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CROSS-LINKED POLYACRYLAMIDE DERIVATIVES (ENZACRYLS) AS WATER-INSOLUBLE CARRIERS OF AMYLOLYTIC ENZYMES

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ABSTRACT

Methods are described for the water-insolubilisation of alpha- and beta-amylase by chemical coupling to two cross-linked copolymers of acrylamide. For one copolymer, coupling was effected either by diazo or isothiocyanato groups, and for the other, acid azide groups were employed. Active derivatives of alpha-amylase were prepared by diazo, isothiocyanato, and acid azide coupling, whereas active derivatives of beta-amylase could be prepared only by diazo and isothiocyanato coupling. The stabilities of the active, copolyacrylamide-bound preparations of amylase were comparable to those of the corresponding, cellulose-bound enzymes. In the case of alpha-amylase, the copolyacrylamide derivatives were more stable than the free enzyme in solution.

INTRODUCTION

We have previously reported[1,2] the water-insolubilisation of amylolytic enzymes by chemical coupling to microcrystalline cellulose. Cellulose derivatives have been used as enzyme carriers by other workers[3–6], but these carriers often lead to strong, physical adsorption of enzymes which must be detached by rigorous washing with buffer and sodium chloride solution[5,7]. Such is the case with amylolytic enzymes. Furthermore, we have shown that prolonged washing with salt may lead to denaturation. Since it is also possible that polysaccharide carriers may undergo unique interactions with glycoside hydrolases, we have considered alternative types of carrier.

Other workers[8–10] have prepared water-insoluble derivatives of alpha-amylase by chemical coupling to cross-linked derivatives of polymethacrylic acid. Although this polymer is hydrophilic overall, hydrophobic interactions are also important, as evidenced by conformational studies in both gel[11] and free solution[12]. It is well known that hydrophilic features of the support may confer stability to the bound enzyme, whereas hydrophobic features have a marked, destabilising effect. Thus, derivatives of polymethacrylic acid were inferior to cellulose derivatives as carriers of

alpha-amylase, both with respect to enzyme activity retained on coupling and also to storage[1]. A further factor against the use of derivatives of polymethacrylic acid in the present studies was their polyionic nature which could have led to electrostatic adsorption of the enzymes.

Most other polymers used for enzyme coupling have either been hydrophobic[13–15] or, like polymethacrylic acid, polyionic[16].

An alternative method for the water-insolubilisation of enzymes is by trapping them in a polyacrylamide gel[17,18]. Water-insoluble preparations of D-glucose oxidase and lactate dehydrogenase were obtained, which had good stability to storage and lyophilisation. It thus seems that polyacrylamide could be a useful carrier for glycoside hydrolases.

Since macromolecules are unable to penetrate the gel matrix, the "trapping" method is unsuitable for such enzymes as amylases which are active against macromolecular substrates. In the present studies, therefore, we have sought to insolubilise alpha- and beta-amylase by chemical coupling to the surface of particles of cross-linked polyacrylamide gel.

EXPERIMENTAL

Crystalline alpha-amylase (*B. subtilis*) and crystalline beta-amylase (sweet potato) were purchased from the Sigma Chemical Company.

4-Nitroacrylanilide. — A solution of 4-nitroaniline (27.6 g, 0.2 mole) in warm, dry ether (1 litre) was stirred continuously for 2 h with acryloyl chloride (9.05 g, 0.1 mole). A further portion of acryloyl chloride (1 ml) was then added. After stirring for a further 2 h, the crystals which separated were collected, and the ether was evaporated to give more solid. The combined crystals and solid were triturated with water (5 × 500 ml) at 80–85°. Recrystallisation of the residue from acetone, followed by ethyl acetate, yielded 4-nitroacrylanilide (10.5 g, 54%), m.p. 239–240° (Found: C, 56.5; H, 4.1; N, 14.7. $C_9H_8N_2O_3$ calc.: C, 56.3; H, 4.2; N, 14.6%).

Preparation and selective reduction of Enzacryl AN. — A solution of acrylamide (3.55 g, 0.05 mole), 4-nitroacrylanilide (0.38 g, 0.002 mole), and *N,N'*-methylenediacrylamide (0.616 g, 0.004 mole) in ethanol (25 ml) at 75° was purged gently with nitrogen for 20 min, and a solution of benzoyl peroxide (100 mg) in chloroform (1 ml) was added. After storage for 12 h at 75°, the resulting copolymer was broken up under ethanol and passed through a coarse, syringe needle. After being washed with nearly boiling ethanol (3 × 500 ml), the copolymer (Enzacryl AN) was allowed to dry in air.

