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MAGNETIC, IMMOBILISED DERIVATIVES OF ENZYMES*

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ABSTRACT

A novel, water-insoluble, stable coating has been attached to various metals, which is capable of covalent attachment to enzymes. The coating (aminobenzoic acid–formaldehyde resin) can be attached to nickel, cobalt, tin, iron, and aluminium. β-D-Glucosidase has been attached to the coated metals by diazotisation of the coating. The stability and usefulness of the resulting, metallic enzyme-preparations is discussed with special emphasis on the value of ferromagnetic supports for enzymes.

INTRODUCTION

Insolubilised enzymes are now firmly established as useful biochemical tools. Various insolubilisation matrices have been used, including adsorption onto ion-exchange materials[1], and covalent attachment of part of the enzyme molecule to sites on a preformed polymer of cellulose *trans*-2,3-carbonate[2,3], poly(allyl cyclic carbonate)[4,5], 3-(4-aminophenoxy)-2-hydroxypropyl ethers of cellulose[6,7], metal chelates of polysaccharides and Celite[8] and of poly(salicylic acids)[9,10], and glass[11]. Polysaccharides are useful supports for these preparations[12] due to their hydrophilicity and the increased stability of the insolubilised enzyme.

We now describe the attachment of β-D-glucosidase (β-D-glucoside glucohydrolase E.C. 3.2.1.21) to various metals by a novel method involving an initial coating of the metal with an aminobenzoic acid–formaldehyde resin followed by diazo-coupling to the tyrosine groups of the enzyme. In this way, further useful properties (high density, ferromagnetism, and particle size and shape), due to the metallic support, can be imposed on the insolubilised-enzyme matrix, as well as the increased stability and reusability of the enzyme noted previously[1,2,5–8,10].

EXPERIMENTAL AND RESULTS

Preparation of enzyme supports. — 4-Aminobenzoic acid (6 g) was dissolved in boiling, distilled water (100 ml). Nickel powder (15 g) was added, followed, after 5 min, by dropwise addition of formaldehyde solution (40%, 0.5–1.0 ml) to the

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boiling solution. The product was filtered, ground lightly, and washed several times with distilled water.

Various other amines and metal powders were also used, and in two preparations 25% glutaraldehyde or acetaldehyde (0.5 ml) were employed. The resulting powders were tested for enzyme-coupling ability (Table I), as described below.

TABLE I

RELATION BETWEEN REACTANTS FOR ENZYME SUPPORT AND THE ENZYME-COUPLING ABILITY OF THE RESULTING POWDER

Preparation	*Amine*	*Metal powder*	*Enzyme-coupling ability*
1	4-Aminobenzoic acid	Ni[a]	+
	3-Aminobenzoic acid	Ni	+
	2-Aminobenzoic acid	Ni	+
	3,5-Diaminobenzoic acid	Ni	+
	2,4-Diaminobenzoic acid	Ni	+
2	4-Aminobenzoic acid[b]	Ni	+
3	4-Aminobenzoic acid	Co	+
4	4-Aminobenzoic acid	Fe[c]	+
	4-Aminobenzoic acid	Al[d]	+
	4-Aminobenzoic acid	Sn	+
	4-Aminobenzoic acid	Cu	−
	4-Aminobenzoic acid	Mg	−
	4-Aminobenzoic acid[e]	Ni	−
	3-Aminobenzoic acid[e]	Ni	−
	4-Hydroxybenzoic acid	Ni	−
	3-Aminobenzenesulphonic acid	Ni	−
	2,5-Diaminobenzenesulphonic acid	Ni	−
	Glycine	Ni	−
	L-Tyrosine	Ni	−
	L-Glutamic acid	Ni	−
	1,3-Diaminobenzene	Ni	−
	4-Aminosalicylic acid	Ni	−
	4-(2-Amino-2-deoxy-D-glucosylamino)benzoic acid	Ni	−
	Isobutyl (4-carboxyphenyl)carbamate	Ni	−
	4-Aminobenzoic acid[f]	Ni	−

[a]An active preparation could also be made using nickel in the form of wire or large particles. [b]Acetaldehyde was used in place of formaldehyde. [c]An active preparation could also be made by using iron filings. [d]An active preparation could also be made by using aluminium sheeting. [e]The reaction was carried out in ethanolic solution instead of in aqueous solution. [f]Glutaraldehyde (25%) was used in place of formaldehyde.

