyme, was not known.

To date, four separate endo- β-glucanases have been found. The exo- β-glucanases are of less importance and have not been studied very extensively. The two endo-barley β-glucan hydrolases from barley are important for rapid hydrolysis of endosperm cell wall material, thus allowing amylase and protease enzymes to come in contact with the cell contents of the endosperm. The function of the laminarinase in the kernel is still uncertain.

Effect of Gibberellic Acid (GA_3) on Cytolytic Activity Development During Malting

Bourne and Pierce (12) studied the development of β-glucan degrading activity through steeping, germination and kilning. The pronounced increase in activity that occurred during germination was reduced by about fifty percent during kilning. Addition of GA_3 to the steep water during the last day of steeping resulted in a further increase in the

activity of the enzymes in samples germinated two to six days.

The effects of GA_3 have been studied in the malting process for some time. It has many and varied effects on the germinating kernel. The effects of GA_3 relating to cytolytic activity are not as well documented as its effects on some other enzyme systems.

MacLeod and Millar (44), in 1962, studied the effect of GA_3 on aleurone and endosperm dissections. Incubation of these tissues with GA_3 enhanced the development of endo- β-glucanase and endo-pentosanase activities relative to water controls. It was suggested that during germination and in the absence of added GA_3, an endogenous gibberellic-like material (72) is translocated from the embryo to the aleurone where it induces secretion of hydrolytic enzymes.

Bendelow (11) studied the effect of GA_3 on two-row and six-row malting varieties. GA_3 increased the cytolytic activity (β-glucan as substrate). A comparison of the cytolytic activity of two row and six-row varieties indicated that two-row varieties increased in cytolytic activity to a greater extent.

Work in this area is continuing, and several groups are studying the effects of GA_3 on separate cytolytic enzymes. This work should provide information relative to the mechanism of synthesis of these enzymes and provide further information regarding the role of cytolytic enzymes during malting.

DEVELOPMENT OF CYTOLYTIC ENDO-ENZYME ACTIVITIES DURING GERMINATION

SECTION I

METHODS AND MATERIALS

Grain Samples

The six varieties used were provided by the Barley Section of the Canadian Grain Commission. The conditions of growth and many physical aspects of the grain are described by MacGregor, LaBerge and Meredith (40). The samples used for malts were from grain harvested at maturity.

Malting Conditions

Individual samples of each variety were steeped at 11°C for 40 hr. with a 1-½ hr. air rest every 8 hr. The samples were then germinated at 11°C for times from 1 to 6 days. Germination was stopped by freezing the samples. The frozen samples were later freeze-dried to approximately 5% moisture. The samples to be treated with gibberellic acid (GA_3) were treated in a similar fashion except for two changes in procedure. After 24 hr. of normal steeping the water was replaced with solution of 4 p.p.m. GA_3 (relative to the water). The sample was then steeped a final 16 hr. with the air rest as before. The second change was that only samples at 2, 3 and 4 days of germination were prepared.

Substrates

Carboxymethyl cellulose -- Soluble sodium carboxymethyl cellulose was a commercial preparation purchased from Nutritional Biochemical Corp.

Barley β-Glucan -- Conquest barley was ground to a fine grist in a Buhler mill and refluxed with boiling 85% aqueous ethanol, .2 kg/l for

1/2 hr. to inactivate enzymes. This material was centrifuged hot, because if allowed to cool, the materials soluble in hot aqueous ethanol precipitated as a sticky sugar-like mixture. The precipitated grist was air-dried. The grist was then mashed for 2 hr. in a mash bath at 40°C with a 0.25% papain solution. The ratio of grist to papain solution used, was 1:8 (w/w). The papain was precipitated by addition of 50 ml. of a trichloroacetic acid solution (27 gm./50 mls. H_2O) to each 400 mls. of each 50 gram mash. All insoluble materials were removed by centrifugation and filtration. To the supernatant solution, 30 g. of ammonium sulfate was added per 100 ml. of filtrate to precipitate the gum. This precipitate was collected by centrifugation and washed at least four times with cold 50% aqueous ethanol. The gum was dissolved in water, frozen and then freeze-dried.

Purity of β-Glucan -- To check the purity of the material, 10 mg. of -glucan were hydrolyzed in 2 ml. of N sulfuric acid. The reaction was carried out in a sealed tube at 110° for 2 hr. The hydrolyzed sample was then diluted to 100 ml. with 0.075M sodium borate buffer pH 7.0. One milliliter of this solution was loaded on to an ion-exchange column and eluted with a linear borate concentration and pH gradient. (0.075M, pH 7.0 --- 0.60M, pH 10). The column effluent was monitored by use of sulfuric acid - orcinol reagent with the colorimetric reading being made at 420 mu. The results of the digestion indicated that trace amounts of xylose and arabinose were present. The percentage of the constituents

by weight was glucose 96.2, xylose 2.4, arabinose 1.4. Reaction of the β -glucan with iodine indicated that some starch was present.

Carboxymethyl-Pachyman -- The pachyman used had been obtained from Seoul, Korea in the form of large fungal bodies (Poria cocos sclerotia) (16). This rock-like material was broken down and milled to a fine grist in a Buhler mill. The grist was refluxed for 1/2 hr. in an ethanol-chloroform mixture (1:2 v/v) to inactivate enzymes and also to extract lipid-like materials (200 g./l.). The mixture was filtered hot, washed with ethanol, and air-dried. This material was insoluble in water and DMSO.

Two different methods were used to prepare the soluble form of pachyman, carboxymethyl-pachyman, from the above prepared material.

The first method (called Method I subsequently) is the same as that described by Clarke and Stone (18). One hundred grams of the above treated pachyman was ground with 240 ml. of 10.5 N sodium hydroxide in a Waring blender to form a very stiff paste. A solution of 50 g. of monochloroacetic acid in 60 ml. of water was added dropwise with constant stirring. The mixture was then heated at 70°C for 4 hr., cooled, and 500 ml. of 2 N hydrochloric acid added with stirring. This changed the viscous solution into a dark-brown gel. Portions of this gel were added to portions of 2 liters of aqueous - ethanolic HCl (20 ml. conc. HCl + 80 ml. EtOH) in a Waring blender. Initially a precipitate formed but changed to a gel-like solution. This product was dialyzed against

distilled water until chloride-free. The resulting product was a very hydrated gel-like material. The water could not be displaced by successive washings with absolute ethanol. Therefore, the material was freeze-dried and kept in a desiccator. The final step involved stirring the material with four liters of acidic methanol (100 ml. 75% HNO_3 made up to one liter with methanol) for 4 hr. The precipitate was collected and dried by suction filtration.

The tan-colored product was soluble to some extent in 0.1 M NaAc. This material gave a viscous solution if 2 grams per 100 ml. were used. However, much of this material was insoluble and was removed by centrifugation. The CMP prepared by this method was hydrolyzed very slowly by malt enzyme.

The second method (called Method II subsequently) was developed because so much of the material prepared by Method I was insoluble. It was decided to selectively take only the material of the pachyman that was soluble in N sodium hydroxide at 80°C. Thirty grams of pachyman with 700 ml. of N sodium hydroxide were heated at 80°C with stirring for 20 min. This solubilized some of the pachyman and produced a very dark-brown solution. The solution was then filtered hot, through glass wool to remove the insoluble materials. The filtrate was cooled to 4°C without any precipitation occurring. The soluble pachyman was then precipitated using acidic ethanol (5 ml. HAc/250 ml. EtOH). The precipitate was collected by centrifugation and washed on a (Buchner) funnel with

acidic-ethanol and ethanol to near neutral pH. It was then dried with acetone.

For carboxymethylation, 50 g. of the above fraction was dissolved in 240 ml. 5.0 N NaOH in a Waring blender for one minute. This produced a dark-brown viscous solution. Twenty-five grams of monochloroacetic acid in 30 ml. of water were added dropwise with continuous stirring. The solution was then incubated at 70°C with occasional stirring for 4 hr. It was cooled to 4°C, and 100 to 125 ml. of 2 N HCl was added. Addition of HCl was stopped when a solid gel formed. Portions of the gel were then mixed vigorously in a Waring blender with an acidic ethanol solution (80 ml. EtOH:20 ml. conc. HCl). A white precipitate formed. The precipitate was collected by suction filtration, then dispersed in 1,200 ml. of water and dialyzed against 4 x 2-liter volumes of distilled water. The dialysate had changed to a gel by the end of the dialysis period. The final purification involved shaking this gelled product in 2.5 liters of acidic methanol (100 ml. HAc/1. of solution) for four hours. The product precipitated as a fibrous material, was collected and then washed with methanol by suction filtration. It was then dried with acetone and ground in a mortar to a fine white powder.

Titration indicated there was approximately one free carboxyl group for every three glucose units. The two changes in Method II from the method of Clarke and Stone (18) Method I, for carboxylation, were the following. A larger volume of a lower concentration of NaOH was used to

initially dissolve the pachyman. The reason for this change was that the larger volume produced a viscous solution rather than a doughy paste, (Method I), thereby facilitating a more homogeneous mixing while adding monochloroacetic acid solution.

The second change was a substitution of acetic acid for nitric acid in the methanol solution at the final purification stage. This change was made as a precaution, as nitric acid is a strong oxidizing agent which could produce changes in the CMP.

The CMP of Method II formed a solid gel at the 1% concentration level, but yielded a suitable viscous solution at 0.55% in sodium acetate buffer at pH 5.3. Also, it was almost completely soluble in the buffer and was very susceptible to enzyme attack by malt enzymes.

Enzyme Extract

The germinated samples after freeze-drying were ground to a fine grist. Two grams of this grist were extracted with 20 ml. of sodium acetate buffer, pH 5.0, at room temperature by gently shaking for 20 min. The extract was then centrifuged, and filtered to remove insoluble materials. This extract was kept at 5°C until used.

Measurement of Endo-Activity

Endo-activity was measured using Ostwald-type viscometers with flow times for water of 10 to 12 sec. All viscometric measurements were carried out in a water bath at 25.0 $\pm$ 0.1°C. Four milliliters of substrate were mixed with a suitable dilution of the extract made up to

0.4 ml. outside the viscometers. Then 4.0 ml. were pipetted into the viscometer with the first of six readings being taken within 90 seconds of mixing (zero time).

The relative viscosity, N_r, representing the ratio of the viscosity of the solution, N, to the viscosity of the solvent alone, N_o, was calculated from the respective flow times, t/t_o. From this, the specific viscosity, N_{sp}, which represents the change in solution viscosity due to solution components other than the solvent, was calculated.

$$N_{sp} = \frac{N - N_o}{N_o} = \frac{t - t_o}{t_o} = N_r - 1$$

The reciprocal of the specific viscosity was computed for each viscometric reading. These values were plotted against the mean time of each reading. The slope of the best line, calculated by least mean squares method, was multiplied by 10^5 to yield a whole number value which represented the relative endo-activity.

RESULTS AND DISCUSSION

The development of barley- β-glucan endo-hydrolase, endo β-1-4 glucanase, and endo β-1-3 glucanase activities are shown in Figures 1, 2 and 3, respectively. The varieties used in this study were grown in field plots at the University of Manitoba in 1970. Further information on the conditions of growth for these samples is found in a paper by MacGregor, LaBerge and Meredith (40). The varieties were harvested at maturity. Samples were later steeped and germinated as described in the Methods section.

The varieties used consisted of two six-row barleys (Bonanza and Conquest) and two two-row barleys (Betzes and Centennial). These four varieties were considered to have good malting quality within their respective row types. Another six-row barley, which was used, was a poor malting variety (Keystone). The sixth variety (906), was a six-row selection from a cross between two-row and six-row barleys.

Barley- β-Glucan Endo-Hydrolase Activity

This endo-activity was assessed using Ostwald-type viscometers. The viscous substrate used, was a 1% solution of barley β-glucan in sodium acetate buffer at pH 5.0.

Trends

The most striking feature with regard to the development of enzyme activity during germination is the very rapid early increase in activity

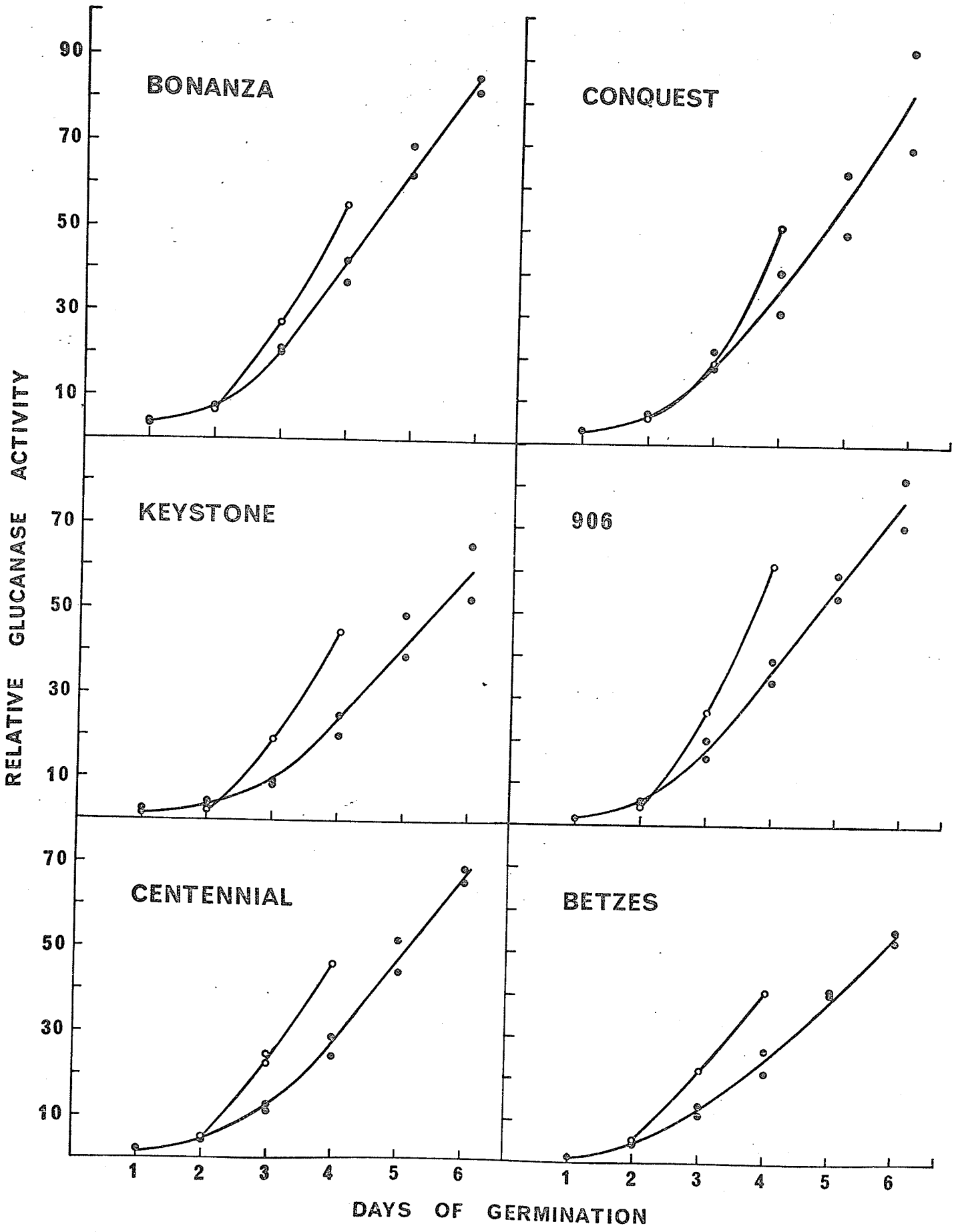
(Fig. 1). This feature is found for all six varieties. Other workers (12, 46, 64, 70) have also demonstrated this rapid increase in activity during germination. In each case, after a certain period of germination a plateau in the activity profile was indicated. This plateau should represent the maximum development of β-glucan hydrolase activity. The length of germination required to reach this activity plateau will depend upon the conditions of germination used, particularly the temperature. Other factors involved are the variety being germinated and the moisture content of the sample at the end of steeping. The possible time difference involved was illustrated by Preece and co-workers. Preece (70) indicated the activity began to level off after 10 days while Preece and Hoggan (64) found this same effect after only 4 or 5 days of germination. The results of Manners and Marshall (46) indicated an even shorter time of 3 to 4 days for the barley- β-glucan endohydrolase activity to level off. The germination temperatures used, were not stated for any of these results -- thus it is difficult to compare these results with those obtained in the present study.

The fact that the plateaus did not occur in the present study is at least partially attributable to the use of a relatively low germination temperature, (11°C), resulting in slower enzyme development. One set of results that did indicate β-glucan hydrolase development for barley germinated at a known temperature was reported by Bourne and Pierce (12). When a sample of Proctor barley was germinated at 14.5°C, this leveling-

Figure 1. Development of barley- β-glucan endo-hydrolase activity during germination. The activity was measured viscometrically for samples -

●——● steeped in water or

O——O steeped in GA_3.


BONANZA
CONQUEST
KEYSTONE
906
CENTENNIAL
BETZES
RELATIVE GLUCANASE ACTIVITY
DAYS OF GERMINATION
90
70
50
30
10
1
2
3
4
5
6

off stage in the barley- β-glucan endo-hydrolase activity required 5 to 6 days. Thus, it may be understandable that this change in the rate of development was not found. The absence of this plateau feature is not considered to be of great significance in the present study, as it is the developmental trends in activity that are being compared. What is of significance, is the relative rates of increase during germination of the β-glucan hydrolase activity because all the varieties used, were malted under identical conditions. By using the same conditions, it is hoped that differences in developmental activities can be associated with the classes of barleys used.

Differences Between and Within Row Types

In comparing the two-row varieties, Betzes and Centennial, with the six-row varieties, Conquest and Bonanza, (Fig. 1), it is evident that the six-row types have higher barley- β-glucan hydrolase activities. The comparison of these four varieties in terms of β-glucan hydrolase is made because they are all considered to have malting quality. The results of this comparison agree with Bendelow's finding (11) that in general six-row barleys have higher levels of hydrolytic enzyme activity than two-row barleys.

The selection, 906, was found to have a barley- β-glucan endo-hydrolase activity profile more similar to the six-row malting varieties than to the two row malting varieties examined. However, its activity was intermediate between the two levels. The poor-malting six-row

variety, Keystone, was found to be considerably lower in β-glucan hydrolase activity than the six-row varieties with good malting quality. Its activity was approximately the same as the two-row varieties studied.

Effect of Gibberellic Acid (GA_3)

As described in the Methods section, separate samples of the six varieties of barley were treated with 4 p.p.m. GA_3 during steeping. No stimulative effect was found for barley- β-glucan endo-hydrolase after 2-day germination at 11°C for any of the varieties. However, after 3 and 4 days of germination all six varieties demonstrated a stimulated effect, with activities above those found in normally germinated samples.

With the six-row varieties, the β-glucan hydrolase activity enhancement for Bonanza and Conquest was not as great as that for Keystone (Fig. 1). This separation within the six-row barleys also represents a separation in terms of malting quality. For the two-row barleys examined, the activity enhancement due to added GA_3 was approximately the same for both.

As can be seen in Fig. 1 the stimulative effect on β-glucan hydrolase activity for the two-row malting varieties is significantly larger than this same effect in β-glucan hydrolase activity for the six-row varieties with malting quality. The six-row selection, 906, from the cross between barleys of the two-row and six-row types showed a greater stimulative effect in β-glucan hydrolase activity than the six-row

malting types. This variety seems to have characteristics of both row types, that is, high β-glucan hydrolase activity, characteristic of six-row barleys and high β-glucan hydrolase response to GA_3, characteristic of two-row types.

Too few varieties were examined in this study to make any firm generalizations about classes. However, in terms of β-glucan endohydrolase activity and for the varieties used, it would appear that the malt of a poor malting barley could be greatly enhanced by addition of GA_3. This assessment is made on the basis of a 4-day germination study with GA_3 added during steeping. Whether this stimulation in activity is related to total synthesis or simply to a release mechanism within the cells is not known. Bendelow (11) found that normal varietal differences in β-glucan hydrolase activities of germinated barleys were reduced by treatment of the barley with added GA_3 during steeping. However, this treatment did not result in complete removal of these differences. From these results Bendelow (11) suggested that GA_3 does affect the synthesis of the β-glucan hydrolase but is not the only controlling factor. Bourne and Pierce (12) also indicate an increase in total activity for GA_3-treated barley above that of the untreated sample. This again suggested some effect on synthesis.

