Water-insoluble Papain Conjugates of Hydrous Titanium(IV) Oxide and of Surface-coating Materials Modified to contain Hydrous Titanium(IV) Oxide

By **John F. Kennedy** * and **Victor W. Pike**, Department of Chemistry, University of Birmingham, P.O. Box 363, Birmingham B15 2TT

Hydrous titanium(IV) oxide, on exposure to a dilute solution of papain (EC 3.4.22.2) at pH 7.0, has been demonstrated to bind the enzyme. The resultant water-insoluble hydrous titanium(IV) oxide–papain conjugate exhibits substantial esterolytic and proteolytic activity, a broad profile of pH *versus* esterolytic activity, and stable activity during storage in aqueous suspension at 4 °C. The foregoing observations are discussed in the light of the known chemical and physical structures of hydrous titanium(IV) oxide. An investigation has revealed that hydrous titanium(IV) oxide may be included in surfaces of emulsion paint or epoxy-resin and still retain an ability to bind papain. This property of hydrous titanium(IV) oxide forms the basis of a novel procedure for the convenient preparation of surface-coating–enzyme conjugates exhibiting catalytic activity and the properties of conjugates prepared by this procedure were investigated. Papain bound to hydrous titanium(IV) oxide that had been included in emulsion paint was found to possess substantial specific esterolytic activity and to maintain activity during storage, whereas papain bound to hydrous titanium(IV) oxide that had been included in epoxy-resin was found to possess lower specific esterolytic activity and to be less stable during storage. Such surface-coating–enzyme conjugates, having enzyme attached to a single surface, may be conveniently operated against continuous feeds of substrate solution. Further, since such conjugates are easily withdrawn from substrate solution, catalysis may be terminated rapidly. It has also been demonstrated that unmodified surfaces of glass, epoxy-resin, and emulsion paint form conjugates with papain by adsorption but that such conjugates are unstable to storage in substrate solution.

WATER-INSOLUBLE derivatives of enzymes offer many potential advantages over the soluble native enzymes. These advantages include the possible re-use or continuous use of the same enzyme, the control of substrate treatment and the non-contamination of treated substrate by soluble enzyme. Current methods for the insolubilisation of enzymes depend mostly on cross-linking, molecular entrapment, adsorption, or covalent binding processes.[1-7] By correct choice from these processes water-insoluble enzymes may often be prepared in the intended physical form. However, the catalytic properties of a water-insoluble enzyme depend critically on many factors including the electronic and steric microenvironment provided by the support and the nature of the linkages between support and enzyme.[1-7] Consequently it is often difficult to select or develop a procedure for the insolubilisation of an enzyme so that the procedure yields the desired combination of physical form, chemical structure, and catalytic properties in the product. Occasionally, as we report here, an established procedure may be adapted in order to obtain a water-insoluble enzyme with the desired combination of properties.

Several hydroxides and hydrous oxides of transition elements have received previous investigation of their ability to bind microbial cells,[8] proteins,[9,10] peptides,[9] and amino-acids.[9] The hydrous oxides of Ti^{IV}, Zr^{IV}, Fe^{III}, V^{III}, and Sn^{II} possess strong affinity for the binding of enzymes and provide microenvironments generally conducive to the maintenance of enzymic activity. Hydrous titanium(IV) oxide has proved to be especially suitable as a support for enzymes since it provides hydrophilicity, porosity, and resistance to biodegradation and is easily prepared by the hydrolysis of the readily available titanium(IV) chloride.

The protease, papain (EC 3.4.22.2), is a thiol-enzyme having considerable commercial importance, for example in beer chill-proofing, and therefore much effort has been directed towards producing suitable water-insoluble

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derivatives of the enzyme.[1-7,11] The present paper reports the successful use of free hydrous titanium(IV) oxide to insolubilise papain and further describes its use in a novel scheme for the production of water-insoluble papains in a physical form which allows their continuous operation and ready recovery from substrate solution. Thus, on exposure to a dilute solution of papain, coatings of epoxy-resin and emulsion paint, which have been premodified to contain hydrous titanium(IV) oxide, have been demonstrated to form chemically stable and catalytically active conjugates of the enzyme. Surfaces of unmodified glass, epoxy-resin, and emulsion paint, on exposure to papain solution, have each been demonstrated to form an unstable conjugate of the enzyme.

EXPERIMENTAL AND RESULTS

Assay of Proteolytic Activity.—In principle, proteolytic activities were determined by monitoring spectrophotometrically for acid-soluble products released by digestion of casein. In control experiments the presence in the assay systems of support materials (uncoupled to enzyme) did not affect the amounts of acid-soluble products released and detected. Substrate solution for each assay was freshly prepared by dissolving casein (Hammarsten, B.D.H. Ltd.; 2.0 g) in warm 0.1M-sodium phosphate buffer (pH 6.5; 95 ml). The solution was then heated at 100 °C for 20 min. The volume of the cooled substrate solution was finally adjusted to 100 ml by adding 0.1M-sodium phosphate buffer (pH 6.5).

(i) *Assay of hydrous titanium*(IV) *oxide–papain conjugate.*[12] Fresh substrate solution (3.0 ml) at 37 °C was added to a known portion, also at 37 °C, of hydrous titanium(IV) oxide–papain conjugate suspended in 0.1M-sodium phosphate buffer (pH 6.5; 3.0 ml) which contained both 10^{-3}-M-L-cysteine hydrochloride and 8×10^{-4}M-ethylenediaminetetra-acetic acid. The suspension was incubated for 10 min at 37 °C and then aqueous 5% w/v trichloroacetic acid solution (3.0 ml) was added to terminate the reaction. After 20 min at room temperature the resultant suspension was centrifuged and the optical density increase at 280 nm (path length 1.0 cm) of the supernatant liquid was measured relative to that of a solution obtained by the same procedure modified to have zero reaction-time.

