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A NEW AND CONVENIENT METHOD FOR ENZYME INSOLUBILISATION USING DIAZOTIZED *m*-DIAMINOBENZENE*

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SUMMARY

A convenient method for enzyme insolubilization is described, which involves diazotization of *m*-diaminobenzene in the presence of a solid support and exposure of the resultant material to a solution of the enzyme. Optimum conditions for the attachment of β-D-glucosidase (EC 3.2.1.21) to cellulose have been established. The method is successful for a wide range of enzymes and a number of different solid supports.

INTRODUCTION

In recent years there has been a remarkable increase in interest in the subject of enzyme insolubilization [1–7], and a variety of methods have been employed for the covalent attachment of enzymes to solid matrices [8–19]. Many methods possess a major disadvantage in that a number of chemical stages are required to introduce into the matrix material the appropriate reactive grouping. In addition, the number of solid supports suitable may be restricted since the material must possess some functional group which can serve as the starting point for the required chemical sequence.

During earlier work in this laboratory on another method of enzyme insolubilization, we observed that small quantities of *m*-diaminobenzene could become strongly absorbed on to cellulose. Diazotization and subsequent exposure to β-D-glucosidase (EC 3.2.1.21) gave a cellulose preparation with significant enzyme activity. This suggested that such a process could provide the basis of a very convenient new method for enzyme insolubilization in which the reactive solid to which the enzyme is attached is produced in only one step.

MATERIALS AND METHODS

m-Diaminobenzene, β-D-glucosidase (emulsin), dextranase (EC 3.2.1.11; ex *Penicillium*), α-chymotrypsin (EC 3.4.4.5; bovine), papain (EC 3.4.4.10), peroxidase (EC 1.11.1.7; horseradish), catalase (EC 1.11.1.6; beef liver) and uricase (EC 1.7.3.2; hog kidney) were all obtained from Koch–Light Laboratories Ltd. "Sigmacell" microcrystalline cellulose (30 μm) was the product of the Sigma Chemical Co. Ltd. Neosyl and glucoamylase (EC 3.2.1.3; fungal) were obtained from A.B.M. Industrial Products

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Ltd. The glucose oxidase (EC 1.1.3.4; fungal) was from the Boehringer Corp. Ltd. Cellex CM (carboxymethyl cellulose) and Bioglas 1000 were the products of Bio-Rad Laboratories. Celite (80–120 mesh) was obtained from B.D.H. Chemicals Ltd, and the DEAE-cellulose used was Whatman DE32.

Glucoamylase was dialysed against 0.2 M acetate buffer (pH 4.5) before use.

Buffers were prepared following the methods described by Dawson et al. [20]. The pH of each buffer was checked on a Radiometer titrator TTT2 pH meter.

Determination of enzymic activities

A constant rate of magnetic stirring was employed throughout each assay. Unless otherwise stated, incubations were at 37 °C. Absorbance of solutions, or supernatants after centrifugation, was determined using a Unicam SP500 spectrophotometer. Enzyme activities were calculated from initial slopes.

β-D-Glucosidase activity [21] was determined at pH 5.0 using *o*-nitrophenyl-β-D-glucopyranoside as substrate. α-Amylase and dextranase were assayed against starch or dextran, respectively, reducing sugar produced being determined using the 3,5-dinitrosalicylic acid method [22]. Glucoamylase activity was determined by measuring the glucose liberated from starch by the glucose oxidase method of Dahlqvist [23]. Chymotrypsin was assayed by the casein method of Laskowski [24] modified to allow the hydrolysis products after precipitation with trichloroacetic acid to be determined using the Folin–Ciocalteu reagent in a manner similar to that described by Anson [25]. Papain was assayed [26] against *N*-α-benzoylarginine ethyl ester using a Radiometer autoburette ABU11, Autotitrator TTT2 and Titrigraph system. Glucose oxidase activity was determined using the peroxidase–2,2′-azino-di-(3-ethylbenzothiazoline-6-sulfonic acid) (ammonium salt) system described by Werner et al. [27].

The percentage enzymic activity reported represents the number of units of activity detected on the solid expressed as a percentage of the activity of the soluble enzyme to which the solid was exposed. The figure is therefore the product of two factors: the percentage of the enzyme offered which had coupled and the efficiency of the solid-phase enzyme as compared with the soluble enzyme. The "percentage enzymic activity", therefore, provides a very useful index of the success of an insolubilization method.

