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ACTIVE WATER-INSOLUBLE DERIVATIVES OF PAPAIN AND OTHER ENZYMES BASED ON PREFORMED DIAZONIUM-TYPE SUPPORTS

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Summary

Papain (EC 3.4.22.2) has been coupled to supports of titanium (IV) oxide and cellulose, which are particulate and pre-coated with diazotised 1,3-diaminobenzene, giving water-insoluble and stable derivatives which possess low proteolytic activity but high esterolytic activity. In addition the reversible binding of zinc (II) at the active site of papain has been exploited to inhibit protectively the enzyme during its linkage to the aforementioned supports, thereby yielding water-insoluble derivatives of papain having superior activity upon reactivation with EDTA. Application of the improved procedure of enzyme coupling to macroporous cellulose particles gave a water-insoluble derivative of papain having further enhanced proteolytic activity. Other properties of the water-insoluble derivatives of papain and of similarly prepared water-insoluble conjugates of urease (EC 3.5.1.5) and cholinesterase (EC 3.1.1.8) with cellulose are also reported.

Introduction

The peptide hydrolase papain (EC 3.4.22.2) has been used widely as a primary agent in several processes, including those of beer chill-proofing and milk clotting. Alternatively, it may be feasible to effect such processes by the proteolytic action of suitable water-insoluble derivatives of papain whose use may be expected to provide several benefits. These benefits may include the opportunity to re-use or continuously use the same enzyme, better control of substrate treatment and the non-contamination of treated substrates by soluble enzyme. The development of variegated and satisfactory procedures for the insolubilisation of papain thus assumes considerable importance.

Contemporary methods of insolubilising enzymes mostly depend on the adsorption, molecular entrapment, cross-linking or covalent binding of enzyme [1—5]. Although advantages are associated with each of these modes of

enzyme insolubilisation, that of covalent binding has proved least problematical and has been the most thoroughly investigated. However, nearly all of the many attempts to insolubilise enzymes by covalent binding have been confined to those supports which are easily activated for the binding of enzyme by their simple chemical derivatisation. Often too, a particular method of enzyme insolubilisation is limited to a group of supports whose structures are related chemically. In exception, Gray et al. [6] have reported a procedure that involves, in principle, the covalent attachment of enzyme to a support coating of diazotised 1,3-diaminobenzene and that thereby enables enzymes to be bound to water-insoluble materials of multifarious chemical and physical structures. The present paper describes the results of an investigation into the suitability of this procedure for the insolubilisation of papain on a selection of supports and a modification to the coupling scheme yielding water-insoluble derivatives of papain having improved catalytic properties. With regard to a need to investigate and expand further the application of (diazotised 1,3-diaminobenzene)-coated supports to the insolubilisation of other enzymes, some properties of both urease (EC 3.5.1.5) and cholinesterase (EC 3.1.1.8) conjugates with (diazotised 1,3-diaminobenzene)-coated cellulose are also reported.

Materials and Methods

Materials. The following were used: papain (EC 3.4.22.2), 1,3-diaminobenzene, cellulose (crystalline, Whatman CC31) coated with diazotised 1,3-diaminobenzene which has fluoroborate as counterion (Enzacryl fluoroborate), urease (EC 3.5.1.5) and urease insolubilised on (diazotised 1,3-diaminobenzene)-coated cellulose (Enzacryl urease), all Koch-Light Laboratories Ltd; cholinesterase (EC 3.1.1.8), Worthington, Biochemical Corp; titanium (IV) oxide powder, batch CLD 786, gift from British Titan Products; macroporous cellulose (cellulose hydroxypropylated at position 6) experimental batch given by Pharmacia Fine Chemicals; microcrystalline cellulose, Sigmacell Type 38, Sigma Chemical Co. Ltd.

Buffers were prepared following the methods described by Dawson et al. [7]. Unless otherwise stated in ensuing descriptions, "buffer" refers to sodium acetate (0.1 M), phosphate (0.1 M) or borate (0.1 M) buffer for procedures performed at pH 4.5, 7.0 or 9.0, respectively.

Estimation of particle densities and sizes. Densities of particles were calculated from their dry weight and the volumes of water displaced when the dried materials were submerged in water and deaerated. Average particle diameters were estimated with a calibrated eye-piece microscope from measurements, in a common direction, of 125 stationary particles chosen randomly from a scattered sample. The total surface area per unit weight of material was calculated from the distribution of particle diameters and the estimated density, assuming the particles to be spherical.

