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ENZYME IMMOBILIZATION ON CELLULOSE DITHIOBIS(THIOFORMATE) AND CELLULOSE *S*-METHYLDITHIOCARBONATE

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ABSTRACT

Two cellulose derivatives, namely, the *S*-methyldithiocarbonate and the dithiobis(thioformate), have been used to immobilize alpha-amylase and trypsin. Properties of the immobilized enzymes are described. Temperature- and pH-stability were little changed. The specific activity relative to that of the free enzyme was ~5% for alpha-amylase and ~17% for trypsin. After six usage-cycles, the alpha-amylase conjugates retained <20% of the original bound-activity, while the trypsin conjugate retained ~30% of such activity.

INTRODUCTION

We have reported on an evaluation of dithiobis(thioformates) (DTTF) and *S*-methyldithiocarbonates (SMD) as reactive groups for the covalent immobilization of biological molecules[1]. We now describe their application to two enzymes (alpha-amylase and trypsin) that are readily available and industrially important. These enzymes have also been immobilized by other procedures[2–6], thus allowing comparisons to be made. Immobilized enzymes continue to attract the attention of workers in fundamental and applied areas[6–8].

RESULTS AND DISCUSSION

Determination of optimal coupling conditions. — A series of experiments showed that 24 h at 25° was most suitable for the coupling of alpha-amylase to cellulose-DTTF and cellulose-SMD, and for trypsin to cellulose-SMD. The pH conditions of coupling were quite different for the alpha-amylase and trypsin (Table I).

Results of model-compound experiments with glycine ethyl ester[1] indicated that pH 8.6 might be more favourable for coupling than pH 6.0. A higher pH might be expected to favour nucleophilic attack on the DTTF and SMD groups, as the attacking amino-groups should be less-protonated. The cases of the model compound and trypsin fit this proposal. Such first-order reasoning is not sufficient for alpha-amylase. The amino acid composition of *B. subtilis* alpha-amylase[9] indicates the

TABLE I

EFFECT OF COUPLING-pH ON THE ACTIVITY OF IMMOBILIZED ALPHA-AMYLASE AND TRYPSIN

pH	*Cell-DTTF–alpha-amylase*		*Cell-SMD–alpha-amylase*		*Cell-SMD–trypsin*	
	Activity[a]	*Protein bound*[c]	*Activity*[a]	*Protein bound*[c]	*Activity*[b]	*Protein bound*[c]
6.0	0.139	2	0.231	3	0.46	0.4
8.6	Nil	Negligible	0.05	Very low	3.52	3.7

[a]Mmol of D-glucose equivalents liberated/min/g of matrix, at pH 6.0, 25°. [b]Mmol of substrate hydrolysed/min/g of matrix, at pH 8.0, 25°. [c]Mg/g of matrix.

TABLE II

SPECIFIC ACTIVITY AND ACTIVITY RETENTION FOR IMMOBILIZED ALPHA-AMYLASE AND TRYPSIN

Enzyme	*Activity*[a]	*Bound protein (mg/g of matrix)*	*Specific activity*	*Activity retention (%)*
Cell-DTTF–alpha-amylase	0.139	2	0.069[b]	4.9
Cell-SMD–alpha-amylase	0.231	3	0.077[b]	5.4
Cell-SMD–trypsin	3.52	3.7	0.95[c]	17.5
Free alpha-amylase			1.43[b]	
Free trypsin			5.44[c]	

[a]As defined in Table I and Experimental. [b]Mmol of D-glucose equivalents liberated/min/mg of protein, at pH 6.0, 25°. [c]Mmol of substrate hydrolysed/min/mg of protein, at pH 8.0, 25°.

presence of 61 lysine and 42 arginine residues per thousand, and the isoelectric point of the enzyme is 5.4. At pH 6.0, a large degree of protonation of the strongly basic lysine (ε-amino, p*K* 10.53) and arginine (guanidino, p*K* 12.48) side-chains would be expected, which could account, at least in part, for the relatively small amount of protein bound compared to the number of SMD or DTTF groups available. However, this reasoning is not consistent with lower protein-binding at pH 8.6 than at 6.0. The effect of local environment on the alpha-amylase must play a crucial role.

