A Simple Reducing Sugar Assay for Measuring β-Glucanase Activity in Malt, and Various Microbial Enzyme Preparations[1]

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ABSTRACT

The reducing sugar method of Dygert, Florida, and Thoma (11) employing neocuproine hydrochloride as color reagent and glycine as the copper chelating agent was adapted to measure reducing sugars liberated from barley β-glucan, lichenin, and laminarin by the action of β-glucanases from malt and *Bacillus amyloliquefaciens*. Studies showed that bacterial β-glucanase gave the same results on barley β-glucan and lichenin, but did not attack laminarin. Malt contained β-glucanases which attacked both lichenin and laminarin. Lichenin and laminarin had high reducing sugar backgrounds which restricted the useful linear range. Reduction with sodium-borohydride virtually eliminated this background. The optimum pH and temperature for bacterial β-glucanase activity on lichenin were 6.5 and 40–45° C, respectively, while malt β-glucanases displayed optima of pH 4.0 and 40° C on lichenin and pH 4.5 and 45° C on laminarin. Km values were 0.053% for bacterial β-glucanase on lichenin, 0.67% for malt β-glucanase on lichenin, and 0.05% for malt β-glucanase on laminarin.

Key words: *Bacillus amyloliquefaciens, β-Glucanase assay, Barley β-glucan, Km value, Laminarin, Lichenin.*

In recent years, interest in the roles of specific enzymes in brewing has increased. One group of enzymes, the β-glucanases, because of their effect on mash filtration, extract yield, wort, and beer viscosity (2, 6, 8, 9, 13, 15, 16, 27) have come under particular scrutiny. Unfortunately, assay procedures have received little attention, and it appears no attempt has been made at any sort of standardization. When we initiated studies with malt and bacterial enzymes, the shortcomings in present methods of β-glucanase assay soon became apparent.

Two substrates, barley β-glucan and laminarin, and two techniques, viscometric and reducing sugar determination, have been employed most often (12, 14, 17, 18, 19, 21, 22). Since extraction of β-glucan from barley is a tedious task (4, 5, 7, 10, 23) we sought to avoid use of this substrate. Further, we found the reproducibility of viscometric assays with barley β-glucan and laminarin to be poor, varying considerably from batch to batch. In addition, laminarin is expensive and the high substrate concentrations necessitated with the viscometric technique makes assay costly.

While screening bacterial β-glucanase on various substrates we found that reducing sugar values on barley β-glucan and lichenin, a commercially available β-(1, 3) (1,4) glucan, were the same. K. Erdal and B. Enevoldsen[3], using two bacterial β-glucanase preparations, also have found that the increases in reducing power paralleled one another using the above two substrates, indicating the same activity. This suggests that lichenin could be used to replace barley β-glucan. However, they found that the products of enzyme hydrolysis were not the same with both substrates.

Parrish *et al.* (23) concluded that the major structural unit in lichenin is the same as that found in cereal glucans [4 β-D-glucopyranosyl units linked by a (1 → 3) bond and two consecutive (1 → 4) bonds]. Peat and co-workers (24) found that lichenin contained more (1 → 3) links, showing that although lichenin closely resembles the cereal glucans it can be differentiated from them in terms of fine structure. Ducroo and Delecourt (10) suggested that β-glucanase from *Bacillus subtilis* and endo-β-glucanase from germinated barley both have affinity for the trimeric G4G3G position of the molecule, cutting the adjacent (1 → 4) linkages. This explains why these enzymes are without action on laminarin and other substrates which contain only β-1,4 or β-1,3 linkages. These enzymes act only on β-glucans or on lichenin. Preece (25) found that the β-glucan he isolated from barley bore a close relation to lichenin, and it is of interest that oats, which yields β-glucan under conditions similar to those described for its isolation from barley, was described as a source of lichenin by Morris (20). Luchsinger *et al.* (18) isolated a laminarinase from malt and found it to have activity against laminarin by both reducing sugar and viscometric assay. It had no activity against barley β-glucan using either assay.