Relations Between Malting Quality and Barley β-Glucan Hydrolase Activity

Within the six-row varieties used there were two varieties with, and one variety without, good malting quality. Bass, Bendelow and

Meredith (8), and later Sparrow and Meredith (74) had shown that reasonably good correlations exist between cytolytic activity (β-glucan as substrate) and various parameters of malting quality. Some of the parameters used to define malting quality in that study were malt extract, cold-water extract and enzyme activity. The results of the present work support those findings, as the malting varieties Bonanza and Conquest had considerably higher barley- β-glucan endo-hydrolase activities than that of the non-malting variety, Keystone.

The relation between barley- β-glucan endo-hydrolase activity and malting quality as suggested by the correlations referred to above, is probably indirect, rather than a direct contribution to fermentable materials. More likely the relation involves the degradation of cell wall-like or matrix materials in the endosperm. Dickson and Shands (20) and Brown and Morris (13), had stated that degradation of cell walls and matrix material was necessary prior to starch degradation. Thus, the barley- β-glucan endo-hydrolase enzyme may be a controlling factor in the rate at which starch is made accessible to the amylase enzymes.

Both Betzes and Centennial are considered to be good malting, two-row barleys. In terms of β-glucan hydrolase activity, after 6 days of germination, the Centennial would appear to be the better of the two, because of a slightly higher activity.

As already mentioned, these two-row varieties are considerably lower in barley- β-glucan endo-hydrolase activity than their six-row malting

counterparts, Conquest and Bonanza. The choice of a two-row or six-row malt for brewing, based on β-glucan hydrolase activity, would probably depend largely upon the proportions of constituents used in the mash. With the present trend to use larger quantities of unmodified materials, the malt requirement would be based primarily upon the amount of enzyme activity the malt could produce. The potentially fermentable materials in this case would be added as unmodified adjuncts. Thus, for this case, a six-row malt would be preferable with its higher enzyme activity.

However, if the mash were to contain a large proportion of malt, then a two-row variety would possibly be preferable. In this case, less stress would be placed upon the requirement from the malt for high enzyme activity and more stress upon the requirement for potentially fermentable materials from the malt itself.

Regardless of the uncertain nature of the β-glucan function in the endosperm, it is suggested from β-glucan hydrolase activity development alone, that the enzyme responsible plays an important role in physiological change during germination. Consequently, it may also play an important role in modification during malting.

Role of GA_3

That GA_3 can effect stimulation of barley- β-glucan endo-hydrolase activity has been shown here, and previously (12, 64, 70). It has also been shown that a natural gibberellin-like material is present in barley

and increases in quantity during germination (72). The general consensus of reports on GA_3 stimulation in barley is that added GA_3 causes enhancement of those effects produced by the endogenous gibberellin-like hormone. The natural gibberellin appears to be produced in the embryo section of the seed and is translocated during germination to the aleurone, where it induces secretion, or de novo synthesis of hydrolytic enzymes. These hydrolytic enzymes subsequently attack and degrade endosperm materials.

If, in fact, the level of natural gibberellin-like material is a controlling factor in the level of barley- β -glucan endo-hydrolase activity, then from the maltsters' standpoint of not wishing to add GA_3, for reasons of economy, barleys may be selected for low GA_3 response. This idea has been discussed by some researchers working in the area of barley selection for malting quality. Bendelow (11) looked at one aspect of this idea. He compared the response due to added GA_3 with the initial level of cytolytic activity (barley- β-glucan endo hydrolase activity) during germination. It appeared that some relation did exist. There were exceptions but in general a trend appeared to be present.

As the natural gibberellin-like material has been reported to increase during germination (72), then it would seem better to test the correlation of the response due to GA_3 with the maximum barley- β -glucan endo-hydrolase activity. This should yield an inverse correlation if the effect of added GA_3 upon β-glucan hydrolase activity is related to synthesis. This in turn would suggest that the normal synthesis of barley- β -glucan endo-hydrolase activity is at least partially controlled by

the level of natural hormone in the seed.

Bourne and Pierce (12) tested the effect of GA_3 (3 p.p.m.) using Proctor barley and reported that the stimulative effect on barley- β -glucan hydrolase activity was of only marginal practical importance. This would be expected if this barley already had a relatively high level of endogenous hormone, as it has already been shown using added GA_3 (11, 44) that above a certain upper threshold level no further stimulative effect occurs.

In conclusion, the possible practical importance of adding GA_3 to good malting, six-row barleys for improved barley- β -glucan endo-hydrolase activity appears to be marginal. With the two-row malting barleys, the enhancement of β -glucan hydrolase activity by added GA_3 was significant at 4 days germination. However, depending upon the stress placed on the two-row malt for high enzyme activity, the need for β -glucan hydrolase activity stimulation by adding GA_3 may be removed if only a nominal activity is required for the mash. Such a nominal requirement for enzyme activity would exist where a high proportion of malt was being used in the mash. The possible significance of adding GA_3 to a poor malting, six-row barley, however, appears to be considerable. The six-row, poor malting barley, Keystone, was found to have considerably higher enzyme activity after being treated with GA_3 during steeping. This suggests that by such treatment it may be feasible to use this poorer malting barley for the mash if it were steeped with GA_3 prior to

germination.

Endo β 1-4 Glucanase Activity

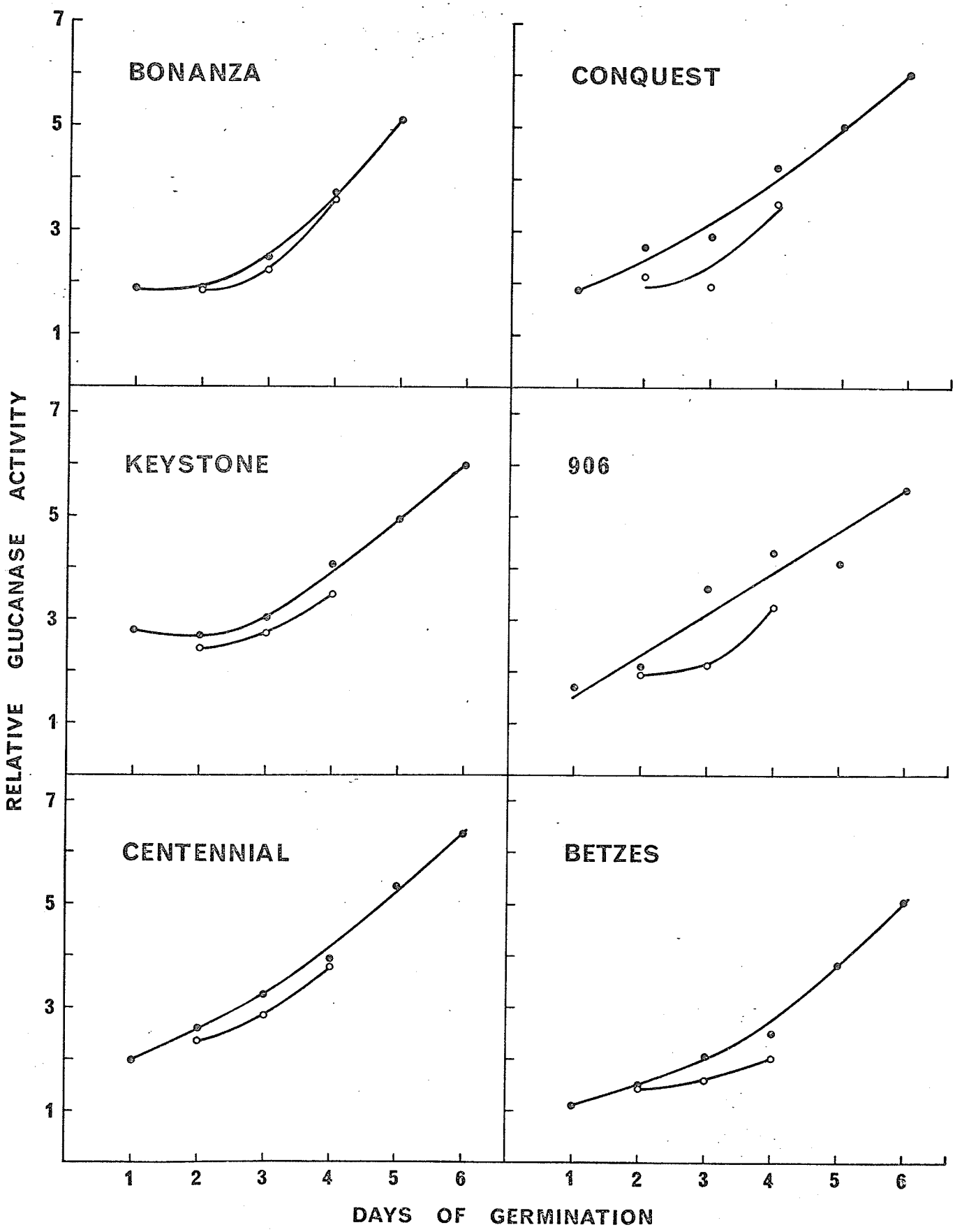
Endo β 1-4 glucanase activity is also frequently referred to as cellulase, or more specifically, as endo cellulase activity. In measuring this activity it is unfortunate that smaller β 1-4 polymers are not readily available as the natural cellulose is very insoluble in water. To get around the problem, derivatives of cellulose such as carboxymethyl and hydroxymethyl cellulose have been prepared which are soluble, and provide a suitable viscous substrate upon which the cellulase enzymes can act. In the present study sodium carboxymethyl-cellulose solution was used at approximately 0.5% concentration to measure the endo-enzyme activity viscometrically.

Trends. The development of the β 1-4 glucanase activity is shown in Fig. 2. In all varieties there is an increase in activity during germination. With the exception of the early growth stages of Bonanza and Keystone, which have an apparent lag, all varieties exhibit a linear increase. Several differences between these activity profiles (Fig. 2) and those for barley-β-glucan endo-hydrolase (Fig. 1) are observed. The lack of differences between row types, between malting and non-malting varieties, and among the varieties altogether is in sharp contrast to the differences found in the β-glucan hydrolase activity profiles. Also the endo β 1-4 glucanase activity increases slowly and appears to be unaffected by gibberellic acid, while the barley β-glucan

Figure 2. Development of endo β1-4 glucanase activity during germination. Activity was measured viscometrically for samples -

●——● steeped in water or

○——○ steeped in GA_3.


ENDO β(1–4) GLUCANASE
BONANZA
CONQUEST
KEYSTONE
906
CENTENNIAL
BETZES
RELATIVE GLUCANASE ACTIVITY
DAYS OF GERMINATION
1
3
5
7
1
2
3
4
5
6

endo-hydrolase activity increased rapidly and was stimulated by gibberellic acid.

In 1951, Lundin (39) compared the cellulase activity of a two-row variety (Herta), and a six-row variety (Stella), and found a difference of six times as much activity for the six-row as for the two-row type of barley, and four and one-half times as much activity in malt. As can be seen (Fig. 2), no such difference is even suggested in the present study.

A study on cellulase by Enebo, Sandegren, and Ljungdahl (22) may be considered of a more comparable nature than Lundin's result (39), to the results presented here (Fig. 2). Enebo et al. (22) germinated different varieties of barley for up to 13 days at a temperature of 12°C. In the first 6 days of germination, their cellulase activity increased in a curvilinear manner with a total increase of four to five times the level present at the first day of germination. After this period, there was a rapid increase in cellulase activity up to 13 days of germination. It seems unlikely, even at 12°C, that malt would be germinated for 13 days without excessive root and plumule growth. Assuming the whole seed was assayed, then this embryonic development may be of significance in the explanation of the cellulase activity reported (22).

A more recent study by Fuji and Horie (24) showed endo β 1-4 glucanase development for barley germinated at 15°C. It was found, that the cellulase activity peaked after 4 days germination at a level approximately 4 to 5 times that present after one day of germination. This activity had decreased slightly after five and one half days of germination.

In the present work, the increase in endo β1-4 glucanase activity was fairly linear with no indication of an activity maximum. Manners and Marshall (46) found a linear increase in activity up to the time of kilning. However, the barley- β -glucan endo-hydrolase activity had already peaked a day and one half earlier, suggesting that germination was further advanced than in the present study. The relative increase in endo β1-4 glucanase activity is small compared to the relative increase measured in barley- β -glucan endo-hydrolase activity. This difference should suggest the relative importance of these two enzyme activities in germinating barley.

Relation between malting quality and β1-4 glucanase activity. The early work on cellulose-degrading enzymes in barley and malt was initiated on the assumption that cellulose was the major constituent of the endosperm cell wall. Consequently, it was thought that the endo-cellulase enzyme would be of considerable significance during malting. As mentioned previously, Brown and Morris (13) had referred specifically to the degradation of "cellulose" in the endosperm cell walls. The fact that no apparent degradation of starch occurred, prior to the breakdown of the cell walls, had also been stressed. These workers' observations suggested the relative importance of the "cellulase" system. As late as 1951, cellulase enzymes were still thought to play a major role in endosperm modification, as Lundin (39) considered that if cellulase activity was low, a poor germination would result.

In 1959, MacLeod and Napier (42) cleared up much of the uncertainty associated with cellulose distribution in the kernel. They indicated the majority of the cellulose was present in the hull and that very little, if any, was present in the endosperm.

The point which appears to have been overlooked, is that synthesis and development are occurring in the embryo simultaneously with the degradation in the endosperm. MacLachlan and Perrault (41), in a study of the apical tissue of germinating pea seedlings, found cellulase activity to be present in this tissue. Its presence is thought to be associated with the retention of an elastic cell membrane during cell enlargement. A similar function for cellulase activity in other plants, including barley, seems quite possible. Also, it would be expected that as the growth of the embryo increases, more cellulase activity would be required. The known presence of cellulose in root and shoot tissue makes this idea seem more feasible than the possibility of cellulose-degrading enzymes playing a role in endosperm modification.

In barley, the increase in β1-4 glucanase activity during germination would be expected for the following reason. The embryo would induce modification of the endosperm which in turn would allow growth of the roots and shoot. With increased root and shoot growth the need for more cellulase activity would occur, to maintain the increased number of cell walls being produced.

Thus, it is proposed that much of the β 1-4 glucanase activity is

a result of endosperm modification rather than a cause of it. If this proposal is correct, then a direct influence of endo β1-4 glucanase activity on endosperm modification would not seem likely. The possibility of indirect effects is not excluded.

Endo β1-3 Glucanase Activity

The endo β1-3 glucanase activities were measured using extracts from samples of the same green malts used for assessment of β1-4 glucanase and barley- β-glucan endo-hydrolase activities. However, this work was done several months later, because a suitable substrate for β 1-3 glucanase viscometric analysis was not initially available. The substrate, CMP, prepared later by Method II, was found to be suitable and produced a viscous solution at 0.55% concentration. During the interval of time, however, between the assessment of the first two enzyme activities, and the β1-3 glucanase activity, the samples of Bonanza and 906 were lost. The development of β1-3 glucanase activity in the remaining samples is shown in Fig. III.

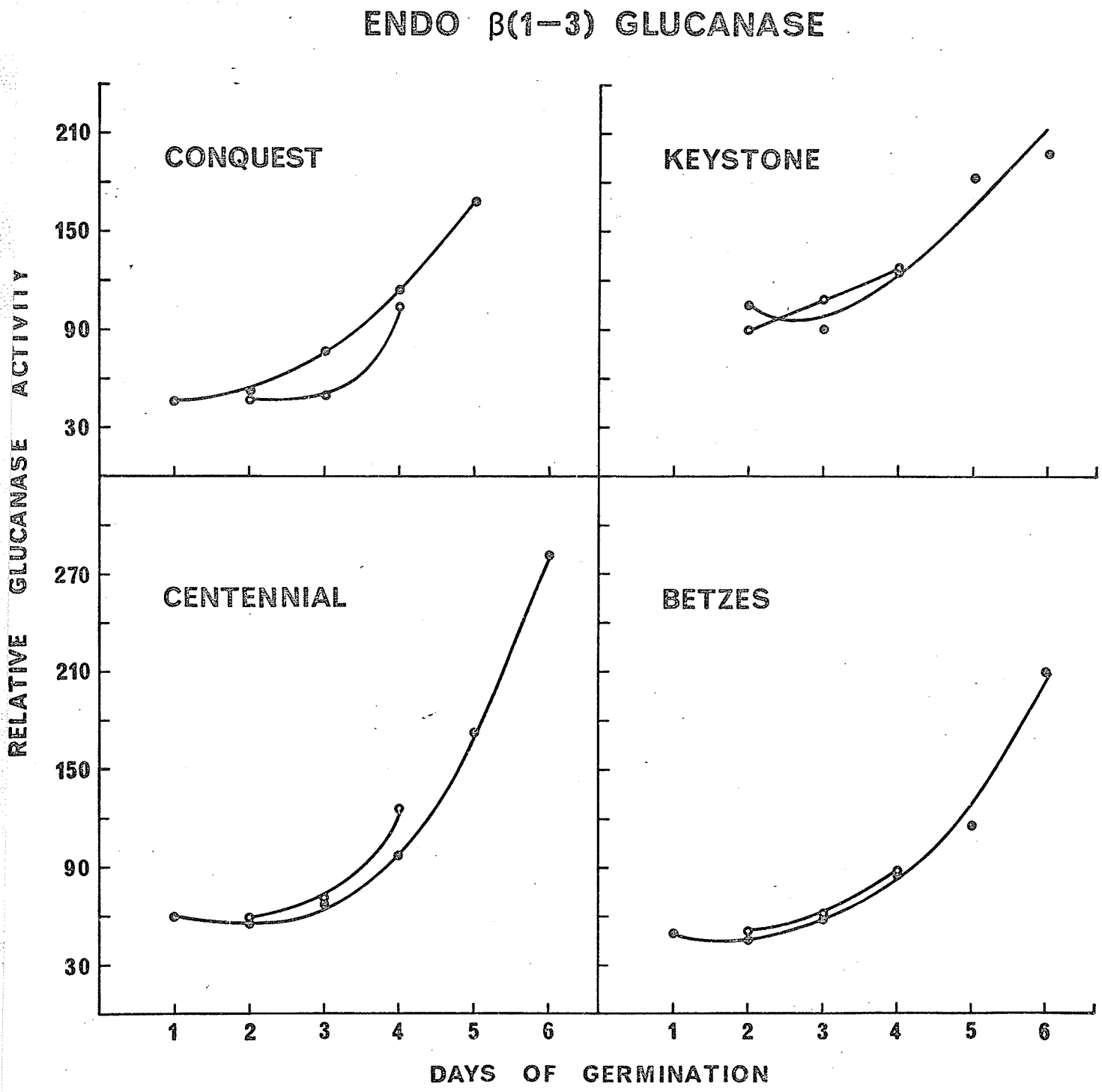
Trends and comparisons. The increase in β 1-3 glucanase activity during germination, Fig. III, is quite significant for the four varieties examined. All varieties appear to exhibit a lag in activity development during the first two to three days of germination. After this period the activity increases very rapidly.

Comparing the β1-3 glucanase activities of the two-row and six-row varieties, one finds that for the two-row varieties, Centennial and

Figure 3. Development of endo β 1-3 glucanase activity during germination. Activity was measured viscometrically for samples -

●——● steeped in water or

○——○ steeped in GA_3.


ENDO β(1–3) GLUCANASE
CONQUEST
KEYSTONE
CENTENNIAL
BETZES
RELATIVE GLUCANASE ACTIVITY
210
150
90
30
270
210
150
90
30
1
2
3
4
5
6
DAYS OF GERMINATION

Betzes, the activity increases more rapidly than for the six-row varieties, Conquest and Keystone. Centennial, particularly, has a considerably higher activity than any of the other varieties after 6 days of germination. Whether it is significant or not, it is noted that Keystone had the highest level of β 1-3 glucanase activity during the first days of germination. Manners and Marshall (46) found an increase of approximately six times the endo β 1-3 glucanase activity during germination. These results were similar to those found in the present study. Both sets of results indicated that no leveling-off trend was occurring at the end of the germination period.