Standard samples of original soluble papain (0—100 μg) were likewise assayed and a calibration curve of optical density at 280 nm against concentration of enzyme was thereby constructed. The initial slope of this curve was used to assess the specific activity of the enzyme from the definition that one unit of proteolytic activity (P) is that which gives an initial increase in optical density at a rate of 1.00 min^{-1} under the conditions of assay. The optical density change occurring during assay of the hydrous titanium(IV) oxide–papain conjugate was related, using the calibration curve, to a weight of soluble papain. This weight was multiplied by the calculated specific activity of the soluble enzyme to give a measure of the activity of the conjugate.

(ii) *Assay of surface–papain conjugates.*[12,13] Each assay was performed in duplicate. Substrate solution was diluted with an equal volume of 0.1M-sodium phosphate buffer (pH 6.5) which also contained 10^{-3}M-L-cysteine hydrochloride and 8×10^{-4}M-ethylenediaminetetra-acetic acid. An aliquot portion (4.0 ml) of the diluted substrate solution was added to the surface–papain conjugate (plate size 2.4 cm × 1.25 cm) and after 18 h incubation at 25 °C a portion (0.10 ml) of the incubated substrate solution was added to aqueous 5% w/v trichloroacetic acid solution (0.50 ml). The resultant precipitate was centrifuged off. A portion (0.20 ml) from the supernatant liquid was added to 0.5M-sodium hydroxide solution (0.40 ml) with thorough mixing. Folin and Ciocalteu's phenol reagent (B.D.H. Ltd.; 0.02 ml) was immediately added and then the solution was mixed thoroughly. After 20 min the optical density of the solution at 660 nm (path length 1.0 cm) was measured relative to that of a solution obtained by the same procedure modified so that the incubate contained the corresponding support material unbound to enzyme.

Standard samples of original soluble papain (0—25 μg) were likewise assayed and a calibration curve of optical density at 660 nm against concentration of enzyme was thereby constructed. The initial slope of this curve was used to assess the specific activity of the enzyme from the definition that one unit of proteolytic activity (F) is that which gives an initial increase in the optical density at a rate of 1.00 min^{-1} under the conditions of assay. The optical density change occurring during assay of the surface–papain conjugate was related, using the calibration curve, to a weight of soluble papain. This weight was multiplied by the calculated specific activity of the soluble enzyme to give a measure of the activity of the conjugate.

Assay of Esterolytic Activity.—(i) *Assay of hydrous titanium* (IV) *oxide–papain conjugate.*[14] The conjugate was assayed by an automated titrimetric method, using Radiometer equipment (Titrigraph, Autotitrator TTT2, and Autoburette ABU 11). A known weight of conjugate was suspended in activating diluent [a solution of both 10^{-3}M-L-cysteine hydrochloride and 4×10^{-4}M-ethylenediaminetetra-acetic acid (1.0 ml)] contained in the magnetically stirred cuvette of the autotitrator and thermostatted at 25 °C. The pH of the stirred suspension was adjusted automatically to the pH of assay (the pH of maximum activity unless otherwise stated) by the addition of standard 0.05M-sodium hydroxide solution. After a steady pH had been attained, a solution of 0.05M-*N*-α-benzoyl-L-arginine ethyl ester hydrochloride (BAEE) in activating diluent (1.0 ml), previously adjusted to the pH of assay with 0.05M-sodium hydroxide solution, was added. The suspension was stirred and the volume of standard 0.05M-sodium hydroxide solution required per min to maintain the pH at the set pH of the assay was recorded over at least 10 min. Under these conditions contributions to the required rate necessitated by non-enzymic hydrolysis of substrate or changes in concentration of absorbed carbon dioxide were found generally to be $<4\%$ of the total rate and correction was made to the overall rate as appropriate. In a control experiment the rate of non-enzymic hydrolysis of substrate was unaffected by the presence of hydrous titanium(IV) oxide unbound to enzyme. Soluble papain, at a final concentration of 0.50 mg ml^{-1}, was assayed likewise. One unit of esterolytic activity (E) is defined as the activity that hydrolyses one μmol of BAEE per min at 25 °C. Thus esterolytic activities were calculated from the corrected rates of addition of standard sodium hydroxide solution.

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[13] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, 1957, **193**, 265.
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(ii) *Assay of surface–papain conjugates.*[15] Each assay was performed in duplicate. The surface–papain conjugate (plate size 2.4 cm × 1.25 cm) was immersed completely in a portion (4.0 ml) of substrate solution [2.5×10^{-4}M-BAEE in 0.1M-sodium phosphate buffer (pH 6.5), which also contained both 10^{-3}M-L-cysteine hydrochloride and 4×10^{-4}M-ethylenediaminetetra-acetic acid], and then incubated at 25 °C. After 18 h the optical density at 253 nm (path length 1.0 cm) of the incubated substrate solution was measured. Account for non-enzymic hydrolysis of substrate was provided by likewise and simultaneously treating a surface (plate size 2.4 cm × 1.25 cm) of the same type having no bound enzyme. The difference between the obtained optical densities was taken to represent the increase in optical density at 253 nm attributable to enzymic hydrolysis of substrate. Contributions to this difference in optical density from the possible leakage of bound protein into substrate solution were estimated from control experiments. It was found that assay responses were sufficiently large to reduce possible but not necessarily incurred errors from this source to $<5\%$ for the activities of papain conjugates of paint surfaces and to $<10\%$ for the activities of papain conjugates of other surfaces.