With this background it was felt that a reducing sugar assay employing lichenin and laminarin as substrates could offer a simple, reproducible means of assaying for β-(1,3) (1,4) and β-(1,3) glucanase in bacterial and cereal preparations. Reducing sugar methods offer both convenience and cost advantages. Both lichenin and laminarin can be obtained commercially, eliminating the necessity for tedious extraction of barley β-glucan. Also, lower substrate concentrations and smaller amounts of substrate can be employed, thus reducing the cost. However, both lichenin and laminarin contain high background reducing values, seriously restricting the linear range of assay. This study presents a method for eliminating these high backgrounds as well as exploring

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conditions for the assay of β-glucanases from *Bacillus amyloliquefaciens* and malt.

EXPERIMENTAL

Preparation of Substrates

Barley β-glucan was prepared by D. G. Kramme according to the procedure of Preece and Mackenzie (26). It was shown to be free of starch by negative iodine reaction and no change in viscosity when its solution was submitted to the action of crystalline α-amylase.

Lichenin and laminarin were purchased from ICN · K&K Laboratories, Inc., Plainview, NY, and Nutritional Biochemical Corporation, Cleveland, OH, respectively.

For use in this study solutions of the substrates up to a concentration of 0.5% were prepared as follows:

Step 1

The required amount of substrate was ground to a fine powder in a dynamill (Tekmor Company) for 2 min with cooling water on the jacket. It was then dissolved in 50–60 ml distilled water by heating at 80–90°C, with stirring, for 20–30 min. After cooling 0.68 g of monobasic potassium phosphate was added and the solution polish filtered through a 0.22 μ Millipore filter. The polished filtrate was adjusted to the desired pH with HCl or NaOH and the volume brought to 100 ml.

Step 2

Solution of the substrate was as described in step 1. After the 20–30 min heating step an amount of sodium borohydride equal to one third the weight of the substrate was mixed in and heating at 80–90°C continued for an additional hour. The solution was adjusted to pH 5.5 with glacial acetic acid to remove excess borohydride then cooled to 40°C. Salts were removed by stirring in Amberlite MB-3 for 30 min. The mixture was then vacuum filtered to remove the resin. The monobasic potassium phosphate was then added and the slightly hazy solution polished, adjusted to the desired pH, and brought to volume as described in step 1.

In this study a 0.15% substrate concentration was employed except in those experiments where the effect of substrate concentration on enzyme activity was studied.

Preparation of Neocuproine Reagents

Reagent "A" (copper reagent) and Reagent "B" (neocuproine) were prepared as described by Dygert *et al.* (11).

Extraction of β-Glucanases From Malt

A modification of the method of Ballance and Meredith (1) was used. Sixty-five g of dynamilled Larker malt were slurried in 150 ml of 0.00085 *M* $CaCl_2$ solution. The slurry was mixed in a Waring Blendor at 4°C for 5 min then centrifuged at 12,000 rpm in a Sorvall RK2B refrigerated centrifuge at 4°C for 30 min. The resultant cake was extracted a second time with 150 ml of $CaCl_2$ solution. The combined supernatants, approximately 300 ml, were ultrafiltered through an Amicon PM10 membrane until 250 ml of permeate were collected. The 50 ml of remaining ultraconcentrate were freeze dried yielding 4.25 g of extract.

Assay Procedure, with reducing sugars determined by method of Dygert *et al.* (11).

Into each of 4 tubes graduated at 25 ml were pipeted 2 ml of substrate solution and the tubes were equilibrated at the desired temperature for 10–15 min. After equilibration the tubes were treated as follows:

1. Buffer Blank—added 1 ml of buffer at appropriate pH.
2. Glucose Standard—added 1 ml of glucose standard in buffer containing 36 μg of glucose.
3. Enzyme Blank—added 4 ml of Neocuproine Reagent "A" then 1 ml of enzyme solution. (Enzyme solution was prepared in buffer).
4. Enzyme Assay—added 1 ml of enzyme solution.

After exactly 15 min incubation, 4 ml of Neocuproine Reagent "A" were added to each tube (except the enzyme blank). After mixing 4 ml of Neocuproine Reagent "B" were added to each tube, the tubes capped, mixed, and placed in a boiling water bath for 12 min to develop color. The tubes were then cooled to room temperature in running tap water and the volumes brought to 25 ml with distilled water. After mixing, the color intensity was determined with a Spectrophotometer at 450 nm.

