IMMOBILIZATION OF β-GLUCANASE AND STUDIES ON ITS DEGRADATION OF BARLEY β-GLUCAN

by

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A commercial fungal β-glucanase preparation was immobilized by various methods including adsorption to DEAE-cellulose, cross-linking with glutaraldehyde, adsorption to a phenol-formaldehyde resin (Duolite) and to perlite followed by fixation with glutaraldehyde, covalent binding to glutaraldehyde-treated, partially hydrolyzed or partially aminolyzed nylon, and covalent binding in the Ugi-reaction to polyisonitrile-nylon. The recovery of enzymatic activity varied from 0.02 to 4.5% and the immobilized enzyme preparations showed specific activities from 1 to 25% of that of the starting material.

DEAE-cellulose-, nylon- and Duolite-β-glucanase were used in packed bed reactors for continuous hydrolysis of barley β-glucan. The Duolite-β-glucanase was most active in depolymerizing this polysaccharide.

The action pattern of insoluble crude β-glucanase (Duolite-β-glucanase) on barley β-glucan was very different from that of the crude enzyme preparation in solution. Comparison with the mode of action of a homogeneous preparation of endo-1,4-β-glucanase in dissolved and immobilized form, respectively, indicated that this change in action pattern was partly due to coimmobilization of enzymes catalyzing consecutive steps in the hydrolysis of barley β-glucan to glucose and partly to steric hindrance of the interaction between the immobilized enzyme and the interior regions of the substrate.

1. INTRODUCTION

A wide range of systems have been described for immobilization of enzymes, but many of these are too complex for practical and industrial use because of the many reaction steps involved, the high cost of chemicals and the purity of enzymes required. The present paper deals with insolubilization of a commercial fungal β-glucanase, which finds widespread use in the brewing industry for depolymerization of 1,3;1,4 mixed-linked β-glucan from barley and malt (4, 7, 18). Various simple and inexpensive immobilization procedures including adsorption to an ion exchange resin (3, 28, 30), attachment to inert supports (11, 14, 22, 23), covalent coupling to derivatives of nylon (9, 13, 15), and intermolecular cross-linking with glutaraldehyde (10, 27) were tried out. The physical form of some of these immobilized enzymes like granular phenol-formaldehyde resin (Duolite) and nylon fibers made them easily applicable in column reactors (25, 26, 29).

Immobilization of α-amylase alters its action pattern from an endo-enzymatic to an exo-enzymatic mode (2, 17, 19), and in the present report similar changes are described for the immobilized β-glucanase.

2. EXPERIMENTAL

2.1. Materials

Crude fungal β-glucanase, Glucanase GV-L, was a liquid culture concentrate manufactured by A/S Grindstedværket, Brabrand, Denmark. Partially purified β-glucanase was prepared by precipitation of the commercial enzyme with 55% saturated ammonium sulphate. Prior to immobilization the precipitate was redissolved in deionized water to give the final volume of the crude glucanase employed unless otherwise stated. Homogeneous endo-1,4-β-glucanase isolated from Glucanase GV-L as described elsewhere (32) was used in a single experiment.

Barley β-glucan, prepared according to the method of PREECE and MCKENZIE (24), was a gift from Mrs. E. MEILING and J. A. SØRENSEN M.sc., Department of Brewing Chemistry, Carlsberg Research Laboratory. Nylon-6,6 powder was a product of I.C.I., nylon-6 BS3

granulate BASF, was a gift from A/S Badilin, Copenhagen, Denmark. Polyisonitrile-nylon-6 powder, containing 45 μmoles of isocyanide groups/g, was a gift from Dr. M. SOKOLOVSKY, Department of Biochemistry, Tel Aviv University, Israel. Perlite 4258 kieselguhr was bought from Dicalite, Belgium. Duolite S-761, (18-40 mesh), (Dia-Prosim, Vitry, Chauny, France) was a gift from A/S Glent & Co., Copenhagen, Denmark. DEAE-cellulose, DE 52 microgranular, was from Whatman, England. Glutaraldehyde (25% (w/v), aqueous, pract.), acetaldehyde (z. synth.), and N,N-dimethyl-1,3-propanediamine (lab. reag.) were obtained from Fluka (Schweiz), Merck (W. Germany), and British Drug Houses (England) respectively.