Portions of substrate solution containing soluble papain at concentrations from 0 to 10 μg ml^{-1} were also incubated at 25 °C for 18 h. The optical density at 253 nm (path length 1.0 cm) of each solution was then measured against that of a substrate solution pre-incubated alone at 25 °C for 18 h. Each reading was corrected by substracting an appropriate measure of the optical density at 253 nm attributable to that of dissolved protein. Corrected values were used to construct a calibration curve of increase in optical density at 253 nm against concentration of papain. By the use of this calibration curve, assay responses for papain–surface conjugates could be related to a weight of soluble papain, originating from the same batch as that used to prepare the conjugates, and to its activity (in *E* units).

Determination of Bound Protein in Water-insoluble Papains.—The quantity of bound protein present in each water-insoluble papain was determined by a previously reported procedure [16,17] adapted from that of Kay *et al.*[18] The procedure involves complete hydrolysis (6M-HCl at 110 °C for 24 h *in vacuo*) of the water-insoluble papain followed by paper chromatography of the hydrolysate to separate amino-acids from possibly interfering materials. Amino-acids were detected by reaction with ninhydrin, eluted with ethanol, and monitored by spectrophotometry at 575 nm. Where necessary correction was made for any background colour that originated from support material and travelled with the same R_F value as the amino-acid spots chosen for elution. Standard amounts of soluble protein, in the presence of appropriate support material, were subjected to the same procedure to provide calibration curves relating total protein content and optical density at 575 nm (path length 1.0 cm). The protein contents of the water-insoluble papains were calculated from the appropriate optical density readings by reference to the calibration curves.

Preparation of Hydrous Titanium(IV) *Oxide.*—Water (10 ml) was added to titanium(IV) chloride (0.5 ml). The stirred mixture was immediately neutralised to pH 7.0 by the addition of 1.0M-ammonia solution. The resultant suspension was centrifuged. Hydrous titanium(IV) oxide was isolated by removal of the supernatant liquid and then washed with water (5 × 25 ml).

Preparation of Hydrous Titanium(IV) *Oxide–Papain Conjugates. Effect of the Presence of* Zn^{II} *during Enzyme-coupling.*—A solution of papain (EC 3.4.22.2; Koch–Light; 5.0 mg ml^{-1}) in 0.1M-sodium phosphate buffer (pH 7.0; 10 ml) containing both 10^{-2}M-L-cysteine hydrochloride and 4×10^{-4}M-ethylenediaminetetra-acetic acid was added to hydrous titanium(IV) oxide freshly prepared from titanium(IV) chloride (0.5 ml). After 1 h with stirring at 4 °C the mixture was centrifuged and the supernatant liquid discarded. The precipitate was then washed with 0.1M-sodium phosphate buffer (pH 7.0; 10 × 25 ml) which contained both 10^{-2}M-L-cysteine hydrochloride and 4×10^{-4}M-ethylenediaminetetra-acetic acid, and was finally stored in the same buffer (25 ml) at 4 °C.

Another hydrous titanium(IV) oxide–papain conjugate was obtained by the same procedure modified so that the solution of papain offered to the support also contained 3.38×10^{-3}M-zinc(II) sulphate but no ethylenediaminetetra-acetic acid.

The protein content (Table 1) and esterolytic and proteolytic activities (Table 2) of each preparation were determined. The specific esterolytic and proteolytic activities of the conjugates and of the soluble papain used in the preparation of the conjugates are shown in Table 3.

TABLE 1

Protein contents of hydrous titanium(IV) oxide–papain conjugates

Method of preparation of conjugate	Protein offered to support* (mg)	Protein bound to support (mg)	% Offered protein bound to support
+ Zn^{II}	45.9	27.6	60.1
no Zn^{II}	45.9	30.8	67.1

* Total weight of protein offered to support of hydrous titanium(IV) oxide prepared from 0.5 ml of titanium(IV) chloride.

Effect of Storage in Suspension at 4 °C *on the Esterolytic Activity of Hydrous Titanium*(IV) *Oxide–Papain Conjugates.*—From the time of their preparation, hydrous titanium(IV) oxide–papain conjugates were stored at 4 °C in 0.1M-sodium phosphate buffer (pH 7.0; 25 ml) containing both 10^{-2}M-L-cysteine hydrochloride and 4×10^{-4}M-ethylenediaminetetra-acetic acid. At intervals during storage, samples of each conjugate were withdrawn and assayed for esterolytic activity (Figure 1).

Stability of Hydrous Titanium(IV) *Oxide–Papain Conjugate.*—After 28 days storage of the hydrous titanium(IV) oxide–papain conjugate, prepared in the absence of Zn^{II}, an assay was performed for the presence of esterolytic activity in the liquid phase only of the storage suspension, but no activity was detected. The separated conjugate was also assayed for esterolytic activity (Figure 1). At the end of the assay (incubation period 20 min) the assay mixture was centrifuged. The supernatant substrate solution was removed and then reassayed for esterolytic activity, but again no activity was detected.

Effect of Lyophilisation on the Esterolytic Activity of Hydrous Titanium(IV) *Oxide–Papain Conjugates.*—A sample of each hydrous titanium(IV) oxide–papain conjugate was

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[17] J. F. Kennedy and A. Rosevear, *J.C.S. Perkin I*, 1974, 757.
[18] R. E. Kay, D. C. Harris, and C. Enterman, *Arch, Biochim. Biophys.*, 1956, **63**, 14.

assayed for esterolytic activity before and after lyophilisation from a solution of both 10^{-3}M-L-cysteine hydrochloride and 4×10^{-4}M-ethylenediaminetetra-acetic acid which had been adjusted to pH 6.5 with 0.1M-sodium hydroxide solution (Table 3).