Optimisation of enzyme coupling ability of enzyme supports. — Several preparations of the enzyme support were made, as described above, using 4-aminobenzoic acid and nickel powder but varying the amount of formaldehyde solution added. The activities of the resulting enzyme preparations, after coupling of the support to β-D-glucosidase, were determined (Fig. 1) as described below.

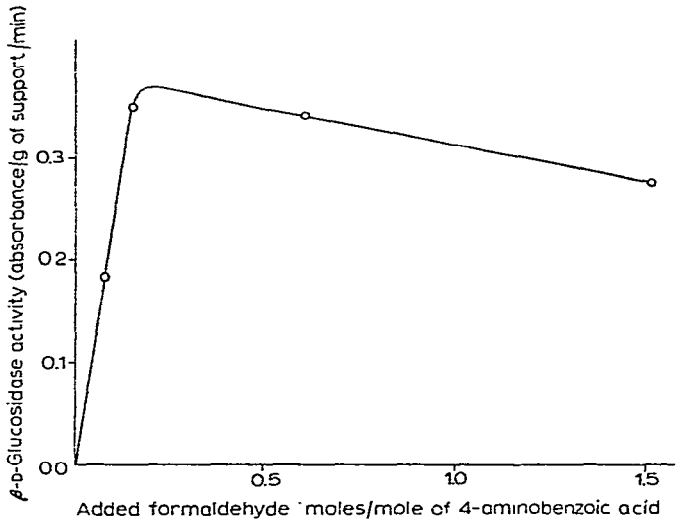


Fig. 1. Activity of preparations of β-D-glucosidase immobilised on diazotised 4-aminobenzoic acid–formaldehyde copolymer-coated nickel: variation of activity with the amount of formaldehyde present during polymerisation.

Coupling of β-D-glucosidase with enzyme support. — The green aminobenzoic acid–formaldehyde–nickel preparation (100 mg) was washed with distilled water (5 × 12 ml; this process was greatly facilitated by the use of a magnet to remove the powder from the solution), added to a mixture of ice-cold M sodium nitrite (5 ml) and ice-cold 0.6–1M hydrochloric acid (5 ml), and stirred for 1–2 min at 0–4°. The diazotised support was washed with 0.1M sodium acetate buffer (pH 4.8, 3 × 15 ml, 0–4°) and coupled to β-D-glucosidase (*ex.* sweet almonds, Koch–Light Laboratories Ltd., 1–4 mg/ml) in 0.1M acetate buffer (pH 4.8, 1–2 ml) for 2–18 h at 0–4°. The supernatant solution was decanted, a saturated solution of 2-naphthol in saturated, aqueous sodium acetate (5 ml) was added, and the mixture was stirred for 2 h at 0–4°. The resulting red powder was washed with 0.1M sodium acetate buffer (pH 4.8, 10 × 15 ml) and with a solution of enzyme substrate (2-nitrophenyl β-D-glucopyranoside, 1 mg/ml) in the acetate buffer before use.

Other conditions for the diazotisation, varying the concentration of hydrochloric acid and time of diazotization, the effect of acetic and perchloric acids, and the effects of bromide and iodide ions, did not increase the coupling ability of the support.

Determination of β-D-glucosidase activity. — The enzyme-coupled product (100 mg) was added to a stirred solution of 2-nitrophenyl β-D-glucopyranoside (1 mg/ml) in 0.1M sodium acetate buffer (pH 4.8, 1.0 ml) at 20° or 37°. The same rate of stirring and temperature were employed in all comparable experiments. At various times, an aliquot (0.1 ml) of the supernatant solution was added to 50mM sodium carbonate (1.0 ml), and the absorbance (*A*) of the solution at 420 nm was determined. The results of testing are shown in Table I and Fig. 1.