Little published information regarding the endo β 1-3 glucanase activity was found. Its natural substrate has not been isolated or located in the barley kernel with any certainty. Taiz and Jones (75) detected this type of activity in the aleurone layer. These workers measured the development of β 1-3 glucanase activity in aleurone layers soaked on water. The amount of this activity released into the imbibing medium was measured. Then the aleurone layers were ground up and extracted, and the amount of activity that could be extracted was measured. The total activity was calculated by adding these two measured activities. It was found that the total activity increased about two and one half times after the aleurone layers had been soaked for 16 hr. About 80% of this activity at 16 hr. imbibition was of the extracted type, indicating that only 20% was being released. Thus, the aleurone cells seem to be holding or storing the active enzymes. These same workers (75), using

histochemical stains, claim to have found a β1-3 glucan material in the aleurone cell walls. One of the stains used was aniline blue which they reported was specific for carbohydrates containing β1-3 linking glucose residues. This histochemical stain did not affect the endosperm cells, indicating it will not bind to barley β-glucan. However, it did stain the aleurone cell walls quite intensely, suggesting the presence of a β 1-3 glucan.

Effect of GA_3. Of the four varieties treated with GA_3, only Centennial showed any indication of stimulation (Fig. III). This occurred on the fourth day of germination and not earlier. The other three GA_3-treated varieties have endo β 1-3 glucanase activities very similar to the untreated samples at corresponding germination times. Manners, Palmer, Wilson and Yellowlees (45) reported a significant increase in β 1-3 glucanase activity from extracts of malt which had been treated with GA_3 as compared to an untreated malt. Whether this difference represents a net synthesis is not known. It may be that much of the aleurone tissue remained intact, even after milling, and the difference in activity is related to the amount of endo β 1-3 glucanase activity released by the addition of GA_3.

Taiz and Jones (75) compared the effect of GA_3 upon stimulation of endo β 1-3 glucanase activity in aleurone layers from barley. Relative to water controls, GA_3 accelerated the release of this activity from the aleurone cells. This was indicated by higher activity in the imbibing

medium and lower extractable amounts of this activity relative to the water controls. No significant increase in total activity was found. The present study supports the suggestion by Taiz and Jones (75) that no increase in total activity occurs with GA_3 treatment.

Relations between malting quality and β 1-3 glucanase activity.

The role of this enzyme in malting is not known. The first reports of a malt enzyme that would degrade laminarin suggested it might attack the β 1-3 linkages of the barley β-glucan (71). However, other work (33) showed that this was not the case. The occurrence of β 1-3 glucanase activity in the aleurone layer (75) suggests that its functions are associated either with the aleurone layer itself or with adjacent tissue. Taiz and Jones treated aleurone layers with GA_3 and after varying periods of time stained the tissue with periodic acid - Schiff's reagent. Photomicrographs showed degradation of the aleurone cell wall material in a polar manner in the direction of the endosperm. These workers suggest that the observed degradation is due to β 1-3 glucanase activity. A β 1-3 glucanase activity might be partially responsible for the observed degradation, but it will not be indicated by the disappearance with time of material that can be stained by periodic acid - Schiff's reagent. This stain will not affect β1-3 linked glucose chains as there are no free adjacent hydroxyl groups between which the periodic acid can cleave. If the β 1-3 glucanases were largely responsible for the observed degradation, then series of β 1-3 linked glucose residues would have to be

separated by at least several 1-4, 1-2 or 1-6 linked residues. These residues could then be cleaved by the periodic acid - Schiff's reagent.

Assuming the enzyme does function in aleurone cell wall degradation, its relation to malting quality would still be unknown. Brown and Morris (13), and Dickson and Shands (20) had indicated that the aleurone cells appeared to remain intact even after extensive degradation had occurred within the endosperm. The endo β 1-3 glucanase activity development appeared to lag up to 3 days before it increased. Thus, this enzyme would not appear to be required for release of other hydrolytic enzymes from the aleurone cells.

It is possible that the endo β 1-3 glucanase enzyme functions to assist in the total conversion of the aleurone layer to soluble sugars for the new plant's food supply. If this is the case, then its relation to malt quality would be minimal because of its late production during growth.

The total endo β1-3 glucanase activity as measured in the present study may not be due entirely to enzymes present in the aleurone layer. Heyn (26) worked with Avena coleoptiles and reported β 1-3 glucanase activity to be present in this tissue. Thus some β 1-3 glucanase activity may also exist in the developing shoot tissue.

The present information available suggests that the endo β 1-3 glucanase does not play a significant role in modification of barley to malt. However, more extensive work will be required to determine its actual function during germination and thereby indicate its true significance

to malting quality.

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PURIFICATION AND CHARACTERIZATION OF CYTOLYTIC ENZYMES

SECTION II

METHODS AND MATERIALS

Preparation of Enzymes

Crude extract -- Seventy-five grams of a commercial green malt were extracted in a Waring Blender with 210 mls of 0.1M trisodium citrate buffer, pH 6.0, containing 0.1M $CaCl_2$ and 0.001M thioglycerol for five minutes in the cold ($4^{\circ}C$). The mixture was then centrifuged at 10,000 x g for 20 minutes. The pellet was returned to the blender and extracted successively with 135 mls and 130 mls of the buffer. The supernatant solutions were combined and filtered through glass wool.

For partitioning on CM-cellulose, the above filtrate was dialyzed against four two-liter volumes of 0.02M sodium acetate buffer at pH 4.75 and containing 0.001M $CaCl_2$ and 0.001M thioglycerol, and this is the same buffer used to equilibrate CM-cellulose columns. This method was described by LaBerge and Meredith (11) for separating β-amylases in barley and malt. Extraction into citrate buffer followed by dialysis into acetate buffer was a better extraction method than direct extraction into acetate buffer as the former method produced extracts with higher cytolytic activity (barley- β-glucan hydrolase and β 1-3 glucanase activity).

If the extract was partitioned on DEAE-cellulose, the filtrate was dialyzed against four two-liter volumes of 0.005M tris-acetate buffer at pH 8.5 containing 0.001M $CaCl_2$, the same buffer as used for equilibrating DEAE-cellulose columns.

Carboxymethyl Cellulose Column Chromatography -- Whatman CM-32 cellulose was prepared as recommended by Peterson (23). After allowing the powder to swell in the water, the fines were removed by decanting. The cellulose was then washed successively for one-half hour each with 0.5M NaOH and 0.5M HCl. The cellulose was equilibrated with 0.02M acetate buffer, pH 4.75, containing 0.001M $CaCl_2$ and 0.001M thioglycerol. A slurry of this ion-exchanger was degassed under vacuum and poured into columns (2 cm x 45 cm).

Partition of the extract on CM-cellulose was accomplished using an increasing sodium ion concentration. The system involved four vessels each containing 500 g of degassed acetate buffer at pH 4.75 containing 0.001M $CaCl_2$ and 0.001M thioglycerol. The arrangement of buffers of various sodium ion concentration were as follows:

vessel number:	4	3	2	1
sodium ion concentration: (moles/l.)	0.75 →	0.02 →	0.15 →	0.02- → to column

All columns were run at 5°C. The gradient was effective for separating α - and β-amylases in barley and malt as shown by LaBerge and Meredith (11) and by MacGregor, LaBerge and Meredith (18). The gradient produces good partitioning characteristics for barley and malt proteins on CM-cellulose.

Diethylaminoethyl (DEAE)-Cellulose Column Chromatography. Whatman DE-32 cellulose was prepared in an analogous manner to the preparation

of CM-cellulose except that DEAE-cellulose was first washed with 0.5M HCl and then with 0.5M NaOH solutions. The DEAE-cellulose was equilibrated with 0.005M Tris-HCl buffer at pH 4.75 and containing 0.001M $CaCl_2$. A slurry of the ion-exchanger was degassed and poured into columns (2 cm x 45 cm).

Partition of the extract on DEAE-cellulose was accomplished by using an increasing chloride ion concentration. A four-vessel gradient system was used with each vessel containing 500 g of degassed 0.005M Tris-HCl buffer at pH 8.5 and containing 0.001M $CaCl_2$. The increased chloride concentration was produced by addition of NaCl to the gradient vessel buffers. The arrangement of buffers of various chloride ion concentration was as follows:

vessel number:	4	3	2	1
chloride ion concentration: (moles/1.)	1.003 →	0.003 →	0.153 →	0.003 → to column

Sephadex G-100 -- A K 100/100 column (Pharmacia) containing Sephadex G-100 was prepared. This column was eluted with an upward flow of 0.02M acetate buffer at pH 4.75 containing 0.001M $CaCl_2$.

Bio-Gel P-100 -- Bio-Gel P-100 (Calbiochem) was washed extensively with water and then equilibrated with 0.02M acetate buffer at pH 4.75. A slurry of the gel beads was degassed and poured into two separate K 25/45 columns (Pharmacia). These columns were connected in series and pumped at a flow rate of 0.25 mls/min. The purpose for using two columns

was to reduce back-pressure and associated packing problems that occur when using single, long columns. The overall length of the combined columns was approximately 60 cm.

Diaflo Membrane Filtration -- The Diaflo apparatus (Amicon Corporation, Lexington, Massachusetts) was used with UM-10 ultra-filtration membranes. These membranes retain molecules of molecular weight greater than 10,000. This system was used to concentrate the enzyme solutions.

Disc Electrophoresis -- The gel formulation and reservoir buffers were described by MacGregor and Meredith (19) for disc electrophoresis at pH 4.75. The electrophoretic gels were run in glass tubes (0.5 mm x 7.5 mm) and also as gel slabs using the Ortec 4200 Electrophoresis system. The slab system was better than the individual tubes when a comparison of sample separations on different solutions was to be made. Individual tubes were more useful when slicing and subsequent analysis of activity in the gel slices were to be carried out. The gels were sliced with a commercial wire gel-slicer that produced slices of 1.5 to 2.0 mm in thickness.

The gels were stained for protein after electrophoresis with Coomassie Blue. The Coomassie Blue was prepared as described by Fishbein (8) by diluting 1% Coomassie Blue solution in ethanol twenty fold with 12% trichloroacetic acid. The gels were stained for 18 hr. in this solution. Destaining was accomplished by incubation of the gels with 12% trichloroacetic solution and intermittent changes of acid.

Nitrogen Determination -- A micro-Kjeldahl digestion was used to hydrolyze the samples. When the final digestion was made up to 100 mls before assaying for nitrogen, the proportions of the reagents were as follows: one keltab (1.5 g. potassium sulfate, 0.0075 g. selenium), 3.0 mls H_2SO_4 and 1.5 mls of 30% H_2O_2. The volume of enzyme solution digested varied (0.5 to 3.0 mls) depending on the nitrogen concentration of the solution.

Estimation of the nitrogen was made using the automated method of Mitchelson and Stowell (21) involving alkaline sodium phenate reagent. Using a range expander to amplify the colorimetric signal, a sensitivity down to 0.5 ug/ml nitrogen was obtained. Most samples digested were diluted to contain 1 to 4 ug nitrogen/ml.

Automated Reducing Sugar Assay -- This method was described by Technicon Instrument Corporation as Clinical method 02 for glucose estimation. The reagents involved were $CuSO_4$-$5H_2O$, neocuproine hydrochloride (salt of 2,9 dimethyl 1,10 phenanthroline) and Na_2CO_3. The use of NaCl described by method 02 was not included in the present assay. The chemical reaction involved reduction by reducing sugars of cupric-neocuproine complex to the highly colored cuprous-neocuproine complex. The colorimetric measurement was made at 420 mu.

The enzyme assay involved incubation of 0.1 ml aliquots of enzyme solution with 1.0 ml of the appropriate substrate for 0.5 to 1.5 hr. at room temperature. The reaction was stopped by addition of a few drops

of M Na_2CO_3 to increase the pH above 9.0. The digest was then assayed for reducing power using neocuproine.

Viscometric Assay -- Endo-enzyme activity was determined by measuring the rate of change of the specific viscosity of an enzyme-substrate reaction mixture at 25°C. Ostwald-type viscometers with flow times of 10 to 12 sec. for water were used to follow the change in viscosity. The assay was initiated by adding 4.0 mls of viscous substrate solution to a volume of enzyme solution that had been made up to 0.4 ml with water. The addition of substrate to enzyme was used as the zero time. The reaction mixture was mixed outside of the viscometer and then 4.0 mls were pipetted into the viscometer; the first of six readings was made within 90 sec. Activity was calculated as 10^5 times the slope of the change in the reciprocal specific viscosity with the mean time of incubation. That is, 1 unit of endo-enzyme activity is represented by an increase in reciprocal specific viscosity of 10^{-5}/sec.

Isoelectric Focusing -- Samples of purified enzyme were electrofocused in acrylamide gels by the method of Wrigley (27). A pH gradient from 7 to 10 was established using LKB Ampholine Carrier Ampholytes under a potential of 350 V for 3 1/2 hrs. at 4°C. The pH gradient was determined by mashing the sliced gels in 1.0 ml of 0.001M $CaCl_2$ and measuring the pH of the final solutions using a micro-electrode.

An enzyme solution was also electro-focused in a sucrose density gradient system with LKB 8100 Ampholine Electrofocusing Equipment. Using

an 8 to 10 pH range, the enzyme solution was electrofocused at 10°C for two days under a potential difference of 300 V. The density gradient was collected in 3.0 ml fractions, and the pH determined using a micro-electrode. Activity determinations were performed on aliquots of the individual fractions using reducing-power assays.

Hydrolysis Time Study of Barley β-Glucan -- One percent solutions of barley β-glucan (5.0 ml each) were incubated with aliquots of enzyme solution at room temperature for times ranging from 2 to 24 hrs. The reactions were stopped by addition of 80% ethanol to the digests. Precipitated material was removed by centrifugation, and each solution was evaporated to dryness in a rotary evaporator. The residue was dissolved in 3.0 mls of water, de-ionized with an Amberlite mixed-bed resin and stored at 4°C until analyzed.

Paper Chromatography -- Whatman 3-MM chromatography paper was used with n-propanol:ethyl acetate:water (14:2:7) as the solvent. After spotting, the chromatograms were irrigated using descending chromatography. Reducing sugars were stained with a saturated 1% solution of $AgNO_3$ in acetone and the spots were developed with alkoli. Excess $AgNO_3$ was removed by washing the chromatogram with 10% sodium thiosulfate.

Ion Exchange of Carbohydrate-Borate Complexes -- The method described by Kesler (6, 7) for the separation of sugars from hydrolysates was used. The column (0.6 cm x 64 cm) was packed with Technicon Type S polystyrene resin. The column was operated at a flow rate of 1.0 ml/min.

and at 55°C. The carbohydrate solutions were loaded in 1.0 ml or less and were partitioned with a two vessel sodium borate gradient.

0.6 M, pH 10 ⟶ 0.075 M, pH 7.0 ⟶ to column

The column was monitored continuously for carbohydrate using sulfuric acid - orcinol reagent (0.1% orcinol in 70% H_2SO_4).

INTRODUCTION

As mentioned initially, one of the objectives of this research was to attempt to separate, purify, and characterize several of the endo-enzymes responsible for degradation of β-glucan polymers in malt. The three enzymes studied were endo β 1-3 glucanase and two barley- β -glucan endo-hydrolases, which will be referred to as M_I and M_{II}.

To characterize an enzyme properly, it is necessary to separate and purify the enzyme so that there is little doubt as to what is producing the chemical change that is being observed. Where crude preparations are used in characterizations associated enzyme systems, naturally present in the organism, may add to or mask the effects produced by the principal enzyme being studied.

The early methods used in attempting to purify enzymes from barley and malt involved precipitation using dehydrating agents. The possibility of separating one protein from many others in terms of solubility is very slight. Another problem associated with the precipitation method is that, if organic solvents are used as dehydrating agents, the possibility of protein denaturation increases greatly. Ammonium sulphate when used was a better precipitating agent than organic solvents because the presence of salt helped to stabilize the proteins.

Aluminum hydroxide columns were also used and effected a separation by selective adsorption. The adsorbed materials were subsequently eluted with salt. This method was not very selective.

In the last twelve to fifteen years the number of techniques that have become available for separation, detection and analyses of proteins has been phenomenal. The understanding of the principles behind many of these methods may be considerably older than the methods, but only in recent years have these principles been put to practical application. However, even with these developments, the complexity of the proteins in living organisms is such as to make the purification of a specific protein a very challenging task.

In the present work, it was hoped that the three endo-enzymes (endo β 1-3 glucanase, and barley- β -glucan endo-hydrolases M_I and M_{II}) could be purified sufficiently to characterize them without interference from associated enzyme systems. The characterization of some of the properties of these enzymes may be useful in subsequent purification procedures. Understanding these enzymes may be useful for explaining a particular behavior or effect and may result in a better comprehension of the complex cytolytic system present in barley. From the information available in Section I, it would appear that the three endo-enzymes being studied here may be of some significance in germinating barley.

Detection of Enzyme Activity

Prior to separation of certain cytolytic enzymes, a method of analyzing solutions for activity had to be selected. As endo-enzymes were being examined, a method for determining endo-enzyme activity specifically was required. The best method available for this purpose was a

reducing viscosity method utilizing a viscous substrate solution for detecting enzyme activity. This requirement was acceptable for CMP, barley β-glucan, wheat pentosan and soluble CM-cellulose. However, viscosity measurements were time-consuming and required reasonably large amounts of substrate.

To avoid using the viscometric method for all column assays, a secondary method of assaying for activity was chosen. This second method involved measurement of reducing power using the neocuproin colorimetric method described in the methods. The reducing-power method measures total reducing groups produced by both exo- and endo-enzymes and is not as specific for endo-enzyme activity as the viscometric method. However, it was an automated method and allowed faster analyses of column fractions and required less substrate than the viscometric method. It was hoped initially, and later confirmed, that the reducing-power assay could be used to monitor the endo-enzyme activities through the various stages of purification provided that viscometric analyses were used later to confirm the presence of endo-enzyme activities in the active column fractions.

Separation of Endo-Enzyme Activities

In attempting to separate several of the endo-enzymes in the cytolytic enzyme system, two methods of separation were initially considered. These involved separation by ion-exchange column chromatography on carboxymethyl cellulose (CM-cellulose) and separation by molecular sieving

on Sephadex G-100.

On CM-cellulose, a separation of three endo β-glucanase enzymes was accomplished using an increase in sodium ion concentration at pH 4.75 to partition the enzymes (Fig. 1a). The column fractions were assayed for reducing-power activity and for reducing-viscosity activity, using four different substrates. The activities detected using barley β-glucan, CMP, and wheat pentosan as substrates are shown in Fig. 1b, c and d. The fourth substrate was soluble sodium carboxymethyl cellulose. However, no activity was detected using this substrate although β 1-4 glucanase activity was present in the crude extract. Because the support system, CM-cellulose, is a substrate for this enzyme, the enzyme probably forms an enzyme-substrate complex and can not be eluted by molar NaCl.

Two separate endo- β-glucan hydrolases were detected using barley β-glucan. With CMP, two separate β 1-3 glucanase enzymes were detected colorimetrically. Only one of these enzymes had endo β 1-3 glucanase activity as determined by viscosity measurements using CMP. The assay for pentosanase indicated reducing-power activity across the entire column effluent. No endo-pentosanase activity was found.

The separation of cytolytic enzymes according to molecular size was considerably less promising. A preparative K 100/100 column of Sephadex G-100 (Pharmacia) was washed with buffer for several days and then loaded with a crude extract. Four separate types of enzymes were detected with similar elution volumes. Thus, no separation of the enzymes

Figure 1. Separation of cytolytic enzymes from extracts of green malt by chromatography on CM-cellulose.

a) Fractionation of proteins of green malt by gradient elution chromatography on CM-cellulose.

——— absorbance of column effluent at 280 mu

- - - - Na^+ gradient (moles/l)

b) Detection of enzymes that degrade barley β-glucan.

0———0 barley- β-glucan hydrolase (reducing-power activity)

●———● barley- β-glucan endo-hydrolase reducing-viscosity activity)

c) Enzymes active on carboxymethyl pachyman.