Insolubilization of β-D-glucosidase onto Bismarck brown

To a solution of *m*-diaminobenzene (50 mg) in HCl solution (1 M, 2.5 ml) at 0 °C was added with stirring a solution of $NaNO_2$ (6%, w/v; 2.0 ml). The mixture was stirred for 30 min at 0 °C and then at 20 °C for a further 30 min. The solid produced was then washed with 0.2 M acetate buffer (pH 5.0, 3 × 5.0 ml). A solution of β-D-glucosidase (5.0 mg) in 0.2 M acetate buffer (0.5 ml) was then added and the mixture stirred at 4 °C for 2 h. β-Naphthol solution (saturated in saturated sodium acetate solution, 2.0 ml) was then added and after 2 h at 4 °C the solid was washed five times alternately with 0.2 M acetate buffer (pH 5.0, 5.0 ml) and a solution containing sucrose (1 M) and NaCl (1 M) in the same buffer (5.0 ml). The solid was finally suspended in the buffer (0.5 ml).

Enzyme insolubilization: general method

$NaNO_2$ (2%, w/v in water; 2.0 ml) was added to a mixture of *m*-diamino-

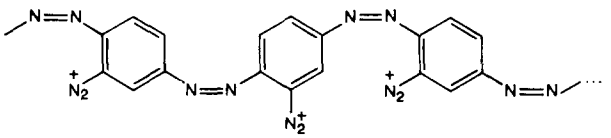
benzene (50 mg) and the appropriate solid (100 mg) in HCl solution (1.0 M; 2.0 ml) at 0 °C and the suspension stirred for 30 min. The solid was washed with 0.2 M acetate buffer (pH 5.0, 3 × 5.0 ml), the enzyme (1.0 mg) in 0.2 M acetate buffer (pH 5.0; 5.0 ml) was added and the mixture stirred for 2 h at 4 °C. β-Naphthol (saturated, in saturated sodium acetate solution, 2.0 ml) was added and stirring continued for 30 min. The suspension was centrifuged and the solid subjected to five cycles of washing with alternately 0.2 M acetate buffer (pH 5.0; 5.0 ml) and a solution containing sucrose (1 M) and NaCl (1 M) in the same buffer (5.0 ml). The solid was finally washed (three times) with the buffer and stored as a suspension in it. A control was prepared by stirring the solid alone (100 mg) with an identical solution of the enzyme for the required time, followed by the same washing procedure. All subsequent controls were prepared in an analogous manner.

Dextranase, α-amylase, glucoamylase, chymotrypsin, papain, catalase, peroxidase, glucose oxidase and uricase were insolubilized onto cellulose using methods essentially similar to that described above. Variations in method are shown in Table I.

Dextranase (5.0 mg) in 0.2 M acetate buffer (pH 6.0; 0.5 ml) was coupled to 100 mg quantities of Biogel P-300, Biogel P-6, CM-cellulose, DEAE-cellulose, Bioglas 1000, celite and neosyl using the method described above. A similar experiment was performed using nylon mesh (25 mg) and a proportionate amount of enzyme.

RESULTS AND DISCUSSION

When *m*-diaminobenzene in acid solution is treated with $NaNO_2$ at low temperature, a red–brown solid, well-known commercially as Bismarck brown, is precipitated [30]. This solid is almost certainly a polymer produced by the coupling of diazotized molecules with others, probably undiazotized. The polymer presumably has a structure similar to that shown below, and when freshly prepared possesses diazo-groups on its surface. By virtue of these diazo groups, Bismarck brown is itself a practicable solid for enzyme insolubilization. β-D-glucosidase is very effectively insolubilized by attachment to this material; the activity detected on the solid represented 59% of the original activity of the soluble enzyme.



Bismarck brown alone however possesses certain disadvantages considered as a solid support for an enzyme. When prepared as described it appears as very fine particles which settle very slowly. Its fine particulate nature renders it unsuitable as a potential column packing. In addition, it appears to have poor mechanical stability which would make re-use difficult. Incorporation into a more convenient solid was therefore desirable. When *m*-diaminobenzene is diazotised in the presence of cellulose, the red solid is produced and simultaneously adsorbed onto the surface of the cellulose. The cellulose in effect undergoes a dyeing process, and the surface coating of Bismarck brown is not removed by washing. The conjugate retains many of the physical properties of the cellulose, e.g. larger particle size and mechanical stability, and

is far more suitable as a solid for enzyme insolubilization. The preliminary experiment in which β-D-glucosidase was attached to the cellulose–Bismarck brown conjugate demonstrated that the Bismarck brown can function effectively as a link between enzyme and solid matrix (Table I).

In order to establish that the method had general applicability, a series of enzymes were then insolubilized onto cellulose using this technique. The results obtained (Table I) show that highly active solid-phase derivatives of α-amylase, dextranase, glucoamylase, papain, glucose oxidase, peroxidase, catalase and uricase were conveniently prepared.