Selective reduction of the nitro groups in Enzacryl AN was achieved with titanous chloride. The copolymer (2 g) was suspended in a solution of titanous chloride (5–6%) in hydrochloric acid (6M, 100 ml), and the temperature was quickly raised to 100°. After 5 min, the mixture was rapidly cooled (ice bath), most of the solution was decanted off, and the polymer was repeatedly washed with distilled water. Finally, the reduced polymer (Enzacryl AA) was ground with distilled water,

passed through a coarse, syringe needle, washed with acetone, and allowed to dry in air.

Radiochemical determination of aromatic amine groups in Enzacryl AA as the hydrochlorides. — Samples of Enzacryl AN and AA were allowed to swell in aqueous sodium hydroxide (2M), exhaustively washed with distilled water, shrunk by drop-wise addition of acetone, and allowed to dry in air.

The prepared samples (0.25 g) of Enzacryl AN and Enzacryl AA were stirred magnetically for 1 h in centrifuge tubes with aliquots (15 ml) of 98mM hydrochloric acid containing radioactive chloride (^{36}Cl, 10 ncuries/ml). After centrifugation, the radioactivities of aliquots (10 ml) of each supernatant, and also of the original hydrochloric acid, were determined by means of a Geiger–Müller counter. After counting, each aliquot was transferred quantitatively to a conical flask, heated on a steam bath to expel carbon dioxide, and titrated with aqueous sodium hydroxide (99 mM).

Treatment of Enzacryl AA with hydrochloric acid leads to insolubilisation of a proportion as aromatic amine hydrochloride. By comparison of the radioactivity of the supernatant with that of the original hydrochloric acid, the total amount of aromatic amine hydrochloride in the polymer was determined. From the control experiment with Enzacryl AN, compensation was made for non-specifically adsorbed chloride (<7% of the total). Enzacryl AA contained 0.27 mequiv. of aromatic amine hydrochloride per gram (59% of the theoretical).

The total amount of hydrochloric acid neutralised by the polymer was determined by titration of the supernatant from the radiochemical determination of Enzacryl AA. This value was equivalent to the insolubilised acid plus an amount equivalent to the sodium carboxylate residues in the polymer (carboxylate residues are produced by hydrolysis of amide residues during acid reduction of Enzacryl AN). The total carboxylate in Enzacryl AA was 1.3–1.4 mequiv./g (corresponding to <10% hydrolysis of total amide).

tert-*Butyl 3-acryloylcarbazate.* — A solution of *tert*-butyl carbazate (2.6 g, 0.02 mole) in distilled water (15 ml) was stirred vigorously at room temperature with sodium hydrogen carbonate (6 g), and acryloyl chloride (1.6 g, 0.02 mole) was added during 5 min.

After 30 min, a further aliquot (1 ml) of acryloyl chloride was added. Stirring was continued for a further 30 min, and the resulting white solid was removed by filtration and washed well with water. Recrystallisation from ethyl acetate–light petroleum (b.p. 40–60°) yielded the title compound (1.8 g, 50%), m.p. 159–160° (Found: C, 51.8; H, 7.6; N, 15.3, $C_8H_{14}N_2O_3$ calc.: C, 51.6; H, 7.6; N, 15.1%).

Preparation of Enzacryl AH. — Acrylamide (3.55 g, 0.85 mole), *tert*-butyl 3-acryloylcarbazate (0.372 g, 0.002 mole), and *N,N'*-methylenediacrylamide (0.616 g 0.004 mole) were dissolved in ethylene glycol (25 ml) and irradiated for 20 h with a Hanovia u.v. lamp (80 watts). The resulting copolymer was broken up, ground thoroughly in a mortar, and washed exhaustively with water. Finally, the copolymer was shrunk by dropwise addition of acetone and allowed to dry in air.

Removal of protecting groups was achieved by shaking with an excess of 2M hydrochloric acid for 4 days at 18–20°. The resulting copolymer (Enzacryl AH) was washed exhaustively with distilled water, shrunk by dropwise addition of acetone, and allowed to dry in air.

Determination of acylhydrazide in Enzacryl AH as acylhydrazide hydrochloride. — The determination was performed as described for the aromatic amine residues in Enzacryl AA. Enzacryl AH contained 0.33 mequiv. of acylhydrazide hydrochloride per g (40% of the theoretical).