One β-glucanase unit (GU) was defined as that amount of enzyme which would liberate 1 μmol of reducing sugar, calculated as glucose, per min under the conditions of the assay.

Reaction Rates

The procedure for studying reaction rates was the same as described for the assay procedure except that the reaction volume was 18 ml instead of 3 ml. Three ml aliquots were removed from the reaction flask at 3-min intervals and reducing sugars determined.

RESULTS

Comparison of Barley β-Glucan, Lichenin, and Laminarin

Several microbial enzyme preparations were checked for β-glucanase activity using barley β-glucan, lichenin and laminarin as substrates. Table I shows the results of these experiments. All the preparations showed similar activity on barley β-glucan and lichenin, while displaying little or no activity on laminarin. For this reason it was felt that lichenin, a commercially available β-glucan could be substituted for barley β-glucan.

Standard Curves

Standard curves were run in water, phosphate buffer (pH 6.5), lichenin, and laminarin. It was found that in water and buffer,

TABLE I
Comparison of Barley β-Glucan, Lichenin, and Laminarin as Substrates[a]

Enzyme	β-Glucanase Units/Gram		
	Barley Glucan	Laminarin	Lichenin
Bacterial preparation 5190-T	4555	0	4414
Bacterial preparation B-1076	4238	0	4200
Trichoderma viride	4301	301	3909

[a]Assay procedure—3 ml of 1% substrate at 40° C, pH 6.5 for 15 min. One β-glucanase unit (GU) is that amount of enzyme which liberates 1 micromole of reducing sugar, calculated as glucose, per min under the conditions of assay.

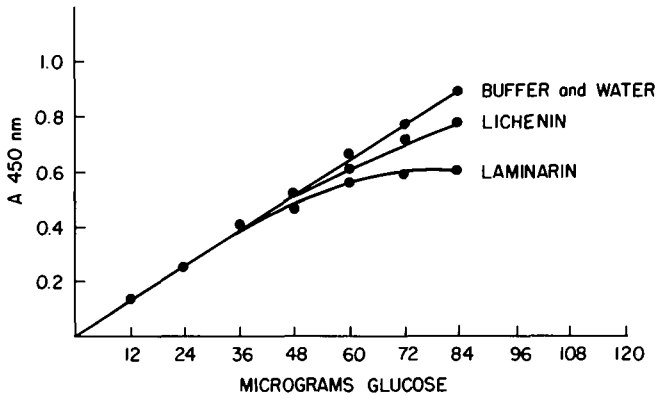

Fig. 1. Standard curves in water, phosphate buffer, lichenin, and laminarin. Varying amounts of glucose in 3 ml of appropriate solution. Lichenin and laminarin used at 0.1% concentration. pH of buffer, lichenin, and laminarin solutions was 6.5.

linearity was lost above 84 μg of glucose or above an absorption of 0.9 at 450 nm. Lichenin showed a blank reducing background of $A_{450\ nm} = 0.33$, thus restricting the linear range to 48 μg of glucose. With laminarin the situation was even more pronounced with a blank background of $A_{450\ nm} = 0.6$, restricting the linearity to 36 μg of glucose. Figure 1 shows the results of these experiments. Figure 2 shows the standard curve obtained with sodium borohydride treated lichenin and laminarin. The blank background has been removed with each substrate and the range of linearity returned to that found with water and buffer.

Studies with β-Glucanase from *Bacillus amyloliquefaciens*

A crude extract from a *Bacillus amyloliquefaciens* fermentation was found to have considerable activity on barley β-glucan and lichenin. The characteristics of this enzyme were studied to determine optimum conditions for assay of the β-glucanase activity. Figure 3 shows the effect of pH and temperature on bacterial β-glucanase activity with lichenin as substrate. Optimum conditions were found to be a pH of 6.5 and a temperature of 40° C.

Figure 4 shows the results of following the reaction rate over the 15 min incubation. Two enzyme concentrations were used. It was anticipated that the lower concentration would show linearity over the incubation time while the higher concentration would display a break in linearity and thus indicate the limit of assay practicality. Such was the case with the higher enzyme level showing linearity to $A_{450\ nm} = 0.5$ after 12 min. This represents approximately 50 μg of reducing sugar, calculated as glucose.