TABLE I

ACTIVITIES OF ENZYMES INSOLUBILIZED BY ATTACHMENT OF CELLULOSE BY DIAZOTIZED *m*-DIAMINOBENZENE

Conditions were as described in Materials and Methods except where stated.

Enzyme	Quantity of enzyme used (mg, except uricase)	pH of coupling	Coupling time (h)	Enzymic activity (%) (see Results)
β-D-Glucosidase				
Test	5.0	5.0	16.0	24
Control	5.0	5.0	16.0	1
α-Amylase				
Test	4.2	6.9	16.0	13
Control	4.2	6.9	16.0	1
Dextranase				
Test	5.0	5.0	16.0	16
Control	5.0	5.0	16.0	0
Glucoamylase				
Test	4.0	8.0	16.0	12
Control	4.0	8.0	16.0	2
Chymotrypsin				
Test	5.0	6.0	16.0	3
Control	5.0	6.0	16.0	0
Papain				
Test	5.0	7.0	0.5	35
Control	5.0	7.0	0.5	0
Glucose oxidase				
Test	5.0	6.0	16.0	18
Control	5.0	6.0	16.0	2
Peroxidase				
Test	2.5	5.0	16.0	17
Control	2.5	5.0	16.0	0
Catalase				
Test	2.5	5.0	16.0	20
Control	2.5	5.0	16.0	0
Uricase				
Test	0.0443 (units)	8.5	18.0	18
Control	0.0443 (units)	8.5	18.0	0

The use of solid supports other than cellulose was then examined; dextranase, which gave good results on cellulose, was used. The production of a satisfactory conjugate between the diazotised diaminobenzene and a solid was very variable. Some solids took up the coloured material very effectively and in these cases subsequent exposure to the dextranase gave highly active solid-phase enzymes. These solids included DEAE-cellulose, Bioglas 1000, celite and neosyl (Table II). Other solids took

TABLE II

ACTIVITIES OF SOLID-PHASE DEXTRANASE BASED ON VARIOUS SOLID SUPPORTS

Conditions were as described in Materials and Methods.

Solid supports	Enzymic activity (%) (see Results)
Biogel P-300	
Test	1.5
Control	1.2
Biogel P-6	
Test	9.6
Control	1.0
Nylon mesh	
Test	3.8
Control	0.2
Cellex CM	
Test	7.2
Control	2.0
DEAE-cellulose	
Test	34.3
Control	0.3
Bioglas-1000	
Test	33.3
Control	3.7
Celite	
Test	21.8
Control	0.0
Neosyl	
Test	28.0
Control	1.0

up little or no colour and subsequently gave products with much lower activities. These included the two Biogels, nylon mesh, CM-cellulose, glass tubes, Ballotini beads and polythene sheets. These last two gave extremely poor enzymic activity.

Clearly the nature of the solid support is extremely important. From these results it appears that the solid must be highly porous to be effective and also have a suitably absorbent surface. However these conditions are likely to be satisfied by a wide enough range of support materials to find extensive use.

Having established in these preliminary tests that the method could be used

for a wide variety of enzymes, and a number of different solid supports, we determined the conditions under which one enzyme in particular, β-D-glucosidase, could be attached to cellulose to give the product with maximum activity.

One of the most critical variables in a process such as this is likely to be the pH of the coupling reaction. The pH-of-coupling profiles obtained for the three enzymes,