Determination of protein content of enzyme-coupled product. — The weighed preparation was left in 5.5M hydrochloric acid (2.0 ml) at 20° for 16 h. The supernatant solution was removed and hydrolysed (110°, 6 h) in a sealed ampoule, then concentrated to ~0.2 ml, and fractionated on previously washed (ethanol–water, 3:1), Whatman No. 1 paper by descending elution with 1-butanol–pyridine–water (6:4:3) for 16 h. The dried chromatograms were sprayed with ninhydrin reagent and developed (60°, 30 min). Selected spots of equivalent R_F values were selected from the chromatograms of the hydrolysed, insolubilised enzymes and hydrolysed, free-enzyme standards, and eluted with ethanol–water (3:1). The absorbances of the solutions at 570 nm were determined. The activity and protein contents of the insolubilised enzymes are shown in Table II.

TABLE II

ACTIVITY AND PROTEIN CONTENT OF THE ENZYME-COUPLED PRODUCTS

Preparation	*Activity (μg of original β-D-glucosidase/g)*	*Protein content (μg of β-D-glucosidase/g)*	*Retention (%) of specific activity*
1	558	1725	32
2	100	n.d.[a]	—
3	480	n.d	—
4	200	1005	20

[a]n.d, not determined.

Analysis of the enzyme support. — An elemental analysis of the 4-aminobenzoic acid–formaldehyde–nickel enzyme support, prepared with the optimal amount of formaldehyde, gave Ni, 90.2; C, 6.23; H, 0.53; N, 0.93. This is equivalent to a 10% coating of $C_{7.83}H_{8.13}NO_{1.96}$ on the nickel powder.

The i.r. spectrum (KBr disc) of this enzyme support is shown in Fig. 2.

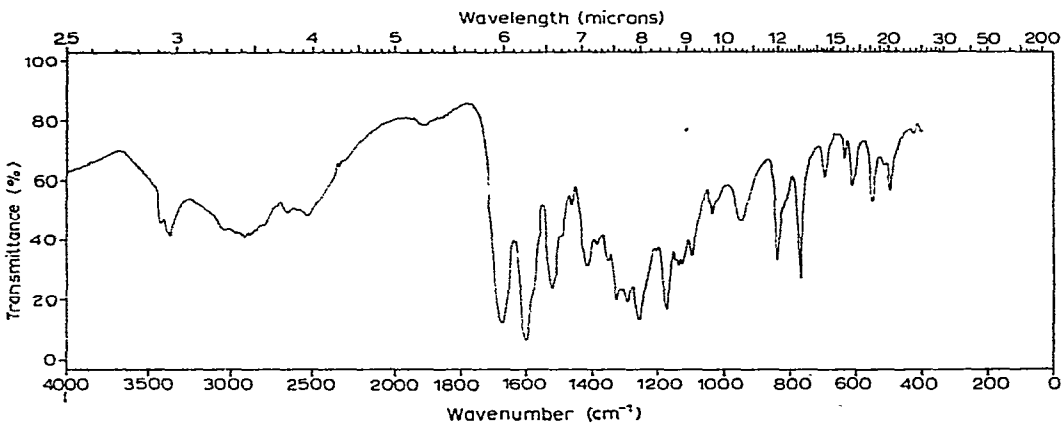


Fig. 2. I.r. spectrum (KBr disc) of the enzyme support (4-aminobenzoic acid–formaldehyde copolymer).

pH-Activity profiles for bound and free β-D-glucosidase. — The insolubilised β-D-glucosidase (100 mg) was washed with buffer [0.2M, 2 × 10 ml, sodium phosphate–citrate (pH 4), or sodium phosphate (pH 4.65, 5.35, 6.10, 6.30, 7.50, or 8.70)] and then suspended in the same buffer (1.0 ml). A solution of 2-nitrophenyl β-D-glucopyranoside (1 mg/ml, 1.0 ml), in buffer identical to that used for the enzyme, was added and the mixture stirred for up to 30 min at 20°. An aliquot (0.1 ml) of the

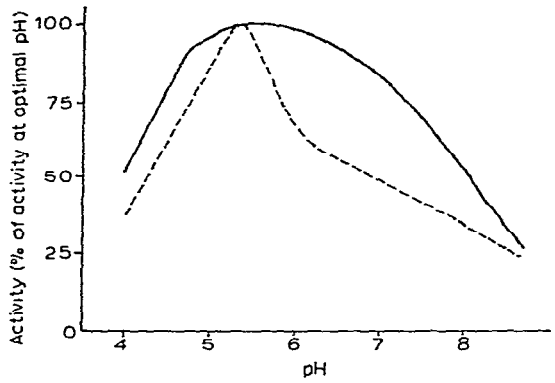


Fig. 3. pH–Activity profiles for free β-D-glucosidase (— — —), and β-D-glucosidase immobilised on 4-aminobenzoic acid–formaldehyde copolymer-coated nickel (———).