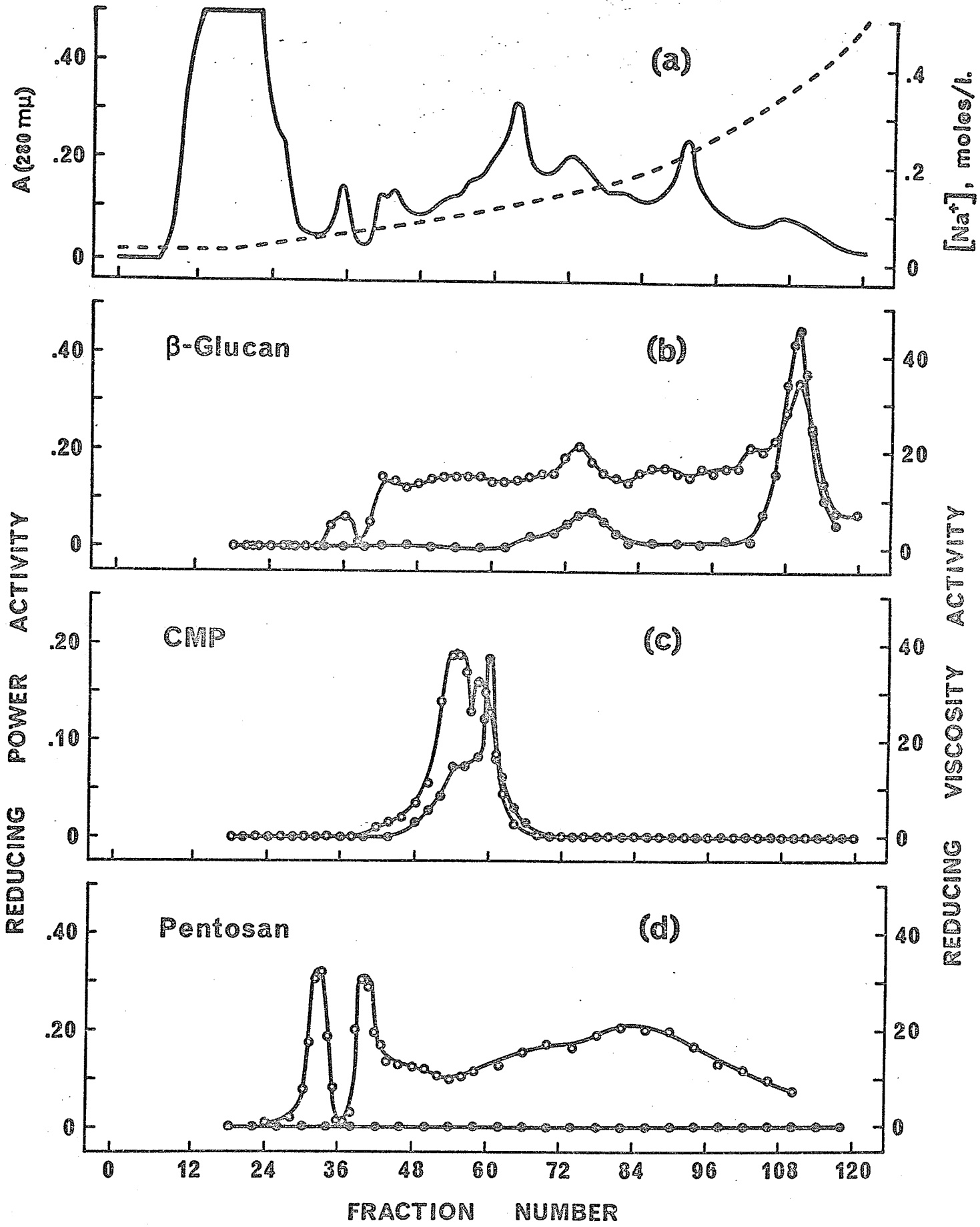
0———0 β 1-3 glucanase (reducing-power activity

●———● endo β 1-3 glucanase (reducing-viscosity activity)

d) Enzymes that attack soluble wheat pentosans.

0———0 pentosanase activity (reducing-power activity)

●———● endo-pentosanase activity (reducing-viscosity activity)


(a)
A (280 mμ)
[Na+], moles/l.
.40
.20
0
.4
.2
0
β-Glucan
(b)
CMP
(c)
Pentosan
(d)
REDUCING POWER ACTIVITY
REDUCING VISCOSITY ACTIVITY
40
20
.10
0
12
24
36
48
60
72
84
96
108
120
FRACTION NUMBER

had occurred. The only apparent advantage of this method was that a β 1-4 glucanase enzyme was eluted from the column.

Following this preliminary work, purification of three endo β-glucanases was initiated, including two barley- β-glucan endo-hydrolase enzymes and an endo β 1-3 glucanase. The purification and characterization of these enzymes will be discussed in separate sections.

Endo β 1-3 Glucanase

As already described, the β 1-3 glucanase activity was separated from barley β-glucan hydrolase activities by chromatography on CM-cellulose (Fig. 1b, c). In the initial study, CMP (Method I) was used as a substrate. Because the enzyme would not very readily attack CMP, incubations were excessively long. For this reason, another substrate, laminarin, was examined. Laminarin is an algal β-glucan containing β 1-3 and β 1-6 linkages. The β 1-6 linkages make up 2 to 10 per cent of the total linkages. It was found that β 1-3 glucanase enzymes could be detected with either CMP or laminarin, but the enzymes attacked laminarin much more rapidly. Thus, a 0.2% laminarin solution was used for the reducing-power assay.

The CMP prepared by Method I (Section I) was used during the early purification of the endo β 1-3 glucanase enzyme to ensure that a β 1-3 linkage-cleaving activity was being observed. When more CMP was prepared (Method II, Section I), it was found to be considerably more susceptible to cleavage by the enzyme. Thus, in the characterization study,

CMP (Method II) was used for both reducing-viscosity and reducing-power assays.

Purification I

Seventy-five grams of green malt grist was extracted as described in the Methods and dialyzed against 0.02M acetate buffer, pH 4.75, containing 0.001M $CaCl_2$ and 0.001M thioglycerol. The final volume of dialysate, after four changes of buffer (2 liters each), was approximately 550 milliliters. One-hundred-and-fifty-milliliter aliquots of this crude extract were separated on three CM-cellulose columns using the sodium ion gradient described in the Methods and illustrated in Fig. 1a. Aliquots of the column fractions were incubated with laminarin to measure reducing-power activity. The results were similar to the double peak for reducing power shown in Fig. 1c. However, the second peak was considerably larger than the first peak, indicating greater reducing-power activity. When the column fractions containing the reducing-power activity were assayed viscometrically, a normal distribution of activity was found with no shoulder of endo-enzyme activity as indicated in Fig. 1c. The viscosity activity peak coincided with the second reducing-power activity peak. No endo-enzyme activity was found under the first reducing-power activity peak suggesting that this activity was due to an exo β 1-3 glucanase or a β 1-3 glucosidase.

Fractions containing the endo β 1-3 glucanase activity were pooled to exclude as much of the reducing-power activity of the first peak as

possible. The fractions containing the first reducing power-activity peak were discarded as it was only the endo-enzyme that was being considered.

The pooled fraction containing the endo β 1-3 glucanase activity was concentrated to one fourteenth of its original volume by membrane filtration in an Amicon cell.

An aliquot of this concentrated enzyme solution was then loaded onto a Bio-Gel P-100 column. The column was eluted with 0.02M acetate buffer, pH 4.75, 0.001M in each of $CaCl_2$ and thioglycerol. The effluent was monitored by ultraviolet light at 280 millimicrons. The absorbance at this wavelength indicated at least four proteins were present and had been eluted in close succession. The peak endo β 1-3 glucanase activity was found in column fractions containing portions of two major protein absorbance peaks.

Tubes containing the endo β 1-3 glucanase activity were pooled and concentrated. To determine the number of proteins that were present, separation on disc electrophoresis was effected by the method of MacGregor and Meredith (19) for low pH disc electrophoresis. The electrophoretic gels were stained for protein with Coomassie Blue. Seven separate protein bands appeared; three of these were stained quite intensely. Thus, it appeared that a different purification procedure was necessary in order to remove the extra proteins from the endo β 1-3 glucanase protein.

Purification II

As ion-exchange chromatography is a very selective method for protein purification, it was decided to incorporate the use of a second ion-exchange method into the purification procedure. Diethylaminoethyl cellulose (DEAE) was tested for its suitability for separating endo β 1-3 glucanase from other extraneous proteins. The DEAE-cellulose was prepared as described. A new citrate extract of green malt was prepared, and this was dialyzed against a similar Tris-HCl buffer as was used to equilibrate the DEAE-cellulose column. Several attempts were made to find a pH and chloride ion concentration (Tris-HCl buffer) at which the endo β 1-3 glucanase enzyme would bind to the DEAE-cellulose ion-exchanger. At pH 7.5, 8.0 and 8.5 with Tris-HCl buffer at a concentration of 0.005M, the endo β 1-3 glucanase activity from the malt extract always eluted in the frontal peak indicating it was passing directly through the column without binding. Figure 2a illustrates the protein separation of the malt extract on DEAE-cellulose at pH 8.5 using the gradient indicated. Figure 2b illustrates the distribution of β 1-3 glucanase activity. The frontal peak, up to the time when the gradient was started, was collected as a unit volume as no separation based on its ion-exchange properties could be expected during this period.

On considering the protein distribution, Fig. 2a, it was noted that a considerable amount of protein had been separated from the β 1-3 glucanase fraction by this procedure. The frontal peak containing the endo

Figure 2. Purification on endo β 1-3 glucanase from green malt. Reducing-power activities are presented as absorbance at 420 mu.

a) Chromatography of green malt extract on DEAE-cellulose.

——— absorbance at 280 mu

- - - - Cl^- ion concentration (moles/l)

b) Activity of β 1-3 glucanase enzymes eluted from DEAE-cellulose (a).

●———● β 1-3 glucanase (0.2% laminarin)

0———0 barley- β -glucan hydrolase (0.2% β -glucan)

c) Rechromatography of β 1-3 glucanase on CM-cellulose

d) Final purification of β 1-3 glucanase on Bio-Gel P-100

β 1-3 glucanase activity was therefore pooled, concentrated and dialyzed with 0.02M acetate buffer, pH 4.75, containing 0.001M $CaCl_2$ and 0.001M thioglycerol. After four changes of dialysis buffer, the dialysate was loaded onto a CM-cellulose column and eluted with the gradient used previously (Fig. 1a). The separation, in terms of protein distribution, is indicated in Fig. 2c. A comparison of Fig. 1a and Fig. 2c indicated that the DEAE-cellulose ion-exchange method had removed most of the protein of the crude malt extract. Only three major protein absorbance peaks were detectable by UV absorbance at 280 millimicrons. The column fractions were analyzed for β 1-3 glucanase activity by the reducing-power assay, and it was found that the activity peak corresponded to the second protein absorbance peak. The presence of endo-enzyme activity in the column fraction containing reducing-power activity was confirmed by viscometric analysis using CMP.

The apparent shift of the endo β1-3 glucanase illustrated in Fig. 2c from the elution position illustrated in Fig. 1c is a result of a slower flow rate that was used for the column separation illustrated in Fig. 2c. Also, the CM-cellulose column used in this separation (Fig. 2c) was not washed with starting buffer between completion of loading and initiation of the elution gradient. The CM-cellulose column that was used in the first separation (Fig. 1c) was washed for one hour with starting buffer in order to elute a large frontal peak from the column. This wash, however, would cause a small movement of the proteins along the ion-exchanger, resulting in an earlier elution than would occur from

a column not receiving the wash. This point is mentioned so as not to misinterpret the change in elution behavior of the enzyme.

The fractions containing the endo β 1-3 glucanase activity from the CM-cellulose separation were pooled, concentrated, and an eight-milliliter aliquot was loaded onto a Bio-Gel P-100 column. The profile of absorbance at 280 millimicrons is shown in Fig. 2d. At highest sensitivity for the UV absorbance spectrophotometer, very little protein was detected. Three protein peaks are just detectable. The endo β 1-3 glucanase activity was located (Fig. 2d), and the fractions containing this activity were again pooled and concentrated.

At each stage of purification, aliquots of the respective enzyme solutions were saved for measurements of activity, determination of nitrogen content and for disc electrophoresis. Disc electrophoresis at pH 4.75 using a slab gel (Fig. 3) illustrates the stages of purification. After elution from Bio-Gel P-100, and concentration, it can be seen that only two protein bands are present at pH 4.75. Electrophoresis of the final purified fraction was rerun in glass tubes. After electrophoresis, three gels were stained with Coomassie blue and three more were sliced with a commercial wire gel-slicer. Slices from the gel were taken sequentially from the top down to the methyl green dye band and incubated with 0.2% laminarin. After a suitable incubation time, the reducing-power activity was measured. The activity was found to be concentrated within two slices from each of the gels. The R_f value of

Figure 3. Disc electrophoresis of endo β 1-3 glucanase at various stages of purification. From right to left the stages are.

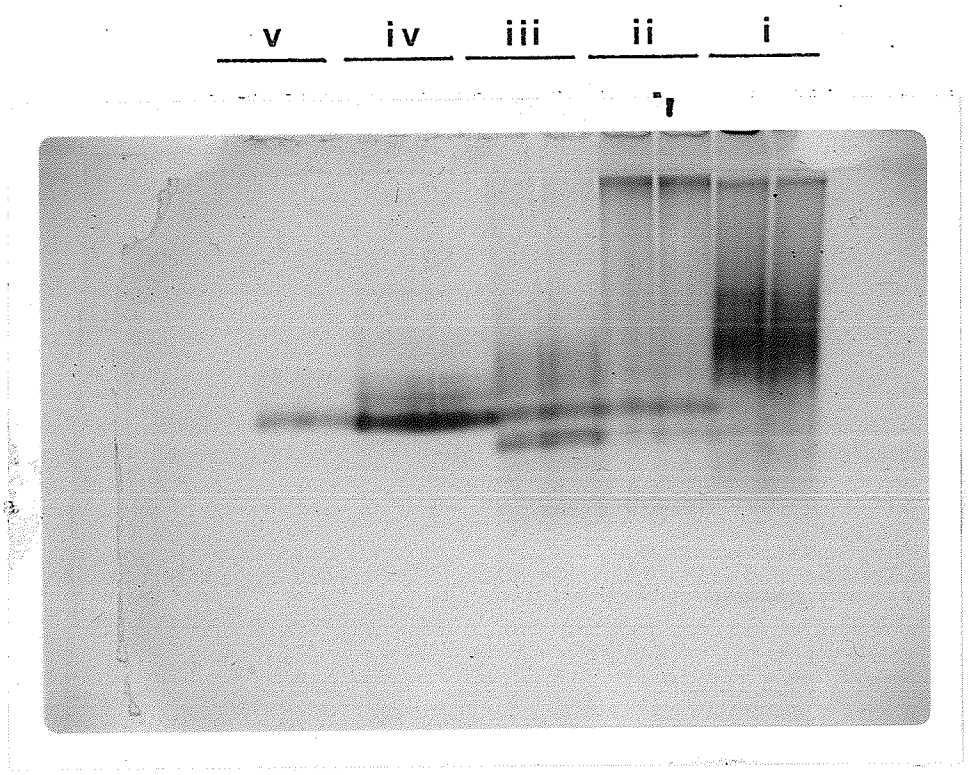
i) crude malt extract in Tris-HCl buffer

ii) after separation on DEAE-cellulose

iii) following dialysis against 0.02M acetate buffer at pH 4.75

iv) following rechromatography on CM-cellulo ɜ

v) final purified enzyme after separation on Bio-Gel P-100


v
iv
iii
ii
i

this activity band relative to the methyl green dye band was compared with the R_f values of the two Coomassie-blue-stained protein bands. The activity band coincided quite closely with the less intense and less mobile protein band. No activity was found at a corresponding position for the major protein band. It was concluded that the endo β1-3 glucanase activity had been separated sufficiently to proceed with characterization.

The purification of endo β1-3 glucanase activity in terms of specific activity and per cent recovery at each stage of purification is presented in Table I.

The purification appears to proceed with little loss of activity up to the stage involving separation at low pH and CM-cellulose. At this stage, a 42% loss in activity occurred. Although the separation on CM-cellulose results in a significant loss in activity, it appears to be a very useful step as a five-fold increase in specific activity from the previous purification step was achieved. The final stage of purification on Bio-Gel P-100 resulted in considerable loss in activity. Whether this occurred during separation or subsequently, due to excessive dilution prior to concentration, is not known. Bio-Gel P-100 is an effective step in terms of removing other proteins from β1-3 glucanase protein as indicated by disc electrophoresis (Fig. 3).

Several possibilities exist for stabilization of this enzyme. Evidence will be present in the Characterization section to show that this

TABLE I

PURIFICATION OF ENDO β 1-3 GLUCANASE

Stage of Purification*	Total Volume	Endo Activity /ml.	Total Activity	% Recovery	N (mg./ml.)	Specific Activity	Relative Specific Activity
(1)	668	465	310,260	100	.546	839	1
(2)	173	1,770	306,210	99	.184	9,620	12
(3)	190	1,535	291,650	94	.144	10,660	13
(4)	30	5,388	161,640	52	.098	54,980	66
(5)	40	1,050	42,000	13.5	.033	31,818	38

* Stage (1). A sodium citrate extract of green malt was dialyzed into 0.005M Tris-HCl buffer at pH 8.5.

continued ...

TABLE I (continued)

Stage (2). The extract was separated on DEAE-cellulose and the frontal peak that contained the activity was concentrated.

Stage (3). The concentrated solution of Stage 2 was dialyzed to 0.02M acetate buffer, pH 4.75.

Stage (4). The fraction was partitioned on CM-cellulose and the fractions containing activity were pooled and concentrated.

Stage (5). The concentrated solution of Stage 4 was separated on a Bio-Gel P-100 column, and the fractions containing activity were concentrated.

enzyme is almost 5 pH units away from its isoelectric point when subjected to chromatography at pH 4.75. It may be better in terms of stabilization of activity to dialyze the concentrated enzyme solution after CM-cellulose chromatography, back to pH 8.5, and then separate it on Bio-Gel P-100. A higher salt concentration may also help to stabilize the endo β 1-3 glucanase enzyme.

Two other groups of workers have attempted to purify an endo β 1-3 glucanase from malt. Luchsinger and co-workers made progress in purification of an endo β 1-3 glucanase in 1963. Luchsinger, Ferrell and Schneberger (13) reported the separation of a laminarinase on CM-cellulose and P-cellulose in a citrate-phosphate buffer. The laminarinase was considered to have endo-enzyme activity because, on hydrolysis of laminarin, large oligosaccharides were produced besides mono- and disaccharides. Specific activities indicated a 57-fold increase in activity.

Luchsinger and Richards (12) separated a crude extract on P-cellulose using three discontinuous sodium phosphate gradients. In this study, the laminarinase was separated from two barley- β -glucan hydrolases. The separation using P-cellulose appears to be very similar to that obtained in the present study on CM-cellulose.

Manners and Marshall (20) reported a purification of endo β 1-3 glucanase from malt. An ammonium sulfate-precipitated fraction was further purified on a Bio-Gel P-60 column equilibrated with 0.001M

acetate buffer at pH 5.3. Two successive separations on Bio-Gel P-60 resulted in a sixty-fold purification of endo β1-3 glucanase activity. The purified enzyme has no action on barley β-glucan, lichenin, or cellodextrin. In the present work, although only a preliminary study was made, the separation of β-glucanase enzymes by molecular sieving did not appear to be feasible. All of the enzymes had approximately the same elution volume. However, the use of polyacrylamide gels of high cross-linkage by Manners and Marshall (20) suggests that dextrans of higher cross-linkage than P-100 may also be very effective for purifying these enzymes.

Characterization

The purified enzyme, containing an additional protein band as shown by disc electrophoresis was used for all characterization experiments. The initial characterization involved finding the optimum pH for reaction of the enzyme with laminarin and CMP.

Effect of pH. Two separate effects were noted in 0.08M Tris-Acetate. The first involved the effect of different substrates on the pH optimum. The two β 1-3 glucan substrates were laminarin and CMP, neither of which are naturally-occurring substrates in barley or malt. As laminarin solutions cannot be assayed viscometrically, the activity was assayed by increase in reducing power for both laminarin and CMP. The results are indicated in Fig. 4. Two distinct pH optima were obtained using the two separate substrates. With laminarin, the pH

Figure 4. Effect of different substrates on pH optimum of endo β 1-3 glucanase activity.