To further verify the practical linear range of the assay the effect

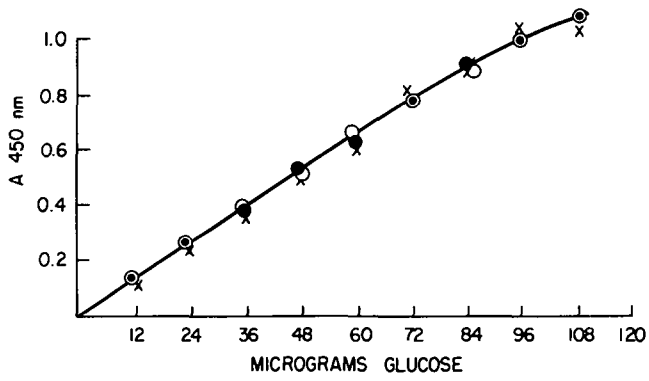

Fig. 2. Standard curve in phosphate buffer and $NaBH_4$ treated lichenin and laminarin. Procedure as described in Fig. 1. •——• Buffer, o——o Lichenin, x——x Laminarin.

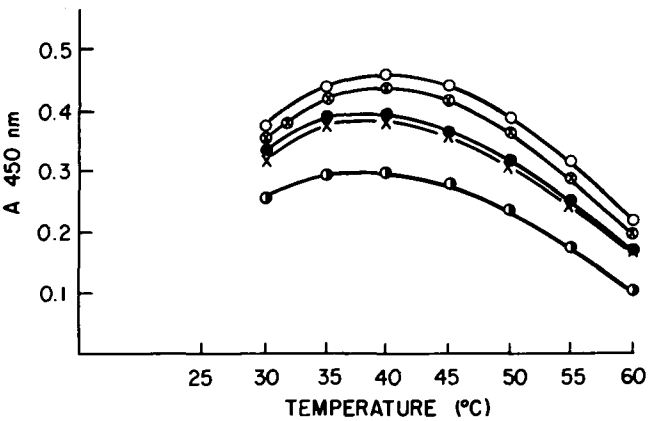

Fig. 3. Effect of temperature and pH on activity of bacterial β-glucanase on lichenin. Enzyme was incubated in 3 ml of 0.1% substrate for 15 min. Phosphate buffer was used at all pH values. x——x pH 5.0, •——• pH 6.0, o——o pH 6.5, •——• pH 7.0, o——o pH 8.0.

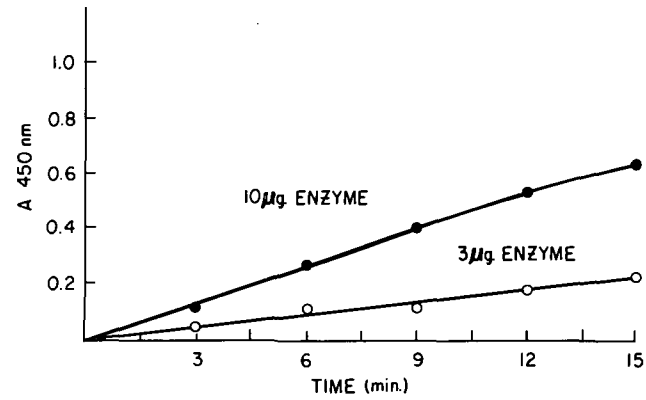

Fig. 4. Reaction rate of bacterial β-glucanase on lichenin. Lichenin concentration was 0.1%, pH was 6.5, and incubation temperature 40° C. Enzyme concentrations were 10 μg •——• and 3 μg o——o per 3 ml of incubation mixture. Three ml aliquots were removed at 3 min intervals and reducing sugars determined.