TABLE III

REUSABILITY TRIAL OF BOUND β-D-GLUCOSIDASE

Activity (%)	(100)[a]	103	122	124	130	127	117	132
Incubations	1	2	3	4	5	6	7	8

[a]Initial preparation.

TABLE IV

STABILITY OF BOUND β-D-GLUCOSIDASE

Treatment			*Activity* (%)	
			Bound enzyme	*Free enzyme*
Stirred with substrate[a]	2 × 1 h[b]	20°	98	—
Stirred with substrate[a]	4 × 1 h[b]	20°	97	—
Stirred with substrate[a]	1 × 18 h	20°	60	—
Stirred with substrate[a]	1 × 60 h	20°	43	—
Acetate buffer[c]	168 h	20°	102	15
Acetate buffer[c]	15 h	37°	92	18
Acetate buffer[c]	15 min	50°	35	75

[a]2-Nitrophenyl β-D-glucopyranoside (1 mg/ml, 2 ml). [b]Washed (3 ×) with 0.1M sodium acetate buffer (pH 4.8, 15 ml) between incubations. [c]0.1M Sodium acetate buffer (pH 4.8, 2 ml).

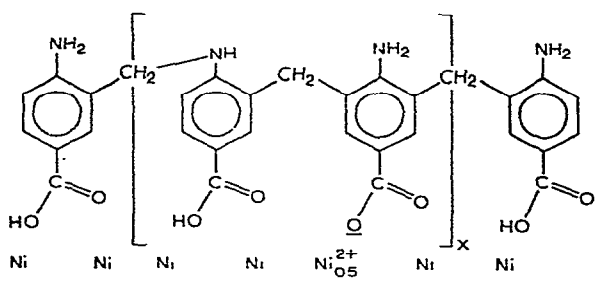
incubate was added to 50mM sodium carbonate (0.5 ml), and the absorbance at 420 nm was determined. Similar determinations of activity were made on free β-D-glucosidase, using the same buffers. The pH-activity profiles of the free and coupled enzymes are shown in Fig. 3.

Stability of bound β-D-glucosidase. — The activity of a sample of bound β-D-glucosidase was determined repeatedly after washing with 0.1M sodium acetate buffer (pH 4.8, 3 × 15 ml, 5 min for each wash), and the results are shown in Table III.

The activity of samples of bound and free β-D-glucosidase were determined after various treatments, and these results are shown in Table IV.

DISCUSSION

Aminobenzoic acids in aqueous solution can be used in a matrix-forming reaction. Formaldehyde, in such reactions, usually gives condensation products with the amino nitrogen under alkaline conditions[13], but in acid it forms methylene bridges[14]. These bridges are formed *o* and *p* to the aromatic amine residue[15], and *m* to the aromatic carboxylic acid[16]. This can be rationalised by examining the postulated structure **1**.



(Order of methylene and *N*-methylene-amine linkages is random)

Nickel surface

1

The optimal molar ratio of formaldehyde to 4-aminobenzoic acid for the preparation of the matrix (Fig. 1) is ~1:5, and as the proportion of formaldehyde is increased there is a gradual loss in enzyme-coupling ability. At low concentrations of formaldehyde, the main reaction is a coupling (**1**) between the aromatic rings, but as the concentration increases, more of the amine functions become blocked by condensation with the excess of formaldehyde. The amount of 4-aminobenzoic acid reacted to form the matrix on the nickel depends to a great extent on the surface area of the nickel, and usually there was a large amount of unreacted 4-aminobenzoic acid. It is assumed that some of the formaldehyde would be reduced by the hydrogen formed by the reaction of the hot benzoic acid with the metal and in this way decrease any gas pockets which might be trapped as the resin matrix was formed. It is possible that the liberated hydrogen might also reduce amine–formaldehyde to give secondary amines[14].