2.2. Preparation of immobilized β-glucanase

Floc of partially hydrolyzed nylon-6,6 (9, 25) or partially aminolyzed nylon-6 (13) was activated with 12.5% glutaraldehyde at pH 8.5 and 0°C for 20 min. After washing, 2.5 g of the activated floc was mixed with 50 ml partially purified β-glucanase at pH 7 and 4°C. After 18 h reaction time the product was washed with 0.5M-NaCl, water and 0.1 M-sodium acetate pH 5.0. In a single experiment aminolyzed nylon-6 floc was replaced by powder.

The coupling capacity of approx. 500 mg of activated nylon was determined by reaction with 20 mg of glycyl-L-leucine in 5 ml of 0.1M-sodium borate pH 9.0.

Polyisonitrile-nylon was coupled with partially purified β-glucanase or glycyl-L-leucine by means of a four-component condensation reaction, the Ugi-reaction, essentially as described by GOLDSTEIN et al (9).

Perlite was coupled to the enzyme by suspending the perlite in 10 ml β-glucanase solution adjusted to pH 7.5, air drying the suspension at 40°C, and resuspending the dried product at pH 7.5 in acetone/water (2:1) containing 0.5% glutaraldehyde for 40 min. After washing with 0.5M-NaCl, the product was stored in water at 4°C.

DEAE-cellulose previously equilibrated with 0.01M-acetate buffer pH 5.0 was used for adsorption of crude β-glucanase, adjusted to pH 5 and diluted 145-fold with water. The en-

zyme-resin was washed with a large surplus of the acetate buffer.

Cross-linking of partially purified β-glucanase was performed by shaking with 0.1-2.0% glutaraldehyde in phosphate buffer pH 7.5 for 2 or 24 h at room temperature. In some experiments the cross-linking was performed in the presence of approx. 80% saturated sodium sulphate. The insolubilized products were washed with 0.5M-NaCl and stored in water at 4°C.

The immobilization of β-glucanase on Duolite was studied in more details. The Duolite (5 g) was rinsed as described previously (23) and incubated with shaking for 1 h with 6 ml crude glucanase. The liquid was removed by filtration and 0.05-2.0% glutaraldehyde in 0.1 M-phosphate buffer pH 7.0 was added to the enzyme-resin. After 20 h at 4°C the enzyme-resin was washed with 0.1 M-acetate buffer pH 5.0 containing 1M-NaCl. After a brief wash with water the enzyme-resin was packed in a chromatographic column and washed for 48 h at 4°C by pumping through a 0.02 M-sodium acetate buffer pH 5.0. In some experiments the enzyme-resin was stabilized by reduction with sodium borohydride before the washing steps. This reagent was added at intervals during 20 min to a suspension of the enzyme-resin in phosphate buffer pH 7.0 at 0°C. The total molar amount of sodium borohydride was normally 20-fold molar excess over the glutaraldehyde. Only when the cross-linking was performed at glutaraldehyde concentrations of 1% or higher was the molar excess of sodium borohydride reduced to 5-fold.

In some experiments adsorption, cross-linking, and reduction, or some of these steps, were performed in the presence of 0.27% barley β-glucan.

Duolite-endo-1,4-β-glucanase was prepared from 10 g of resin and 3 mg of the pure enzyme (32) essentially as described above. To protect the enzyme, all steps, including the NaBH$_4$-reduction, was performed in the presence of 0.27% barley β-glucan, and the 48 h wash took place at 4°C.