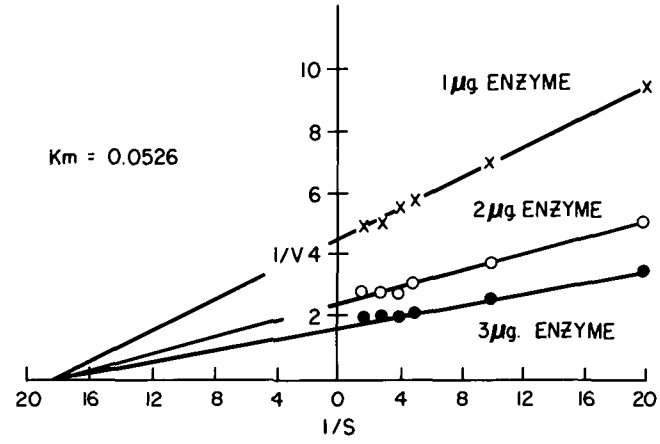

Fig. 5. Lineweaver-Burk plots of bacterial β-glucanase activity *vs.* lichenin concentration. Lichenin concentrations from 0.05% to 0.5% were employed. Three enzyme concentrations, 1, 2, and 3 μg/3 ml incubation mixture were investigated. Incubation conditions were 40° C at pH 6.5 for 15 min.

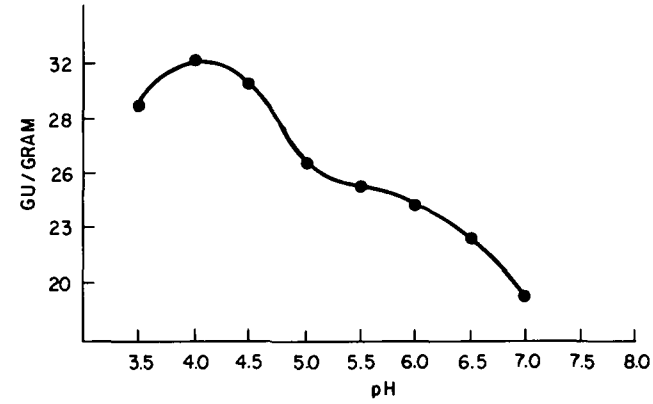

Fig. 6. Effect of pH on malt β-glucanase activity on lichenin. Incubation conditions—0.1 mg malt extract in 3 ml 0.1% lichenin for 15 min at 40° C. One β-glucanase unit (GU) is that amount of enzyme which will produce 1 μmol of reducing sugar, calculated as glucose, per min under conditions of assay.

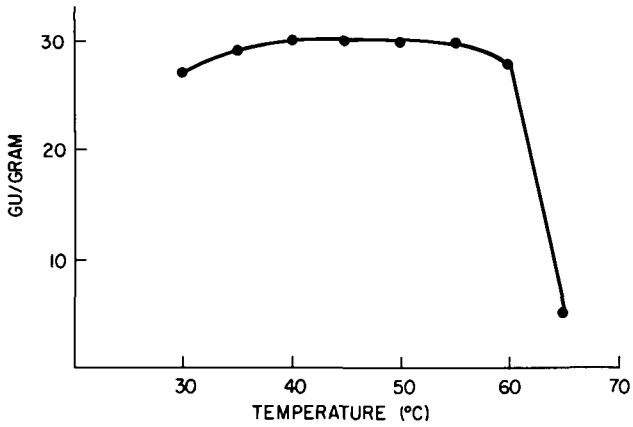

Fig. 7. Effect of temperature on malt β-glucanase activity on lichenin. Incubation conditions—0.1 mg malt extract in 3 ml 0.1% lichenin for 15 min at pH 4.0. Determination of reducing sugar and definition of GU as described for Fig. 6.

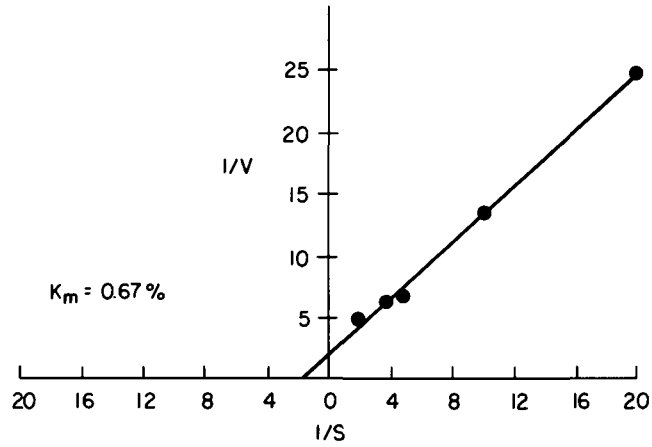

Fig. 8. Lineweaver-Burk plot of malt β-glucanase activity *vs.* lichenin concentration. 0.1 mg of malt extract incubated in 3 ml lichenin solution (0.05–0.5% concentration) for 15 min at 40°C and pH 4.0.