Determination of enzymic activities. All incubations of water-insoluble enzymes with substrate were performed with stirring and all spectrophotometic measurements were carried out on precentrifuged solutions, using a Unicam SP500 spectrophotometer.

Using a Radiometer Autoburette ABU11, Autotitrator TTT2 and Titrigraph system, papain and papain derivatives were assayed for esterolytic activity, against *N*-α-benzoyl-L-arginine ethyl ester (BAEE), by titrating at 25° C the acid released at the pH of maximum activity on incubation in the presence of L-cysteine ($5 \cdot 10^{-3}$ M) and EDTA ($4 \cdot 10^{-4}$ M) [8]. EDTA was omitted for the assay of zinc (II)-papain and its derivatives. One unit of esterolytic activity is defined as the activity that hydrolyses 1 μmol of substrate per min at 25° C.

Proteolytic activities of papain and papain derivatives were assayed by spectrophotometric determination of the acid-soluble products released from Hammarsten casein at pH 6.5 and 37° C in the presence of L-cysteine ($5 \cdot 10^{-3}$ M) and EDTA ($4 \cdot 10^{-4}$ M) [9]. One unit of proteolytic activity is defined as the activity that gives an increase of $A^{1cm}_{280nm} = 1.00$ per min (final solution volume, 5.0 ml).

Urease activity was determined by titrating (bromocresol green-methyl red indicator) the ammonia released at 25° C from urea (3.0%, w/v) in sodium phosphate/citrate buffer (0.15 M), containing EDTA (10^{-3} M) and bovine serum albumin (0.28 mg/ml) [10]. One unit of urease activity is defined as the activity that releases 1 μmol of ammonia per min at 25° C.

Cholinesterase activity was determined by measuring the absorbance increased on incubation at 25° C with a solution of butyrylthiocholine iodide (0.1 M) in deoxygenated sodium phosphate/citrate buffer (0.1 M) and subsequent treatment with 5,5′-dithio-bis-(2-nitrobenzoic acid) solution [11]. One unit of cholinesterase activity is defined as the activity which produces an increase of $A^{1cm}_{412nm} = 1.00$ per min (final solution volume, 5.25 ml).

Preparation of (diazotised 1,3-diaminobenzene)-coated supports for papain insolubilisation. Enzacryl fluoroborate (3.0 g) was prepared for coupling to papain by adding at 0° C buffer (60 ml) of the pH desired for coupling papain. The mixture was stirred, then centrifuged and the supernatant liquid discarded.

3.0 g macroporous cellulose and titanium (IV) oxide (3.0 g) were each coated with diazotised 1,3-diaminobenzene by treatment with HCl (1 M; 60 ml) and 1,3-diaminobenzene (1.5 g) and after 5 min, sodium nitrite solution (2%, w/v; 160 ml) was added, all at 0° C. After 30 min stirring at 0° C, followed by centrifugation, the solid was repeatedly washed at 0° C with buffer (150 ml), of the pH required for coupling papain, until the supernant was clear on centrifugation.

General procedure for coupling papain [6]. Papain solution (2.0 mg/ml; 15.0 ml) in activating buffer (containing L-cysteine · HCl ($5 \cdot 10^{-3}$ M) of one pH to be used throughout the procedure) was added to the freshly prepared (diazotised 1,3-diaminobenzene)-coated support (3.0 g) and the suspension was stirred at 4° C. After 2 h a saturated solution of 2-naphthol in saturated sodium acetate (60 ml), was added and the suspension was stirred at 4° C for 4.5 h. After centrifuging, the solid was washed alternately five times with activating buffer (60 ml, aq.) and a solution of both sucrose (1 M) and NaCl (1 M) in activating buffer (60 ml). The solid was then washed twice with a solution of both L-cysteine · HCl (10^{-3} M) and EDTA ($4 \cdot 10^{-4}$ M) in water (60 ml) which had been adjusted to pH 6.5 with NaOH solution (1.0 M).

Modified procedure for coupling papain. The general coupling procedure was modified by omitting EDTA from all solutions except wash solutions and by

incorporating zinc (II) sulphate ($1.35 \cdot 10^{-3}$ M) in the buffer used for coupling. Assay of the zinc (II) sulphate/papain solution for esterolytic activity in the absence of EDTA was used to verify the complete inhibition of the enzyme before its insolubilisation.