Specific activity and activity retention. — The specific activities of alpha-amylase and trypsin samples are shown in Table II, together with the activity retention, expressed as a percentage of the specific activity of the free enzyme. The retention values for alpha-amylase lie within the range (1–16%) for alpha-amylase immobilized by other methods[3–4], while the value for trypsin compares with 16–25% retention with BAEE as substrate[5].

Relative reactivities of Cellulose-DTTF and Cellulose-SMD towards alpha-amylase. — The following equations illustrate the reactions of Cellulose-DTTF and Cellulose-SMD with enzyme.

$$\text{Cell-O-C(=S)-S-S-C(=S)-O-Cell} + NH_2\text{-Enz} \rightarrow \text{Cell-O-C(=S)-NH-Enz} + CS_2 + S + \text{Cell-OH}$$

$$\text{Cell-O-C(=S)-S-CH}_3 + NH_2\text{-Enz} \rightarrow \text{Cell-O-C(=S)-NH-Enz} + CH_3SH$$

On the above basis, equal weights of cellulose-DTTF of d.s. 1.0 and cellulose-SMD of d.s. 0.55 should couple protein in the ratio of 1 : 1. Table II shows the ratio to be 2 : 3 (*i.e.*, cellulose-SMD couples alpha-amylase more effectively than cellulose-DTTF) and also indicates that retention of activity does not parallel the higher degree of coupling.

One factor contributing towards higher reactivity of cellulose-SMD is probably steric. The cellulose-DTTF would consist of cross-linked chains, whereas the active groups in cellulose-SMD should protrude from the chains and be more accessible to the incoming enzyme. Another possibility is that the percentage of sulphur is not an exact reflection of reactive cellulose-DTTF and/or cellulose-SMD groups.

pH–Activity relationships. — Fig. 1 shows that, apart from some narrowing of the activity profiles, the immobilized alpha-amylases behave similarly to the free enzyme at pH values between 3.6 and 9.0. After storage for 24 h in the various buffers before assay, the activity profiles of free and immobilized alpha-amylase narrow further at pH values below 6.0, but remain similar at pH values above 6.0 (Fig. 2), indicating the need to avoid the lower pH values.

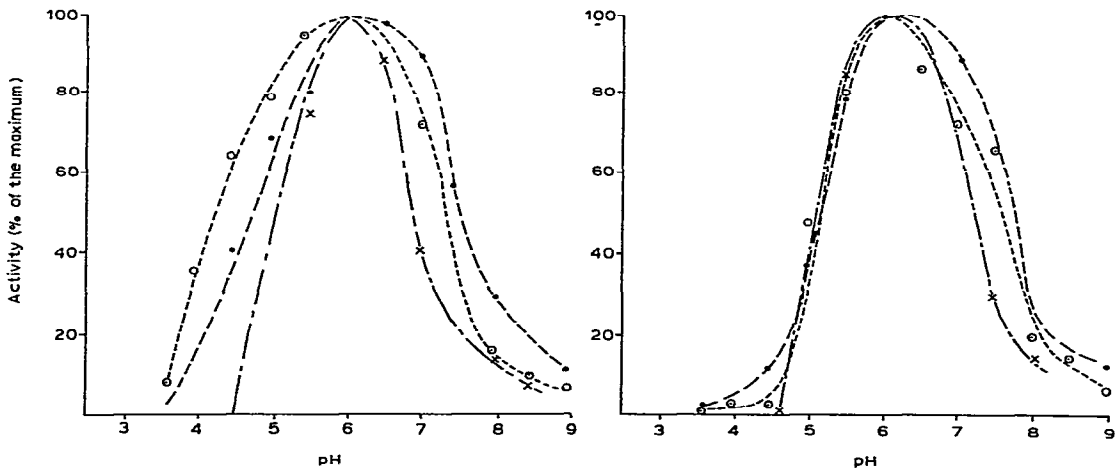

Fig. 1 (left). pH–Activity profiles of the free and immobilized alpha-amylases: ⊙ -------, free alpha-amylase; x — — —, Cell-DTTF–alpha-amylase; ● ———, Cell-SMD–alpha-amylase.