TABLE 2

Initial esterolytic and proteolytic activities of hydrous titanium(IV) oxide–papain conjugates

Method of preparation of conjugate	Esterolytic activity offered to support * (*E* units)	Esterolytic activity bound to support (*E* units)	% Offered esterolytic activity bound to support	Proteolytic activity offered to support * (*P* units)	Proteolytic activity bound to support (*P* units)	% Offered proteolytic activity bound to support
$+Zn^{II}$	86.2	53.3	61.8	60.6	9.09	15.0
no Zn^{II}	86.2	60.0	69.6	60.6	12.7	21.0
	88.0	57.2	65.0	60.6	10.3	17.0

* Total activity offered to support of hydrous titanium(IV) oxide prepared from 0.5 ml of titanium(IV) chloride.

The pH versus *Esterolytic Activity Profile of Hydrous Titanium*(IV) *Oxide–Papain Conjugate.*—Values of the esterolytic activity of papain and of hydrous titanium(IV) oxide–papain conjugates were determined at pH values in the range 3.5—9.0 (Figure 2).

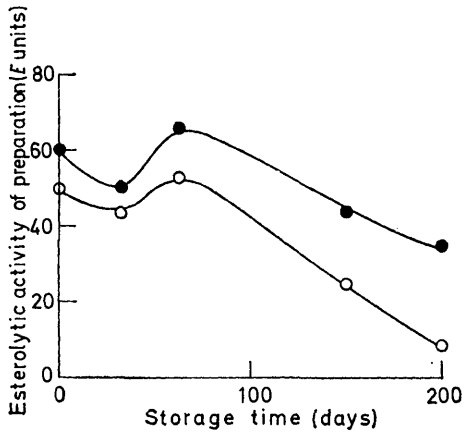


FIGURE 1 The effect of storage in suspension at 4 °C on the esterolytic activities of hydrous titanium(IV) oxide–papain conjugates: (i) conjugate prepared in the absence of Zn^{II}, ●; (ii) conjugate prepared in the presence of Zn^{II}, ○

Preparation of Surfaces of Glass, Epoxy-resin, Modified Epoxy-resin, Emulsion Paint, and Modified Emulsion Paint.—(i) *Glass surfaces.* Glass plates (2.4 cm × 1.25 cm) were cut from microscope slides, washed successively with chloroform, acetone, and water, and then dried. All glass plates which were subsequently to be coated were scored on one side to aid the adhesion of the coating.

(ii) *Epoxy-resin surfaces.* Glass plates (2.4 cm × 1.25 cm) were each coated on one side with epoxy-resin by applying a freshly prepared mixture of hardening agent (Araldite Hardener HY 951; 2 parts by volume) and epoxy-resin (Araldite Epoxy-resin MY 753, batch number 11/3921; 10 parts by volume). Each plate was allowed to harden at room temperature for 72 h. Finally each plate was washed in stirred, frequently changed distilled water for 48 h.

(iii) *Modified epoxy-resin surfaces.* Modified epoxy-resin surfaces were prepared by the procedure described for the preparation of epoxy-resin surfaces except that lyophilised hydrous titanium(IV) oxide (20 mg per glass plate) was also included in the applied mixture.

(iv) *Emulsion paint surfaces.* Glass plates were each coated on one side with emulsion paint [Berger, Jenson and Nicholson Ltd; Magicote Vinyl Silk Finish (Honey)], allowed to dry at room temperature for 48 h, and then washed in stirred, frequently changed distilled water for 48 h.

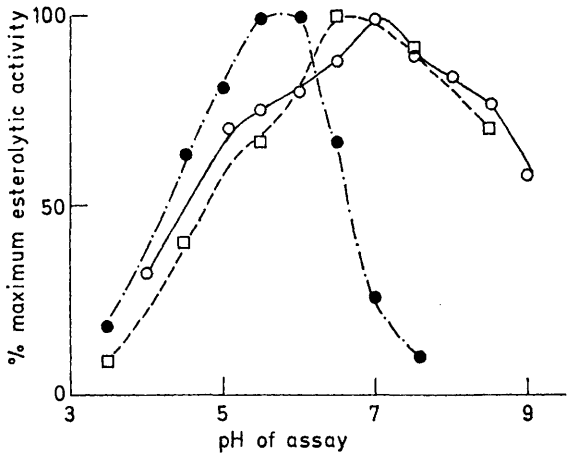


FIGURE 2 The profiles of pH *versus* esterolytic activity of: (i) papain, ●; (ii) hydrous titanium(IV) oxide–papain conjugate prepared in the presence of Zn^{II}, ○; (iii) hydrous titanium(IV) oxide–papain conjugate prepared in the absence of Zn^{II}, □

(v) *Modified emulsion paint surfaces.* Hydrous titanium(IV) oxide, originally prepared from titanium(IV) chloride (0.50 ml), was mixed with emulsion paint (*ca.* 0.80 g). Glass plates were each coated on one side with the mixture (*ca.* 40 mg) following the same process as that employed to prepare surfaces of unmodified emulsion paint.

