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EXTRACTION OF *SORGHUM VULGARE* AND *HORDEUM VULGARE* α-GLUCOSIDASE*

TERENCE GEORGE WATSON and LAWRENCE NOVELLIE

National Chemical Research Laboratory, Council for Scientific and Industrial Research, P.O. Box 395, Pretoria, Republic of South Africa

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Key Word Index—*Sorghum vulgare*; millet; *Hordeum vulgare*; Gramineae; barley; α-glucosidase; enzyme extraction.

Abstract—Sorghum and barley grain α-glucosidase are both insoluble in water. They are, nevertheless, active in the insoluble state. Limited extraction of the enzyme from certain "normal" varieties of sorghum was achieved with sodium chloride under alkaline conditions and this could be enhanced by the addition of papain. In contrast, barley grain did not release α-glucosidase activity under these conditions. Maximum liberation of α-glucosidase from sorghum grain was achieved using a combination of 8 M urea and 0·1 M sodium sulphite. Barley grain did not respond similarly, the enzyme being resistant to extraction at low urea concentrations and completely denatured at high concentrations. The α-glucosidase from these two cereals appear to differ significantly in structural stability and in mode of attachment to insoluble grain matrix.

INTRODUCTION

THE PRESENCE of α-glucosidase (E.C. 3.2.1.20, α-D-glucoside glucohydrolase) activity in barley grain has long been recognized.[1] The enzyme was shown to be insoluble in water but rendered partially soluble during the germination of the barley. Extraction from malted barley could be improved by using alkaline conditions.[2] Other ungerminated cereals have later been found to be sources of α-glucosidase activity, e.g. buckwheat,[3] and two forms of the enzyme have been isolated from rice.[4] An acid α-glucosidase, previously only described in mammalian sources, has been found recently in sweet corn.[5] Two main types of sorghum occur, differing in the water solubility of their malt amylases which are freely soluble in the "normal" varieties but insoluble or only partially soluble in the "birdproof" varieties. In this paper, a detailed study of the extraction of α-glucosidase from "normal" sorghum grain is presented and comparisons drawn with that of barley grain.

RESULTS AND DISCUSSION

Water was a poor extractant of sorghum grain α-glucosidase, releasing, after 1 hr at 0°, less than 2% (0·06 nmol maltose consumed/min/mg of grain) of the enzyme

* Part XXIII in the series "Kaffircorn Malting and Brewing Studies". For Part XXII see DAIBER, K. H., MALHERBE, L. and NOVELLIE, L. (1973) *Brauwissenschaft*, **26**, 220.

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activity found by the direct incubation of milled grain with buffered maltose (3·38 nmol/min/mg). Extraction was improved to a limited extent (0·36 nmol/min/mg) under alkaline conditions, pH 9·3 in 0·1 M glycine/NaOH buffer, especially in the presence of sodium chloride. Optimum conditions were obtained by extraction at pH 9·3 for 1 hr at 30° with a salt concentration of 1 M, when 0·75 nmol/min/mg were released. An attempt to further enhance extractability was made by the addition of various surface active agents, basic amino acids, proteins and peptone, reducing compounds and proteolytic enzymes, as used by Novellie[6] for the extraction of insoluble amylases from sorghum malt. Papain (5%) proved to be the most effective. Extraction at 30° released 1·43 nmol/min/mg of α-glucosidase activity after 1 hr and 1·60 nmol/min/mg after 2 hr. Nevertheless this was less than 50% of the direct method of assay. The action of papain was not entirely due to its enzymatic effect, proteolytic or otherwise, 1·08 nmol/min/mg of activity were released after incubation for 1 hr at 30° with a boiled preparation of papain. The non-enzyme component was found to be non-dialysable. Peptone did not improve extraction.

The limited success of NaCl under alkaline conditions suggested that some, but not all, of the α-glucosidase activity was associated with the globulin fraction of the grain protein, the albumin fraction being virtually free of enzyme activity. The remainder, then, was associated with the prolamine and glutelin fractions which together account for about 70% of sorghum grain protein.[7] The protein of these fractions may be conveniently solubilized by 70–80% ethanol in the case of prolamine and 0·1–0·2% NaOH in the case of glutelin,[7–9] conditions which do not lend themselves to enzyme extractions. Ethanol, at a concentration of 70%, completely destroyed enzyme activity and *n*-butanol[10] resulted in only weak activity.

Urea is a good protein solubilizing agent and has been used at an optimum concentration of 2 M for the isolation of β-amylase from barley.[11] However, Daftary and Pomeranz[12] have shown that only 25% of sorghum proteins could be extracted with 3 M urea, although 8 M urea was more successful. Haikerwal and Mathieson[7] achieved complete solubilization of sorghum flour protein using 8 M urea and 0·1 M sodium dodecyl sulphate. In the present investigation, 8 M urea alone at 0° (Fig. 1) did not release more enzyme than NaCl at high pH. However, denaturation in 8 M urea at 0° for 1 hr was negligible. Extraction could not be improved by the use of sodium dodecyl sulphate, as complete enzyme denaturation resulted.