Determination of the protein contents of water-insoluble papains. Samples of water-insoluble papains were washed with water thoroughly, dried, weighed and hydrolysed with HCl (6 M) in vacuo for 24 h at 110°C. Standard papain solutions were similarly hydrolysed, in the presence of proportionate quantities of support material, to provide standards for the determination of the protein contents of sample hydrolysates, by the chromatographic procedure of Kay et al. [12]. Descending chromatography of hydrolysate was on Whatman No. 1 paper using the upper phase of a water/*n*-butanol/acetic acid mixture (5:4:1, v/v) for irrigation. Bands R_F 0.07 and R_F 0.20 provided estimates of protein content in close agreement; the average result is reported.

Investigation of water-insoluble derivatives of urease and of cholinesterase. The pH vs. activity profiles of Enzacryl urease (maximum activity $7.45 \cdot 10^{-2}$ units/mg of support) and of cholinesterase insolubilised on (diazotised 1,3-diaminobenzene)-coated cellulose (prepared by the general procedure for enzyme insolubilisation of Gray et al. [6]) were determined and compared to the determined pH vs. activity profiles of native urease (maximum specific activity, 1.02 units/mg) and native cholinesterase (maximum specific activity 1.44 units/mg), respectively.

Results and Discussion

A common shortcoming of the insolubilisation of enzymes by covalent attachment to water-insoluble supports is an accompanying reduction in enzyme specific activity attributable to the chemical modification of enzyme functional groups essential for catalysis or substrate binding. This effect may be obviated through the insolubilisation of an inactive zymogen [13] or enzyme-inhibitor complex [14—16]; a subsequent reactivating stage yields the water-insoluble enzyme with enhanced specific activity.

Since papain is reversibly inhibited in combination reactions by some divalent cations [17,18] we predicted that such reactions would provide a means of protecting papain from chemical disruption at the active site, and concomitant inactivation, during covalent insolubilisation. Using a zinc (II) system as inhibitor, zinc (II) sulphate, ($1.35 \cdot 10^{-3}$ M) was demonstrated to inhibit completely solutions of papain (2.0 mg/ml) in buffers (pH range 4.5—9.0) containing L-cysteine ($5 \cdot 10^{-3}$ M). (Modification of these conditions may be necessary for papain preparations of different activity and purity). The presence of L-cysteine was necessary to ensure that the inhibition of papain occurred solely by the reaction of the active site with zinc (II) and not by the oxidation of the essential sulphydryl group, and was essential for complete solubilisation of zinc (II) in zinc (II) sulphate/papain solutions above pH 7. The observation that papain is inhibited fully by reaction with zinc (II) in the presence of excess L-cysteine accords with the known preference of zinc (II) to react with the active site of papain rather than the sulphydryl group of L-cysteine [18]. It was found that zinc (II)-inhibited papain may be activated

fully by the addition of EDTA in a quantity stoicheiometrically equivalent to the total amount of zinc (II) present. These observations enabled a modified procedure to be devised for the insolubilisation of papain. The (diazotised 1,3-diaminobenzene)-coated support is treated with a solution of papain (2.0 mg/ml), zinc (II) sulphate ($1.35 \cdot 10^{-3}$ M) and L-cysteine ($5 \cdot 10^{-3}$ M) in buffer. Subsequent wash solutions then provide sufficient EDTA to activate fully the bound zinc (II)-papain.

Of the modified and unmodified schemes of enzyme insolubilisation the former gives the highest esterolytic and proteolytic activities when papain is insolubilised on (diazotised 1,3-diaminobenzene)-coated cellulose at pH values from 4.5 to 9.0, and the highest esterolytic activity when papain is insolubilised on (diazotised 1,3-diaminobenzene)-coated titanium (IV) oxide at pH 7.0 (Table I). Zinc (II)-papain derivatives of the pre-coated cellulose, macroporous cellulose and titanium (IV) oxide supports, prepared by the modified scheme but omitting EDTA, exhibited low levels of esterolytic activity relative to the corresponding fully activated preparations (Table II), thus demonstrating that a high proportion of zinc (II) had remained bound to papain during protein coupling and subsequent washes. It may be concluded that zinc (II), when bound at the active site of papain during attachment of the enzyme to coatings of diazotised 1,3-diaminobenzene in the pH range 4.5—9.0, increases the potential catalytic activity of the resultant water-insoluble enzyme. This result is not a consequence of an induced increase in protein content for the water-insoluble enzyme, since the presence of zinc (II) at the protein-coupling stage did not augment significantly the final protein contents of the papain derivatives of (diazotised 1,3-diaminobenzene)-coated cellulose (Table III). Thus the efficacy of the addition of zinc (II) at the coupling stage, is a direct result of a mechanism acting to protect the active site of papain from chemical modification.