Coupling of Papain to Surfaces.—Surfaces were pre-washed with 1M-hydrochloric acid and then water or with water

TABLE 3

Specific esterolytic and proteolytic activities of papain and hydrous titanium(IV) oxide–papain conjugates

Preparation	Specific esterolytic activity (*E* units/mg protein)	Specific proteolytic activity (*P* units/mg protein)
Soluble papain	1.88	1.32
Hydrous titanium(IV) oxide–papain conjugate (prepared with Zn^{II} present)	1.93 (0.00 after lyophilisation)	0.329
Hydrous titanium(IV) oxide–papain conjugate (prepared with Zn^{II} absent)	1.95 (0.13 after lyophilisation)	0.412

alone. Papain was then coupled to each type of surface by submerging the appropriate plate (2.4 cm × 1.25 cm) in a solution of papain (0.50 mg ml^{-1}) in 0.1M-sodium phosphate buffer (pH 7.0; 4.0 ml) which contained both 10^{-3}M-L-cysteine hydrochloride and 4×10^{-4}M-ethylenediaminetetra-acetic acid for 1.5 h at 4 °C. Each plate was washed thoroughly with distilled water (10 × 5.0 ml) and then initial esterolytic and proteolytic activities were measured (Table 4). Each activity reported for surface-coating–papain conjugates is the measured activity of the appropriate coated glass plate corrected by subtraction of the activity calculated from control experiments to be present on the reverse (uncoated) side of the plate.

TABLE 4

Initial esterolytic and proteolytic activities of surface–papain conjugates

Surface	Equivalent weight of esterolytically active papain per unit surface area (μg cm^{-2})	Esterolytic activity per unit surface area 10^3 (*E* units/cm^2)	Equivalent weight of proteolytically active papain per unit surface area (μg cm^{-2})	Proteolytic activity per unit surface area 10^5 (*F* units/cm^2)
Glass	0.167	0.314		
Epoxy-resin	0.666	1.25		
Emulsion paint	1.66	3.12		
Modified epoxy-resin	0.505	0.950	Not detected	Not detected
Modified emulsion paint	2.17	4.08	0.217	2.68

Effect of Storage at 4 °C *in Substrate (BAEE) Solution on the*

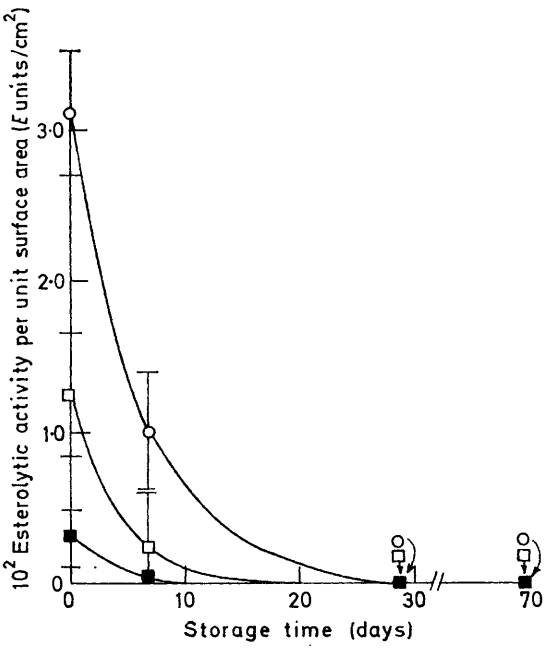


FIGURE 3 The effect of storage at 4 °C in substrate (BAEE) solution on the esterolytic activities of papain conjugates of unmodified surfaces of: (i) glass, ■; (ii) epoxy-resin, □; (iii) emulsion paint, ○

Esterolytic Activities of Surface–Papain Conjugates.—After initial assay, surface–papain conjugates were each washed with 1.0M-sodium phosphate buffer (pH 6.5; 3 × 5.0 ml) which contained both 10^{-3}M-L-cysteine hydrochloride and 4×10^{-4}M-ethylenediaminetetra-acetic acid and then stored in substrate (BAEE) solution (5.0 ml) at 4 °C. At intervals the surface–papain conjugates were withdrawn from their storage solutions, washed as before storage, dried by absorbing excess moisture onto tissue paper, and reassayed for esterolytic activity (Figures 3 and 4). Estimates were made of the protein present in representative samples of each surface–papain conjugate after 70 days storage. For those conjugates which still possessed bound protein, estimates of the specific esterolytic and proteolytic activities were calculated on the assumption that no significant loss of protein occurred during storage (Table 5). This assumption is probably valid for those conjugates which exhibited near constant activity during storage but may be invalid for those conjugates which lost activity during storage.

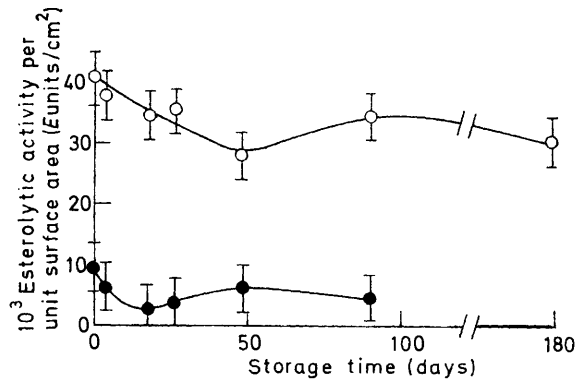


FIGURE 4 The effect of storage at 4 °C in substrate (BAEE) solution on the esterolytic activities of papain conjugates of surfaces of: (i) emulsion paint, pre-modified to contain hydrous titanium(IV) oxide, ○; (ii) epoxy-resin, pre-modified to contain hydrous titanium(IV) oxide, ●

TABLE 5

Specific esterolytic and proteolytic activities of papain and of surface–papain conjugates

Preparation	Specific esterolytic activity (*E* units/mg protein)	Specific proteolytic activity 10^3 (*F* units/mg protein)
Soluble papain	1.88	124
Papain conjugate of modified epoxy-resin surface	0.0673	Not detected
Papain conjugate of modified emulsion paint surface	0.180	1.18