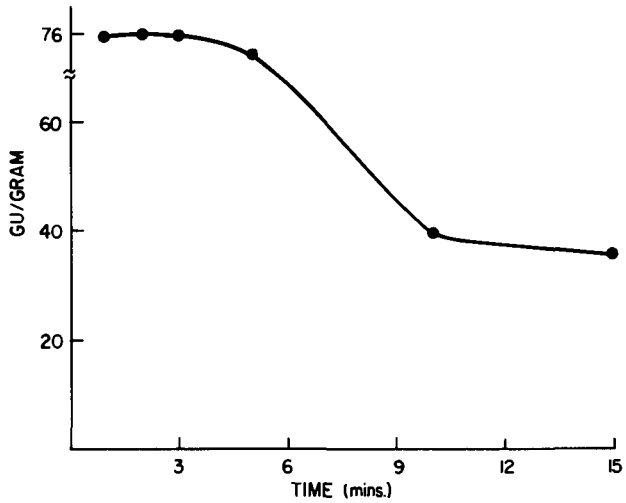

Fig. 9. Determination of β-glucanase activity of malt on lichenin using initial reaction rates. 0.7 mg of malt extract were incubated in 21 ml of 0.1% lichenin at pH 4.0 and 40° C. Three ml aliquots were removed at 1, 2, 3, 5, 10, and 15 min incubation for reducing sugar determination.

of varying the enzyme concentration was also studied. Concentrations from 1 to 10 μg of enzyme were used in the assay. Linearity was obtained through 5 μg, this concentration, giving an Absorbance at 450 nm = 0.5, thus confirming the results of the rate study. For reproducibility then, an amount of enzyme should be chosen for assay which will give a final Absorbance between 0.1 and 0.5.

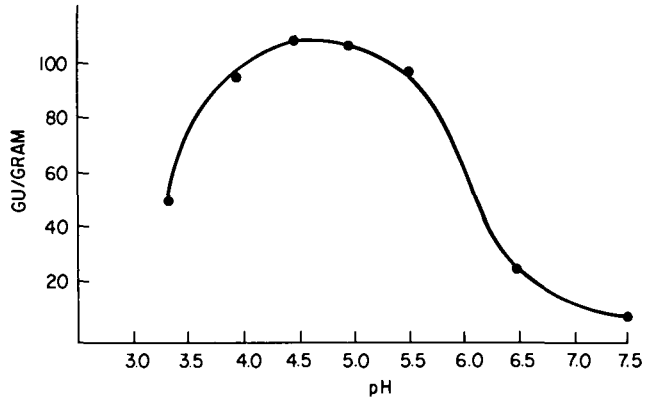

Fig. 10. Effect of pH on malt β-glucanase activity on laminarin. 0.1 mg of malt extract incubated in 3 ml of 0.1% laminarin for 15 min at 40°C.

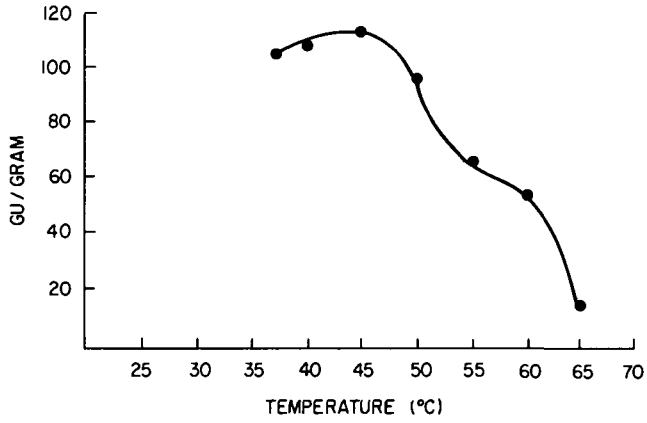

Fig. 11. Effect of temperature on malt β-glucanase activity on laminarin. 0.1 mg of malt extract incubated in 3 ml of 0.1% laminarin for 15 min at pH 4.5.