TABLE I

ESTEROLYTIC AND PROTEOLYTIC ACTIVITIES OF PAPAIN INSOLUBILISED ON (DIAZOTISED 1,3-DIAMINOBENZENE)-COATED SUPPORTS

(Diazotised 1,3-diaminobenzene)-coated support of enzyme	Coupling conditions	Esterolytic activity per unit weight of support (units/g)	Percent of offered esterolytic activity exhibited	Proteolytic activity per unit weight of support (units/g)	Percent of offered proteolytic activity exhibited
Cellulose	pH 4.5, +Zn (II)	2.59	19.1	0.164	2.18
	pH 4.5, no Zn (II)	1.52	10.9	0.147	1.94
	pH 7.0, +Zn (II)	3.93	28.8	0.251	3.22
	pH 7.0, no Zn (II)	2.26	16.6	0.189	2.43
	pH 9.0, +Zn (II)	2.71	20.3	0.210	2.84
	pH 9.0 no Zn (II)	2.04	14.4	0.149	1.84
Titanium (IV) oxide	pH 7.0, +Zn (II)	1.55	10.8	0.109	1.33
	pH 7.0, no Zn (II)	1.08	7.5	n.d.	n.d.
Macroporous cellulose	pH 7.0, +Zn (II)	2.03	14.2	0.333	4.05

n.d., not determined.

TABLE II

ESTEROLYTIC ACTIVITIES OF INSOLUBLE ZINC (II)-PAPAINS UNTREATED BY EDTA

Preparations and assay conditions were as described in Materials and Methods.

(Diazotised 1,3-diaminobenzene)-coated support of zinc (II)-papain	Enzyme coupling pH	Esterolytic activity per unit weight of support (units/g)	Percent of offered esterolytic activity exhibited
Cellulose	4.5	0.529	3.92
	7.0	0.967	7.11
	9.0	0.855	6.38
Titanium (IV) oxide	7.0	0.200	1.50
Macroporous cellulose	7.0	0.200	1.50

Cysteine and histidine residues, or their respective free amino acids become modifed at their side-chain functional groups in reaction with some diazonium salts [21—26]. Thus, since the active site residues L-cysteine-25 and L-histidine-159 are essential for the catalytic action of papain [19,20] reactions between these residues and diazotised 1,3-diaminobenzene coatings may occur and be partly responsible for loss of potential activity during the insolubilisation of papain by the unmodified procedure of Gray et al. [6]. Indeed Löeffler and Schneider [27] have correlated the modification of one L-histidine residue with loss of activity in the reaction of papain with the diazonium species, 5-diazonium-1-H-tetrazole. Putatively, in zinc (II)-papain, zinc (II) is bound to L-cysteine-25 which in turn is only 0.34 nm distant from L-histidine-159

TABLE III

PROTEIN CONTENTS AND ESTIMATED SURFACE AREAS PER UNIT WEIGHT OF WATER-INSOLUBLE PAPAINS

Procedures were as described in Materials and Methods.

(Diazotised 1,3-diaminobenzene)-coated support of enzyme	Average particle diameter (cm) $\times 10^3$	Particle density (g/cm^3)	Estimated total surface area per unit weight (cm^2/g)	Coupling conditions	Protein bound per unit weight of support (mg/g)	Percent of offered protein coupled
Cellulose	2.90	1.43	755	pH 4.5, +Zn (II)	0.980	10.9
				pH 4.5, no Zn (II)	0.897	10.1
				pH 7.0, +Zn (II)	1.23	11.5
				pH 7.0, no Zn (II)	1.21	11.9
				pH 9.0, +Zn (II)	1.27	13.0
				pH 9.0, no Zn (II)	1.30	13.2
Titanium (IV) oxide	20.4 *	3.26	65.3	pH 7.0, +Zn (II)	0.508	5.13
Macroporous cellulose	2.46	1.79	708	pH 7.0, +Zn (II)	1.15	11.6

* Measured after use. Uncoated titanium (IV) oxide had an average particle diameter of $3.42 \cdot 10^{-2}$ cm.