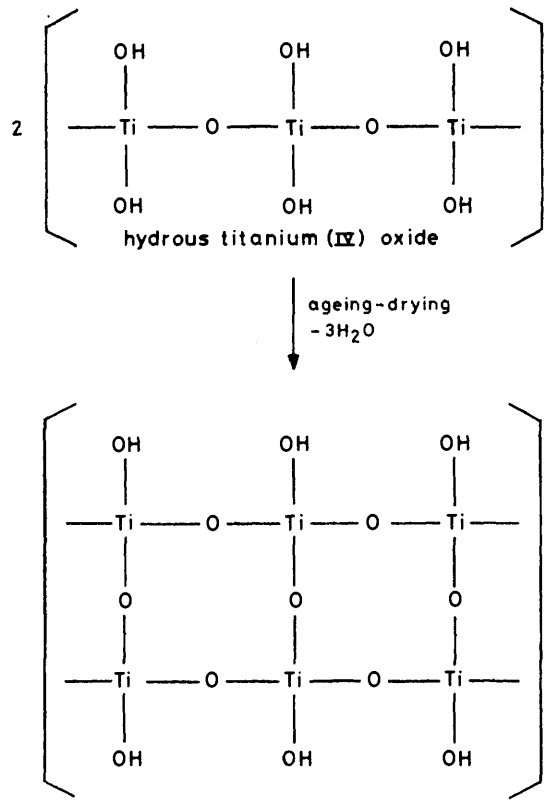
DISCUSSION

It has been reported that freshly precipitated hydrous titanium(IV) oxide binds certain microbial cells (*Saccharomyces cerevisiae, Escherica coli, Serratia marcescens*, and *Acetobacter*),[8] the enzyme, D-glucose oxidase (EC 1.1.3.4),[10] and the peptide, lathumycin[9]. Knowledge of the structure of freshly prepared hydrous titanium(IV) oxide is extensive and has been reviewed previously.[10] The structure has been assigned the empirical formula TiO-

$(OH)_2$[19] and features a linear polymeric chain of alternating oxygen and titanium atoms.[20] Two hydroxy groups are known to be pendant to each titanium atom of the chain (Scheme).[20] On this basis interactions have been postulated [10,21] to arise through displacement of the hydroxy groups or surface-adsorbed water [20,22] of hydrous titanium(IV) oxide by ligands on the entering moiety (cell, protein, or peptide). This has seemed reasonable since examples of similar chelation, often involving hydroxy groups as ligands to species having a single titanium(IV) atom, are profuse.[23-25] In addition to hydroxy groups, proteins and peptides may possess side-chain amino, carboxy, and thiol groups and these must be considered as possible candidates to act as ligands to titanium(IV) in hydrous titanium(IV) oxide. In a related study [9] both carboxy and amino groups have been implicated in binding the amino-acids, L-glutamic acid and L-lysine, to zirconium(IV) hydroxide. In view of these considerations hydrous titanium(IV) oxide is expected to exhibit a general affinity for the binding of protein and therefore would be predicted to serve as a potential support for the water insolubilisation of papain.

Our investigations demonstrate that hydrous titanium(IV) oxide, on contact with a dilute solution of papain at pH 7.0 and at 4 °C, binds the enzyme (Table 1). Usefully high loading of hydrous titanium(IV) oxide with protein occurs under the reported conditions of enzyme-coupling although these conditions may not be optimal. We therefore next investigated the catalytic properties of such conjugates and endeavoured to obtain some information on the degree of interaction of hydrous titanium(IV) oxide with those amino-acid residues in papain which are essential for catalytic activity.

In a previous study [11] of the insolubilisation of papain onto pre-formed diazonium-type supports it was found that the active-site of papain may be protected from chemical modification and concomitant inactivation during insolubilisation of the enzyme by previously complexing the active-site with Zn^{II}. Subsequently ethylenediaminetetra-acetic acid was employed to generate greater activity than may be obtained by a parallel procedure omitting Zn^{II}. The L-cysteine-25 and L-histidine-159 residues of papain are essential for its catalytic activity [26,27] and thus the protective action of Zn^{II} was postulated to result from steric shielding and/or deactivation of one or both essential amino-acid residues from electrophilic attack by diazonium groups. In the present study the protection of the active site of papain by complexation with Zn^{II} before coupling of the enzyme to hydrous titanium(IV) oxide affected little the protein content (Table 1) or esterolytic and proteolytic activities of the product (Table 2). This evidence alone strongly indicates that neither the L-cysteine-25 nor L-histidine-159 residue plays a significant role in binding papain to hydrous titanium(IV) oxide and may imply that L-cysteine and L-histidine residues are not utilised generally in binding proteins to hydrous titanium(IV) oxide. The L-tyrosine residues of papain are known to undergo chemical modification without affecting the specific esterolytic activity of the enzyme.[28,29] Therefore, the possibility that papain binds through L-tyrosine hydroxy ligands to



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hydrous titanium(IV) oxide is not only chemically feasible but also accords with the observation that specific esterolytic activity is totally retained by papain protein on attachment to hydrous titanium(IV) oxide (Table 3).

