Carbohydrate Research, 48 (1976) 255–264

PURIFICATION OF MALTED-BARLEY ENDO-β-D-GLUCANASES BY ION-EXCHANGE CHROMATOGRAPHY· SOME PROPERTIES OF AN ENDO-BARLEY-β-D-GLUCANASE*

DAVID J. MANNERS AND GLYNN WILSON†

Department of Brewing and Biological Sciences, Heriot-Watt University, Edinburgh EH1 1HX (Great Britain)

(Received September 1st, 1975, accepted for publication, September 5th, 1975)

ABSTRACT

Two endo-β-D-glucanases which act, respectively, on (1→3)-β-D-glucans and barley β-D-glucan have been isolated from malted barley, and purified by ion-exchange chromatography. The latter enzyme is highly specific for barley β-D-glucan, and has no action on either (1→3)- or (1→4)-β-D-glucans It will also act on dyed barley-β-D-glucan Certain group-specific reagents inhibit the endo-barley-β-D-glucanase and the endo-(1→3)-β-D-glucanase to similar extents

INTRODUCTION

Extracts of malted barley contain a complex system of enzymes capable of hydrolysing β-D-glucosidic linkages[2–4]; at least five enzymes can be distinguished according to their action pattern and specificity[2]. Purification of these enzymes by gel filtration on Biogel P-60 is limited in two ways Firstly, only relatively small amounts of starting material can be purified; secondly, it is difficult to achieve a simple, complete separation of the two endo-β-D-glucanases of low molecular weight, namely, an endo-barley-β-D-glucanase (defined as endo-β-D-glucanase activity towards barley β-D-glucan) and an endo-(1→3)-β-D-glucanase. We now describe a procedure for the separation and purification of these enzymes by ion-exchange chromatography. This method results in high yields of purified enzyme, and can be scaled-up as a batch procedure to obtain relatively large amounts of highly purified protein which are needed for studies on enzyme structure and for the exploration of enzyme–substrate relationships *in vivo*[5] Some properties of the endo-barley-β-D-glucanase purified by this method are also described.

*Studies on β-glucanases Part III For Part II, see Ref 1

†Present address The Rockefeller University, New York, N Y 10021, U S A.

EXPERIMENTAL

Enzyme source — Malted barley (Var Golden Promise, 1973 harvest) was a gift from A K. MacWilliam of Drybroughs & Co Ltd , Edinburgh

Analytical methods — Reducing sugars were determined by a modified Nelson method[6] Protein was determined by a modified Lowry method[7], calibrated against bovine serum albumin The protein content of column fractions was monitored by measuring the extinction at 280 nm

Substrates and enzyme assays — The preparation of substrates and the measurement of β-D-glucanase activities by both reductometric and viscometric assay procedures have been described in detail elsewhere[1] In experiments with purified endo-barley-β-D-glucanase, 0 5 ml of lichenin solution (0 5%) was incubated for 1 h at 37° with 0 3 ml of sodium acetate buffer (0 1M, pH 4 8) and 0 2 ml of appropriately diluted enzyme solution, the reaction being stopped by the addition of the Nelson copper reagent[6].

Inhibitor assays — The source of enzyme inhibitors and the conditions for their reaction with enzyme solutions have been described previously[1]

Chromatography — Malted-barley extracts were fractionated by ion-exchange chromatography at 0–4°, on columns of DEAE- or CM-cellulose (DE52 and CM52 grades, respectively, Whatman Biochemicals Ltd , Maidstone, Kent), prepared according to the manufacturer's instructions For the large-scale me hod, ion-exchange resins were prepared as thick pads, in Buchner funnels, to give a total bed-volume of ~680 ml Gel filtration was carried out on columns of Biogel P-30 or P-60 (Bio-Rad Laboratories Ltd , Bromley, Kent) Descending paper chromatograms on Whatman No 1 paper were developed in ethyl acetate–pyridine–water (10 4 3) Reducing sugars were detected with alkaline silver nitrate[8]