[28,29]. Therefore during the coupling of papain to coatings of diazotised 1,3-diaminobenzene, zinc (II) preserves potential activity by protecting one or both of the essential residues from chemical modification. This protective action is postulated to occur through steric shielding and/or deactivation of one or both of the essential sulphydryl and imidazole moieties towards electrophilic attack by diazonium groups.

The protein contents of papain derivatives of (diazotised 1,3-diaminobenzene)-coated cellulose prepared at one pH showed no significant variation with the type of protein-coupling procedure (Table III), suggesting that reactions between active site residues and diazotised 1,3-diaminobenzene make an insignificant contribution to the total binding of papain. Since L-lysine and L-tyrosine residues in papain react readily with 5-diazonium-1-H-tetrazole [27], diazo-coupling reactions between coatings of diazotised 1,3-diaminobenzene and the plentiful L-lysine and L-tyrosine residues of papain are expected to be predominantly responsible for attachment of the enzyme to the supports. The greater reactivity of L-lysine than L-tyrosine and L-tryptophan residues of papain with 5-diazonium-1-H-tetrazole at pH 9.0 [27] is consistent with the binding of a higher proportion of offered papain to (diazotised 1,3-diaminobenzene)-coated cellulose at pH 9.0 than at lower pH values (Table III), since increased protonation of the ϵ-amino group of L-lysine at lower pH would be expected to result in lowered reactivity towards positively charged diazonium groups.

That the (diazotised 1,3-diaminobenzene)-coated titanium (IV) oxide derivative of papain had a significantly lower weight of bound protein per unit weight than the corresponding cellulose and macroporous cellulose derivatives of papain (Table III) reflects the respective surface areas per unit weight available for enzyme attachment: the larger particle size and higher density of the (diazotised 1,3-diaminobenzene)-coated titanium (IV) oxide derivative result in a surface area per unit weight an order smaller than for the other derivatives (Table III). The low esterolytic activity per unit weight of the (diazotised 1,3-diaminobenzene)-coated titanium (IV) oxide derivative (Table I) is therefore a consequence of the low protein content per unit weight of the preparation.

Specific esterolytic and proteolytic activities of the water-insoluble papains were calculated by division of the appropriate percentage of offered activity exhibited by the percentage of offered protein bound.

The specific esterolytic activities of the water-insoluble papains were greater than that of the native papain from which they had been derived, irrespective of the procedures used to couple the enzyme (Tables I and III). This suggests that, during enzyme insolubilisation in the absence of Zn (II), any loss of specific esterolytic activity resulting from chemical modification at the active site, (which may be assumed to occur in view of the protection afforded by zinc (II) when bound at the active site), is more than counter-balanced by some other factor. Such a factor may involve the selective insolubilisation of only those enzyme mocecules which are catalytically viable. Increases in the specific esterolytic activity of papain on insolubilisation by other methods have also been reported [30,31].

Papain derivatives of (diazotised 1,3-diaminobenzene)-coated cellulose and titanium (IV) oxide supports possessed proteolytic to esterolytic activity ratios

and specific proteolytic activities lower than the respective values of the native papain from which they had been derived (Tables I and III). Papain insolubilised on (diazotised 1,3-diaminobenzene)-coated macroporous cellulose exhibited a proteolytic to esterolytic activity ratio and a specific proteolytic activity both significantly higher than did the other papain derivatives although again they were much lower than the respective values of the native enzyme (Tables I and III). These results, and work in which macroporous cellulose and crystalline cellulose were used as carbonate derivatives for enzyme insolubilisation [32—34], strongly indicate that steric restrictions on the access of macromolecular substrates to the active sites of bound enzyme molecules operate to a lesser degree in the (diazotised 1,3-diaminobenzene)-coated macroporous cellulose derivative of papain than in the corresponding crystalline cellulose derivatives.

Other effects may be partly responsible for the low specific proteolytic activities and the low ratios of proteolytic to esterolytic activity recorded. A preferential reduction of proteolytic rather than of esterolytic activity has also been observed for diazo-coupling reactions between papain and a diazotised copolymer of 4-aminophenyl-L-alanine and L-leucine [35] and between succinyl-papain and 5-diazonium-1-H-tetrazole [27]. Such observations indicate that in reactions of papain with diazonium salts, the sub-sites which are involved in the binding of polypeptide substrates, but are remote from the active site involved in esterolysis [36], undergo chemical modification and that such modification is an important factor causing a disproportionate reduction in proteolytic activity.

