WATER-INSOLUBILISATION OF GLYCOSIDE HYDROLASES WITH CROSS-LINKED POLY(ACRYLOYLAMINOACETALDEHYDE DIMETHYL ACETAL) (ENZACRYL POLYACETAL)

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(Received August 4th, 1971; accepted for publication, September 8th, 1971)

ABSTRACT

The preparation of water-insoluble derivatives of alpha-amylase and dextranase by chemical binding with a polyaldehyde derived from cross-linked poly(acryloylaminoacetaldehyde dimethyl acetal) (Enzacryl Polyacetal) is described. The water-insoluble derivatives could be used repeatedly against their respective substrates. Bound alpha-amylase and at least a proportion of the bound dextranase molecules were more stable to heat denaturation than are the corresponding free enzymes.

INTRODUCTION

Water-insolubilisation of enzymes is commonly achieved by covalent binding of the enzyme with reactive derivatives of cellulose[1–3] or other polysaccharides such as agarose[4,5] and cross-linked dextran[6]. Unfortunately, polysaccharide carriers may physically adsorb glycoside hydrolases. This complicates covalent coupling and necessitates rigorous, and sometimes harmful, washing procedures[7,8].

Recently, we have described a new type of carrier for enzyme insolubilisation[9], namely, cross-linked poly(acryloylaminoacetaldehyde dimethyl acetal) (Enzacryl Polyacetal). Acid treatment of this polymer leads to the generation of aldehydrol groups which are effective in enzyme binding within an intensely hydrophilic environment of acetal, aldehydrol, and hemiacetal groups. This polymer could be a useful support for glycoside hydrolases. The insolubilisation of two such enzymes, alpha-amylase and dextranase, is described in this paper.

EXPERIMENTAL

Materials. — Crystalline alpha-amylase (*B. subtilis*) was obtained from the Sigma Chemical Company Ltd., and dextranase (*Cytophaga*) from Dextran Products Ltd., Scarborough Ontario. Enzacryl Polyacetal (a copolymer of acryloylaminoacetaldehyde dimethyl acetal and *N,N'*-methylenediacrylamide in the molar ratio 8:1) was obtained from Koch–Light Laboratories Ltd.

Activation of Enzacryl Polyacetal. — Enzacryl Polyacetal (2 g) was suspended in hydrochloric acid (0.25M, 40 ml) at 30°. After 48 h, the suspension was neutralised

with 0.25M sodium hydrogen carbonate and the polyaldehyde (Enzacryl CHO) washed repeatedly with distilled water. The aldehydrol content of the polymer, determined by the method of Bryant and Smith[10], was 4.97 mequiv./g (65.6% of the theoretical).

Enzacryl CHO could be stored under distilled water for several weeks without deterioration. When required, dry polymer was obtained by equilibrating with ethanol, shrinking the suspended polymer by dropwise addition of ether, and drying *in vacuo*.

Water-insolubilisation of alpha-amylase. — Enzacryl CHO (100 mg) was allowed to swell for 1 h in phosphate buffer (25mM, pH 7.5, 10 ml). After centrifugation of the suspension, the supernatant was discarded, and a solution of alpha-amylase (5 mg) in phosphate buffer (25mM, pH 7.5, 0.25 ml) was added. Coupling was allowed to proceed for 18 h at 0–5° with magnetic stirring.

The water-insolubilised alpha-amylase derivative was washed twice with phosphate buffer (25mM, pH 7.5, 15 ml), twice with a solution of soluble starch (1%) in the same buffer, and twice with buffer alone. Each washing was carried out at 0°, with vigorous magnetic stirring and was of 30-min duration. The derivative was finally suspended in phosphate buffer (10 ml) and stored at 0–5°.

Water-insolubilisation of dextranase. — Dextranase (5 mg) in phosphate buffer (25mM, pH 7.5, 0.25 ml) was coupled with Enzacryl CHO, following the method described for alpha-amylase. The washing procedure was modified, in that a solution (1%) of dextran ($\overline{M}_w$ 70,000) was substituted for starch solution.