Enzyme extraction — Malted-barley flour was extracted by stirring with 0 2M sodium acetate buffer (pH 5 0, 3 litres/kg of flour) for 3 h at 0–4° After centrifugation and dialysis against 0 02M sodium acetate buffer (pH 5, 24 h, 0–4°), protein was precipitated by the addition of solid ammonium sulphate to 80% saturation A solution of the resulting precipitate in sodium acetate buffer (0 01M, pH 5) was dialysed against the same buffer (48 h, 0–4°) and freeze-dried This 0–80% ammonium sulphate fraction (E_1) was the starting material for subsequent purification procedures

Ion-exchange chromatography of malted-barley extract — (*a*) *On DEAE-cellulose* A column (30 × 2 5 cm) of DEAE-cellulose equilibrated in citrate–phosphate buffer (0 02M, pH 8 0) was used to fractionate 500 mg of E_1 dissolved in the same buffer Protein was eluted from the column by a salt gradient (0→M NaCl) in 0 02M citrate–phosphate, followed by a pH gradient (8 0→3 0) of 0 02M citrate–phosphate containing M NaCl Fig 1 shows that all the activity towards barley β-D-glucan and laminarin was eluted at the beginning of the salt gradient Endo-(1→4)-β-D-glucanase activity (*i e* , activity towards CM-cellulose by viscometric assay), which was extremely low in the sample of malted-barley flour used in these experiments, was also unadsorbed by the DEAE-cellulose In contrast, a large proportion of the protein (E_{280nm})

and one peak of activity towards cellobiose (β-D-glucosidase) were bound to the column Fractions eluted at the beginning of the salt gradient were combined, and dialysed against 0 02M sodium acetate (pH 4 8, 24 h at 0–4° with two changes of acetate buffer)

(*b*) *On CM-cellulose* The dialysed solution from (*a*) was applied to a column (30 × 2 5 cm) of CM-cellulose equilibrated in 0 02M sodium acetate buffer at pH 4 8 Protein was eluted from the column with a gradient of 0 02–1 0M sodium acetate (pH 4 8). The results are shown in Fig 2 Complete separation of the endo-(1→3)-β-D-glucanase and endo-barley-β-D-glucanase activities was obtained, the latter enzyme being strongly bound to the ion-exchange resin

(*c*) *Batchwise ion-exchange procedure* A solution containing 2 g of Fraction E_1 in 500 ml of 0 02M citrate–phosphate buffer (pH 8) was poured on to a pad of DEAE-cellulose previously equilibrated with the same buffer The DEAE-cellulose was then washed with 2 litres of equilibrating buffer, the eluant being recycled through the ion-exchange resin to give Fraction E_2 Table I shows that 75% of the endo-barley-β-D-glucanase and 98% of the endo-(1→3)-β-D-glucanase activities were recovered in Fraction E_2 In contrast, 66% of the total protein was bound to the DEAE-cellulose Fraction E_2 was dialysed in an Amicon TCF-10 ultrafiltration cell, using a Diaflo PM-10 membrane (Amicon Ltd , High Wycombe, Bucks), and 0 02M sodium acetate buffer (pH 4 8) as dialysis buffer The resulting solution (Fraction E_3) was poured on to a pad of CM-cellulose previously equilibrated with 0 02M sodium acetate buffer (pH 4 8) Protein was eluted from the pad by successive elution with 2 litres of equilibrating buffer (Fraction E_4), 2 litres of 0 25M sodium acetate (pH 4.8, Fraction E_5), and 2 litres of M sodium acetate (pH 4 8, Fraction E_6) After collection, each fraction was recycled through the CM-cellulose The recovery of protein, endo-(1→3)-β-D-glucanase, and endo-barley-β-D-glucanase (viscometric assays) is shown in Table I Virtually all of the endo-(1→3)-β-D-glucanase was recovered in Fraction E_5, whereas the endo-barley-β-D-glucanase was more firmly bound to the ion-exchange resin and was then completely eluted with M acetate The specific activities of the endo-(1→3)-β-D-glucanase in Fractions E_1 and E_5 were 8 3 and 42 3, respectively, and of the endo-barley-β-D-glucanase in Fractions E_1 and E_6 were 1 9 and 15 2, respectively. The specific activity of both enzymes in E_1 is about twice that of the initial extract