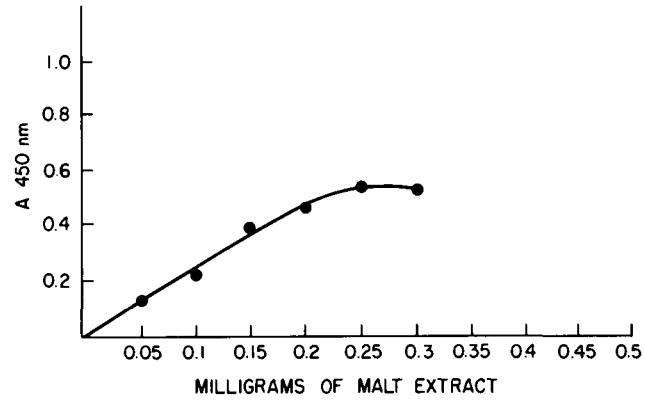

Fig. 12. Effect of malt β-glucanase concentration on laminarin. Varying amounts of malt extract were incubated in 3 ml of 0.1% laminarin for 15 min at 45°C and pH 4.5.

The effect of lichenin concentration on bacterial β-glucanase activity was studied. Lichenin concentrations from 0.05 to 0.5% were used. Three enzyme concentrations were employed, 1, 2, and 3 μg. The results are shown in Fig. 5 in the form of Lineweaver-Burk plots. A Km value of 0.053% was found indicating that a 0.1% lichenin concentration is about minimum for the described assay procedure.

Bacterial β-glucanase was tested using laminarin as the substrate. Enzyme concentrations from 1 to 10 μg showed no increase in reducing sugar production indicating that bacterial β-glucanase does not attack laminarin.

Studies with β-Glucanase from Malt

Figures 6–9 show the results of studies on malt β-glucanase activity on lichenin. Figure 6 shows the results of running the assay at various pH values. The temperature of assay was 40°C. Optimum pH for activity was 4.0.

The optimum temperature for activity was found by running the assay at pH 4.0 and varying the temperature of incubation. A broad peak resulted (Fig. 7) with little difference in activity between 40° and 55°C. At 65°C the enzyme was rapidly inactivated. Increasing the temperature should increase the reaction rate. Since no increase in activity occurred between 40°C and 55°C it was concluded that the increased reaction rate at higher temperature was offset by the increased rate of enzyme denaturation. For this reason 40°C was chosen as the preferred temperature for assay.

Studying the effects of varying enzyme concentration pointed to significant differences between malt β-glucanase and bacterial β-glucanase on lichenin. The malt enzyme showed linearity over an Absorbance range of 0.1 or an increase in reducing sugar value equivalent to 10 μg glucose, while the bacterial enzyme displayed linearity over an Absorbance range of 0.5 or 50 μg of glucose. It is possible that malt β-glucanase is very specific in its action and does not release many smaller oligosaccharides, resulting in only a small increase in reducing sugar value. A study of the effects of varying lichenin concentration confirms the difference. A Lineweaver-Burk plot (Fig. 8) shows that the malt enzyme has a Km value of 0.67%, much higher than the bacterial β-glucanase and much too high for the reaction velocity to approach a maximum at the 0.1% substrate concentration employed in these studies.

Since attempts to prepare 1.5–2.0% solutions of lichenin proved unsuccessful a determination of initial velocity was used to approximate the activity of the malt extract. Samples were removed from the incubation mixture at 1, 2, 3, 5, 10, and 15 min and reducing sugars determined. Figure 9 shows that the reaction was linear for the first 3 min and the calculated activity for the malt extract was 76 GU/g.