The specific proteolytic activities and the ratios of specific proteolytic activity to specific esterolytic activity of hydrous titanium(IV) oxide–papain conjugates were substantially lower than the respective values of the soluble papain from which the conjugates were prepared (Table 3). A preferential reduction of proteolytic activity rather than of esterolytic activity occurs during the insolubilisation of papain and other proteolytic en-

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zymes by many procedures.[9,11,16,17,30,31] Generally this phenomenon has been attributed to the operation of steric restrictions on the access of *macromolecular* substrates to the active sites of bound enzyme. The existence of this effect is supported by the observation that replacement of *crystalline* by *macroporous* cellulose (a material porous to macromolecules) as the support, in many methods of enzyme insolubilisation, results in water-insoluble enzymes with greater activity towards macromolecular substrates.[11,16,17,30] It has also been postulated [11] that the sub-sites of papain, which are involved in binding polypeptide substrates but are remote from the active site involved in esterolysis,[32] may be chemically modified in the bound enzyme, thereby preferentially reducing activity towards macromolecular substrates. Thus, for example, *soluble* succinylpapain on treatment with 1*H*-tetrazole-5-diazonium ion loses a greater proportion of proteolytic activity than of esterolytic activity.[28] However, despite the possible operation of such effects, the proportion of specific proteolytic activity retained by papain protein on attachment to hydrous titanium(IV) oxide is higher than corresponding proportions reported to be retained by papain on attachment to many other supports.[11] Hydrous titanium(IV) oxide, freshly prepared from titanium(IV) chloride and ammonia solution, as described here, not only exhibits surface hydrophilicity but is also known to be porous.[33] The presence of porosity and surface hydrophilicity in hydrous titanium(IV) oxide–papain conjugates predictably reduces steric restriction on the access of macromolecular substrates to active sites compared to the steric restriction operating in enzyme conjugates of other less porous supports. For this reason the exhibition of favourable activities towards macromolecular substrates is expected to be a general property of hydrous titanium(IV) oxide–enzyme conjugates. Such a property may have great importance since the major areas of interest of enzymic reactions to industry are in the hydrolyses of high molecular weight substrates.

The operational lifetime of a water-insoluble enzyme is an important factor determining its commercial viability. Hydrous titanium(IV) oxide–papain conjugates retain a large proportion of their esterolytic activity after 200 days storage at 4 °C in suspension (Figure 1). Such good stability to storage again probably reflects the surface hydrophilicity and porosity of hydrous titanium(IV) oxide. Although freshly prepared hydrous titanium(IV) oxide is amorphous, on ageing some conversion to the anatase crystal form is detectable [19] and such structural alterations may be responsible for oscillation in the catalytic activity of hydrous titanium(IV) oxide papain–conjugate on ageing. However, no active soluble papain was found to be liberated from hydrous titanium(IV) oxide–papain conjugate during storage in buffer or in substrate solution therefore indicating that interactions between hydrous titanium(IV) oxide and papain protein are adequately stable.

The lyophilised forms of water-insoluble enzymes are often most convenient for storage and transportation. However lyophilisation of the hydrous titanium(IV) oxide–papain conjugates was found to result in 90—100% destruction of esterolytic activity (Table 3). The drying or ageing in air of hydrous titanium(IV) oxide is known to result in cross-linking of the linear polymeric chains of alternating titanium and oxygen atoms through loss of water to give a structure having a titanium atom: hydroxy group ratio of 1 (Scheme).[20] Such drastic structural changes, involving formation of a less hydrophilic microenvironment and almost certainly structural distortion to bound enzyme, may be expected also to occur during lyophilisation of hydrous titanium(IV) oxide–papain conjugates and may account for the observed loss of esterolytic activity on lyophilisation. This is further implied by the observation that a serious loss of activity occurs on lyophilisation of a hydrous titanium(IV) oxide–dextranase (dextranase, EC 3.2.1.11.) conjugate.[34]

The profile of pH *versus* esterolytic activity of hydrous titanium(IV) oxide–papain conjugate, compared with that of papain, shows a substantial shift in the alkaline limb to higher pH (Figure 2). It has been suggested that hydrous titanium(IV) oxide consists of positively charged particles below pH 2.5 and of negatively charged particles above pH 3.5 since ^{89}Sr has been found to be more strongly adsorbed on hydrous titanium(IV) oxide above pH 3.5 than below.[35] This evidence may suggest that hydrous titanium(IV) oxide perturbs the profile of pH *versus* esterolytic activity of papain to higher pH by acting as a polyanionic surface in the pH range of papain activity, effecting a decrease in the local pH at the surface of the water-insoluble enzyme compared with that in the bulk solution. Chemical modification and conformational distortion of papain are also expected to contribute to the perturbation of the profile of pH *versus* esterolytic activity of the enzyme on binding to hydrous titanium(IV) oxide.

The foregoing results demonstrate that hydrous titanium(IV) oxide efficiently binds papain forming a conjugate which is stable and has favourable catalytic properties. Nevertheless, in order to extract full benefit from the use of a water-insoluble enzyme to catalyse a chemical reaction it is necessary both to be able to recover conveniently water-insoluble enzyme from treated substrate solution and, for many applications, to be able to treat a feed of substrate solution continuously. Columns and stirred tanks are both reactor-types specifically designed to meet these two requirements. However, freshly prepared hydrous titanium(IV) oxide is both amorphous and finely particulate and may therefore be unsuitable for use in either reactor-type. Although both filtration and

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centrifugation are adequate for the complete removal of hydrous titanium(IV) oxide from suspensions, both processes require substrate solution to be treated in batches, are unsuitable for rapid separations, and may be inconvenient for separations from large volumes of suspensions. Thus the ease of operation and recovery of hydrous titanium(IV) oxide–enzyme conjugates is somewhat limited by their physical form. The rapid removal of immobilised enzyme from substrate solution at the end of reaction is feasible if the water-insoluble enzyme has the physical form of a flat surface-coating based on an inert support; the immobilised enzyme may then be mechanically withdrawn from substrate solution. Therefore, the possibility of binding papain to hydrous titanium(IV) oxide included in surface-coating materials attached to glass was investigated. Surfaces of epoxy-resin and emulsion paint, pre-modified to contain hydrous titanium(IV) oxide, and control surfaces of unmodified glass, epoxy-resin, and emulsion paint were prepared. Each fully hardened surface was exposed to a dilute solution of papain at pH 7.0 and after thorough washing, was found to exhibit initial esterolytic activity (Table 4).