Further purification of endo-(1→3)-β-D-glucanase and endo-barley-β-D-glucanase — Partially purified enzymes obtained from the batchwise ion-exchange method can be further purified by gel filtration on columns of Biogel P-30 or P-60[1,2], or by a combination of gel filtration and ion-exchange chromatography on columns of CM-cellulose This latter procedure is particularly useful if small amounts of endo-(1→3)-β-D-glucanase are obtained on elution with M acetate, *i e* , in Fraction E_6 Ion-exchange chromatography of 2 g of Fraction E_2 resulted in complete separation of the two endo-β-D-glucanases The use of larger amounts (10 g) of starting material resulted in contamination of Fraction E_6 with small proportions of endo-(1→3)-β-D-glucanase It has been estimated that the present procedure yields *ca.* 300 mg of

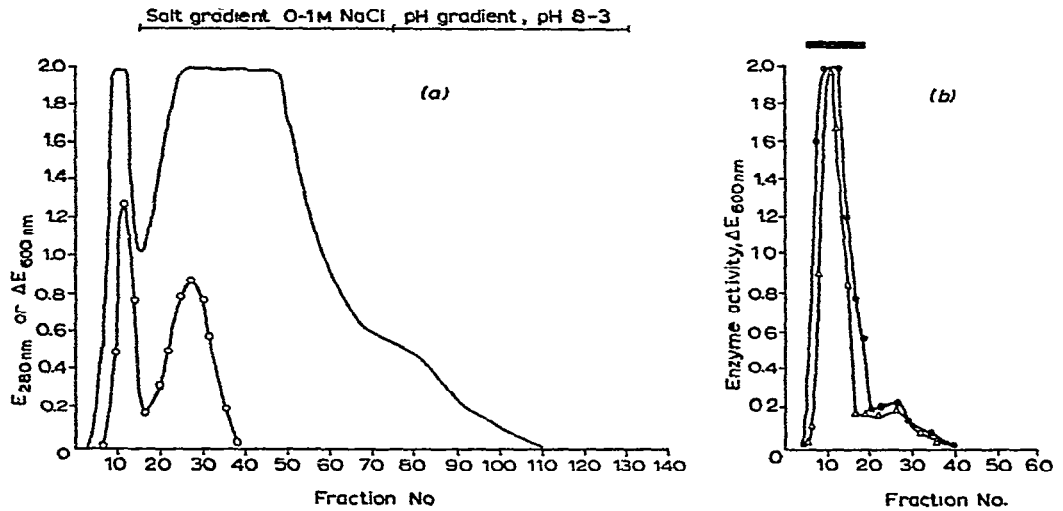

Fig 1 Fractionation of a malted-barley extract on DEAE-cellulose (*a*) protein, ———, cellobiase, O—O, (*b*) laminarinase, ●—●, endo-barley-β-D-glucanase, Δ—Δ The heavy bar denotes those fractions that were combined for CM-cellulose fractionation