Fig. 2 (right). pH–Activity profiles of the free and immobilized alpha-amylases after storage for 24 h in buffers of various pH values: ⊙ -------, free alpha-amylase; x — — —, Cell-DTTF–alpha-amylase; ● ———, Cell-SMD–alpha-amylase.

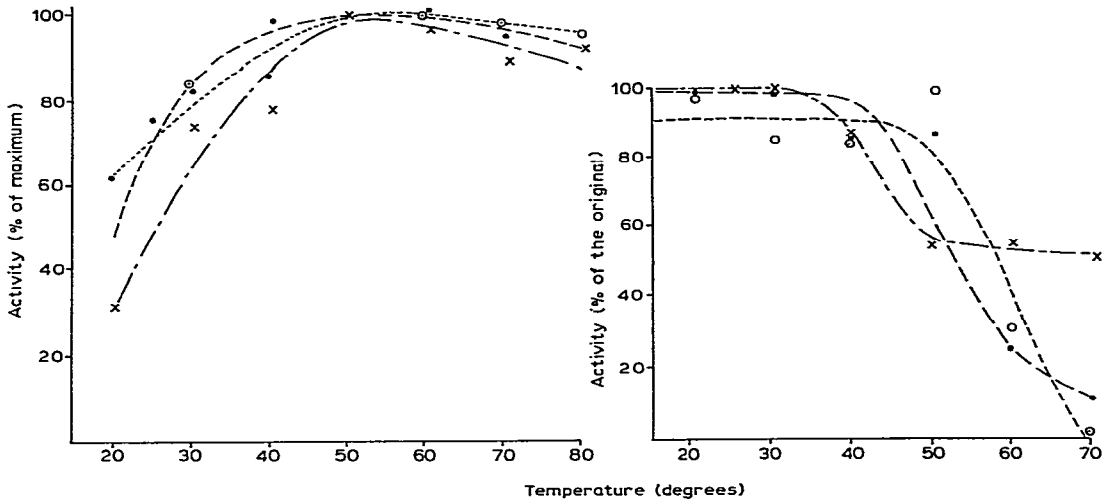

Fig. 3 (left). Temperature–activity profile of the free and immobilized alpha-amylases: ⊙ -------, free alpha-amylase; x — — —, Cell-DTTF–alpha-amylase; ● ————, Cell-SMD–alpha-amylase.

Fig. 4 (right). Temperature-stability curves for the free and immobilized alpha-amylases: ⊙ -------, free alpha-amylase; x ————, Cell-DTTF–alpha-amylase; ● ————, Cell-SMD–alpha-amylase.

Temperature–activity relationships. — Temperature–activity profiles for the two immobilized alpha-amylases were very similar to those of the free enzyme (Fig. 3). Temperature-stability studies show some increase in stability above 50° for the cellulose-DTTF derivative, but no significant stabilization was observed for the cellulose-SMD–alpha-amylase (Fig. 4).

Storage-stability of the immobilized alpha-amylases. — The air-dried forms of both DTTF and SMD alpha-amylases lost ~25% activity on storage for five months, this activity being lost in months four and five. The buffer-suspended samples lost activity at the rate of ~15% of the original per month. Under the same conditions, the free enzyme lost, respectively, 10% and 15% activity in the five months. A sample of the cellulose-DTTF–alpha-amylase stored in buffer for five months was filtered and assayed for remaining protein content. It was found that 25% of the original bound-protein had been lost, whereas 80% of the enzyme activity had been lost. The storage trials thus imply some reaction between the enzyme and the excess of DTTF/SMD groups on extended contact. Probably the remaining enzyme is bound more tightly, but less favourably, to the cellulose matrix.

Repeated uses of the immobilized enzymes. — A crucial test for an immobilized-enzyme preparation is the extent to which it can be reused, and in this respect the alpha-amylase preparations were disappointing (Table III).