Determination of bound protein. — Samples (50 mg) of the water-insoluble enzyme derivatives were hydrolysed with aliquots (0.5 ml) of 6M hydrochloric acid at 110° for 18 h. Each hydrolysate was centrifuged, and the supernatant was decanted from the small amount of solid residue which was washed with distilled water. The combined hydrolysate and washings were evaporated to dryness, maintained for 18 h *in vacuo* over a mixture of sodium hydroxide and calcium chloride, and then re-constituted with distilled water (0.2 ml). Hydrolysates of the free enzyme (5 mg) and the non-coupled carrier (100 mg) were treated similarly.

The re-constituted hydrolysates were subjected to ascending paper chromatography using the organic phase of 1-butanol–acetic acid–water (4:1:5). The precise chromatographic procedure, including pre-treatment of chromedia and quantitative ninhydrin estimation of the separated components, was carried out as described by Kay, Harris, and Entenman[11]. Enzyme hydrolysates gave rise to two bands of ninhydrin-positive material (R_F 0.50 and 0.35) which were clearly separated from bands due to the carrier and which could be extracted into a mixture of ethanol–water (3:1). The enzyme concentration in samples prior to hydrolysis was calculated by relating absorbance (575 nm) to enzyme (mg) on standard graphs obtained by chromatographic assay of known amounts of the free enzymes.

Activity of water-insoluble enzymes on initial and repeated use. — An aliquot (0.2 ml) of the suspension of water-insoluble alpha-amylase was centrifuged and the supernatant was discarded. The derivative was then re-suspended in a solution (1%) of soluble starch in phosphate buffer (25mM, pH 6.9, 6 ml) at 25°. Digestion was

allowed to proceed for 30 min with constant-speed, magnetic stirring. The course of hydrolysis was followed by assaying centrifuged samples with the dinitrosalicylate reagent of Bernfeld[12]. Re-solubilised activity was determined by prolonged incubation of the supernatant solution of a centrifuged sample of digest taken after 20 min.

On conclusion of the incubation, the alpha-amylase conjugate was washed briefly with phosphate buffer. The digestion and washing procedure was then repeated seven times, re-solubilised activity being determined on alternate occasions of use.

The activity of the dextranase conjugate, on initial and successive occasions of use, was similarly determined except that digestion was carried out on a solution (1%) of dextran ($\overline{M}_w$ 70,000) in acetate buffer (25mM, pH 5.5, 6 ml) at 40°.

The results, for both the alpha-amylase and dextranase derivatives, are presented in Table I.

TABLE I

ACTIVITY OF ALPHA-AMYLASE AND DEXTRANASE CONJUGATES ON INITIAL AND REPEATED USE[a]

Use No.	*Enzacryl CHO–alpha-amylase*		*Enzacryl CHO–dextranase*	
	Enzyme units[b] in digest	*Enzyme units re-solubilised*	*Enzyme units[c] in digest*	*Enzyme units re-solubilised*
1	303	148	17.3	5.7
2	217		12.9	
3	207	48	10.5	1.0
4	175		10.5	
5	165	29	7.5	1.3
6	164		7.5	
7	148	19	7.6	0.9
8	150	24	7.3	

[a]Initially, alpha-amylase and dextranase conjugates contained 1.03 and 1.04 mg of protein, respectively. Correcting for re-solubilisation, the respective activities were initially not less than 1 and 4% of the activities of the soluble enzymes. [b]One alpha-amylase unit liberates reducing sugar equivalent to 1 μmole of maltose/min at 25°. [c]One dextranase unit liberates reducing sugar equivalent to 1 μmole of isomaltose/min at 40°.

Stability of water-insoluble alpha-amylase and dextranase derivatives to heat denaturation. — Samples of the alpha-amylase and dextranase derivatives were suspended in phosphate buffer (25mM, pH 6.9) and acetate buffer (25mM, pH 5.5), respectively, and incubated at 50°. Control incubations were performed with solutions of alpha-amylase and dextranase, each dissolved in the appropriate buffer. Enzyme activity was determined at intervals against buffered starch or dextran solution as previously described.

Storage of water-insoluble alpha-amylase and dextranase derivatives. — The activity of samples of the alpha-amylase and dextranase derivatives was re-determined after storage at 0–5° for 5 months in phosphate buffer (25mM, pH 6.9) and acetate buffer (25mM, pH 5.0), respectively.