The colours of coatings of diazotised 1,3-diaminobenzene depended characteristically on their underlying supports, dark brown on macroporous cellulose, red-brown on cellulose and light brown on titanium (IV) oxide. Although these colour differences may arise partly through different depths of coating, they also offer a clue to the reasons for differences between the pH vs. activity profiles of papain insolubilised on the pre-coated cellulose and on titanium (IV) oxide supports (Fig. 1). Each support may impose special features on the structure of the coating which, through effects on the microenvironmen-

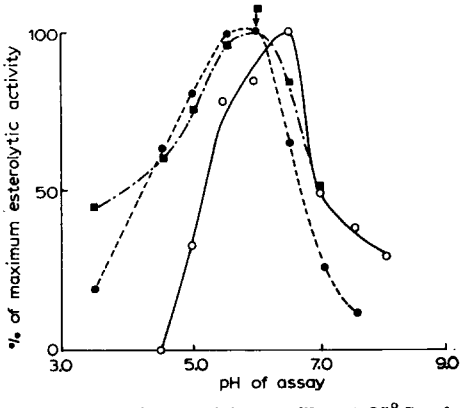

Fig. 1. The pH vs. activity profiles at 25°C, of papain (●- - - - -●), papain insolubilised (at pH 7.0 in the presence of zinc (II)) on (diazotised 1,3-diaminobenzene)-coated cellulose (■· · · ·■), and papain insolubilised on (diazotised 1,3-diaminobenzene)-coated titanium (IV) oxide (○———○).

tal pH and ionic properties at the solid-liquid interface, in turn influence the observed pH vs. activity profile of bound enzyme. Since the pH vs. activity profile of succinyl-papain is significantly altered on reaction with 5-diazonium-1-H-tetrazole [27], chemical modification of bound enzyme is predictably an important factor perturbing the pH vs. activity profile of papain on attachment to (diazotised 1,3-diaminobenzene)-coated supports. Shifts to lower pH were observed for the acid limbs of the pH vs. activity profiles of similarly immobilised urease and cholinesterase (Figs. 2 and 3, respectively). Thus perturbations to the pH vs. activity profiles of enzymes insolubilised on (diazotised 1,3-diaminobenzene)-coated supports may advantageously alter the pH range of enzyme action and are to be generally expected. The cholinesterase derivative of (diazotised 1,3-diaminobenzene)-coated cellulose was found to have a maximum activity of 0.106 unit/mg, which represents 7.36% of the activity offered in its preparation.

Lyophilisation of a (diazotised 1,3-diaminobenzene)-coated cellulose derivative of papain (enzyme coupled at pH 7.0 as zinc (II)-papain) used a 24.4% reduction in esterolytic activity but the preparation retained 42.0% of its remaining esterolytic activity on subsequent storage at 4.0°C for 280 days. Column packings of papain derivatives of pre-coated macroporous cellulose and titanium (IV) oxide supports, showed good stability to elution with substrate (BAEE) solution at pH 6.5 and 20°C (Table IV), although the flow rates were somewhat inferior to those of columns of the corresponding uncoated supports (Table V). The flow rates of columns of both the (diazotised 1,3-diaminobenzene)-coated titanium (IV) oxide derivative and uncoated titanium (IV) oxide decreased with use (Table V). This behaviour was probably a result of particle attrition during column operation, since the average particle size of the coated column packing, after use, was found to be less than that of the uncoated titanium (IV) oxide support before use (Table III). Even allowing for the lower esterolytic activity per unit weight of the papain derivative of