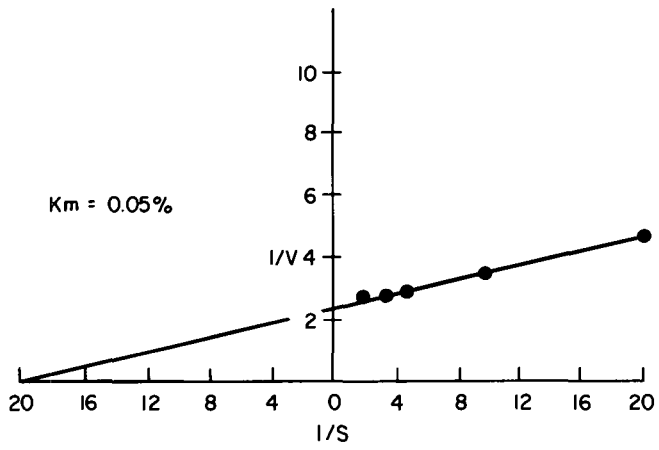

Fig. 13. Lineweaver-Burk plot of malt β-glucanase activity *vs.* laminarin concentration. 0.1 mg of malt extract incubated in 3 ml of laminarin solution (0.05–0.5% concentration) for 15 min at 45°C and pH 4.5.

Data presented in Figs. 10–13 show the results of studies with malt β-glucanase using laminarin as substrate. Figure 10 presents the results of varying the pH of reaction on β-glucanase activity employing an incubation temperature of 40°C and a substrate concentration of 0.1%. Optimum pH was found to be 4.5. The curve is somewhat broader than that obtained with lichenin as a substrate and the activity on laminarin is somewhat higher than that obtained with lichenin (115 GU/g *vs.* 76 GU/g).

Using a pH of 4.5 and varying the temperature (Fig. 11) an optimum of 45°C was observed. This β-glucanase appeared to be more heat sensitive than the one active on lichenin since activity fell off rapidly above 50°C.

Figure 12 shows the results of varying the β-glucanase concentration. Substrate concentration was again 0.1% and the reaction was carried out at a pH of 4.5 and a temperature of 45°C. Linearity was observed over an Absorbance range equal to 0.5 which corresponds to a change in reducing value equivalent to 50 μg of glucose.

Varying the concentration of laminarin produced the Lineweaver-Burk plot shown in Fig. 13. A Km value of 0.050% was found indicating that the 0.1% substrate concentration used was sufficient to maintain maximum reaction velocity under the conditions of assay.

DISCUSSION AND CONCLUSIONS

Lichenin and laminarin are commercially available β-glucans. Lichenin is a β-(1,3) (1,4) glucan while laminarin is a β-1,3 glucan. Previous studies (3,10,18) of pH optima and heat stability of malt β-glucanases suggested that the enzyme which hydrolyzed laminarin was different from that which hydrolyzed barley β-glucan. In our studies a β-glucanase from *Bacillus amyloliquefaciens* was found to have high activity against barley β-glucan but no activity against laminarin. Results with a lichenin substrate were identical to those obtained on barley β-glucan leading us to conclude that lichenin could be substituted for barley β-glucan. These results also suggested that lichenin and laminarin could be used as substrates to distinguish between β-(1,3) (1,4) glucanases and β-1,3 glucanases. However, both lichenin and laminarin have very high reducing sugar values leading to high substrate blank background which seriously restrict the useful linear range for activity. When one adds to this background that due to reducing sugars found in the crude enzyme preparations, then the useful range is further restricted and in the case of laminarin is virtually eliminated. In the present study we were able to reduce substrate background in lichenin to an $A_{450\ nm}$ of less than 0.05 and to less than 0.003 with laminarin by reduction with $NaBH_4$. Extraction and ultrafiltration of the crude enzyme preparations significantly lowered the reducing sugars in the enzymes.

With substrate backgrounds removed studies were carried out to determine the optimum conditions for assaying β-glucanases from *Bacillus amyloliquefaciens* and malt. Optimum pH, temperatures, and substrate concentrations were determined for the various enzymes and a reproducible assay procedure developed. The bacterial β-glucanase had a higher optimum pH than the malt enzymes, 6.5 *vs.* 4.0–4.5. Experiments with malt on lichenin and laminarin showed different pH and temperature optima as well as significantly different Km values, confirming the presence of at least two β-glucanases in malt. Km values of 0.05% for bacterial β-glucanase on lichenin and for malt β-1,3 glucanase on laminarin indicated the use of substrate concentrations as low as 0.1% for maintaining maximum reaction velocities. A Km value of 0.67% for malt β-(1,3) (1,4) glucanase on lichenin suggested the use of a significantly higher substrate concentration to ensure maximum reaction velocity. It was also found that the *Bacillus amyloliquefaciens* preparation contained more β-(1,3) (1,4) glucanase than malt.

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