DISCUSSION

Previous workers have used monomeric dialdehydes to couple enzymes t aminoethylcellulose[5,13] and to partially hydrolysed 6-nylon[14]. Dialdehydes have als been used to directly cross-link enzymes[15,16]. Enzyme insolubilisation using aldehyde is thus well-established.

The Enzacryl-insolubilised alpha-amylase and dextranase could be repeatedl re-used with fair recovery of activity (see Table I). However, even after eight occasion of use, some re-solubilisation of enzyme activity was always detected. Similar result have been reported by Gilbert and Crutchfield[5], who found that rennin, coupled t aminoethylcellulose with glutaraldehyde, was partially re-solubilised on contact wit milk. Also, we have observed[9] that trypsin, coupled to Enzacryl CHO, was partiall re-solubilised on successive re-use with casein solution, although the same derivativ could be used repeatedly to digest N^{α}-L-benzoylarginine ethyl ester without re-solubil sation of activity. Most recently, we have shown that urease derivatives based o Enzacryl CHO did not dissociate on repeated re-use against urea[17]. It seems that th size of the substrate, rather than its charge or chemical identity, is the critical factc in promoting dissociation. Affinity of the enzyme for a macromolecular substrate i probably sufficient to cause significant partitioning between the carrier and aqueou phase. Enzacryl CHO–enzyme conjugates could not be dissociated by repeate washing with dilute mineral acid, as indicated by protein estimation before and afte treatment.

Richards and Knowles[18] have suggested that enzyme insolubilisation wit dialdehydes (glutaraldehyde) first involves random condensation of dialdehyde mole cules to give a mixture of unsaturated aldehydes. Primary amino-groups on the enzym may then undergo Michael addition across the conjugated double bonds, or reac with the aldehydrol groups to give aminol and possibly azomethine linkages. Onl the aldehydrol binding procedure is easily reversible and this is probably the mor important in the case of Enzacryl CHO. Michael addition seems less likely, becaus the lower reactivity of the active methylene groups in the acylaminoacetaldehyd side-chains is less conducive to preliminary condensation than in the case of a simpl dialdehyde.

The proportion of the free solution activity retained by insolubilised alpha amylase and dextranase (1 and 4%, respectively) is characteristic of enzymes activ against polymeric substrates. Loss of activity is believed to be caused by steric an diffusional limitations. Thus, on successive occasions of use, re-solubilised activity i always considerably greater than the corresponding fall in activity of the conjugates

alpha-Amylase was chosen to evaluate Enzacryl CHO, since comparative dat are available on conjugates of this enzyme with polymethacrylic acid[19], cellulose[7] and cross-linked polyacrylamides[10]. The dramatic increase in stability to hea denaturation (see Fig. 1) and good storage properties (64% after 5 months) ar consistent with results obtained with cellulose and polyacrylamide derivatives.

Insolubilised dextranase was initially heat-denatured more rapidly than solubl

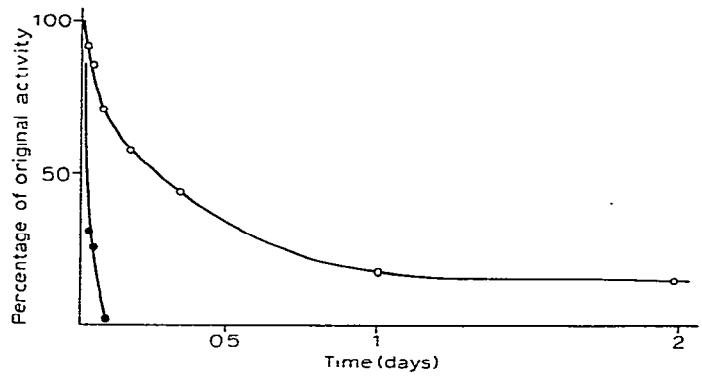

Fig. 1. Loss in activity of soluble alpha-amylase and Enzacryl CHO–alpha-amylase at 50°; ●——●, soluble alpha-amylase; O——O, Enzacryl CHO–alpha-amylase.

dextranase (see Fig. 2), but a proportion of the dextranase molecules appear to be stabilised. This phenomenon may arise because some enzyme molecules are bound to the carrier in a different way from others. Variation in the number of enzyme-to-carrier linkages or different orientations relative to the carrier surface may be involved. It could be that supporting the enzyme at several different points may help in preserving its tertiary structure. The activity retained by insolubilised dextranase on storage (16% after 5 months) could also be consistent with stabilisation of only a proportion of the bound enzyme molecules.