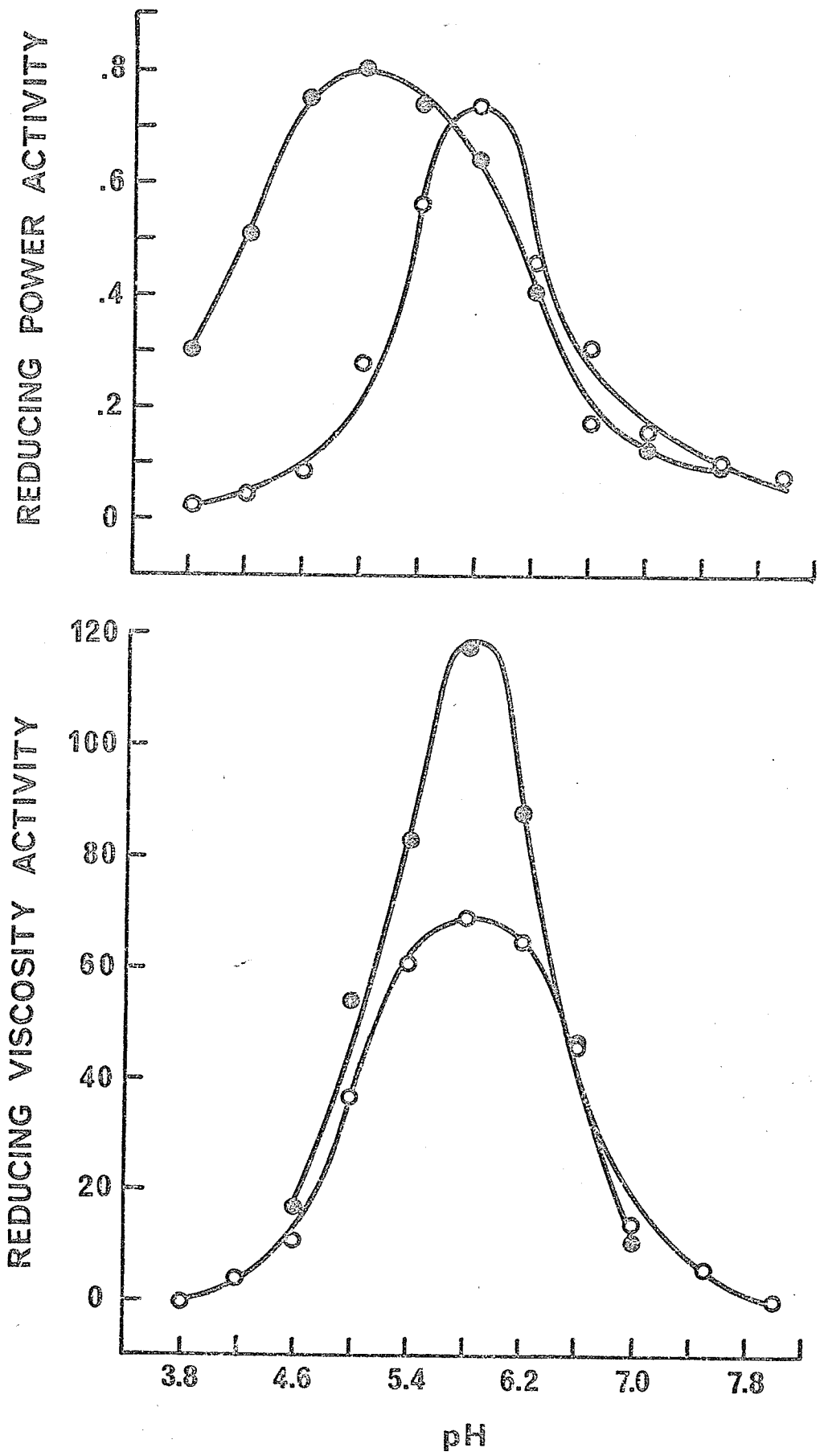
●——● 0.2% laminarin

O——O 0.55% CMP

Figure 5. Effect on pH on endo β 1-3 glucanase activity.

O——O with substrate and buffer

●——● with substrate, buffer and 0.1M NaCl


REDUCING POWER ACTIVITY
.8
.6
.4
.2
0
REDUCING VISCOSITY ACTIVITY
120
100
80
60
40
20
0
3.8
4.6
5.4
6.2
7.0
7.8
pH

optimum appears to be at 5.0, while with CMP the optimum pH appears to be 5.8 to 5.9. The initial impression is that two enzymes are present. The separation by electrophoresis at pH 4.75 and subsequent analysis of activity within the gel, however, did not support this conclusion, as only one activity band was found. Isoelectric focusing on acrylamide gel provided no evidence to support the presence of two enzymes. In fact, the isoelectric focusing experiments, to be discussed presently, supported the presence of one β 1-3 glucanase enzyme.

The difference in pH optima observed for β 1-3 glucanase activity on the two substrates may be due to a substrate effect. The laminarin may have from 2 to 10 per cent of its glucose residues linked by β 1-6 linkages with the remainder of the residues linked by β 1-3 linkages. The presence of these β 1-6 bonds would probably have a pronounced effect on the conformation of the polymer in solution.

The CMP, although composed entirely of β 1-3 linkages, has dissociable side groups in the form of carboxymethyl functions. The presence of these side groups alone may have an effect on the polymer conformation, but in addition these side groups are ionic and will be affected by pH. This dissociation constant estimated by titration of the CMP appears to be approximately 3.7. This would mean that in the pH range from 5.0 to 6.0 that CMP would be largely dissociated. Thus, a combination of steric hindrance by the side group and ionic interaction between CMP and the β 1-3 glucanase enzyme may result in a shift in the pH optimum.

The pH optimum of 5.8 to 5.9 measured by reducing power for the β 1-3 glucanase on CMP was confirmed by the reducing-viscosity method (Fig. 5). It was noted that the substrate viscosity (N_{sp}), measured in the pH range from 3.8 to 8.0, increased by two and one half times between pH 3.8 and 4.6 but, thereafter, remained relatively stable. Thus, it did not appear that any change had occurred in the CMP in the range 5.0 to 6.0 to account for the shift in activity.

Another sample of purified β1-3 glucanase was used to repeat the experiment in order to test the effect of adding sodium chloride. A very pronounced effect was observed. The enzyme activity in this second sample was 20 to 30% higher than that of the first sample. However, a very sharp pH optimum was observed with 0.1M NaCl in the reaction mixture. The NaCl specifically, or the increased ionic strength of the solution, appears to have stimulated the endo β1-3 glucanase activity, but only in the vicinity of the pH optimum. The reason for this effect is difficult to explain. The effect of NaCl and various other ions on the activity of endo β 1-3 glucanase will be discussed later but the present results strongly support the occurrence of a pH optimum for activity on CMP at 5.8 to 5.9.

Manners and Marshall (20) had also assessed the pH optimum for a purified endo β1-3 glucanase from malt acting on CMP. These workers reported a pH optimum of 5.4 in acetate buffer for this enzyme. The pH optimum curve these workers reported was the normal dumbbell-shaped

distribution curve as was observed in the present study. A somewhat different result was obtained by Luchsinger, Ferrell and Schneberger (13). These workers found the pH optimum for their purified laminarinase occurred at approximately 4.6. The pH-activity distribution curve reported was atypical with a sharp peak of activity being reported on the top of an otherwise normal curve. The laminarinase appeared to have endo-substrate activity as indicated by the presence of large oligosaccharide products. However, small products, including glucose, were also found. The laminarinase was prepared from germinated barley. From these two sets of results (13, 20) and the results presented here, two contingencies may be postulated.

It is possible that the endo β 1-3 glucanase protein varies slightly in amino acid structure from variety to variety, resulting in small changes in pH optima. Significant differences of pH optima have been observed, however, and this would seem to suggest a rather major structural change in the vicinity of the enzyme active site. Such changes would not really be expected among varieties.

The second possibility is that an associated β 1-3 glucanase activity is present in the partially purified enzyme fractions being used for pH-optimum studies. Where a reducing-power assay was used, an active exo β 1-3 glucanase enzyme could produce an apparent pH optimum different from that of the endo-enzyme activity that is supposedly being measured. Where an assay specific for endo-enzyme activity was used, such a change should not be observed.

Effect of salts and thiol. The effects of sodium chloride, calcium chloride and thioglycerol (thiol) on the endo 1-3 glucanase activity were tested. The results are presented in Table II. CMP (0.55%) was used as the substrate in 0.1M Tris-Acetate at pH 5.9

The activities were estimated viscometrically. The per cent activity was calculated from triplicate results relative to a blank where water was added in place of the reagent. Although Luchsinger et al. (13) had reported a 40 to 50% stimulation by addition of NaCl at 0.01 to 0.02M concentration to the original acetate buffer, no such stimulation was found in the current study. However, a stimulative effect by 0.1M NaCl was strongly suggested in the pH-optimum study for this enzyme (Fig. 5). The study on the effect of salts and thiol was carried out at the optimum pH for CMP and differed from the system used in the pH study only in that the Tris-acetate buffer concentration for the substrate was increased from 0.08 to 0.1M. If the change in stimulation by 0.1M NaCl is related to this change in ionic strength, it would suggest a very high sensitivity to salt over the narrow range of 0.08 to 0.10M sodium acetate. Manners and Marshall (20) reported no significant stimulation of activity by NaCl.

The effect of $CaCl_2$ appears to be of an inhibitory nature. It is not certain whether this is an apparent inhibition due to a possible substrate effect or a true enzyme inhibition. The divalent calcium ion may result in a strong binding between dissociated carboxyl functions

TABLE II

EFFECT OF SALTS AND THIOL ON ENDO β1-3 GLUCANASE ACTIVITY

Reagent	Concentration	% Activity Relative to the Blank with Water
Blank	-	100
NaCl	.025 M	100
NaCl	.050 M	102
NaCl	.100 M	105
$CaCl_2$	.025 M	94
$CaCl_2$	.100 M	23
Thioglycerol	.001 M	95
Thioglycerol	.01 M	95

on the CMP. A decrease in the initial viscosity was observed when $CaCl_2$ was added relative to the water blank. That this change in the initial viscosity may account for the apparent inhibition is negated by the fact that a similar decrease in initial viscosity was observed when NaCl was added. As can be seen, in Table II, no decrease in activity due to NaCl was observed. This suggests that a true inhibition rather than an apparent inhibition was produced by $CaCl_2$.

Thioglycerol produced a small inhibition at 10^{-3}M concentration. However, no further decrease in activity was observed when the level of thioglycerol was increased tenfold.

Manners and Marshall (20) reported a 42% stimulation by $CaCl_2$ at 0.05M concentration for the β 1-3 glucanase activity. This activity was measured on laminarin by the increase in reducing power. The possibility that it was an exo-enzyme that was being stimulated in activity appears to be nullified by the fact that this enzyme fraction had little effect on di- and trisaccharides. Once again, there appear to be significant discrepancies between the results of Luchsinger et al. (13), Manners et al. (20), and the present result.

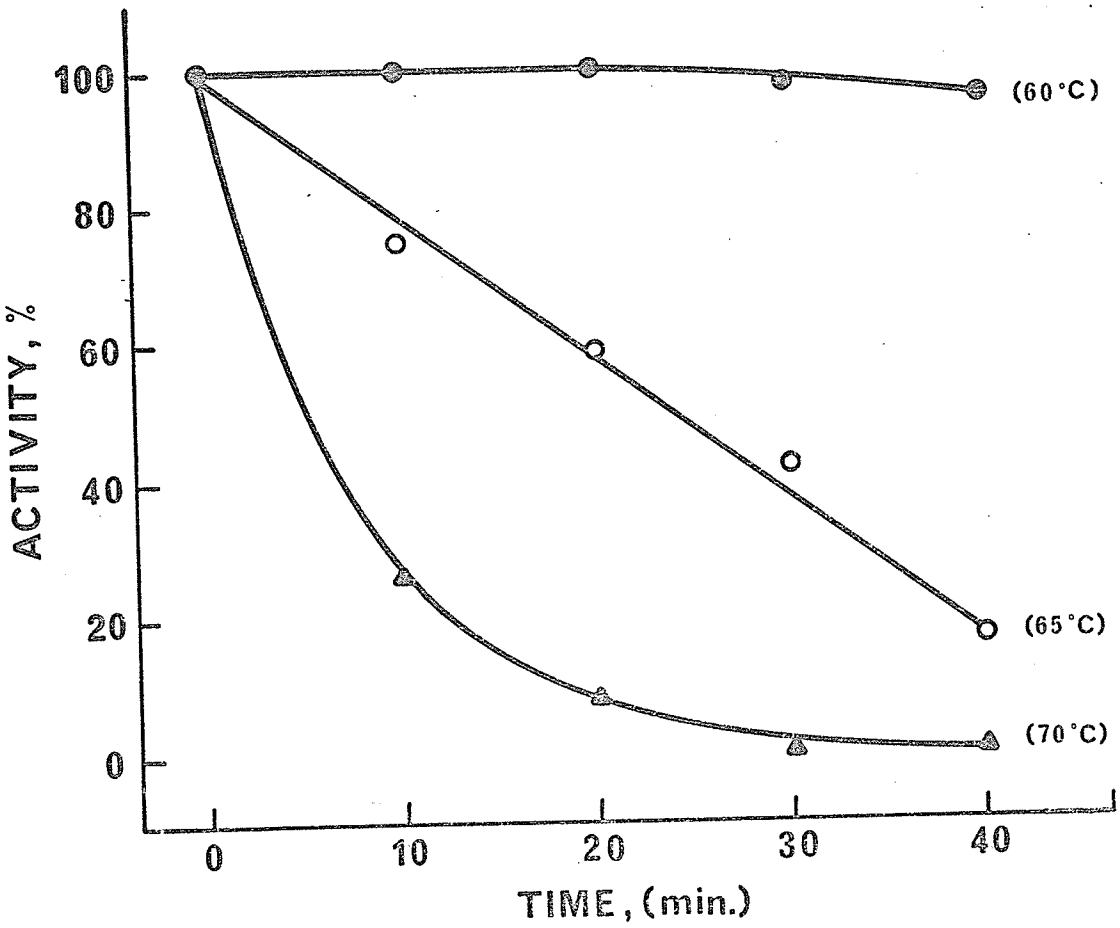
Heat stability study. The β 1-3 glucanase activity for this particular study was assessed by increased reducing power on both CMP and laminarin. The results coincided very closely with one another. The heat stability of the enzyme at 60°, 65° and 70° is indicated in Fig. 6. The enzyme is very stable at 60°C for 40 min., but is rapidly inactivated

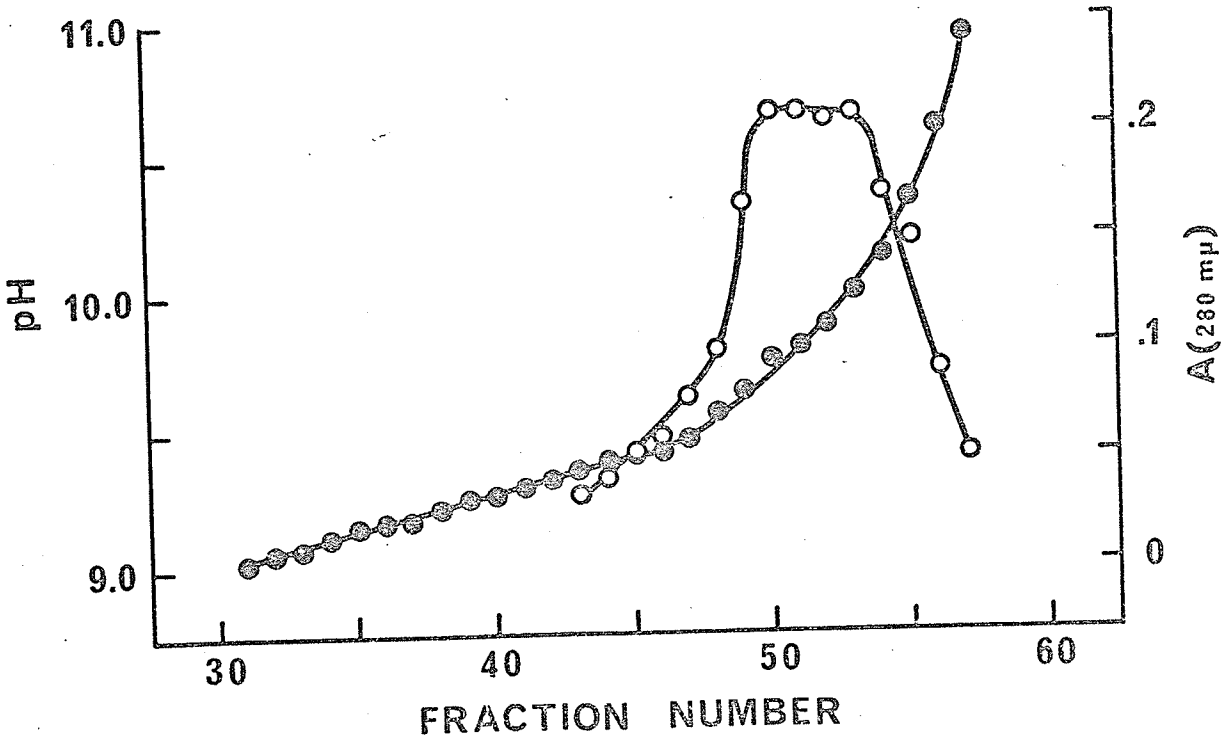
Figure 6. Heat stability of endo β 1-3 glucanase. The endo β 1-3 glucanase activity was measured relative to the activity of an unheated enzyme aliquot. Activity was measured by the reducing-power assay.

Figure 7. Isoelectric focusing of endo β 1-3 glucanase in a sucrose density gradient.

●——● pH gradient

0——0 β1-3 glucanase activity measured in absorbance units at 420 mu (not 280 mu)


100
80
60
40
20
0
ACTIVITY, %
(60°C)
(65°C)
(70°C)
0
10
20
30
40
TIME, (min.)


11.0
10.0
9.0
pH
.2
.1
0
A(280 mμ)
30
40
50
60
FRACTION NUMBER

in a linear manner at 65°C. If this enzyme is of importance during mashing it should be quite active for a reasonable length of time.

Isoelectric point. The isoelectric point of the endo β 1-3 glucanase enzyme appeared to be unusual. The fact that it did not bind to DEAE-cellulose at pH 8.5 suggested that its isoelectric point was above 8.5. Also, when disc electrophoresis was carried out on the purified enzyme at pH 8.9, there were no protein bands apparent after staining with Coomassie Blue. After determining the isoelectric point for the enzyme the reason for this behaviour was obvious.

Isoelectric focusing was initially carried out in acrylamide gels as described in the methods. The pH range of the ampholyte used was 7.0 to 10.0. Gels were stained with Coomassie Blue after incubating the gel in 12% TCA for several days to remove the ampholyte. A sharp protein band was found within 1 mm. of the high pH end of the gel. Other gels were sliced into slices of approximately 2 mm., mashed in separate tubes and incubated with 1 ml. of 0.001M $CaCl_2$. The pH of each solution was measured using a micro electrode, and aliquots were incubated with 0.2% laminarin. The activity was found to be in the last slice of the gel at the high pH end. The pH of the gel, however, was only about 9.3, while the range should have been 7 to 10. It was concluded that only an apparent pH was being observed because of dilution. The actual pH may have been somewhat higher.

The enzyme was also analyzed in a sucrose-density-gradient

isoelectric-focusing system. The range for the ampholyte system used was 8.0 to 10.0. After 2 days and using 300 V potential difference, fractions were collected, the pH was measured, and the fractions were tested for activity. The results are indicated in Fig. 7. The activity loss amounted to greater than 90% after 2 days. The broad activity peak indicated the protein had not finished concentrating. The manufacturer of this electrophoresis equipment (LKB Produkter, Sweden) recommended a period of 5 to 6 days to reach the equilibrium for proteins in the pH range of 8 to 10. This procedure, however, was not followed because of the anticipated loss in activity. The results, however, are sufficient to indicate an isoelectric point for this enzyme of approximately 9.8. Such a protein would be expected to have a very unusual amino acid composition. The unusually high isoelectric point should be a good characteristic for recognition of this enzyme, as very few proteins have such a high isoelectric point.

Summary

In summary, the endo β 1-3 glucanase was purified to the point of having only one apparent contaminating protein, as indicated by disc electrophoresis at pH 4.75. It had a pH optimum of 5.0 on laminarin and 5.8-5.9 on CMP. The enzyme was not significantly affected by NaCl in the range of 0.025M to 0.10M, but was inhibited quite strongly by $CaCl_2$ at 0.1M. Thioglycerol had a small inhibitory effect at 10^{-3}M concentration. This enzyme was quite stable at 60°C for 40 min. It also had an

unusually high isoelectric point compared to most proteins.