2.3. β-glucanase packed bed reactor

Reactor studies were performed in laborato-ry scale using a thermostated Pharmacia K 16/20 chromatographic column equipped with adaptors. The substrate solution (0.03% barley β-glucan in 0.02M-sodium acetate pH 5.0) was fed downwards by means of a peristaltic pump, the flow rate being varied to change the substrate residence time.

2.4. Analytical procedures

β-glucanase activity was measured by the amount of reducing sugar released from barley β-glucan as determined by the method of SOMOGYI and NELSON as described by HODGE and HOFREITER (12). The assay mixture, 6 parts of 0.67% barley β-glucan in water, 1 part of 0.2 M-sodium acetate pH 5.0 and 1 part of enzyme in solution or suspension, was incubated 30 min with shaking at 30°C. The reaction was stopped by mixing 400 μl of reaction mixture with 400 μl of low alkalinity copper reagent (12) or by boiling. The SOMOGYI and NELSON procedure was completed and the absorbance read at 546 nm. One unit of β-glucanase catalyzes the release of reducing power equivalent to 1 μmole glucose/min at the conditions stated above.

The contents of high molecular weight barley β-glucan in effluents from Duolite-β-glucanase reactors were measured on 1 ml aliquots by a micro version of a precipitation technique described earlier (7).

Total carbohydrate contents were determined by the phenol-sulphuric acid procedure, using glucose as standard (5).

Amino acid analyses were performed on protein hydrolysates on the Durrum D-500 amino acid analyzer, except for samples containing ε-aminocaproic acid (from nylon-6), which were analyzed on a modified Spinco Model 120 amino acid analyzer (31). Crude enzyme, partially purified enzyme, and immobilized enzyme derivatives were hydrolyzed in 6M-HCl in vacuo at 110°C for 24 h.

2.5. Action pattern

Barley β-glucan (0.03% in 0.02 M-sodium acetate pH 5.0) was hydrolyzed at 30°C in packed bed enzyme reactors. In parallel, the substrate was hydrolyzed in a batch-wise manner by a

corresponding preparation of soluble enzyme, using an enzyme concentration adjusted to hydrolyze a similar number of glucosidic bonds per substrate molecule as obtained with the insolubilized enzymes. The reaction was stopped by acidification and the composition of the barley β-glucan hydrolysates was analyzed by gel filtration chromatography as described by Enevoldsen and Schmidt (6).

3. RESULTS AND DISCUSSION

3.1. Immobilization of β-glucanase

The recovery of β-glucanase activity after coupling of partially purified enzyme to different types of nylon varied from 0.02 to 0.15%, with the highest activity being obtained by coupling to partially aminolyzed nylon (Table I). The use of more concentrated or more diluted enzyme in the coupling process or coupling in the presence of saturated sodium sulphate did not significantly improve the yields. Crude β-glucanase could be coupled with similar yields, but the leakage of soluble enzyme was higher.

The commercial β-glucanase contains several enzymes capable of catalyzing the hydrolysis of β-glucosidic bonds, but the endo-1,4-β-glucanase described elsewhere is responsible for more than half of the total activity against barley β-glucan as determined by the release of reducing sugar (32). This enzyme has an isoelectric point around pH 4.5 (32) and thus the higher recovery of activity in the preparation of the aminolyzed nylon-β-glucanase might be due to the electrostatically favoured binding of the anionic endo-1,4-β-glucanase to the cationic support material. In contrast, the acid hydrolyzed nylon-β-glucanase showed a much lower enzymatic activity although the corresponding activated anionic support showed a higher coupling capacity for protein than the polycationic partially aminolyzed nylon. However, this difference in activity between the two types of derivatives could hardly be explained as only resulting from a general microenvironmental effect due to the charges on the matrices since both types only showed an increase in activity of around 15% in the presence of 0.5M-KCl.