The enzymes were partially resolubilized on successive use, as shown by (*a*) detection, in the first two uses of the alpha-amylase preparations, of slight enzyme activity in the supernatant buffer after filtration, and (*b*) almost negligible bound-protein remaining in the SMD–alpha-amylase samples, and only 50% remaining

TABLE III

REPEATED-USE DATA FOR IMMOBILIZED ALPHA-AMYLASE AND TRYPSIN SAMPLES

Use No.	*Cell-DTTF–alpha-amylase % Activity*	*Cell-SMD–alpha-amylase % Activity*	*Cell-SMD–trypsin % Activity*
1	100	100	100
2	44.9	58	100
3	23.8	8.3	78.5
4	18	3.9	32
5	18	3.9	32
6	18	1.8	32

in the trypsin samples, after six use-cycles. Six washings with buffer alone diminished the bound protein, by a maximum of 30%, showing that resolubilization in presence of substrate was the predominant cause of activity loss. This has been observed previously for immobilized enzymes acting on macromolecular substrates, *e.g.*, trypsin on casein, alpha-amylase on amylose, and dextranase on dextran[5]. Trypsin has been used repeatedly to hydrolyse BAEE with little or no loss[5], and the use of this small substrate is probably the reason for the better retention of activity by cellulose-SMD–trypsin.

CONCLUSIONS

The aim of this study was to obtain stable enzyme–polymer conjugates of reasonable activity. It would appear that the thiocarbamate linkage involved might not be as stable as indicated by model-compound experiments that involved pH values from 3.6 to 9.0. However, the added complication that the matrix, enzyme, and substrate are polymeric does not allow direct comparison with model-compound experiments. The use of a spacer molecule might result in increased enzyme-retention. Although the final enzyme-activity after six usage-cycles is probably not high enough to be of practical use in, for example, industry, further work could lead to useful, supplementary information. The binding of more alpha-amylase at pH 6.0 than at pH 8.6, and the reverse phenomenon for trypsin, poses an interesting problem, as does the increased loss of alpha-amylase from the matrix in the presence of substrate. The SMD and DTTF groups are not highly reactive, and selective removal of the more-reactive nucleophiles might be expected from a solution passed through a column of cellulose-SMD or cellulose-DTTF.

EXPERIMENTAL

Alpha-amylase (four times crystallized, from *B. subtilis*) was obtained from Sigma, and trypsin (lyophilized, 221 U/mg) from Worthington. Starch (soluble according to Zulkowski) was obtained from Merck, and *N*-(α)-benzoyl-L-arginine

ethyl ester hydrochloride (BAEE) from Sigma. The dinitrosalicylic acid assays were performed with a Cecil CE404 colorimeter using filter 5 (Ilford type), u.v. spectra were recorded with a Perkin–Elmer Double Beam model 124 spectrometer, and i.r. spectra with a Hitachi model EPI-G spectrometer.

Preparation of cellulose dithiobis(thioformate) (Cellulose-DTTF). — The method was similar to that used by Trimnell *et al.*[10]. (Calc. for dithiobis(thioformate), d.s. of 1: S, 29.5. Found: S, 27.9 and 29.2.) The i.r. spectrum (KBr disc) of the product showed strong absorptions near 8.0 and 9.6 nm, typical of dithiobis(thioformates)[1,10].

Preparation of cellulose S-methyldithiocarbonate (Cellulose-SMD). — The xanthation procedure was similar to that adopted by Trimnell *et al.*[10] for the preparation of starch xanthates. The procedure for the precipitation of the xanthate salt of cellulose, and for subsequent conversion into the *S*-methyl derivative, was based on the work of Muller and Purves[11]. (Calc. for *S*-methyldithiocarbonate, d.s. of 1: S, 27.6. Found: S, 15.6.) The i.r. spectrum (KBr disc) showed absorptions at 8.3 and 9.4 nm, characteristic of polysaccharide *S*-methyldithiocarbonates[12].