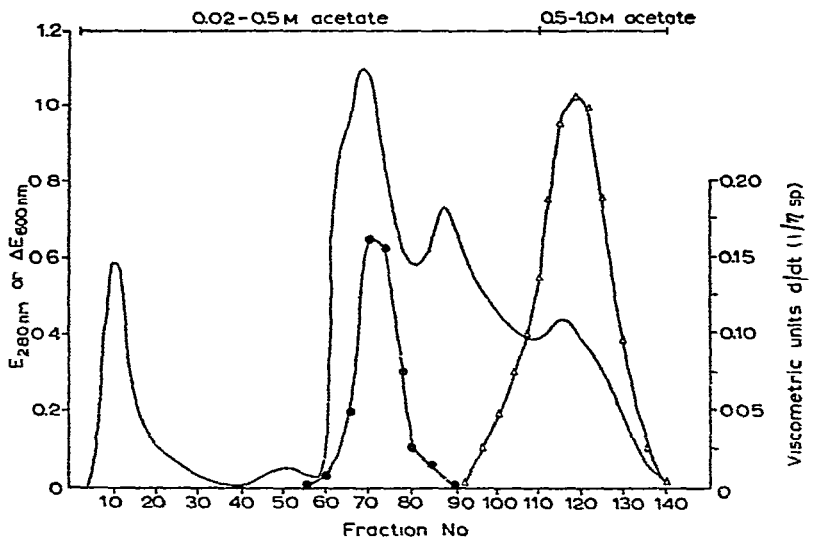

Fig 2. CM-cellulose chromatography of combined fractions from a column of DEAE-cellulose protein, ———, endo-(1→3)-β-D-glucanase, ●—●; endo-barley-β-D-glucanase, Δ—Δ

TABLE I

BATCHWISE ION-EXCHANGE CHROMATOGRAPHY OF MALTED BARLEY EXTRACT

Stage	*Total protein*		*Endo-(1→3)-β-D-glucanase*		*Endo-barley-β-D-glucanase*	
	(mg)	*Yield (%)*	*Total units*[a]	*Yield (%)*	*Total units*[a]	*Yield (%)*
Ammonium sulphate						
0–80% (E_1)	1500	100	1.25×10^4	100	2.8×10^3	100
DEAE-Cellulose (E_2)	505	34	1.22×10^4	98	2.1×10^3	75
Amicon concentrate (E_3)	381	26	1.09×10^4	87	1.9×10^3	68
CM-Cellulose						
(*a*) 0.02M acetate (E_4)	32	2	N.D.[b]	0	N.D.	0
(*b*) 0.25M acetate (E_5)	224	15	9.48×10^3	76	N.D.	0
(*c*) M acetate (E_6)	123	8	N.D.	0	1.87×10^3	67

[a]Unit defined as d/dt ($1/\eta_{sp}$) of 0.1 [b]N.D., none detected

purified endo-(1→3)-β-D-glucanase per kg of barley flour, compared with 100 mg/kg obtained by gel filtration[1]

Properties of endo-barley-β-D-glucanase. — (*a*) *Enzyme purification* The enzyme was prepared by the batchwise ion-exchange method, final purification being achieved by gel filtration on Biogel P-30. Fig 3 shows the elution pattern of the purified enzyme on Biogel P-30 The enzyme solution was concentrated by ultrafiltration on a Diaflo PM-10 membrane to give a stock solution of 2 8 mg of protein/ml, this represented a 105-fold increase in specific activity relative to the initial extract

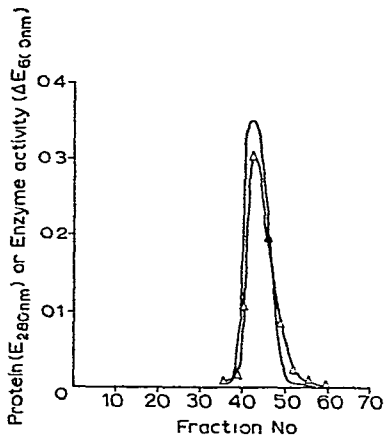

Fig 3 Final purification of endo-barley-β-D-glucanase on Biogel P-30, column size, 80 × 3 5 cm, 4-ml fractions were collected Distribution of protein, ———, activity towards lichenin, Δ—Δ

(*b*) *Effect of digest pH and temperature* The enzyme showed an optimum pH of 4 8, using 0 05M citrate–phosphate buffers At pH 4 8, the optimum assay temperature (1-h digest time) was 39°

(*c*) *Effect of metal ions* A 5-fold dilution of the enzyme solution was pre-incubated in a 1 1 mixture with mM solutions of various metal ions for 30 min at 25°. Samples (0 2 ml) of each mixture were then incorporated into standard digests With the following metal ions, the activity relative to a control ranged from 101 to 106% Ca^{2+}, Ba^{2+}, Mg^{2+}, Zn^{2+}, Sr^{2+}, Co^{2+}, and Mn^{2+} We conclude that the various metal ions have little or no effect on the endo-barley-β-D-glucanase activity, in contrast to the endo-(1→3)-β-D-glucanase which is activated to a significant extent by some ions[1]