Among the nylon-supported preparations β-glucanase covalently bound to polyisonitrile-nylon via protein carboxyl groups displayed the highest specific activity. The protein contents of all nylon-β-glucanase preparations was much lower than expected from the coupling capacity determined with glycyl-L-leucine (Table I). In the literature we were unable to find any figures for protein contents of enzymes immobilized with partially hydrolyzed or aminolyzed nylon floc or powder as matrix, but pure trypsin coupled in the Ugi-reaction to polyisonitrile-nylon via protein amino groups in amounts as high as 153 mg/g of conjugate (9). The glycyl-L-leucine coupling capacity we found for this matrix was in agreement with the literature value (9) and it was higher than the peptide coupling capacities found for the other nylon-supports (Table I). A protein content of polyisonitrile-nylon-β-glucanase of 2.6 mg/g, therefore, was unexpectedly low and might indicate that other compounds consume polymer reactive sites in competition with the active enzymes. Thus, the Ugi-reaction may only be well suited for immobilization of relatively pure enzymes.

The immobilization of partially purified β-glucanase by intermolecular cross-linking using glutaraldehyde resulted in higher recoveries of activity than obtained for nylon-β-glucanases (Table I). Recoveries of 2.9% were obtained by cross-linking 2 h at 2.0% glutaraldehyde and for 24 h at 0.5% glutaraldehyde. However, the recovery of β-glucanase activity was very sensitive to the concentration of glutaraldehyde and the reaction time. Thus reaction for 2 h at 0.5% glutaraldehyde concentration and for 24 h at 0.1% or 2% glutaraldehyde resulted in activity recoveries below 0.8%. The specific activity of glutaraldehyde immobilized β-glucanase could be increased with only a small decrease in yield of bound active enzyme by cross-linking in 80% saturated sodium sulphate for 24 h at 0.13% glutaraldehyde (Table I). In this solvent cross-linking at higher concentrations of glutaraldehyde or for a shorter period of time also resulted in poor yields. Glutaraldehyde treatment of the crude β-glucanase consistently led to recoveries of less than 0.6% of the starting activity.

Table I

Immobilization of partially purified β-glucanase

Derivative	Details on the coupling	Recovered as immobilized β-glucanase		Specific activity of immobil- ized protein[c] (%)	Protein content (mg/g)	Gly-Leu[d] (μmol- es/g)
		Activity[a] (%)	Protein[b] (%)			
Hydrolyzed nylon-β-glucanase, floc		0.02	1.9	1.1	2.5	0.43
Aminolyzed nylon-β-glucanase, powder		0.15	1.2	13	1.8	1.94
Aminolyzed nylon-β-glucanase, floc		0.08	0.6	13	0.9	1.80
Polyisonitrile-nylon-β-glucanase	via protein NH₂	0.04	0.4	10	2.6	17.0
Polyisonitrile-nylon-β-glucanase	via protein COOH	0.05	0.2	25	1.5	-
Cross-linked β-glucanase	0.5% g.a., 24 h	2.9	26	11	-	-
Cross-linked β-glucanase	0.13% g.a., satd. Na₂SO₄, 24 h	2.4	11	22	-	-
Perlite-β-glucanase	0.5% g.a.	4.5	84	5.4	-	-

a) In per cent of the activity added in the coupling step
b) Measured by amino acid analysis and given in per cent of the amount of protein present in the coupling step
c) Expressed as per cent of the specific activity of the starting material
d) Measured by amino acid analysis

g.a., glutaraldehyde

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Table II

Preparation and properties of Duolite-β-glucanase and Duolite-endo-1,4-β-glucanase