Total carboxylate (produced on shaking with dilute hydrochloric acid in the deprotection step) in Enzacryl AH was 0.1–0.3 mequiv./g (corresponding to 2% hydrolysis of total amide).

Preparation of water-insoluble alpha-amylase derivatives. — (*a*) *By coupling alpha-amylase to Enzacryl AA activated with nitrous acid.* Enzacryl AA (100 mg) was stirred with dilute hydrochloric acid (2M, 5 ml) at 0°. Ice-cold, aqueous sodium nitrite (2%, 4 ml) was added, stirring was continued for 15 min, and the diazo derivative of Enzacryl AA was washed four times with phosphate buffer (75mM, pH 7.6–7.7, 15 ml). After decantation of the final washings, a solution of alpha-amylase (2.5 mg) in phosphate buffer (75mM, pH 7.6–7.7, 0.5 ml) was added. Coupling was allowed to proceed for 48 h at 0–5° with stirring. A solution of phenol (0.01%, 10 ml) in aqueous acetate was then added, and, after a further 15 min, the water-insoluble alpha-amylase derivative was recovered by centrifugation. A control experiment was performed in which Enzacryl AH, preswollen in phosphate buffer (75mM, pH 7.6–7.7) but not treated with nitrous acid, was put through the above coupling procedure.

Both the water-insoluble alpha-amylase derivative and control were subjected to alternate washing with phosphate buffer (20mM, pH 6.9, 15 ml) and a solution of sodium chloride (0.5M, 15 ml) in the same buffer. Each washing was of 20-min duration with vigorous stirring. Five of the above washing cycles were necessary to wash the control to marginal amylolytic activity (<5% of that in the diazo-coupled derivative). Finally, the diazo-coupled alpha-amylase derivative was washed twice with phosphate buffer (15 ml) and, after decantation, resuspended in the same buffer (10 ml).

(*b*) *By coupling alpha-amylase to Enzacryl AA activated with thiophosgene.* Enzacryl AA (100 mg) was stirred with phosphate buffer (3.5M, pH 6.8–7.0, 1 ml) until a thick suspension was obtained. Stirring was then made very vigorous, and a solution of thiophosgene (10%, 0.2 ml) in carbon tetrachloride was added. After 20 min, a further aliquot (0.2 ml) of thiophosgene solution was added; 20 min later, the isothiocyanato derivative of the Enzacryl AA was washed once with acetone (15 ml), twice with 0.5M sodium hydrogen carbonate (15 ml), and twice with 50mM borate buffer (pH 8.6, 15 ml). After decantation of the final washings, a solution of alpha-amylase (2.5 mg) in 50mM borate buffer (pH 8.6, 0.5 ml) was added. Coupling was allowed to proceed for 48 h at 0–5° with stirring. The water-insoluble alpha-amylase derivative was then subjected to the washing procedure with phosphate buffer and buffered sodium chloride solution described in (*a*). Finally, the isothio-

cyanato-coupled alpha-amylase derivative was resuspended in phosphate buffer (10 ml).

(*c*) *By coupling alpha-amylase to Enzacryl AH activated with nitrous acid.* Enzacryl AH (100 mg) was treated with nitrous acid as described for Enzacryl AA. After the acid azide derivative had been washed four times with borate buffer (50 mM, pH 8.6, 15 ml), a solution of alpha-amylase (2.5 mg) in borate buffer (50 mM, pH 8.6, 0.5 ml) was added, and coupling was allowed to proceed for 48 h at 0–5° with stirring. As previously, a control experiment was performed in which Enzacryl AH, not activated with nitrous acid, was put through the coupling procedure. Three cycles of washing with phosphate buffer and buffered sodium chloride solution were sufficient to wash the control to zero amylolytic activity. Finally, the acid azide-coupled alpha-amylase derivative was resuspended in phosphate buffer (10 ml).

Preparation of water-insoluble beta-amylase derivatives. — (*a*) *By coupling beta-amylase to Enzacryl AA activated with nitrous acid.* Beta-amylase (5 mg) in phosphate buffer (75 mM, pH 7.6–7.7, 1 ml) was coupled with the diazo derivative of Enzacryl AA, following the procedures described for alpha-amylase. The washing procedure was also similar to that described for alpha-amylase except that acetate buffer (20 mM, pH 4.8) was used. Five washing cycles were necessary to decrease the amylolytic activity in the control to zero. The diazo-coupled beta-amylase derivative was finally suspended in acetate buffer (20 mM, pH 4.8, 10 ml).