(*d*) *Effect of bovine serum albumin* (*BSA*) BSA (50–500 μg) was added to standard digests containing a 10-fold dilution of enzyme solution With 500 μg of BSA, a 5% increase in activity was observed The stimulation of the endo-barley-β-D-glucanase by BSA is therefore much smaller than that of the endo-(1→3)-β-D-glucanase[1]

(*e*) *Specificity* The purified enzyme had no activity towards laminarin (reducing-power assay) or CM-pachyman and CM-cellulose (viscometric assay) Its action on lichenin and barley β-D-glucan was tested by incubating 0 1 ml of enzyme solution with 2 5 mg of substrate in 0.4 ml of 0 02M acetate buffer (pH 4 8) for 24 h at 37° The major product obtained from both substrates was a trisaccharide, identified as 3-*O*-β-cellobiosyl-D-glucose from the mobility of an authentic sample on paper and from its electrophoretic mobility on paper (0 05M sodium borate buffer, pH 10; 10 V/cm for 1 h) Traces of a higher oligosaccharide (tentatively identified as a tetrasaccharide) were also observed (paper chromatography) in digests

(*f*) *Inhibition of endo-barley-β-D-glucanase* The effect of various inhibitors was examined using the same experimental conditions as those described for the inhibition of malted-barley endo-(1→3)-β-D-glucanase[1] The reductometric assay method with lichenin as substrate was used, except for the *N*-bromosuccinimide reaction, where the viscometric-assay procedure with barley β-D-glucan was employed. A 1 5 dilution of the stock enzyme solution was used in a pre-incubation mixture of equal volumes of enzyme and inhibitor solution For reaction with *N*-bromosuccinimide and 2-hydroxy-5-nitrobenzyl bromide, the dilutions of the stock enzyme solution were 1 10 and 1 2, respectively The results of the inhibitor tests are shown in Table II

TABLE II

INHIBITION OF ENDO-BARLEY-β-D-GLUCANASE

Inhibitor	*Conc in mixture with enzyme (mM)*[a]	*Inhibition (%)*
Cyanate ion	200	14
Iodoacetate ion	10	41
Mercuri-*p*-carboxyphenyl chloride	0 2	30
Mercuri-phenyl nitrate	2	98
N-Bromosuccinimide	5	99
N-Acetylimidazole	92[b]	86
2-Hydroxy-5-nitrobenzyl bromide	10[c]	96

[a]Preincubation time of 30 min unless otherwise stated No inhibition was observed with the following compounds D-glucono-1,5-lactone (10mM), EDTA (50mM), and *N*-ethylmaleimide (mM) [b]Preincubation time, 1 h [c]Preincubation time, 2 h

(*g*) *Action on dyed barley-β-D-glucan* Barley β-D-glucan was dyed with Reactone Red 2B [kindly provided by Ciba–Geigy (U K) Ltd , Manchester] by the method of Zitting and Linko[10]. The glucan (10 g) in 200 ml of M sodium hydroxide was stirred with 2 g of dye for 18 h at room temperature The solution was then acidified with 3M hydrochloric acid, and the dyed glucan was precipitated by the addition of an equal volume of ethanol The precipitate was repeatedly dissolved in hot water and then reprecipitated with ethanol, until the supernatant solution was colourless

Digests were prepared containing 0 5 ml of dyed β-D-glucan (0 5%), 0 3 ml of sodium acetate buffer (0 1M, pH 4 8), and 0 2 ml of a 1 10 dilution of stock enzyme

solution, and incubated at 37° At intervals, 2 ml of ice-cold ethanol was added to the individual digests, which were then cooled at 0° for 10 min before centrifugation The extinction at 500 nm of the supernatant solutions was then measured against a control which contained distilled water in place of enzyme The results are shown in Fig 4