Deri-vative	Details of the preparation				Recoveries of activity (%)a				Properties after 48 h of washing		
	Glutar-aldehyde conc.(%)	Barley β-glucan present		NaBH$_4$-reduc-tion	After ad-sorption to Duolite	After cross-linking	After reduc-tion	After 48 h of washing	β-glucanase units/g	Immobi-lized protein(%)	Specific activityb (%)
		at ad-sorption	at cross-linking								
I	0.05	–	–	–	40	4.4	–	0.90	0.29		
II	0.05	–	–	+	34	5.7	4.2	0.63	0.20		
III	0.05	+	–	–	66	2.5	–	1.3	0.52		
IV	0.05	+	+	+	66	2.5	2.2	0.93	0.31		
V	0.05	+	+	+	52	9.3	4.0	0.59	0.19		
VI	0.20	+	+	+	–	–	3.9	0.60	0.19	11	2.4
VII	1.0	+	+	+	–	–	1.3	0.71	0.23	12	2.2
VIII	2.0	+	+	+	–	–	1.8	0.70	0.23	12	2.7
IX	0.5	–	–	+	–	–	–	0.41	0.13	13	2.5
Xc)	0.05	+	+	+d)	–	0.73	0.67	0.39	0.11		

a) In per cent of the activity added in the adsorption step
b) Specific activity of immobilized protein in per cent of the specific activity of the starting material
c) Immobilization of pure endo-1,4-β-glucanase
d) Reduced in the presence of 0.27% barley β-glucan.

When the partially purified β-glucanase was adsorbed to perlite prior to glutaraldehyde treatment, a recovery of 4.5% was obtained. With crude enzyme the yields were about 2.5%.

Unfortunately, both glutaraldehyde-cross-linked β-glucanase and perlite-β-glucanase are paste-like and not suitable for application in enzyme-reactors. For this purpose Duolite S-761 was superior. This highly porous and hydrophilic granular phenol-formaldehyde resin readily adsorbs proteins from dilute solution (22, 23). Subsequently the conjugate can be stabilized with glutaraldehyde as described in Table II. While the activities were relatively high after the adsorption step a large loss of enzymatic activity occurred during the cross-linking with glutaraldehyde. Barley β-glucan seemed to protect the enzyme to some extent both during adsorption and cross-linking, although the final activity of Duolite-β-glucanase prepared at low concentration of glutaraldehyde was not improved, when barley β-glucan was present.

The non-reduced Duolite-β-glucanase prepared at low glutaraldehyde concentrations could not be washed completely free of leaking activity, while reduced Duolite-β-glucanase slowly was freed of soluble activity. Reduced β-glucanase preparations immobilized at glutaraldehyde concentrations above 0.1% (Table II, derivatives VI-VIII) were rapidly washed free of leaking activity indicating that the reduction by NaBH₄ stabilized reversibly formed protein-glutaraldehyde-linkages. During the 48 h wash only about 10-15% of the protein content was removed suggesting that the much higher activity loss of 50-90% (Table II) originated from inactivation of the bound enzyme or selective loss of enzyme with high β-glucanase activity.

3.2. Immobilized β-glucanase packed bed reactors

Nylon-, Duolite-, and DEAE-cellulose-β-glucanase were tried out in packed bed reactors for continuous hydrolysis of barley β-glucan (Fig. 1). The Duolite-β-glucanase reactor released approx. 6 times as many reducing sugar groups as nylon-β-glucanase. DEAE-cellulose-
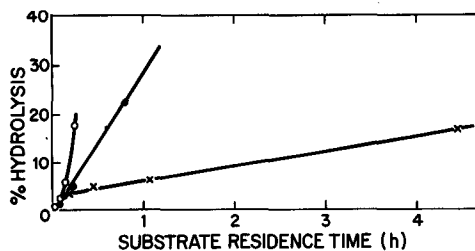

Fig. 1. Continuous hydrolysis of barley β-glucan (0.03% in 0.02M-sodium acetate pH 5.0) at 21° C. Duolite-β-glucanase (derivative II, Table II) (O). DEAE-cellulose-β-glucanase (●). Partially acid hydrolyzed nylon-β-glucanase (Table I) (x). Degree of hydrolysis is calculated from the contents of reducing sugar and total carbohydrate in the effluent using glucose as standard.

β-glucanase constantly released detectable, though very small amounts of soluble β-glucanase and has not been subjected to further study.