Certain structural grain proteins are known to be extensively crosslinked by disulphide bonds and not solubilized by powerful dissociating agents such as 8 M urea.[13] They may only be dispersed after disruption of the disulphide bonds. Since the α-glucosidase activity was not liberated by urea alone it seemed possible that the enzyme was either joined to structural protein by –S–S– linkages or was a part of a molecule itself attached to structural protein in this way.

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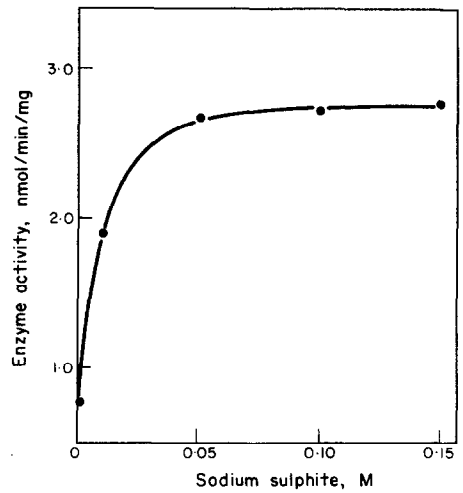

FIG. 1. THE EFFECT OF SODIUM SULPHITE CONCENTRATION ON THE EXTRACTION, FOR 2 hr, OF SORGHUM GRAIN α-GLUCOSIDASE IN 8 M UREA AT 0° AND pH 7.

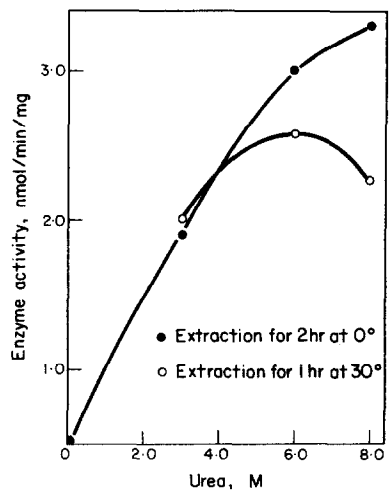

FIG. 2. THE EFFECT OF UREA CONCENTRATION ON THE EXTRACTION OF SORGHUM GRAIN α-GLUCOSIDASE IN 0·1 M SODIUM SULPHITE AT pH 7.

Several agents were used to split the disulphide bonds: sodium dithionite, Na_2SO_3, mercapto-ethanol and dithio-erythritol were largely ineffective in the absence of urea. However, their effectiveness increased as the urea concentration was raised, the most effective combination being 8 M urea and 0·1 M Na_2SO_3 (Figs. 1 and 2). To avoid enzyme denaturation at such high urea concentrations the reaction had to be carried out in ice for an optimum time of 2 hr (Table 1). Cecil and Loening[14] also found that the reaction of disulphide groups of insulin with sulphite was urea dependent. The two inter-chain disulphide bonds were easily cleaved by sulphite alone, urea being necessary for cleavage of the intrachain bond. Subsequent studies have shown this to be the case with many proteins. The reaction with sulphite was pH dependent, with greatest cleavage at pH 6·5–7·0.[14] A similar broad pH optimum of around 7·0 was found in the present study on sorghum grain.

TABLE 1. EXTRACTION OF *Sorghum vulgare* GRAIN α-GLUCOSIDASE WITH 8 M UREA + 0·1 M SODIUM SULPHITE

Temp	Time (hr)	Enzyme activity (nmol/min/mg) Extract	Residue	Total
30	1	2·29	1·20	3·49
0	1	3·04	2·08	5·12
0	2	3·33	1·82	5·15
0	3	3·15	1·69	4·84
0*	2	3·73	1·68	5·41

* With addition of 1% Triton X205.

The protein disulphide groups are probably cleaved in the following manner $RS{\cdot}SR + SO_3^{2-} \rightleftharpoons RS{\cdot}SO_3^- + RS^-$ (1) presumably allowing the release of a soluble enzyme-rich fraction from the insoluble protein matrix of the grain.

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Extractions were also performed with 3 M urea and 0·1 M sulphite at pH 9 for 1 hr at 30° in the presence of phenylmercuric hydroxide, which might be expected to aid extraction by allowing reaction (1) to go to completion[15] $\phi HgOH + RS^- \rightarrow RSHg\phi + OH^-$ (2). However, the activity extracted using phenylmercuric hydroxide was only 0·03 nmol/min/mg compared with 1·23 nmol/min/mg without its addition. This suggests that the active centre of the α-glucosidase also contains an -SH group which becomes inactivated through combination with the mercury.

Extraction with 8 M urea containing 0·1 M Na_2SO_3, pH 7, was increased by the addition of Triton detergent (Table 1) possibly acting as an enzyme stabilizer.[16] Under these optimum conditions *ca* 70% of the known enzyme activity present, i.e. in the extract and residue combined, was rendered soluble. The extracted activity was higher than the activity detected in the grain by direct analysis and the total activity some 60% higher, suggesting that even in a finely milled grain not all enzyme sites are accessible to substrate.