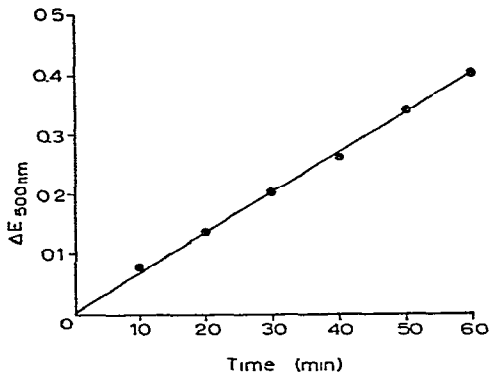

Fig 4 Rate of release of dye from barley β-D-glucan, dyed with Reactone Red 2B, by the purified endo-barley-β-D-glucanase

DISCUSSION

Ion-exchange chromatography provides a convenient method for the purification of the endo-(1→3)-β-D-glucanase and endo-barley-β-D-glucanase from malted barley Fractionation of extracts of malted barley on DEAE-cellulose at pH 8 0 resulted in the separation of a large proportion of extraneous protein from these enzymes, and also in the separation of the two β-D-glucosidases present Complete separation of the two endo-β-D-glucanases was effected by chromatography on CM-cellulose, the endo-barley-β-D-glucanase being strongly bound to this support A batchwise, ion-exchange procedure can be used as a rapid method for the separation and partial purification of endo-(1→3)-β-D-glucanase and endo-barley-β-D-glucanase, from relatively large amounts of starting material, final purification then being achieved by gel filtration In addition to the simplicity of this batch procedure, there was a three-fold increase in yield (mg of purified enzyme/kg of malt flour) of endo-(1→3)-β-D-glucanase compared with the gel-filtration method[1]

The present experiments have confirmed previous observations[1,2] on the number and nature of the endo-β-D-glucanases and β-D-glucosidases present in extracts of kilned malt Only a single peak of endo-barley-β-D-glucanase activity has been observed, whereas Luchsinger *et al*[9] obtained evidence for the presence of two endo-barley-β-D-glucanases in extracts from unkilned malt As one of these enzymes was reported to be heat-labile[9], it is probable that this activity is either destroyed during the kilning process, or is converted into the heat-stable form, and is thus not observed in samples of kilned malt

The endo-barley-β-D-glucanase purified by ion-exchange chromatography was free from any contaminating carbohydrase activities The enzyme had a pH optimum in the region of pH 4 8 and an optimum assay temperature of 39° These properties are similar to those of the endo-(1→3)-β-D-glucanase[1] However, the two enzymes differ in that the endo-barley-β-D-glucanase is not significantly stimulated by metal ions or by BSA

Substrates containing only (1→3)- or only (1→4)-β-D-glucosidic linkages were not attacked by the purified endo-barley-β-D-glucanase, whereas β-D-glucans containing both types of linkage were attacked Since the major product was the trisaccharide 3-*O*-β-cellobiosyl-D-glucose, and both lichenin and barley β-D-glucan contain ~70% of (1→4)- and ~30% of (1→3)-linkages, it follows that enzyme action involves the hydrolysis of (1→4)-β-D-glucosidic linkages The endo-barley-β-D-glucanase thus has a specificity similar to that of an enzyme from *Bacillus subtilis*[11], and belongs to the group EC 3 2 1 73[12] This group differs from other β-D-glucanases (*e g* , from *Rhizopus arrhizus*) which act on barley β-D-glucan, but also on laminarin and belong to the group EC 3 2 1 6[12]

Although Zitting and Linko[10] originally showed that a malt extract would release dye from dyed β-D-glucan, the results in Fig 4 appear to be the first demonstration of a highly purified endo-barley-β-D-glucanase attacking this substrate As this method is relatively sensitive, it might prove a convenient means of assaying crude extracts of malted cereals for endo-barley-β-D-glucanase activity, provided that the β-D-glucosidases have no significant effect on the dyed β-D-glucan, both in the presence and absence of endo-acting enzymes