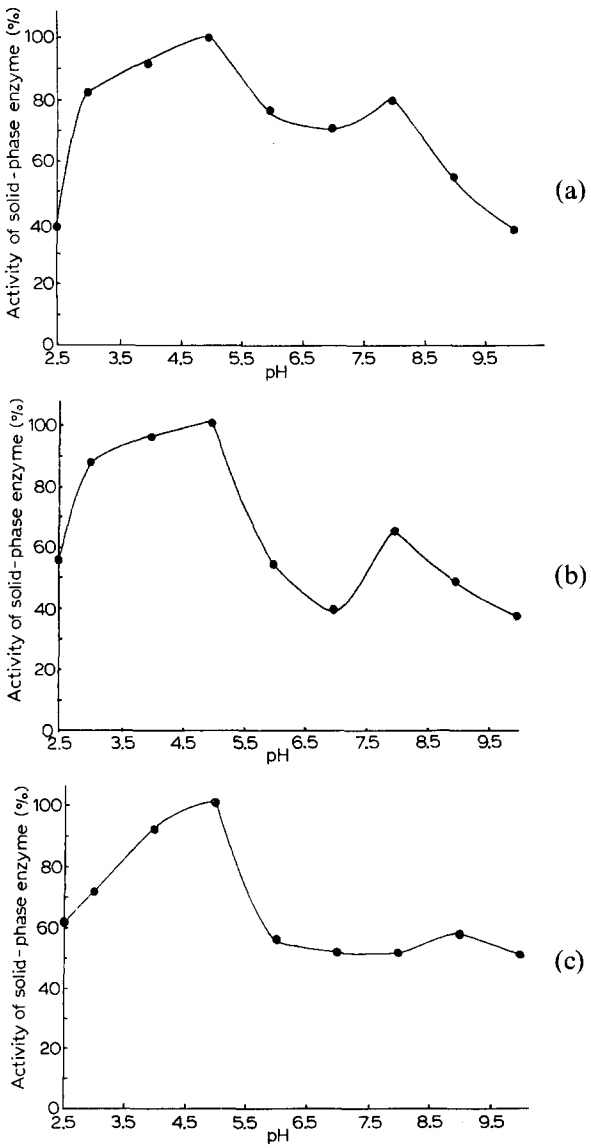


Fig. 1. Dependence on pH of coupling of the activities of solid phase-enzymes prepared from cellulose–diaminobenzene. Conditions were as described in Materials and Methods except that couplings were carried out for 2 h in a range of buffers, all 0.2 M; citrate–phosphate (pH 2.5), citrate (pH 3.0 and 4.0), acetate (pH 5.0), phosphate (pH 6.0, 7.0 and 8.0), borax–boric acid (pH 9.0) and borax–NaOH (pH 10.0). The concentration of enzyme used in each case was 5.0 mg/ml. a, β-D-glucosidase; b, dextranase; c, papain. The enzymic activity is expressed as a fraction of that obtained at pH 5.0.

β-D-glucosidase, dextranase and papain (Fig. 1, Panels a, b and c) are very similar. Each gives the preparation with maximum activity at pH 5. The profiles for dextranase and β-D-glucosidase show subsidiary peaks at pH 8 while that for papain shows a plateau in this region. The similarity of these profiles for three different enzymes suggests that such a pattern may be a general feature of the reaction of all proteins with this type of solid. In addition to indicating that pH 5 would normally be the optimum for the preparation of insolubilized enzymes, the profiles also show that considerable activities may be obtained even when the coupling pH is as high as 10. Such an observation may be very important for enzymes such as uricase for which reaction at pH 5 is impracticable due to its low solubility at this pH.

Further experiments established the following points. The optimum quantity of *m*-diaminobenzene was 50 mg/100 mg of cellulose. Great difficulty was encountered in washing the solid when more than this amount was used. Apparently the cellulose can readily accommodate the Bismarck brown produced from up to 50 mg of *m*-diaminobenzene. A similar result was obtained for celite when glycoamylase was insolubilized onto this solid.

Although the quantity of $NaNO_2$ used for the diazotization of the *m*-diaminobenzene is not critical, experience has shown that the use of quantities lower than that given produces a solid which is less easily washed.

The coupling reaction is quite rapid. Under the conditions described, attachment of the enzyme appears to be complete within 2 h. Significantly longer reaction times generally lead to lower activities, probably due to spontaneous inactivation of the enzymes.

The amount of enzyme offered to the solid to give a product with maximum activity may ultimately determine the value of the method. The results shown in Table III indicate that, as might be expected, the total activity of the product increases as the amount of enzyme offered is increased. The maximum is obtained at about 8.0 mg of enzyme under the conditions used. Interestingly the use of 10 mg of enzyme gave a rather lower result. This may be due to considerable overcrowding of the enzyme molecules on the surface of the solid leading to decreased efficiency. The enzymic

TABLE III

DEPENDENCE OF ENZYMIC ACTIVITY OF β-D-GLUCOSIDASE INSOLUBILIZED ONTO CELLULOSE ON THE QUANTITY OF ENZYME USED

Conditions were as described in Materials and Methods, varying quantities of enzyme being used.

Amount of enzyme offered		Total activity detected on solid (units)	Enzymic activity detected (%)
Activity (units)	Weight (mg)		
30	1.0	31.0	103
90	3.0	66.5	74
120	4.0	82.6	69
150	5.0	84.0	56
240	8.0	87.0	36
300	10.0	73.7	25

activities of the solid products, expressed as a fraction of the total enzymic activity offered, decreased steadily from the 100% obtained when only 1.0 mg was used.

On the basis of these results it is thus possible to describe a set of optimum conditions for β-D-glucosidase attached to cellulose. Maximum enzymic activity is obtained when the conjugate prepared from 100 mg of cellulose and 50 mg of *m*-diaminobenzene is exposed to 8 mg of enzyme at pH 5 for 2 h. It is probable that these conditions would produce high if not maximum activity for many other enzymes attached to various solid supports.

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