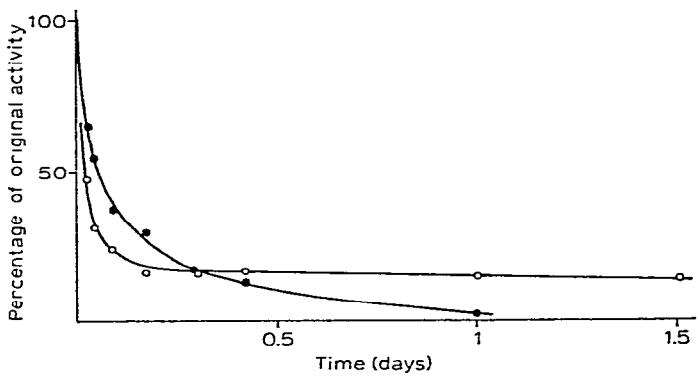

Fig. 2. Loss in activity of soluble dextranase and Enzacryl CHO–dextranase at 50°; ●——●, soluble dextranase; O——O, Enzacryl CHO–dextranase.

Our results appear to indicate that Enzacryl CHO will be a useful addition to the range of carriers available for glycoside hydrolases. The feature of slow enzyme-release, with simultaneous regeneration of aldehyde groups, could be invaluable in industrial situations when dealing with partially soluble substrates. Also, denaturation losses could usefully be offset by periodically coupling fresh enzyme.

ACKNOWLEDGMENT

We thank Koch–Light Laboratories Ltd. for a studentship (T. H. T.)

REFERENCES

1 F. MICHEEL AND J. EWERS, *Makromol. Chem.*, 3 (1949) 200.
2 M. A. MITZ AND L. J. SUMMARIA, *Nature (London)*, 189 (1961) 576.
3 W. E. HORNBY, M. LILLY, AND E. M. CROOK, *Biochem. J.*, 98 (1966) 420.
4 J. PORATH, R. AXEN, AND S. ERNBACK, *Nature (London)*, 215 (1967) 1491.
5 M. L. GREEN AND G. CRUTCHFIELD, *Biochem. J.*, 115 (1969) 183.
6 R. AXEN AND J. PORATH, *Nature (London)*, 210 (1966) 367.
7 S. A. BARKER, P. J. SOMERS, AND R. EPTON, *Carbohyd. Res.*, 8 (1968) 491.
8 S. A. BARKER, P. J. SOMERS, AND R. EPTON, *Carbohyd. Res.*, 9 (1969) 257.
9 R. EPTON, J. V. MCLAREN, AND T. H. THOMAS, *Biochem. J.*, 123 (1971) 21P.
10 W. M. D. BRYANT AND D. M. SMITH, *J. Amer. Chem. Soc.*, 57 (1935) 57.
11 R. E. KAY, D. C. HARRIS, AND C. ENTENMAN, *Arch. Biochem. Biophys.*, 63 (1956) 14.
12 P. BERNFELD, *Methods Enzymol.*, 1 (1955) 149.
13 A. F. S. A. HABEEB, *Arch. Biochem. Biophys.*, 119 (1967) 264.
14 P. V. SUNDARAM AND W. E. HORNBY, *FEBS Lett.*, 10 (1970) 325.
15 F. A. QUIOCHO AND F. M. RICHARDS, *Proc. Nat. Acad. Sci. U. S.*, 52 (1964) 833.
16 K. OGATA, M. OTTESEN, AND I. SVENDSEN, *Biochim. Biophys. Acta*, 159 (1968) 403.
17 R. EPTON, J. V. MCLAREN, AND T. H. THOMAS, unpublished results.
18 F. M. RICHARDS AND J. R. KNOWLES, *J. Mol. Biol.*, 37 (1968) 231.
19 G. MANECKE, *Pure Appl. Chem.*, 4 (1962) 507.
20 S. A. BARKER, P. J. SOMERS, R. EPTON, AND J. V. MCLAREN, *Carbohyd. Res.*, 14 (1970) 287.