The results of inhibitor tests (Table II) provide further information on the activity of the enzyme and suggest the identity of amino acid residues which may be essential for activity D-Glucono-1,5-lactone, which is a competitive inhibitor of β-D-glucosidases[13], did not inhibit the enzyme There was also no inhibition by EDTA, suggesting that metal ions are not required for activity Inhibition by mercuri-*p*-carboxyphenyl chloride and mercuri-phenyl nitrate might indicate that thiol groups are required for activity, although *N*-ethylmaleimide was not an inhibitor The effects of mercuri-phenyl nitrate must be interpreted with care, as it reacts with groups other than thiols in α-amylase[14] and catalase[15] Although Luchsinger[16] observed that a heat-stable endo-barley-β-D-glucanase was rapidly inactivated in the absence of glutathione and cysteine, 67% of the endo-barley-β-D-glucanase activity was recovered during the batchwise ion-exchange method without the use of these reagents Inhibition by cyanate[17] and iodoacetate may not be diagnostic of a particular amino acid residue, however, it is interesting to note that malted-barley endo-(1→3)-β-D-glucanase is inhibited to a similar extent by these reagents The possibility that tryptophan residues are required for activity is shown by the inhibition by 2-hydroxy-5-nitrobenzyl bromide[18] and *N*-bromosuccinimide[19] Inhibition by *N*-acetylimidazole[20] may indicate a requirement for tyrosine. Similarities between the inhibition of the endo-barley-β-D-glucanase and the endo-(1→3)-β-D-glucanase[1] from malted barley are therefore apparent It is thus possible that these enzymes, although acting

on different substrates, may possess common features at their active centres, with similar residues participating in both substrate binding and catalysis

ACKNOWLEDGMENTS

We are indebted to Mr J Y Brodie for technical assistance, and to the Science Research Council for financial support.

REFERENCES

1 D J Manners and G Wilson, *Carbohyd Res*, 37 (1974) 9–22
2 D J Manners and J J Marshall, *J Inst Brew*, 75 (1969) 550–561
3 W W Luchsinger, *Cereal Sci Today*, 11 (1966) 69–77.
4 E J Bass and W O S Meredith, *Proc Amer Soc Brew Chem*, (1960) 38–47
5 G N Bathgate, G H Palmer, and G Wilson, *J Inst Brew*, 80 (1974) 278–285
6 J F. Robyt and W J Whelan, in J A Radley (Ed), *Starch and its Derivatives* Chapman and Hall, London, 4th edition, 1968, p 432
7 G L Miller, *Anal Chem*, 31 (1959) 964
8 W E Trevelyan, D P Procter, and J S Harrison, *Nature (London)*, 166 (1950) 444–445
9 W. W Luchsinger and A W Richards, *Arch Biochem Biophys*, 106 (1964) 65–70
10 A Zitting and M Linko, *Acta Chem Scand*, 25 (1971) 298–300
11 E A Moscatelli, E A Ham, and E L Rickes, *J Biol Chem*, 236 (1961) 2858–2862
12 Enzyme nomenclature, Recommendations (1972) of I U P A C and I U B, Elsevier, Amsterdam, 1973
13 E T Reese, A. H Maguire, and F W Parrish, *Can J Biochem*, 46 (1968) 25–34
14 C T Greenwood and A E Milne, *Stärke*, 20 (1968) 101–107
15 M R Sohler, M A. Siebert, C W. Kreke, and E S Cook, *J Biol Chem*, 198 (1952) 281–291.
16 W W Luchsinger, *Cereal Chem*, 39 (1962) 225–235
17 G R Stark, *Methods Enzymol*, 11 (1967) 590–594
18 H R Horton and D E Koshland, *J Amer Chem Soc*, 87 (1965) 1126–1132
19 T C Spande and B Witkop, *Methods Enzymol*, 11 (1967) 498–506
20 T F. Riordan, W E C Walker, and B L Vallee, *Biochemistry*, 4 (1965) 1758–1765