TABLE 2. EXTRACTION OF *Hordeum vulgare* GRAIN α-GLUCOSIDASE

Extracting soln	Time (hr)	Enzyme activity (nmol/min/mg) Extract	Residue	Total
Direct assay	0	—	—	0·38
0·1 M Na_2SO_3, pH 7·0	1	0	0·31	0·31
3 M urea + 0·1 M Na_2SO_3, pH 7·0	1	0	0·31	0·31
6 M urea + 0·1 M Na_2SO_3, pH 7·0	1	0	0·11	0·11
8 M urea + 0·1 M Na_2SO_3, pH 7·0	1	0	0	0
Water	3	0	—	—
1 M NaCl, pH 9·3	3	0	—	—

Extractions carried out at 0°. — Not determined.

Comparative experiments were also carried out using barley grain. The activity of α-glucosidase was *ca* 10 × less, as determined by the direct method, in the barley grain and in no case was any extraction achieved (Table 2). In addition, high urea concentrations caused considerable denaturation of the insoluble enzyme. At 8 M urea no activity could be detected in either the extract or the residue. It would appear that the barley enzyme is both more resistant to extraction and more sensitive to denaturation by urea than the sorghum grain α-glucosidase. Similar sensitivity of barley enzymes to urea has been found by Shinke and Mugibayashi[11] who report inactivation of β-amylase at urea concentrations above 2 M. What, in fact is most surprising is not the sensitivity of the barley enzyme to high urea concentrations but the exceptional resistance of the sorghum α-glucosidase to denaturation. Whether this is a general property of bound sorghum enzymes remains to be seen. Enzyme characteristics also differ markedly in respect of the pH optimum of reaction with maltose, the sorghum enzyme having a pH optimum of 3·75 compared with that from barley grain of 4·6 as determined directly on milled grain.

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EXPERIMENTAL

Grain. Sorghum; Barnard's Red. Barley; Australian Two Row.

Milling. Grains (10 g) were milled in a Janke and Kunkel beater type mill for 2 × 1 min with a 1 min interval.

Extraction. Sorghum (0·25 g) or barley grain (0·5 g) were extracted by shaking at 5 min intervals in 10 ml of solvent. Time and temp. of extractions and details of solvents employed are given in Results. Urea was de-ionized before use on a mixed bed resin, IR 120(H^+)/Rexyn 201(OH). The extracts were separated from the residues by centrifugation at 10000 rpm for 10 min. Extracts were dialysed overnight in 0·02 M citrate phosphate buffer pH 7 at 2° before assay of α-glucosidase activity.

Enzyme assay. The α-glucosidase activity was determined using 0·5 ml of dialysed extract. To the test was added 3·5 ml 0·2 M citrate–phosphate buffer (pH 3·75 for sorghum and pH 4·6 for barley), 0·5 ml of maltose (Merck biochemical grade) 67·5 mg/ml in citrate-phosphate, sufficient to achieve substrate saturation without substrate inhibition. To the control was added 4 ml of citrate-phosphate buffer. Incubation was at 30° for 1·5 hr. Reaction was stopped by addition of 5 ml Tris-phosphate pH 7, 0·3 M to both tubes, and the tubes held in ice. In addition, 0·5 ml of citrate–phosphate were added to the test and 0·5 ml maltose soln to the control. Glucose was estimated using the glucose oxidase method (Boehringer Test kit).

Direct enzyme assay. Direct assays were carried out on the grains without prior extraction. Milled grain (0·25 g sorghum; 0·5 g barley) was added directly to 45 ml of 0·2 M citrate phosphate buffer (pH 3·75 (sorghum), pH 4·6 (barley)) and pre-incubated for 1 hr at 30°. Maltose soln (5 ml of an aq. soln containing 75 mg/ml) was then added and the flask incubated at 30° for 1·5 hr. 1 hr prior to the end of incubation a second flask, the control, was prepared containing grain suspended in 45 ml buffer to be incubated for 1 hr at 30°. Continuous stirring was employed throughout the incubations. At the end of the incubation time 50 ml of Tris-phosphate buffer at 0° was added to both flasks. In addition, 5 ml of maltose solution was added to the control flask. The contents of both flasks were centrifuged at 10000 rpm for 10 min and the extracts stored at 0° until assayed for glucose. The pre-incubation period of 1 hr allows for the release from the grain of any glucose not produced as a result of α-glucosidase activity. It is not advisable to incubate the control for a further 1·5 hr without maltose as some amylase activity may be present, causing production of maltose which could then act as a substrate for the α-glucosidase leading to an erroneously high glucose determination. It is especially important to follow the procedure outlined above when using malted samples containing high amylase activity. Direct enzyme analysis was also carried out on the insoluble residues remaining after extraction. The residues were washed 2 × with 30 ml cold dist. H_2O before incubation.

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