Barley- β -Glucan Endo-Hydrolase M_{II}

Purification

From the preliminary work relating to the separation of the endo- β -glucanases, it was decided that a separation by chromatography on CM-cellulose would be a good initial purification procedure for barley- β -glucan endo-hydrolase M_{II}. An extract of green malt was dialyzed against 0.02M acetate buffer, pH 4.75, to prepare the extract for chromatography on CM-cellulose. The sodium-ion gradient and the resulting separation of proteins are indicated in Fig. 8a.

Analysis of the column fractions by reducing-power and reducing-viscosity assays using β-glucan as substrate were carried out, and the results are indicated in Fig. 8b. Two reducing-viscosity activities were found. The second peak (Fig. 8b), which had the higher activity of the two, was designated endo-hydrolase M_{II}. Endo-hydrolase M_{II} was more tightly bound to CM-cellulose than most of the other proteins and was eluted relatively free from other proteins (Fig. 8a, b). A peak of activity that appeared to be due to an exo- β -glucanase or a β -glucosidase was eluted just prior to the endo-hydrolase M_{II}. The small shoulder peak was found on all chromatograms. It was detected with reducing-power assays and not with reducing-viscosity assays, strongly suggesting that the activity was due to an exo- β -glucanase or a β -glucosidase.

Figure 8. Purification of barley- β -glucan endo-hydrolases M_I and M_{II} from green malt. Reducing power activities are presented as absorbance at 420 mu.

a) Chromatography of green malt extract on CM-cellulose.

——— absorbance at 280 mu

- - - - Na^+ concentration (moles/1)

b) Barley- β -glucan hydrolase activity eluted from CM-cellulose (a).

●——● barley- β -glucan endo-hydrolase activity (reducing-viscosity activity)

O——O barley- β -glucan hydrolase activity (A 420 mu)

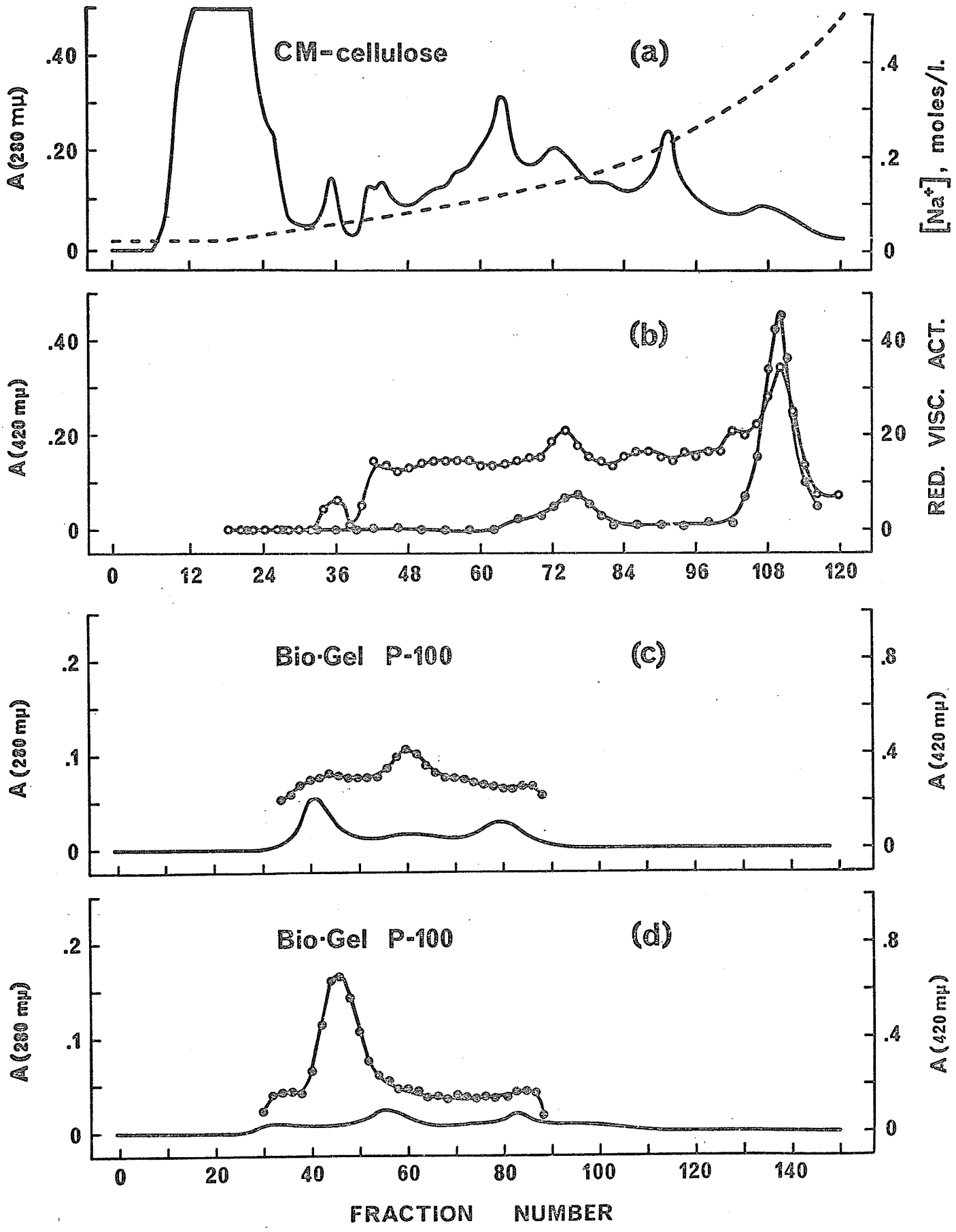
Endo-hydrolases M_I and M_{II} were eluted from the CM-cellulose column at fraction numbers 75 and 110, respectively.

c) Rechromatography of endo-hydrolase M_I on Bio-Gel P-100

●——● reducing-power activity due to endo-hydrolase M_I.
(0.2% β-glucan)

d) Rechromatography of endo-hydrolase M_{II} on Bio-Gel P-100

●——● reducing -power activity of endo-hydrolase M_{II}.
(0.2% β -glucan)


CM-cellulose
(a)
A (280 mμ)
[Na+], moles/l.
.40
.20
0
.4
.2
0
(b)
A (420 mμ)
RED. VISC. ACT.
40
20
0
0 12 24 36 48 60 72 84 96 108 120
Bio-Gel P-100
(c)
A (280 mμ)
A (420 mμ)
.2
.1
0
.8
.4
0
Bio-Gel P-100
(d)
A (280 mμ)
A (420 mμ)
0 20 40 60 80 100 120 140
FRACTION NUMBER

An unusual feature of the reducing-power assays (Fig. 8b) is that an apparent shift in base line occurred after fraction 45. The CM-cellulose gradient system was the same as that used by LaBerge and Meredith (11) and by MacGregor, LaBerge and Meredith (18) to separate the α- and β-amylases of barley and malt. The amylases, reported by these workers (11, 18), would have been eluted successively across column fractions 48 to 110, and these fractions coincide with the increased absorbance of the base-line that remains constant following the change in absorbance. As indicated in Methods and Materials (Section I), the β-glucan solution, when tested with iodine, produced a small positive test for starch. Thus, it appears that a limit degradation product of starch might be responsible for the base-line shift. Evidence will be presented later that this limit product was maltose.

The endo-hydrolase M_{II} activity was pooled to exclude most of the shoulder exo-enzyme activity peak and concentrated by Diaflo membrane filtration. A portion of the concentrated solution was loaded on a Bio-Gel P-100 column. The column effluent was monitored at 280 mu. The chromatogram is illustrated in Fig. 8d. At highest sensitivity for the spectrophotometer, very little protein was detected in the column effluent. The activity was located (Fig. 8d) using the reducing-power assay and confirmed as endo-hydrolase M_{II} using the reducing-viscosity assay.

The endo-hydrolase M_{II} activity was concentrated and found to be stable at $4^{o}C$ over a two-week period. The possibility of a further

purification on DEAE-cellulose did not seem to be feasible. The results obtained with crude extracts (Fig. 2b) indicated that β-glucan hydrolase activity eluted across a large number of column fractions. A significant loss in barley β-glucan hydrolase activity also occurred during the separation.

Two other groups of workers have attempted to purify barley- β -glucan hydrolase from barley or malt. Luchsinger (14) and Luchsinger and Richards (12) separated two endo- β -glucanases from germinated barley that were active on barley β-glucan. The enzymes were differentiated by chromatographic characteristics on P-cellulose (12) and by heat stability (14). The more heat-stable component, A_{II}, (12) appears to correspond to the endo-hydrolase M_{II} of the present study in terms of chromatographic characteristics on anion-exchange columns (P-cellulose and CM-cellulose).

Manners and Marshall (20) separated malt β-glucanases and found one barley- β -glucan endo-hydrolase. Another endo-enzyme was thought to have been inactivated during kilning. However, a heat-stable component was purified by Bio-Gel P-60 chromatography, and this enzyme may be similar to endo-hydrolase M_{II} of the present study and endo- β -glucanase A_{II} described by Luchsinger and Richards (12).

Characterization

It is not likely that an exo- β -glucanase or a β -glucosidase would have the same chromatographic characteristics as endo-hydrolase M_{II}

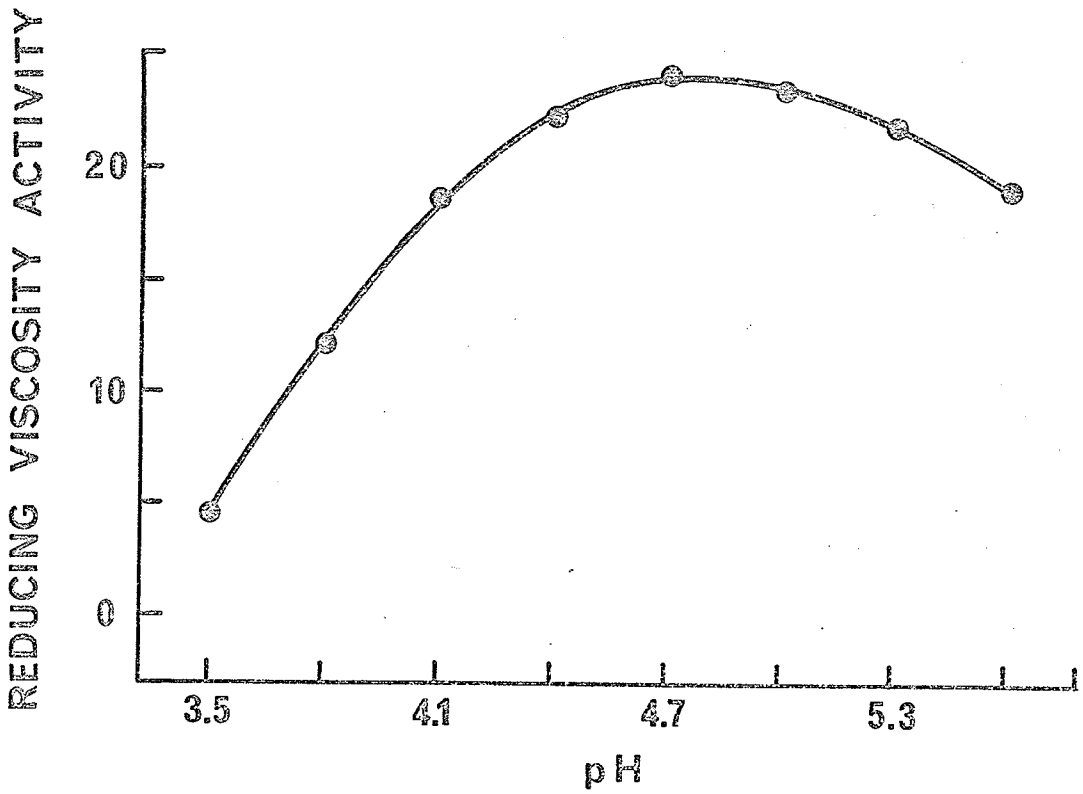
on both CM-cellulose and Bio-Gel P-100. Manners and Marshall (20) indicated that two β-glucosidases from malt had molecular weights of 35,000 and greater than 60,000 compared to about 15,000 for endo- β - glucanase. Therefore, the β-glucosidases are considerably larger than the endo-enzyme and should be separated by chromatography on Bio-Gel P-100 if any of these enzymes were pooled with the endo-enzyme following chromatography on CM-cellulose. To be certain that only endo-hydrolase M_{II} activity was being observed, viscometric assays were used for characterization studies.

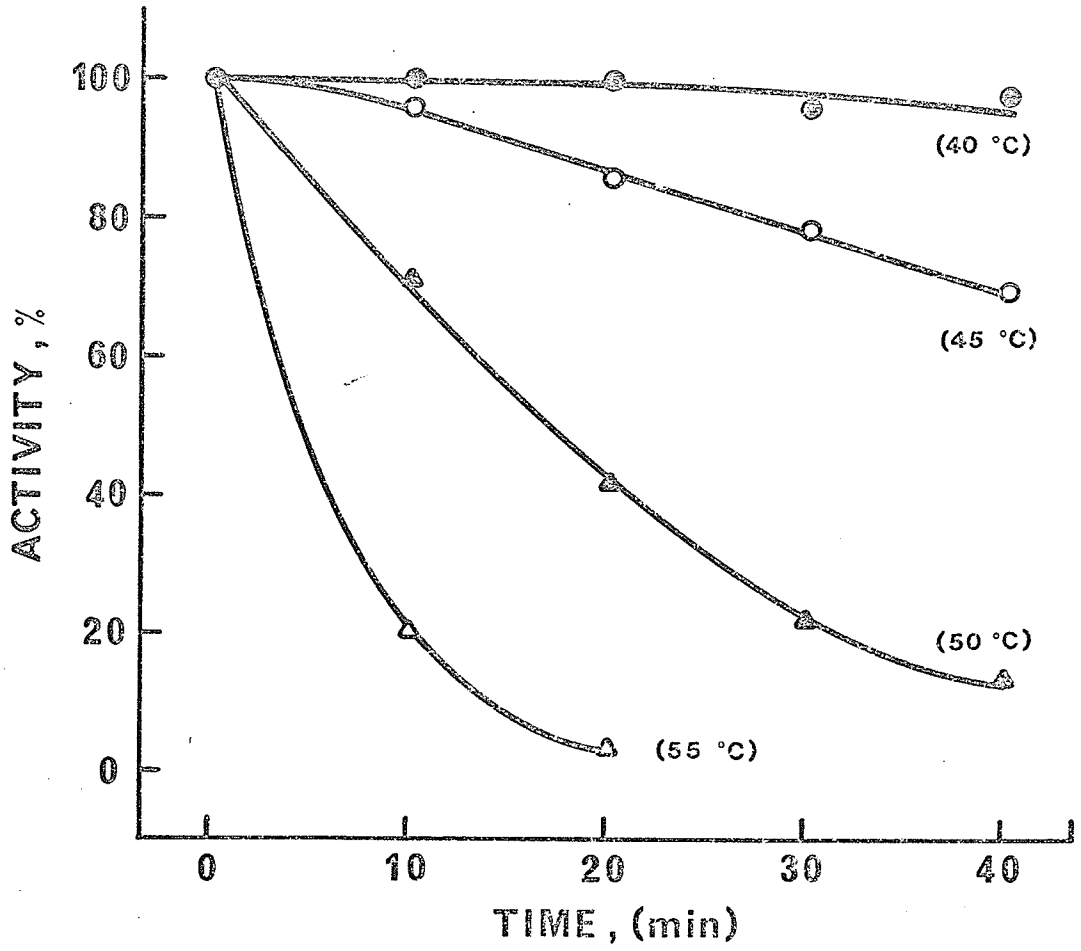
Effect of pH. To measure the effect of pH on endo-hydrolase M_{II} activity, 1% solutions of barley β-glucan in 0.1M acetate buffer were prepared in the pH range from 3.5 to 5.6. The effect of pH on the endo-hydrolase M_{II} activity is shown in Fig. 9. Each point represents the mean value of three separate activity determinations. A broad pH optimum was found suggesting that the enzyme is quite active over the pH range from 4.5 to 5.0. The peak activity appears to be approximately 4.8. As the addition of 0.1M NaCl had sharpened the pH optimum curve considerably for endo β1-3 glucanase, similar tests were performed with this enzyme. However, no sharpening of the pH optimum was produced by 0.1M NaCl.

In 1960, Bass and Meredith (3) separated several β-glucanase fractions on alumina. The pH optimum of one fraction for viscosity-reducing activity was found to be 4.7. A relatively flat activity curve was

Figure 9. Effect of pH on barley- β-glucan endohydrolase M_{II} activity.

Figure 10. Heat stability of barley- β-glucan endohydrolase M_{II}. The percent activity was measured relative to the activity of an unheated enzyme aliquot.


REDUCING VISCOSITY ACTIVITY
20
10
0
3.5
4.1
4.7
5.3
pH


ACTIVITY, %
100
80
60
40
20
0
(40 °C)
(45 °C)
(50 °C)
(55 °C)
0
10
20
30
40
TIME, (min)

observed across the range of pH values from 4.5 to 5.0. This is similar to the results of the present study on endo-hydrolase M_{II}. Luchsinger (14) reported a value of 4.55 for the pH optimum of the heat-stable A_{III} endo- β -glucanase. The pH-optimum curve was relatively flat between 4.3 and 4.7 suggesting that this enzyme may also be endo-hydrolase M_{II}.

Effect of salts and EDTA. The various chemicals listed in Table III were tested for their possible effects on barley β-glucan endo-hydrolase M_{II} activity. A 1% barley β-glucan solution, made up in 0.1M acetate buffer at pH 4.8, was used for the viscometric analysis.

The endo-hydrolase M_{II} activity was stimulated to a small degree by 0.1M NaCl but inhibited by $CaCl_2$. This suggests that the Cl^- ion is not producing a predominant effect, as both stimulation and inhibition occur for salts in which Cl^- is the common ion. Nor is the stimulative effect likely to be due to the specific ion effect of Na^+ as Na^+ ions were present at the 0.1M concentration in the substrate buffer. Thus, it appears that the stimulative effect is due to a change in the protein conformation caused by the increased ionic strength on addition of NaCl.

The addition of $CaCl_2$, although resulting also in an increase in ionic strength, produced an inhibitory effect. This appears to be due to the Ca^{++} ion. As the concentration of $CaCl_2$ increased, the activity decreased. Throughout the purification $CaCl_2$ was present in all buffers at 0.001M concentration. It would also be present in the enzyme fraction used for this study. This fact may explain the possible stimulative

TABLE III

Effect of Salts and EDTA on
Barley- β -Glucan Endo-Hydrolase M_{II} Activity

Reagent	Concentration	% Activity Relative to the Blank with Water
H_2O (Blank)	-	100
NaCl	.1 M	108
EDTA	.005 M	103
EDTA	.014 M	100
$CaCl_2$	.025 M	91
$CaCl_2$	.050 M	88
$CaCl_2$	.100 M	80

effect that was noted with the addition of EDTA. The EDTA may have bound up the small amount of Ca^{++} ion present. No comparable work was done by Luchsinger and his co-worker on the effect of salts on the A_{III} endo β-glucanase.

Heat stability. Endo-hydrolase M_{II} enzyme solutions were incubated in a water bath at fixed temperatures for 40 min. Aliquots were removed at 10-min. intervals, and the endo-enzyme activity was determined. The stability of the enzyme at the various temperatures is illustrated in Fig. 10. At 45°C the enzyme was slowly inactivated with a 25% loss after 40 min. This temperature was specifically considered as certain mashing procedures use an initial 45°C mash for the first half hour, after which the temperature is increased to 65°C. It can be seen that this enzyme would be quite active during the initial 30 min. but would lose activity very rapidly as the temperature was increased to 65°C. Where an initial 65°C mashing procedure is used, this enzyme would be active for only a few minutes.

Again, endo-hydrolase M_{II} appears to be similar to Luchsinger's (14) heat-stable A_{III} component. Luchsinger reported that A_{III} endo β-glucanase required 5 hr. incubation at 40°C to produce 50% inactivation.