The elemental analysis is consistent with structure **1** since this structure requires $C_8H_7NO_2$. The i.r. spectrum (Fig. 2) of the nickel–enzyme support shows distinct

differences from that of 4-aminobenzoic acid. These are most apparent in the C-H in-plane deformation region (1000–1300 cm^{-1}) and the C-H out-of-plane deformation region (800–950 cm^{-1}), due to the change in the benzene substitution pattern, and in the N-H stretching (3300–3500 cm^{-1}) and deformation (1600–1650 cm^{-1}) regions. There is also absorbtion at 1380 cm^{-1}, possibly due to the carboxylate ion. It appears that the resin was held to the nickel by electrostatic bonds.

The polymeric matrix on the metal contained free amino groups which could be used to insolubilise enzymes through diazo coupling with tyrosine groups in the enzyme (*cf.* refs. 6 and 7). In this paper, β-D-glucosidase was selected as a typical enzyme that was easily obtainable and rapidly assayed.

Although the specific activity of the insolubilised β-D-glucosidase is reasonably high (32%, Table II), the protein content is low compared with other insolubilised-enzyme preparations. The high density and low surface area/weight ratio of the metal-powder support permits only relatively low amounts of enzyme to be bound for a given weight of metal.

The pH-activity profile of the bound β-D-glucosidase (Fig. 3) shows a broadening effect relative to that of the free enzyme. This effect is most evident on the alkaline side. The movement of a pH-activity optimum has been noticed before with bound trypsin[17] and β-D-glucosidase[1,2] where the optimum was moved to a more alkaline pH with a negatively charged, solid support[17], was broadened to acid pH with a positively charged support[1], and was left unchanged by a neutral support[2]. For the bound β-D-glucosidase, the partial, or total, ionisation of the carboxylic acid groups would give rise to a negatively charged matrix, thereby raising the local pH. This is a further confirmation of the electrostatic nature of the metal–matrix bond. The small, broadening effect on the acid side of the pH-activity optimum cannot be readily explained, but a similar effect has been noticed for a cationic support[1].

The reusability results (Table III) show an initial increase in enzyme activity up to about the fourth re-incubation. This indicates that there is little or no physical absorbtion of active β-D-glucosidase on the support after the routine washing procedure, but there may be an initial, gradual elution of inactive β-D-glucosidase or enzyme inhibitor which, until removed, decreased the apparent activity of the matrix-bound enzyme.

The effect of moderately fast stirring on the enzyme matrix (Table IV) was to diminish its specific activity by approximately 1% per hour. This loss is not significant in the present work where the incubations were usually only for 3–5 minutes, and the loss could be diminished by less-vigorous stirring. The activity loss was probably due to gradual removal of the coating by friction.

The stability of the bound enzyme to incubation in buffer (Table IV) showed the usual high retention expected of insolubilised enzymes[2,18] at the lower temperatures (20°, 37°), but greatly decreased, relative activity at the higher temperature (50°). This is due to the low melting-point on the nickel coating ($\simeq 60°$), leading to a breakdown of the matrix at moderately high temperatures.

Insolubilised enzymes are useful where their increased stability, reusability, and

recoverability, relative to the native enzyme, are more important than the extra work and expense of the coupling operation. They are easily recoverable from solution by simple filtration or centrifugation operations, or can be used as column packings in continuous-flow operations. Their repeated use offsets the higher cost of manufacture. There are some instances, however, where they are difficult or impossible to recover, such as when they are in a colloidal suspension or when other undissolved particles are present. Separation under these conditions may be achieved only if the difference in sedimentation rate of the insolubilised enzyme and undissolved or colloidal particles is very large. Enzymes attached to the matrices described in this paper would be easily removed from these solutions because their rate of sedimentation is much higher than for most commonly found particles. In particular, their magnetic properties would be utilised to effect ready removal. The speed and ease of removal by a magnetic field ensures that any washing or incubation process can be finished promptly and efficiently. This is useful where a strict control of solution temperature or reaction time is required. Also, no internal stirrer is necessary as the nickel–enzyme matrix will respond to an external magnetic stirrer. The advantages of being able to attach enzymes to such comparatively easily machineable metals as nickel and aluminium, as shown by the attachment of β-D-glucosidase to nickel wire and aluminium sheet, has many possible applications in the biological engineering industry, For example, the metal support could be in the form of small spheres for continuous-flow column reactions or affinity chromatography separations, as a metal paddle in stirred reactions, or in the form of tubing for reactions involving rapid transport of material.

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