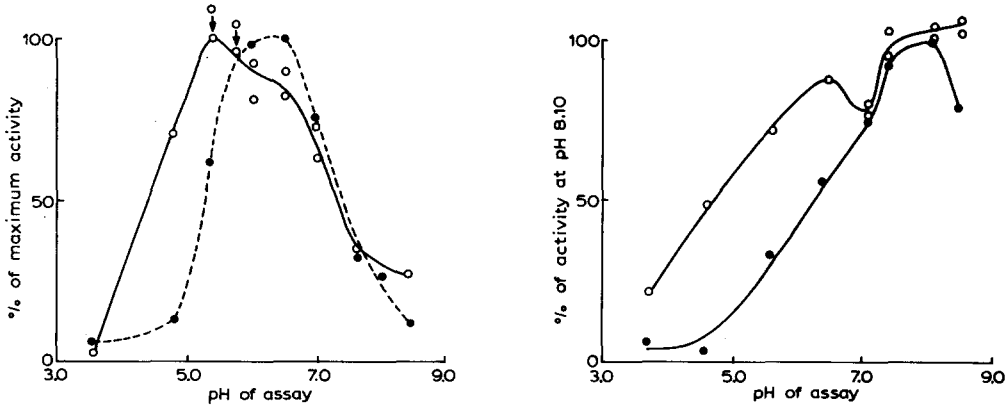

Fig. 2. The pH vs. activity profiles at 25°C, of urease (●· · · · · ●) and urease insolubilised on (diazotised 1,3-diaminobenzene)-coated cellulose (Enzacryl urease) (○———○).

Fig. 3. The pH vs. activity profiles at 25°C, of cholinesterase (●———●) and cholinesterase insolubilised on (diazotised 1,3-diaminobenzene)-coated cellulose (○———○).

TABLE IV

THE STABILITIES OF WATER-INSOLUBLE PAPAINS IN COLUMNS ELUTED WITH SUBSTRATE (BAEE) SOLUTION AT 20°C

The liquid phase of each column consisted of BAEE ($3.5 \cdot 10^{-4}$ M) in sodium phosphate buffer (0.1 M; pH 6.5) containing EDTA ($4 \cdot 10^{-4}$ M) and L-cysteine (10^{-3} M). Each column spent approx. 60% of the total time under flow.

(Diazotised 1,3-diaminobenzene)-coated support of enzyme	Time at 20°C (days)	Initial esterolytic activity per unit weight of support (units/g)	Final esterolytic activity per unit weight of support (units/g)	Percent of initial esterolytic activity lost
Titanium (IV) oxide	35	1.55	0.41	76.7
Macroporous cellulose	26	2.03	0.33	83.8

coated titanium (IV) oxide, compared to that of the corresponding macroporous derivative, the higher density of titanium (IV) oxide enables greater esterolytic activity to be packed per unit volume of column (Table VI). The papain derivative of coated macroprous cellulose, during column operation, retained its particle size and showed no deterioration in flow rate, and is thus a highly satisfactory column-packing material.

During incubations of the water-insoluble papains with substrate (BAEE) solution no leakage of free active papain was detectable by monitoring for continued conversion of substrate upon removal of the water-insoluble enzyme. This not only demonstrates that the washing procedure in the preparation of the water-insoluble papains removes any physically adsorbed enzyme but also indicates the chemical integrity of the preparations. Thus diazotised 1,3-diaminobenzene, when coated on an organic or inorganic support, may be used to produce water-insoluble papains, the properties of which are influenced by the

TABLE V

EFFECT OF COATINGS OF DIAZOTISED 1,3-DIAMINOBENZENE AND EFFECT OF COLUMN USE ON THE FLOW RATES OF SUPPORT MATERIALS

Each column was operated as described under Table IV. The pressure heads were identical for all columns.

Column packing	Column inside diameter (cm)	Column length (cm)	Initital flow rate (ml/h)	Flow rate after 11 days (ml/h)
Titanium (IV) oxide	0.40	10.1	85.6	25.7
Papain insolubilised on (diazotised 1,3-diaminobenzene)-coated titanium (IV) oxide	0.40	10.1	12.3	6.83
Macroporous cellulose	0.80	27.0	23.7	27.4
Papain insolubilised on (diazotised 1,3-diaminobenzene)-coated macroporous cellulose	0.80	21.0	6.55	7.58

TABLE VI

THE ACTIVITIES PER UNIT BED-VOLUME IN COLUMNS OF WATER-INSOLUBLE PAPAINS

Column packing material	Esterolytic activity per unit weight (units/g)	Proteolytic activity per unit weight (units/g)	Dry weight of packing material per unit volume of column bed (g/ml)	Esteroltyic activity per unit volume of column bed (units/ml)	Proteolytic activity per unit volume of column bed (units/ml)
Papain insolubilised on (diazotised 1,3-diaminobenzene)-coated macroporous cellulose	3.93	0.251	0.123	0.483	0.0309
Papain insolubilised on (diazotised 1,3-diaminobenzene)-coated titanium (IV) oxide	1.55	0.109	1.04	1.64	0.113

underlying support, include generally good chemical and catalytic stability, and augur well for their potential application.

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