The endo- β-glucanase studied by Manners and Marshall (20) must also have been fairly heat-stable. Although these workers did not test the heat stability of endo- β-glucanase directly, the enzyme survived

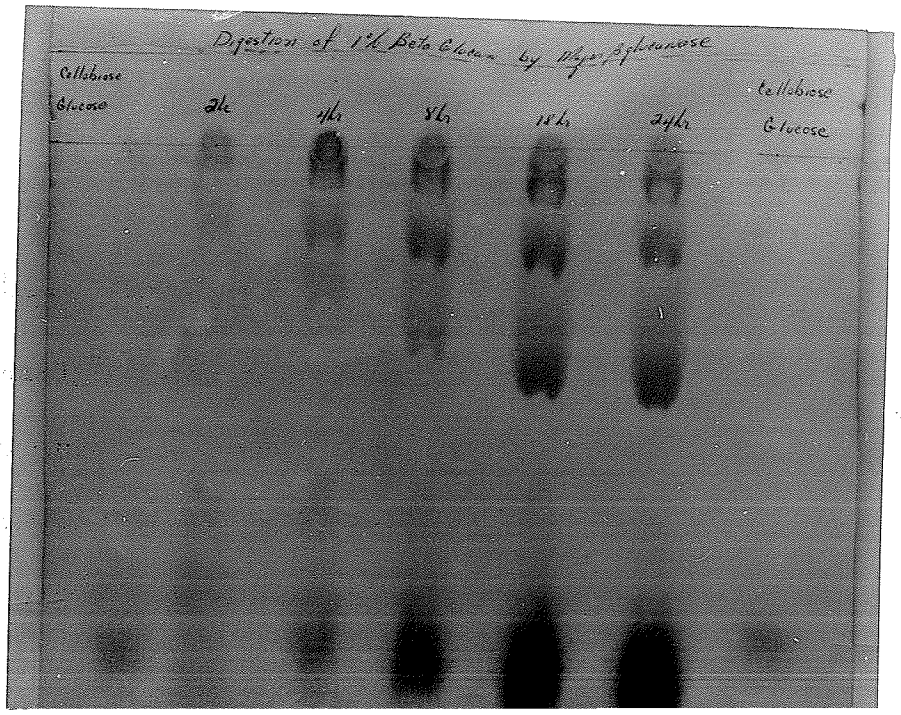
kilning with very little inactivation.

Hydrolysis of barley β-glucan by endo-hydrolase M_{II}. A time study of the hydrolysis of barley β-glucan was performed using barley-β-glucan endo-hydrolase M_{II}. The digestion procedure was described in Methods and Materials. The enzyme solution used for this study was purified on CM-cellulose from a green malt extract.

The occurrence of two separate endo-hydrolase enzymes that will degrade barley β-glucan has been demonstrated in the present study and previously by Luchsinger (14) and Luchsinger and Richards (12). The two enzymes may have different linkage specificities. Parrish, Perlin and Reese (22) found that endo-β-glucanases from sources other than barley degraded barley β-glucan in specific, but different, modes of attack. It does not seem likely that two enzymes are present to perform similar functions. For this reason, the hydrolysis products produced by M_{II} were separated by two methods and can be compared to the products produced by M_I that will be described later.

Separation of enzyme hydrolysis products was initially performed using paper chromatography, and silver nitrate was used to detect reducing sugars. The products that were separated by this method are shown in Fig. 11. Two standards, glucose and cellobiose, were partitioned on each side of the chromatogram. Although not initially detectable, glucose appears to increase as the digestion time increases. However, even at 24 hr., glucose is not the major product. This suggests

Figure 11. Paper chromatographic separation of the hydrolysis products of barley-β-glucan endo-hydrolase M_{II} acting on β-glucan for different lengths of time. Cellobiose and glucose standards were also partitioned.


Digestion of 1% Beta Glucan by Wyer β-glucanase
Cellobiose
Glucose
2hr
4hr
8hr
14hr
24hr
Cellobiose
Glucose

that little exo-enzyme activity is present. The spots of constant intensity at all hydrolysis times at the disaccharide level were due to maltose and this will be demonstrated shortly. Its presence, and the fact that the amount present appears to be constant, is the reason for the high base-line found by the reducing-power assay on CM-cellulose column effluents. The sugar that increases predominantly appears to be a trisaccharide. Higher oligosaccharides are apparent and increase as the length of the digestion increases. The main hydrolytic enzyme is obviously an endo-enzyme.

Enzyme hydrolysis products were separated and, where possible, determined quantitatively using ion-exchange chromatography of borate-sugar complexes to separate the products followed by detection of the products with sulfuric-orcinol reagent. The same digests were used with this method as were used for paper chromatography. The separation of monosaccharides and disaccharides that can be accomplished by this method are shown in the upper two illustrations of Fig. 12. The peaks were in general very well resolved, and such chromatograms are very reproducible.

The time-study hydrolysis products of endo-hydrolase M_{II} as separated by borate ion exchange are indicated in Figs. 12 and 13. As the hydrolysis time increased, several changes occurred. After 2 hr. digestion, the major products were maltose, likely derived from the action of α-amylase on starch, and several products eluted between 2.5 and 4.5 hr. by the borate buffer. As the length of the digestion time was

Figure 12. Separation by borate-ion-exchange column chromatography of standard sugars and endohydrolase M_{II} hydrolysis products of barley β-glucan.

The standard sugars were as follows:

1. sucrose
2. cellobiose
3. raffinose
4. maltose
5. ribose
6. mannose
7. fructose
8. arabinose
9. galactose
10. xylose
11. glucose
12. gentiobiose

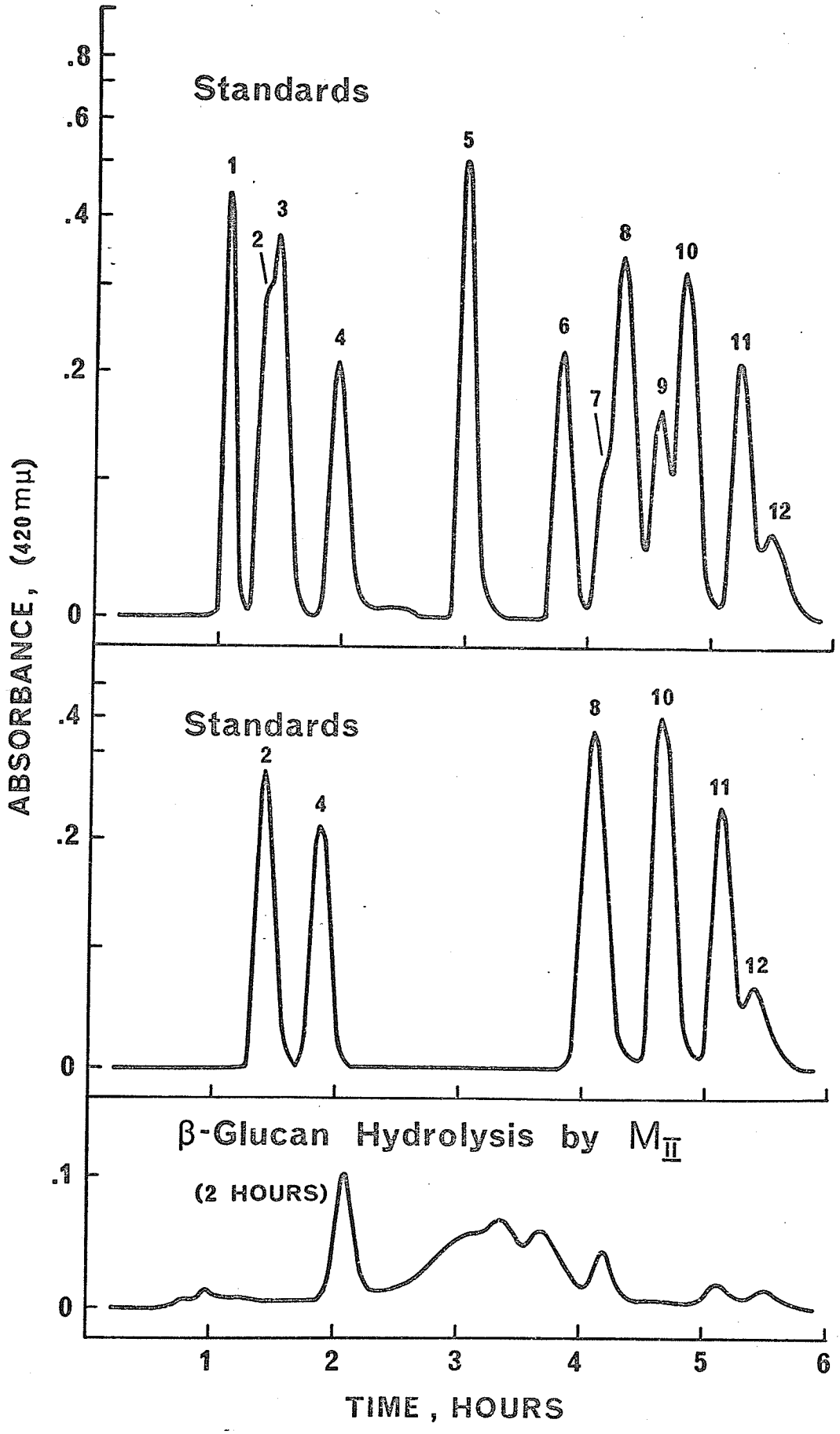
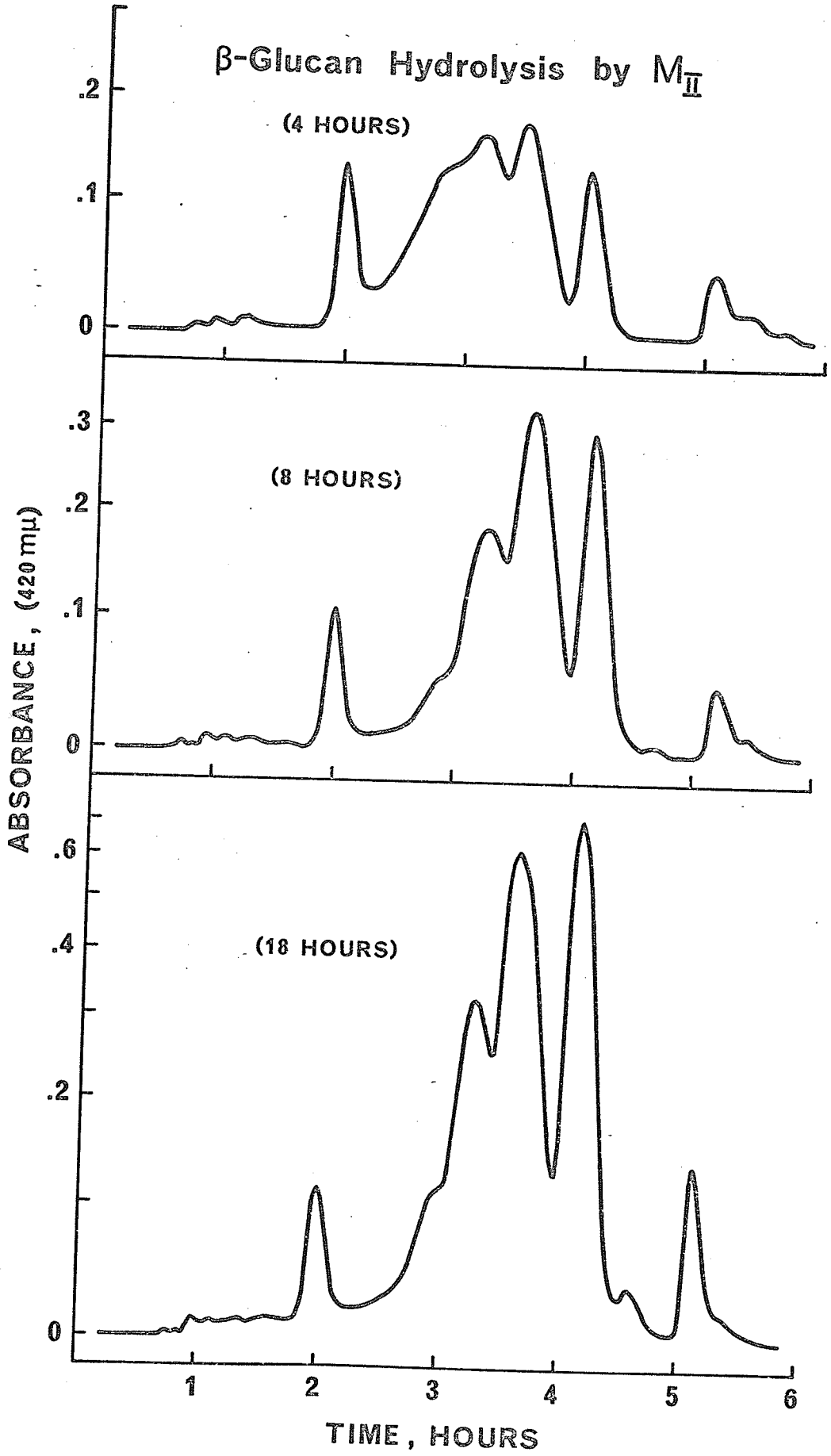

Standards
1
2
3
4
5
6
7
8
9
10
11
12
.8
.6
.4
.2
0
Standards
2
4
8
10
11
12
.4
.2
0
β-Glucan Hydrolysis by M II
(2 HOURS)
.1
0
1
2
3
4
5
6
TIME , HOURS
ABSORBANCE, (420 mμ)

Figure 13. Changes in the action pattern of endohydrolase M_{II} after various times of hydrolysis. Hydrolysis products were separated by borate-ion-exchange column chromatography.


β-Glucan Hydrolysis by M II
(4 HOURS)
(8 HOURS)
(18 HOURS)
ABSORBANCE, (420 mμ)
.2
.1
0
.3
.2
.1
0
.6
.4
.2
0
1
2
3
4
5
6
TIME, HOURS

increased, the unidentified products eluting between 2.5 and 4.5 hr. by the borate buffer increase very rapidly. The limit products after 24 hr. of endo-hydrolase activity appear to be those eluted at 3.0, 3.3, 3.6 and 4.2 hr.

As was observed on paper chromatograms, the maltose concentration was constant. This suggested that the starch contaminant present in the β-glucan substrate was completely hydrolyzed. Although maltose was not a product of β-glucan hydrolysis, its presence was not entirely undesirable. The maltose peak was a useful marker and may be useful further as a good internal standard.

Glucose was present initially as indicated by the small peak at approximately 5 hr. elution time. The fact that it was present and increased in amount over the 18-hr. hydrolysis suggests that a small amount of β-glucosidase or exo- β-glucanase activity was present in the digestion mixture.

As the exact principle of the borate complex is not understood, the separation achieved by this method was compared with that on paper. A 24-hr. digestion of β-glucan with endo-hydrolase M_{II} was performed as described previously. The sample was then streaked on a paper chromatogram, and separated. The sides of the chromatogram were cut off and stained for reducing groups. Using these marker strips, the center of the chromatogram was cut to separate the different products. The strips were then eluted from the paper with water and individually separated on the borate ion-exchange column. The R_g values of the

individual spots on the paper chromatogram and the relative elution position of these spots on the ion-exchange column are indicated in Fig. 14.

Unfortunately, standards were not readily available for identifying these components. However, the action patterns do show a progressive hydrolysis of barley β-glucan to the limit hydrolysis products.

Analysis of the digestion products produced by unpurified or semi-purified malt enzymes have been described by many earlier workers (2, 24, 25, 26). Preece, Garg and Hoggan (25) detected thirteen separate products in a β-glucan digest. None of the products was larger than a tetrasaccharide. It was later suggested by Luchsinger and Richards (15) that many of these products (25) were due to transglucosylation reactions.

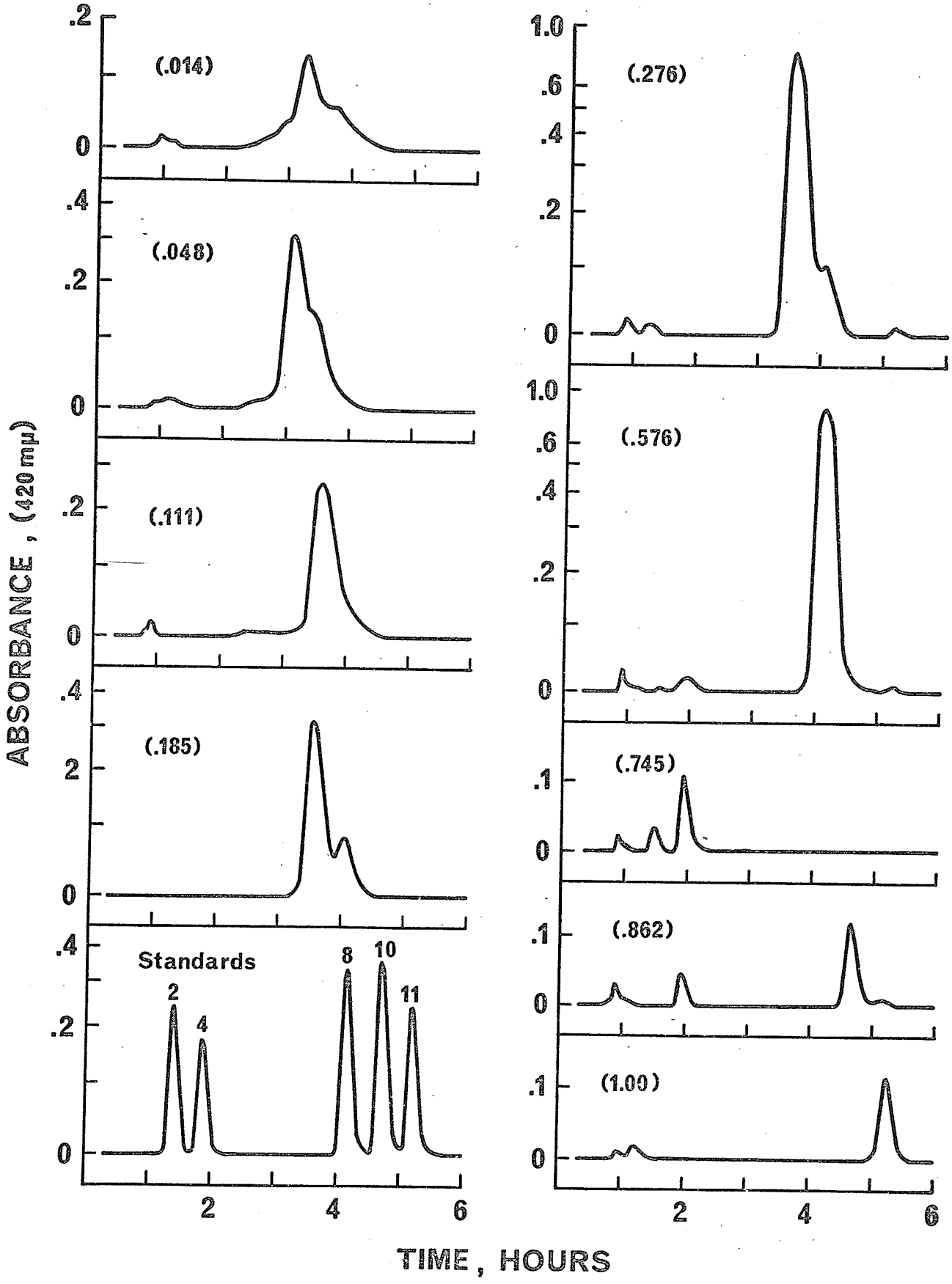
Chen and Luchsinger (4) examined the hydrolysis products of a purified, heat-stable A_{II} endo- β-glucanase. The products were separated by paper chromatography. Chemical analysis of two of the more mobile spots revealed that these were a trisaccharide and tetrasaccharide. These products were identified by Luchsinger, Chen and Richards (16) as 3-O- β-D-cellobiosyl-D-glucose and 3-O- β-D-cellotriosyl-D-glucose, respectively. The two products represented a minimum of 53% and 26%, respectively, of the original β-glucan.

Similar results were obtained for the hydrolysis of lichenin by a malt endo- β-glucanase. This work was carried out by Cunningham and Manners (6). The hydrolysis of lichenin, which has a similar structure

Figure 14. Borate-ion-exchange properties of endohydrolase M_{II} hydrolysis products eluted from paper chromatograms. The Rg values in parentheses correspond to the midpoints of the chromatographic strips.