(*b*) *By coupling beta-amylase to Enzacryl AA activated with thiophosgene.* Beta-amylase (5 mg) in borate buffer (50 mM, pH 8.6, 1 ml) was coupled with the isothiocyanato derivative of Enzacryl AA, as described for the corresponding derivative of alpha-amylase. The washing procedure and preparation of the final suspension of isothiocyanato-coupled beta-amylase was as described for diazo-coupled beta-amylase.

(*c*) *By coupling beta-amylase to Enzacryl AH activated with nitrous acid.* Beta-amylase (2.5 mg) in borate buffer (50 mM, pH 8.6, 0.5 ml) was coupled with the acid azide derivative of Enzacryl AH, as described for the corresponding alpha-amylase derivative. The washing procedure was similar to that employed for the other beta-amylase derivatives, except that only three washing cycles were necessary to decrease the amylolytic activity in the control to zero. The preparation of the final suspension of azide-coupled beta-amylase was as described for diazo-coupled beta-amylase.

Determination of amylolytic activity. — Alpha-amylase activity was determined against 1% soluble starch in phosphate buffer (20 mM, pH 6.9), and beta-amylase activity against 1% soluble starch in acetate buffer (20 mM, pH 4.8). Steady stirring was maintained for all determinations. As in previous studies[1,2], the initial rate of starch hydrolysis was determined by following the rate of formation of reducing sugar by assay of aliquots with the dinitrosalicylate reagent[19]. Absorbance changes (520 nm) thus recorded were related to reducing sugar by means of a standard graph relating absorbance to known amounts of maltose.

Determination of bound enzymes. — Samples of the water-insoluble enzyme derivatives and the non-coupled carriers were hydrolysed with 6M hydrochloric acid

for 18 h at 110°. The pH of aliquots (0.5 ml) of the hydrolysates was then adjusted to 14 by addition of the minimal quantity of 6M sodium hydroxide, and the solutions were lyophilised. The residues were then allowed to stand for 5 days over conc. sulphuric acid, after which they were re-constituted to a final volume of 1 ml, simultaneously being neutralised with concentrated hydrochloric acid. This procedure was necessary to ensure removal of ammonia and, in the case of hydrolysates of Enzacryl AH, hydrazine. The colour yield (570 nm) produced on ninhydrin assay[20] of the neutralised hydrolysates was then measured, and the colour yield produced on assay of hydrolysates of the non-coupled carriers was subtracted. The original enzyme concentration in the water-insoluble enzyme hydrolysates was calculated from standard graphs relating colour yield (570 nm) to known amounts of a hydrolysate of the free enzyme.

Stability of water-insoluble alpha- and beta-amylase to storage in buffer suspension. — The activity of the various preparations was redetermined after storage at 0–5° for 3 months, and the percentage of the original activity was calculated.

Stability of water-insoluble alpha-amylase to lyophilisation. — Aliquots (0.5 ml) of the three water-insoluble alpha-amylase suspensions were centrifuged and, after removal of most of the supernatant, lyophilised. After storage overnight at 0–5°, the samples were reconstituted with the appropriate buffer (0.5 ml) and their activities re-determined.

Stability of water-insoluble alpha- and beta-amylase to heat denaturation at 45°. — Suspensions of each enzymically active alpha-amylase derivative in phosphate buffer (20mM, pH 6.9) were incubated for 4 days at 45°, and their activity was determined at intervals (Fig. 1) against a solution of soluble starch. A control incubation was performed with a solution of free alpha-amylase in the same buffer.

A similar experiment was performed with suspensions of the enzymically active beta-amylase derivatives and a control solution of free beta-amylase in acetate buffer (20mM, pH 4.8).

DISCUSSION

In the preparation of water-insoluble enzyme derivatives, chemical coupling has most commonly been achieved by means of aromatic amine[1,2,13–15] or acid hydrazide[3–6] residues incorporated in the carrier. Activation with nitrous acid leads to diazonium and acid azide residues, respectively, which are effective in covalent bonding with enzymes. The simplicity of these methods probably accounts for their popularity. On superficial consideration, it might seem that these methods are not applicable to carriers based on polyacrylamide, since nitrous acid might be expected to react with amide residues in the copolymer matrix. However, polyacrylamide, in contrast to monomer amides, is refractory to nitrous acid.