The degree of hydrolysis as measured by the increase in reducing power reflects the number of glucoside bonds being hydrolyzed, but not necessarily to what extent the molecular weight of the polysaccharide substrate is reduced. To obtain this information the residual amounts of ammonium sulphate precipitable polysaccharide were determined in effluents from the enzyme reactor, and a proportional relationship
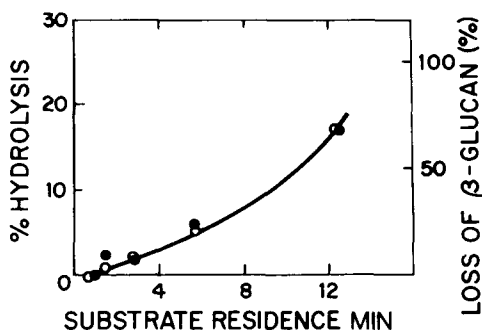

Fig. 2. Continuous hydrolysis of barley β-glucan (0.03% in 0.02M-sodium acetate pH 5.0) at 21° C by a Duolite-β-glucanase (derivative II, Table II) packed bed reactor. Degree of hydrolysis (O) is calculated as in Fig. 1. The loss of barley β-glucan (●) is expressed as the decrease in the content of ammonium sulphate precipitable barley β-glucan.

was observed between increase in reducing power and loss of precipitable substrate. The Duolite-β-glucanase reactor could within reasonable time remove most of the high molecular weight barley β-glucan (Fig. 2) and its thermal stability was investigated at 30°C and 45°C. At 30°C the enzyme reactor continuously removed about 50% of the precipitable barley β-glucan with no decrease in activity during 20 h. At 45°C 70% of the precipitable barley β-glucan was removed at the start of the experiment, and only 40% after 4 h of operation. Thus the immobilization did not improve the temperature stability of the glucanase preparation to any significant extent.

3.3. Action pattern

When barley β-glucan was hydrolyzed to the same extent by Duolite-β-glucanase and the corresponding unmodified crude β-glucanase, very different products were obtained (Fig. 3). Gel filtration of the effluent from the packed bed reactor indicated it essentially contained
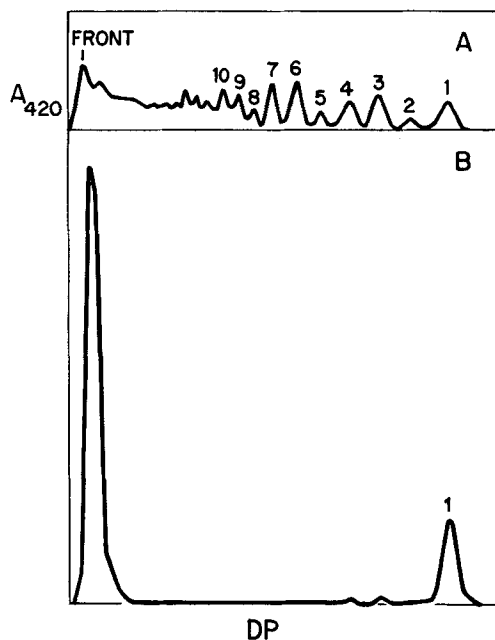

Fig. 4. Gel filtration of barley β-glucan hydrolyzed by dissolved pure endo-1,4-β-glucanase (A, degree of hydrolysis: 6.2%) and insoluble pure endo-1,4-β-glucanase (B, degree of hydrolysis: 7.2%). The numbers indicate degree of polymerization of oligosaccharides.