Assays for enzymic activity. — Determinations of bound protein and washing of immobilized-enzyme samples after coupling were performed as previously described[13]. Enzyme assays were performed by adding known weights (4–10 mg) of immobilized enzyme to the buffered substrate, followed by shaking in a water-bath thermostatted at 25°. Bound-alpha-amylase activity was expressed as the number of millimoles of glucose equivalents liberated from starch/min/g of matrix at pH 6.0 and 25°, as measured by the dinitrosalicylic acid assay[14]. Bound-trypsin activity was assayed by using *N*-(α)-benzoyl-L-arginine ethyl ester hydrochloride as substrate, and expressed as millimoles of substrate hydrolysed/min/g of matrix at pH 8.0 and 25°, following the method of Bergmeyer[15].

Coupling of alpha-amylase to cellulose-DTTF. — To a suspension of the DTTF (200 mg) in phosphate buffer (67mM, pH 6.0, 20 ml) was added alpha-amylase (4 mg) in the same buffer (1 ml). The mixture was shaken at 25° in a water bath for 24 h. The supernatant liquid was filtered through sintered glass, and the immobilized enzyme was subjected to a series of washings with buffer[13] at room temperature. The immobilized alpha-amylase was resuspended in acetate buffer (16mM, pH 6.0, 20 ml) for assay, or stored at 4° after air-drying. The effectiveness of the washing procedure to remove adsorbed enzyme was checked on a control sample of cellulose (200 mg) which had been treated in a similar way to the DTTF. No enzymic activity remained. Another control of DTTF (200 mg) was shaken with the phosphate buffer alone at 25° for 24 h and subjected to the washing procedure described above, to test for possible interference, caused by the derivatization, in the dinitrosalicylic acid assay; no such interference was noted.

Coupling of alpha-amylase to cellulose-SMD. — The coupling and washing procedures were as described for cellulose-DTTF. Controls of cellulose and SMD were also treated as described above.

Properties of the immobilized alpha-amylase. — *(a) pH–Activity relationships.* Incubations of the air-dried samples of immobilized alpha-amylases (10 mg) were

performed in the pH range 3.6 to 9.0, and the activity was expressed as the percentage of the optimum activity. The results were compared to the pH–activity profile of the free enzyme (Fig. 1). A similar series of samples was stored at 4° for 24 h before assay (Fig. 2).

(*b*) *Temperature stability.* Samples (10 mg) of immobilized enzymes and free-enzyme samples were incubated in the pH 6.0 buffer (2 ml) at temperatures from 20–70° with shaking for 1 h, after which they were assayed for activity at the standard 25°. Activity was expressed as percent of that originally present (Fig. 4).

(*c*) *Storage stability.* The activity of each sample stored at 4° in air-dried form was determined each month for 5 months. Similarly, activities of samples stored in suspension at 4° (200 mg in 20 ml of 16mM acetate buffer, pH 6.0) were determined.

Coupling of trypsin to cellulose-SMD. — To a suspension of cellulose-SMD (100 mg) in borate buffer (0.07M, pH 8.6, 20 ml) was added a solution of trypsin (8 mg) in the same buffer (1 ml). The mixture was shaken at 25° for 24 h and then filtered, and the product was washed as described above for alpha-amylase. The washed samples were suspended in borate buffer (0.05M, pH 8.0, 20 ml) containing $CaCl_2$ (0.02M) for assay, or stored at 4° after air-drying. Control samples of cellulose and cellulose-SMD were also subjected to the same washing and storage treatments.

Assay of tryptic activity was performed[15] on 4-mg samples of immobilized enzyme. Due to the hygroscopic nature of the *N*-(α)-benzoyl-L-arginine hydrochloride, its concentration was determined[16] by the change in absorbance at 254 nm on hydrolysis by free trypsin at pH 8.0.

Repeated uses of the immobilized alpha-amylases and trypsin. — Immobilized enzyme samples were assayed, filtered by using a small, sintered-glass funnel, washed at 25° with the appropriate assay buffer (2 × 2 ml), and resuspended in buffer for further assay of activity. Six such use-cycles were employed for each immobilized-enzyme sample.

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