By copolymerisation of acrylamide, 4-nitroacrylanilide, and *N,N'*-methylenediacrylamide, a cross-linked polymer, Enzacryl AN, was obtained which contained aromatic nitro groups. Reaction with titanous chloride in hydrochloric acid led to

Enzacryl AA, which contained aromatic amino groups (0.27 mequiv./g). Activation of the latter was achieved both by diazotisation and by conversion into the isothiocyanato group with thiophosgene. Subsequently, coupling was carried out with both alpha- and beta-amylase.

Diazo groups couple predominantly with the aromatic residues of the enzyme[21], whereas isothiocyanato groups couple with the amino groups of lysine and the terminal amino groups of the protein chains[22]. Such versatility is most desirable in the preparation of water-insoluble enzymes, since one or the other modes of coupling may either destroy, or lead to serious steric hindrance of, the enzymically active site. Thus, in previous work[2], we have shown that gamma-amylase may be coupled to the diazotised 3-(*p*-aminophenoxy)-2-hydroxypropyl ether of cellulose with retention of enzyme activity, whereas, on coupling to the corresponding 2-hydroxy-3-(*p*-isothiocyanatophenoxy)propyl ether of cellulose, inactivation resulted.

Copolymerisation of acrylamide, *tert*-butyl 3-acryloylcarbazate, and *N,N'*-methylenediacrylamide led to a copolymer which, on shaking with dilute hydrochloric acid, gave a copolymer, Enzacryl AH. Enzacryl AH thus contained acid hydrazide residues (0.33 mequiv./g). Nitrous acid treatment of the latter copolymer led to the generation of the acid azide group which, like the isothiocyanato group, is effective in coupling at the free amino residues of the enzymes[4]. Water-insoluble enzymes based on Enzacryl AH were of interest, since the absence of aromatic amino groups in this polymer meant it was relatively more hydrophilic than Enzacryl AA.

Active derivatives of alpha-amylase were obtained by all three coupling procedures (see Table I). The activities retained on coupling compared favourably with that of alpha-amylase coupled to other carriers[1,8–10]. We have previously demonstrated that the more an enzyme is dispersed over a carrier surface the greater is the activity retained on coupling. The present results confirm this hypothesis; the activity retained on coupling is greater for the water-insoluble preparations containing least alpha-amylase.

TABLE I

PROTEIN COUPLED AND ENZYME ACTIVITY IN THE WATER-INSOLUBLE ALPHA- AND BETA-AMYLASE PREPARATIONS

Enzyme	*Enzyme units[a]/mg of free protein*	*Carrier activated*	*Functional site active in protein binding*	*Bound protein (mg/100 mg of carrier)*	*Enzyme units/mg of bound protein*	*Enzyme activity (%) retained on coupling*
Alpha-amylase	1118	Enzacryl AA	Diazo	1.06	68.7	6.1
			Isothiocyanato	0.62	106.8	9.5
		Enzacryl AH	Acid azide	0.22	178.3	16.0
Beta-amylase	431.2	Enzacryl AA	Diazo	3.2	6.4	1.5
			Isothiocyanato	2.6	3.4	0.8
		Enzacryl AH	Acid azide	0.9	Inactive	Inactive

[a]One amylase unit is that which liberates reducing sugar equivalent to 1 μmole of maltose at 20° at optimum pH.

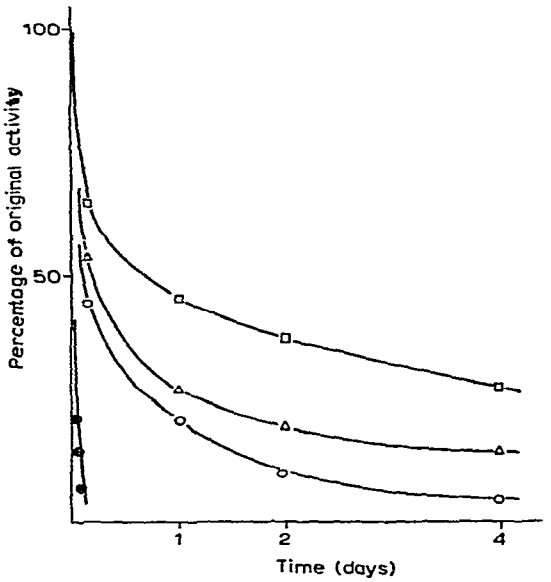

Fig. 1. Heat denaturation of soluble and water-insoluble forms of alpha-amylase on incubation in phosphate buffer at 45°. ●———●, Soluble alpha-amylase; ○———○, water-insoluble, diazo-coupled alpha-amylase; △———△, water-insoluble, isothiocyanato-coupled alpha-amylase; □———□, water-insoluble, acid azide-coupled alpha-amylase.