The standards were as follows:

2. cellobiose	10. xylose
4. maltose	11. glucose
8. arabinose	


ABSORBANCE, (420 mμ)
(.014)
(.048)
(.111)
(.185)
Standards
2
4
8
10
11
(.276)
(.576)
(.745)
(.862)
(1.00)
TIME, HOURS

to barley β-glucan, yielded a trisaccharide and two tetrasaccharides as the major hydrolysis products. The trisaccharide was identified as 4- β-glucosyl-laminaribiose, and this is the same trisaccharide identified by Luchsinger et al. (16). The relative mobility on paper chromatograms of the major components in the present study suggested that the hydrolysis products were a trisaccharide and tetrasaccharide, supporting the findings of Luchsinger et al. As suggested by these workers, it appears that a specific linkage is being cleaved within the β-glucan molecule. The fact that all the products had a β 1-3 linked glucose at the reducing end (16) indicated that the linkages cleaved were β1-4 linkages. Although the exact structure of the products in the present study would be required for absolute proof, it seems likely that the major products are the same as those described by Luchsinger et al. (16).

Summary

In summary, barley- β-glucan endo-hydrolase M_{II} was purified by CM-cellulose and Bio-Gel P-100 chromatography. The enzyme had a broad pH optimum with highest activity at pH 4.8. Activity was stimulated by NaCl and inhibited by $CaCl_2$. EDTA had little effect on activity. The enzyme was quite stable at 40°C, but above this temperature, lost activity quite rapidly. The hydrolysis of barley β-glucan by endo-hydrolase M_{II} produced four major products, and the relative mobility on paper chromatograms of two of these was consistent with the view that the products were a trisaccharide and tetrasaccharide.

Barley- β -Glucan Endo-Hydrolase M_I

Purification

The purification of endo-hydrolase M_I was initiated with a separation of a crude green malt extract on CM-cellulose as had been done previously with endo-hydrolase M_{II}. Partition of the proteins was effected using the gradient illustrated in Fig. 8a. The column effluent was monitored at 280 mu, and the protein elution profile is indicated in Fig. 8a. The total β-glucan hydrolase activity eluted from the CM-cellulose column is indicated in Fig. 8b. The endo-hydrolase M_I enzyme is responsible for the first reducing-viscosity peak in Fig. 8b.

The endo-hydrolase M_I peak was pooled, concentrated by Diaflo membrane filtration and subjected to chromatography on a Bio-Gel P-100. The effluent from the column was monitored, and the protein absorbance at 280 mu is indicated in Fig. 8c. Two major protein bands had been separated. To locate the endo-hydrolase M_I activity, a reducing-power assay was used to check each column fraction for β-glucan-hydrolase activity. The activity was located between the two major protein peaks and these fractions were tested further, viscometrically, to ensure that this activity was due to endo-hydrolase activity. The active fractions were pooled and concentrated. The endo-hydrolase M_I activity was extremely low, even off CM-cellulose, and only small amounts were prepared. Instead of risking the loss of the limited amount of enzyme during attempts at further purification, the enzyme was characterized following

chromatography on Bio-Gel P-100. A small fraction of the purified enzyme was submitted to disc electrophoresis. The concentrated enzyme solution was too dilute to indicate any protein bands on the electrophoretic gel when stained with Coomassie Blue.

Although many workers have studied barley- β-glucan endo-hydrolases from crude malt extracts, only a few groups have tried to purify the enzymes (12, 13, 14, 20). Two endo β-glucanases that degraded barley β-glucan were isolated from germinated barley by Luchsinger et al. (12, 13, 14). One of the endo β-glucanases was heat-labile and designated as A_I. This endo-glucanase enzyme was less active than the other endo-glucanase A_{II} fraction and eluted first from the anion-exchange column (14). In this respect, endo-glucanase A_I is similar to endo-hydrolase M_I isolated in the present study. Further evidence will be presented in the characterization section illustrating further similarities. Manners and Marshall (20) purified malt endo β-glucanase. However, these workers used kilned malt as compared to germinated barley used in the present study and used by Luchsinger et al. (12). The kilning probably destroyed the heat-labile enzyme so that Manners and Marshall did not detect a second endo- β-glucanase enzyme.

Characterization

To remove any possible interference effect due to exo-enzyme activity that may be measured by the reducing-power assay, all activity measurements were made using the reducing-viscosity assay.

Effect of pH. One percent barley β-glucan solutions were prepared in 0.1M acetate buffer over the pH range from 3.5 to 5.6. The viscometric activity of the M_I hydrolase enzyme at the different pH values is shown in Fig. 15. Each of the points represents the mean value of three separate determinations. A very broad pH optimum exists, and the curve is relatively flat over the pH range from 4.4 to 5.3. The peak occurs at approximately 4.8. Addition of 0.1M NaCl to the extracts did not affect the activity. Endo-hydrolase M_I and M_{II} have very similar pH optima.

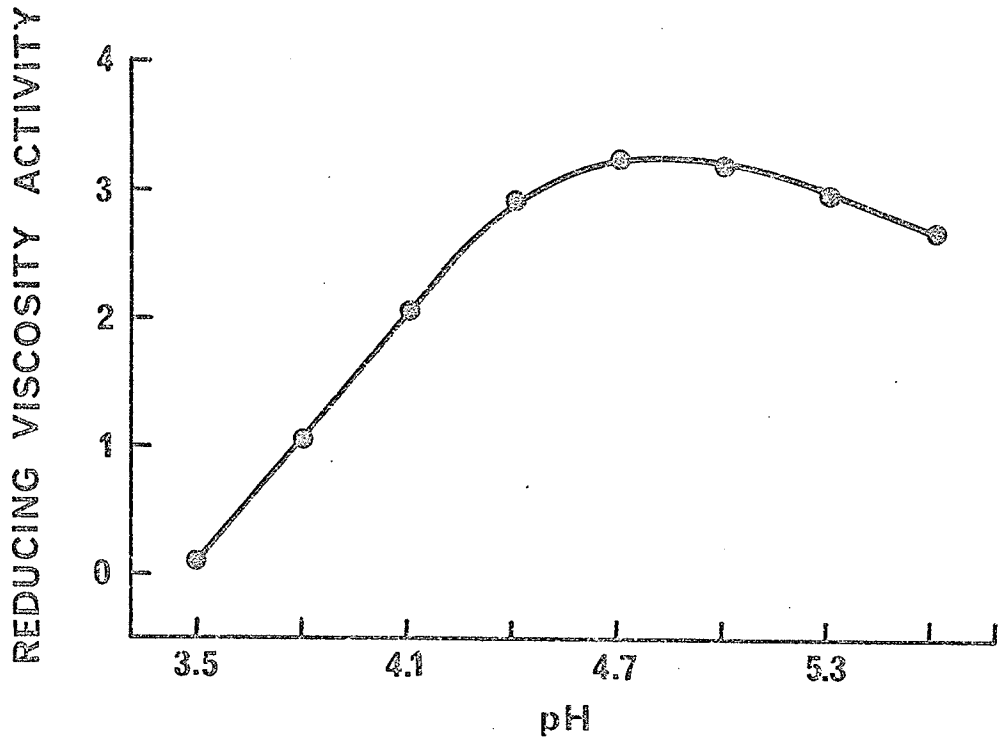
The endo- β-glucanase A_I fraction reported by Luchsinger (14) had a pH optimum of 4.75. This agrees very well with the optimum pH of 4.8 for the endo-hydrolase M_I. Bass and Meredith (3) separated several fractions on an alumina column, and the β-glucan hydrolase activity was relatively steady across a pH range of 4.5 to 5.0.

Effect of NaCl, Thiol and EDTA. Sodium chloride, thioglycerol (thiol) and EDTA were each tested separately for possible stimulation or inhibition effects on the activity of barley- β-glucan endo-hydrolase M_I. The results are indicated in Table IV.

The enzyme was not stimulated or inhibited by any of the materials tested. Ferrell and Luchsinger (7) tested the effect of adding various salts to an albumin fraction prepared by dialysis of a crude extract. The albumin fraction contained most of the heat-labile endo- β-glucanase and little of the more heat-stable endo- β-glucanase. As KCl, NaCl or

Figure 15. Effect on pH on barley- β-glucan endohydrolase M_I activity.

Figure 16. Heat stability of barley- β-glucan endohydrolase M_I. The percent activity was estimated relative to the activity of an unheated enzyme aliquot.


REDUCING VISCOSITY ACTIVITY
4
3
2
1
0
3.5
4.1
4.7
5.3
pH

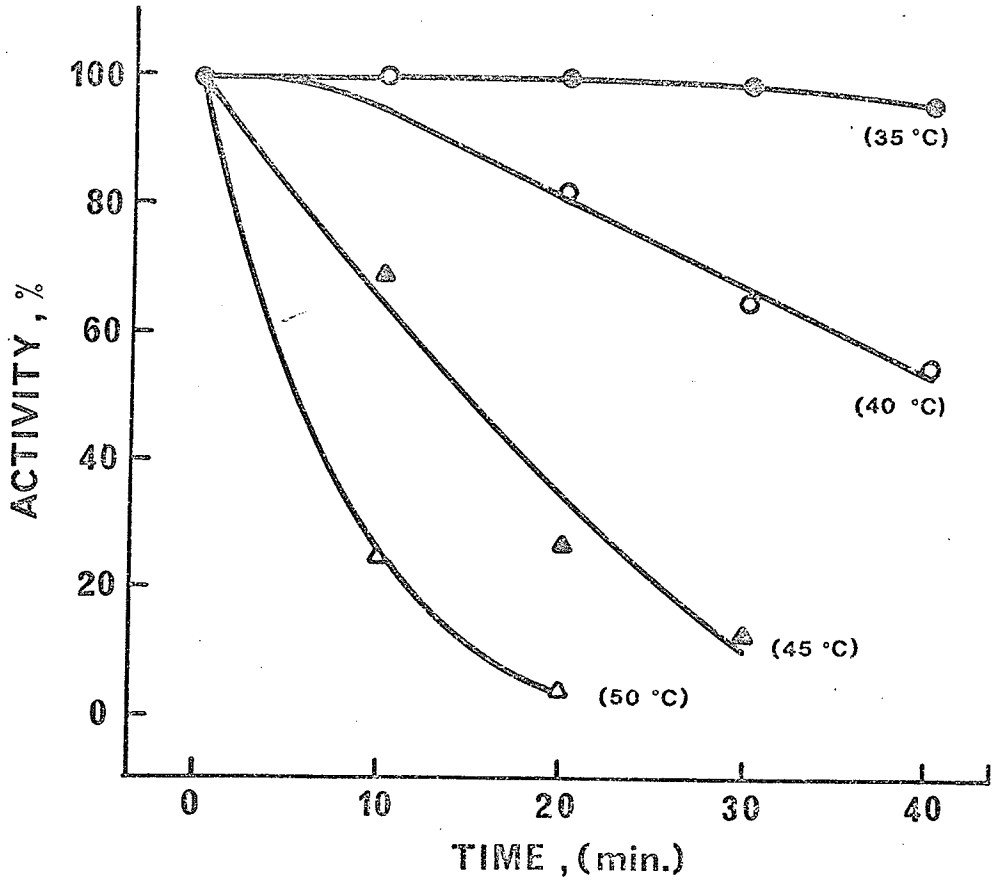

ACTIVITY, %
100
80
60
40
20
0
(35 °C)
(40 °C)
(45 °C)
(50 °C)
0
10
20
30
40
TIME, (min.)

TABLE IV

Effect of NaCl, Thiol and EDTA on
Barley- β -Glucan Endo-Hydrolase M_I Activity

Reagent	Concentration	% Change Relative to Blank with Water
Blank	-	100
NaCl	0.1 M	100
EDTA	.005 M	100
EDTA	.014 M	100
Thiol	.002 M	100

$(NH_4)_2SO_4$ were added at low concentrations (up to 0.04M) to this albumin fraction, a pronounced stimulative effect was observed for each salt. Above 0.08M no further increase in the stimulative effect occurred, and in fact, the activity decreased slightly from the optimum value observed. The fact that the enzyme reaction mixture contained approximately 0.1M acetate buffer may be the reason that no stimulative effect was noted by NaCl in the present study. As EDTA produced no effect, the enzyme probably does not require a divalent ion for its activity. Lack of response to added thiol suggests that sulfhydryl groups are not involved in enzyme action.

As suggested by Ferrell and Luchsinger (7), stimulation by salt is associated more with the general ionic strength of the solution than with a specific ion requirement. A particular range of ionic strengths may result in a change in the conformation of the enzyme structure making the active site more accessible to the substrate. Further work will be necessary to determine whether the enzyme has a specific ion requirement for activity or stability.

Heat stability. Portions of the endo-hydrolase M_I enzyme solution were maintained for 40 min. at several different temperatures. Aliquots were removed at 10-min. intervals, and the activities of these samples were measured by the viscosity-reducing assay using a 1% β-glucan solution as substrate. The results of this study are shown in Fig. 16. The endo-hydrolase M_I enzyme is very heat-sensitive. At 45°C, 80% of the

activity was lost within 25 min. At 50°C, only 4% of the original activity remained after 20 min. Because this enzyme is so sensitive to heat, it is questionable that the enzyme would be active at all after kilning. If some activity did remain, the activity would probably be lost quite rapidly during a 45°C mash. Thus, this enzyme is not expected to play a major role in breakdown of β-glucan during mashing.

The A_I fraction reported by Luchsinger (14) lost 50% of its activity at 40°C after 40 min. This characteristic of the A_I fraction agrees quite well with the results obtained for the M_I hydrolase of the present study.

Hydrolysis of barley β-glucan by endo-hydrolase M_I. Analysis of the products of β-glucan hydrolysis by endo-hydrolase M_I were examined as another possible way of differentiating between the endo-hydrolases M_I and M_{II}. The initial work was carried out using the enzyme fractions separated by chromatography on CM-cellulose. At the commencement of this work, it was hoped that a future comparison could be made with the hydrolysis products, using completely pure enzyme fractions.

The digests were prepared with 1% β-glucan as described in the Methods and Materials section using the endo-hydrolase M_I fraction. Paper chromatography was initially used with the propanol:ethyl acetate: water elution solvent, to separate the products. However, the enzyme fraction was not very active and only a few products could be detected

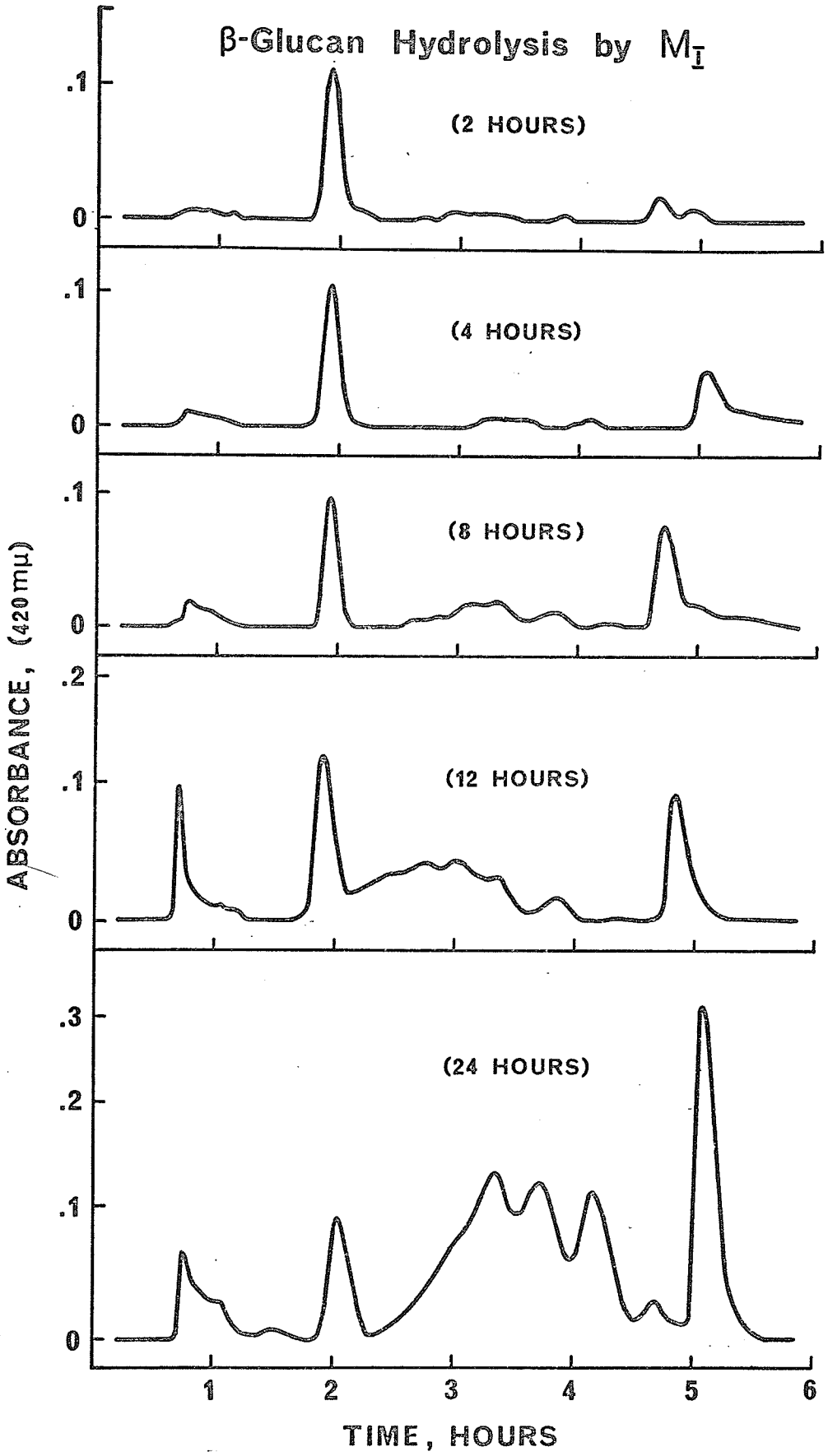
on the paper when it was stained with silver nitrate for reducing sugars.

The samples were also separated as borate derivatives by ion-exchange chromatography, and the column effluent was monitored at 420 mu with sulfuric-orcinol reagent. The separation of the hydrolysis products by this method is shown in Fig. 17. Maltose was detected, as previously, and was eluted by borate buffer after two hours.

After 2-hr. of hydrolysis, glucose and several other products were present. As the hydrolysis time increased, the amounts of three or four oligosaccharides increased. These products appeared to be the same as those produced by the endo-hydrolase M_{II}. The increasing amounts of glucose suggested that an active exo- β-glucanase or a β-glucosidase was present. The peak occurring after 1 hr. elution is probably due to high-molecular-weight oligosaccharides or dextrins. Their presence indicates a considerable decrease in molecular size of the β-glucan substrate as the separated peaks represent materials soluble in 80% ethanol. There is little doubt that the major activity is due to an endo-enzyme, specifically endo-hydrolase M_I. The products eluted by borate buffer from 2.5 hr. to 4.5 hr. appear to represent the major β-glucan limit digestion products of the M_I activity after 24 hours incubation of enzyme with substrate. Although no standards were available to identify the products, these action patterns are extremely useful in following the production of digestion products.

Separation of β-glucanase hydrolysis products of malt enzymes has been carried out by several groups of workers, as already discussed with

Figure 17. Changes in the action pattern of barley-β-glucan endo-hydrolase M_I after various lengths of incubation with β-glucan. Hydrolysis products were separated as borate derivatives by ion-exchange chromatography.


β-Glucan Hydrolysis by M_I
(2 HOURS)
(4 HOURS)
(8 HOURS)
(12 HOURS)
(24 HOURS)
ABSORBANCE, (420 mμ)
TIME, HOURS
0
.1
.2
.3
1
2
3
4
5
6

regard to the endo-hydrolase M_{II}. With regard to an enzyme similar to endo-hydrolase M_{II}, Luchsinger, Chen and Richards (16) reported two major products, a tri- and tetrasaccharide were produced and represented a minimum of 78% of the β-glucan in the digest. Two other products of higher molecular weight were also reported. The major products produced by M_I (Fig. 17) would appear to be the same as those produced by M_{II} (Fig. 13) and from the separation of M_{II} hydrolysis products on paper, the products seemed to be tri- and tetrasaccharides.

A point for speculation is that if the products produced by the M_I and M_{II} endo-hydrolases are the same, indicating the same type of cleavage, why then are two endo-enzymes necessary?

Summary

In summary, barley- β-glucan endo-hydrolase M_I was partially purified by chromatography on CM-cellulose and Bio-Gel P-100. This enzyme exhibited a broad pH optimum with highest activity at 4.8. The enzyme was not affected by EDTA, thioglycerol or NaCl. The enzyme was relatively heat-labile. The limit hydrolysis products, as separated by borate ion-exchange chromatography, appear to be tri- and tetrasaccharides similar to the limit hydrolysis products of endo-hydrolase M_{II}.

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