On storage in substrate (BAEE) solution at 4 °C papain conjugates of each of the unmodified glass, epoxy-resin, and emulsion paint surfaces were each found to exhibit a near exponential decrease in esterolytic activity (Figure 3). After 70 days storage the same conjugates had lost all detectable activity (Figure 3) and protein (<0.1 μg cm^{-2} detected). It may be inferred from these results that surfaces of glass, epoxy-resin, and emulsion paint, on exposure to a dilute solution of papain under the reported conditions, adsorb the enzyme and that such adsorbed enzyme is released into solution on subsequent storage of the conjugates in substrate (BAEE) solution. Desorption most probably occurs as a result of substrate–enzyme interactions. In accord with the observations reported here, Messing [36] has similarly observed the adsorption of papain onto glass and, during storage of the formed conjugate, loss of initial caseinolytic activity. Such desorption behaviour renders the conjugate unsuitable for any application which requires controlled and continuous treatment of substrate yielding product free of soluble enzyme.

Protein was detected to remain on both a papain conjugate of an epoxy-resin surface modified to contain lyophilised hydrous titanium(IV) oxide (14.1 μg cm^{-2}) and a papain conjugate of an emulsion paint surface modified to contain hydrous titanium(IV) oxide (22.7 μg cm^{-2}), after the conjugates had been stored for 70 days in substrate (BAEE) solution at 4 °C. Thus, by comparison with the respective behaviours of papain conjugates of unmodified surfaces of epoxy-resin or emulsion paint in storage, it may be concluded that hydrous titanium(IV) oxide, when included in surfaces of epoxy-resin or emulsion paint, retains ability to bind protein.

That surfaces of modified emulsion paint apparently bind greater quantities of protein per unit surface area of glass support than do surfaces of modified epoxy-resin may be the consequence of a combination of several effects. First the same area of coated glass plate may offer different effective surface areas for binding papain, depending on the coating, since surface irregularity and porosity depend on surface-type. Secondly the surface-availability of hydrous titanium(IV) oxide depends on both the concentration of added hydrous titanium(IV) oxide and the thickness of surface-coating. Thirdly, *lyophilised* hydrous titanium(IV) oxide was incorporated into epoxy-resin whereas *wet* hydrous titanium(IV) oxide was incorporated into emulsion paint: structural changes occur on lyophilisation of hydrous titanium(IV) oxide and it seems probable that the intrinsic ability of lyophilised hydrous titanium(IV) oxide to bind papain differs from that of wet hydrous titanium(IV) oxide.

The papain conjugate of modified epoxy-resin was found to possess only low initial esterolytic activity and no detectable initial proteolytic activity (Table 4). The esterolytic activity of the conjugate was found to decrease substantially during storage in substrate (BAEE) solution at 4 °C (Figure 4). Some of the decrease in esterolytic activity may have been due to the desorption of papain that may have been weakly adsorbed onto the epoxy-resin surface and not bound to surface-embedded hydrous titanium(IV) oxide. Otherwise the observed decrease in esterolytic activity of the conjugate represents a true decrease in the specific esterolytic activity of bound protein. The specific esterolytic activity of papain protein bound to the modified epoxy-resin surface (calculated assuming that no desorption of enzyme occurred during storage) is a very small percentage of that of original soluble papain (Table 5) or that of papain bound to free hydrous titanium(IV) oxide (Table 4). Thus an epoxy-resin microenvironment has a very detrimental effect on the catalytic activity of papain. It may be predicted that extra steric restrictions on the diffusion of substrate and extra hydrophobic interactions prevail in the epoxy-resin microenvironment compared to those acting in the hydrous titanium(IV) oxide microenvironment and that these are responsible for the low specific activities.

In contrast, papain protein bound to emulsion paint, modified to contain hydrous titanium(IV) oxide, was found to possess both greater specific esterolytic activity and greater specific proteolytic activity than papain protein bound to modified epoxy-resin (Table 5). A possible explanation of these observations is that emulsion paint provides a more hydrophilic microenvironment than does epoxy-resin. Even so, both the specific esterolytic and specific proteolytic activities of papain protein bound to modified emulsion paint (Table 5) are substantially lower than the corresponding values of papain protein bound to free hydrous titanium(IV) oxide (Table 3) and this suggests that extra steric restrictions on the diffusion of substrate also prevail in the emulsion paint microenvironment. The exceptionally good stability of the esterolytic activity of the papain conjugate of modified emulsion paint during storage in substrate (BAEE) solution at 4 °C (Figure 4) is further evidence that

[36] R. A. Messing, *Enzymologia*, 1970, **38**, 39.

the emulsion paint microenvironment provides some hydrophilicity. Only a slight decrease in esterolytic activity of the conjugate was observed during the initial stage of storage and this observation probably indicates that weak adsorption of papain is excluded by the presence of papain which is more strongly bound to hydrous titanium(IV) oxide distributed in the emulsion paint. This particular water-insoluble enzyme is then very satisfactory with regard to its esterolytic activity, stability to storage, ease of operation and ease of separation from substrate solution. Its method of preparation has the particular advantages that the surfaces (of any size or shape) may be pre-formed conveniently and enzyme-coupling takes place under mild, neutral conditions without the chemical destruction of enzyme remaining in solution. It is envisaged that other procedures for the insolubilisation of enzymes may be developed employing the principle, here demonstrated, that an additive which has ability to bind enzyme may retain this ability when distributed in a surface-coating.

We thank the S.R.C. and Koch-Light Laboratories Ltd. for a CAPS studentship (to V. W. P.).

[7/1863 *Received*, 24*th October*, 1977]