Fig. 3. Gel filtration of barley β-glucan hydrolyzed by dissolved crude β-glucanase (A, degree of hydrolysis: 11%), and insoluble crude β-glucanase (B, degree of hydrolysis: 13%). The numbers indicate degree of polymerization of oligosaccharides.
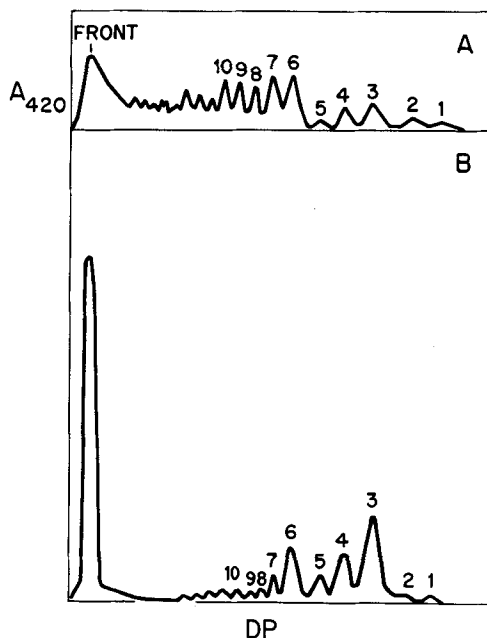
two components, viz. glucose (13%) and high molecular weight polysaccharide (87%). In contrast, the dissolved enzyme gave rise to a series of products comprising the whole range from high molecular weight polysaccharides to oligosaccharides, disaccharide and glucose. In agreement with this effluent composition 18% and 91% of the ammonium sulphate precipitable carbohydrate disappeared by hydrolysis in the enzyme reactor and in solution, respectively. Glucose was likewise the only low molecular weight product in two other enzyme-reactor experiments, where 5% and 20% of the glucosidic bonds in barley β-glucan had been hydrolyzed.

Two factors are considered contribute to the changed pattern observed with the immobilized enzyme. First the free access of substrate to the active site of the endo-β-glucanase may be sterically impeded from the matrix-attachment. Such an effect has been noticed with insolubilized α-amylase which in comparison with dissolved α-amylase is more effective in at-

tacking the ends of the carbohydrate chains of starch substrates (2, 17, 19). Second, the commercial glucanase contains in addition to endo-β-glucanase both β-glucosidase and exo-β-glucanase activity which catalyze consecutive reactions in the degradation of barley β-glucan to glucose. These enzymes are thus coimmobilized on Duolite and from the literature (8, 20, 21) it is known that coimmobilized enzymes may catalyze consecutive reactions with formation of the final product of increased rate compared to the corresponding dissolved enzyme systems.

The possible presence of a steric effect causing immobilized endo-1,4-β-glucanase to hydrolyze chiefly glucosidic bonds near the ends of barley β-glucan was investigated by attaching pure endo-1,4-β-glucanase to Duolite. The composition of digestion mixtures of barley β-glucan reflected different action patterns of immobilized and dissolved endo-1,4-β-glucanase (Fig. 4). The immobilized enzyme essentially produced oligosaccharides and high molecular weight polysaccharides, whereas the dissolved enzyme gave rise to all sizes of products. These effluent compositions are consistent with a preferential attack by immobilized endo-1,4-β-glucanase at the outermost parts of the substrate molecules. However, the composition of digests of barley β-glucan from a packed bed reactor of pure Duolite-endo-1,4-β-glucanase and crude Duolite β-glucanase was entirely different (Figs. 4 and 3). The formation in the latter case of glucose as the only low molecular weight product might be ascribed both to the presence of coimmobilized enzymes catalyzing the consecutive steps in the hydrolysis of barley β-glucan to glucose and to release of oligosaccharides by the action of the endo-1,4-β-glucanase.

Thus, the experiments demonstrate that it is possible to immobilize β-glucanase as derivatives with recoveries of 0.02-4.5% of the original activity and specific activities ranging from 1-25% of the specific activities of the starting materials. Although these values are much lower than the values obtained for some proteolytic enzymes, they compare reasonably well with the values obtained upon im-

mobilization of the counterpart of endo-1,4-β-glucanase, the endo-1,4-α-glucanase or α-amylase, where activity recoveries of about 2% have been reported with specific activities ranging from 4-42% (1, 17). The transformation of the action pattern of β-glucanase upon immobilization from an endo-enzymatic to a more exo-enzymatic type might complicate the practical use of immobilized β-glucanase derivatives in the brewing industry.

ACKNOWLEDGEMENTS

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