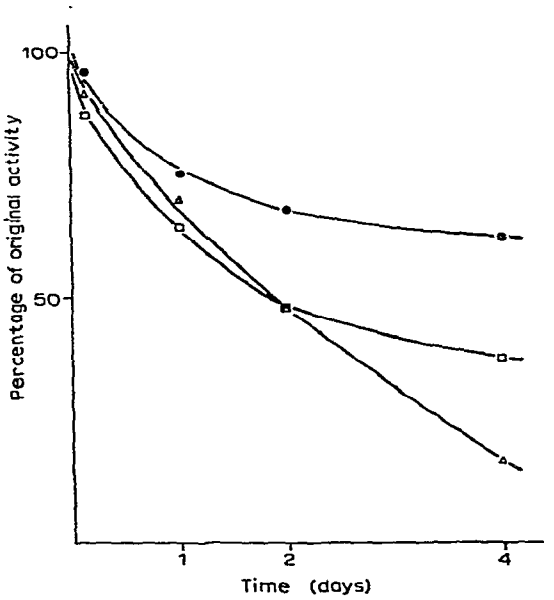

Fig. 2. Heat denaturation of soluble and water-insoluble forms of beta-amylase on incubation in acetate buffer at 45°. ●———●, Soluble beta-amylase; △———△, water-insoluble, diazo-coupled beta-amylase; □———□, water-insoluble, isothiocyanato-coupled beta-amylase.

Although beta-amylase was readily coupled by all procedures, the acid azide-coupled derivative was inactive, and the activity retained on diazo and isothiocyanato coupling to Enzacryl AA was lower than for the corresponding cellulose derivatives[2]. A possible explanation may be that the beta-amylase molecules tend to couple such that their active site is easily hindered. Previously, beta-amylase was coupled *via* activated 3-(*p*-aminophenoxy)-2-hydroxypropyl ethers of cellulose and would have been further away from the carrier matrix than in the present work. In the inactive, acid azide-coupled beta-amylase derivative based on Enzacryl AH, the enzyme must be particularly close to the polymer matrix, and this may account for the lack of activity. That steric effects are more important with Enzacryl AH is evident from the relatively smaller amounts of both alpha- and beta-amylase coupled to this carrier.

A feature of the water-insoluble polyacrylamide derivatives is that they were more easily washed free of physically attached enzyme than the corresponding cellulose derivatives. This was particularly noticeable with enzyme derivatives based on Enzacryl AH.

On storage for 3 months in buffer suspension, the activity retained by the acid azide-coupled alpha-amylase (85%) was rather better than diazo-coupled alpha-amylase (73%), isothiocyanato-coupled alpha-amylase (67%), diazo-coupled beta-amylase (73%), and isothiocyanato-coupled beta-amylase (51%). Similarly, the activity retained after lyophilisation and reconstitution of suspensions of acid azide coupled alpha-amylase (84%) was better than diazo-coupled alpha-amylase (28%) and isothiocyanato-coupled alpha-amylase (44%). The enhanced stability of acid azide-coupled alpha-amylase probably arises because it is attached to the more hydrophilic copolymer.

The stabilities of the water-insoluble alpha- and beta-amylase derivatives to heat denaturation were similar to those of the corresponding cellulose-based derivatives of these enzymes. The water-insoluble alpha-amylase preparations (see Fig. 1) exhibited a very large increase in stability over the free enzyme in solution. It is interesting to note that the most stable alpha-amylase derivative is again that involving Enzacryl AH, the more hydrophilic carrier. The difference in stability between the bound and soluble forms of beta-amylase (see Fig. 2) was much less marked, free beta-amylase being the more stable. An unusual point observed in this experiment was that the beta-amylase used was more stable in solution than samples of the same enzyme from batches supplied for previous work[2].

Our results seem to indicate that glycoside hydrolases may be usefully coupled to water-insoluble copolymers of acrylamide. Further work is projected, both to improve the carriers and to extend our studies to other enzymes.

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