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Selective Removal of Enzymes from Substrate and Products. An Alternative to Immobilization for Enzymes Acting on Macromolecular or Solid Substrates

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ABSTRACT

A new approach for the control and interruption of enzymatic reactions via selective enzyme immobilization has been developed. The technique was exemplified by the use of three model enzymes with the corresponding macromolecular substrates: α-amylase/starch, trypsin/insoluble collagen, and alkaline phosphatase/plasmid DNA. Prior to incubation with its substrate, each enzyme was provided with *de novo* thiol-groups by a two-step reaction involving *N*-succinimidyl-3-(2-pyridyldithio) propionate (SPDP) and DTT. The chemical modification was achieved such that at least 80% of the native enzyme activity was preserved in all cases.

In order to interrupt rapidly the reactions in which the enzymes were used, the modified enzyme was immobilized by reaction via its thiol groups on a thiolsulfinate-agarose derivative. The gel-bound enzyme could then be easily removed from unreacted substrate and product by filtration or centrifugation. Comparative studies showed that the immobilized enzymes had much lower activities in the reactions studied than the corresponding soluble ones. The potential for enzyme reuse was also demonstrated with the α-amylase derivatives, which were quantitatively

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released and eluted in fully active form from the agarose. We have shown that it is possible to achieve practically complete enzyme immobilization in short times and thus to control the progress of the reactions. Because of its simplicity and high efficiency, this approach may represent an interesting alternative for biotechnological processes involving macromolecular or solid substrates.

Index Entries: α-Amylase; trypsin; alkaline phosphatase; starch; Azocoll; plasmid DNA; solid-phase thiolsulfinates; enzyme removal.

Abbreviations: BAEE, N-α-benzoyl-L-arginine ethyl ester; DTT, 1,4-dithio-DL-threitol; mPU, milli-phosphatase units; pNPP, *p*-nitrophenyl-phosphate; Py-S-S-, Dithiopyridyl-; SPDP, *N*-succinimidyl-3-(2-pyridyldithio) propionate; SH-, thiol-; TSI-, thiolsulfinate-.

INTRODUCTION

Since the introduction of the first stable and reusable water-insoluble enzyme derivatives in the late fifties, "immobilized enzymes" have been the subject of extensive theoretical and applied research. This has led to the creation of a technology that offers exciting possibilities in several fields *(1,2)*. Among the main advantages of immobilized enzymes as compared with the corresponding native soluble ones are: ease of reutilization, enhanced stability, and the possibility of controlling and interrupting the reaction by simply separating them from the reaction mixture. However, there are still major limitations for immobilized enzymes in applications involving macromolecular and insoluble particulate substrates, such as starch, cellulose, RNA, DNA, and so on. Steric hindrance and diffusion resistance owing to the solid phase make the catalysis difficult with the soluble form of such substrates and more or less impossible with the insoluble ones *(3,4)*.

Owing to these problems in the use of enzymes bound to solid phases in combination with high-mol-wt substrates, another way to achieve immobilization has been attempted, namely, the use of soluble enzymes physically confined within a semipermeable membrane. Despite its simplicity, the method has not been extensively used owing to inherent disadvantages, such as reduction in the reaction rate as a result of diffusion resistance through the membrane, the risk of substrate adsorption to the membrane when working with low substrate concentrations, and enzyme inactivation owing to high shear forces over long periods of time *(4)*. To improve the stability and retention of the soluble enzyme within these membrane reactors, the enzymes have been coupled to soluble polymers. However, these derivatives are severely diffusion-limited and exhibit very low activity toward high-mol-wt substrates *(5)*. Above all, the main limitation to the use of these

water-soluble conjugates is the requirement for laborious purification protocols to separate excess reagent and enzyme *(3–5)*.

Therefore, the native, soluble enzyme is still the preferred choice in applications where the substrate is of polymeric type. After performing their job, the enzymes are usually denatured by procedures, such as pH shift or heat treatment, or are removed by precipitation or solvent extraction. The use of any of these methods can result in considerable damage to the product. To eliminate these deleterious effects, we have investigated a more selective approach to achieve both enzyme removal and reaction control. The principle is simple and consists of substituting the soluble enzyme with free thiols so that it can be efficiently captured by a solid phase containing thiol-reactive structures. This is added to the reaction mixture when the enzymatic reaction is to be interrupted.

MATERIALS

Sepharose 4B, PD-10 columns (Sephadex G-25), *N*-succinimidyl-3-(2-pyridyldithio) propionate (SPDP), Phadebas amylase test, and CNBr-activated Sepharose 4B, were supplied by Pharmacia Biotech (Uppsala, Sweden). Epichlorohydrine (1-chloro-2,3-epoxy-propane), 1,4dithio-DL-threitol, N α-benzoyl-L-arginine ethyl ester (BAEE), Azocoll (insoluble collagen), α-amylase (EC 3.2.1.1) from porcine pancreas, type I-A, trypsin (EC 3.4.21.4) from porcine pancreas, type IX, and alkaline phosphatase (EC 3.1.3.1) from bovine intestinal mucosa, type VII-N, were from Sigma (St. Louis, MO). Bradford's reagent was from Bio-Rad (Richmond, CA). The restriction enzymes (*Eco*RI, *Hin*dIII) and T4 ligase were purchased from Pharmacia Biotech (Uppsala, Sweden), and used according to the manufacturer's recommendations. The plasmids used were pUC 18 *(6)* and pHSG399 *(7)*. The *Escherichia coli* strain used was DH5α, which was purchased from Gibco (Gaithersburg, MD). All other chemicals used were reagent- or analytical-grade.

METHODS

General Methods

Preparation of Thiolsulfinate-Agarose

TSI-agarose was prepared by stepwise oxidation of gel-bound thiol groups first to disulfide with potassium ferricyanide and afterwards to thiolsulfinate moieties with magnesium monoperoxy-phthalate, as described in ref. *(8)*. The concentration of gel-bound thiosulfinate groups obtained was 450 μmol/g of dry gel as titrated with reduced glutathione *(8)*. The concentration of remaining thiol groups in TSI-agarose as determined with 2,2′ dipyridyldisulfide (2-PDS) *(9)* was 28 μmol/g of dry gel.

Protein Determination

Protein concentrations were determined by the Bradford's method *(10)* using the individual enzymes as standards. The protein concentrations of the standards were determined by total amino acid analysis. The amino acid analysis was performed at the Institute of Biochemistry, Uppsala University, and the results were processed neglecting the contribution of tryptophan, which was not analyzed.

Determination of Enzymatic Activities

α-Amylase activity was measured by determining the reducing sugars released, essentially according to Bernfeld, using 2% starch in 0.04 *M* Tris, pH 7.0, 10 m*M* calcium chloride at 25°C *(11)*. One amylase unit was defined as the amount of enzyme catalyzing the release of reducing sugars equivalent to 1 mg of maltose/min under the above-mentioned conditions.

In the adsorption experiments performed in the presence of starch degradation products, amylase activity was determined by the Phadebas assay, according to the manufacturer's instructions *(12)*.

Alkaline phosphatase activity was measured spectrophotometrically with *p*-nitrophenylphosphate (pNPP) as substrate at 25°C in 0.1 *M* Tris, pH 8.5, containing 1 m*M* magnesium chloride. The rate of formation of *p*-nitrophenolate was followed at 405 nm *(13)*. One unit of phosphatase activity (one phosphatase unit, 1 PU) was defined as the amount of enzyme catalyzing the hydrolysis of 1.0 μmol of substrate/min under the above-mentioned conditions.

Trypsin activity was determined spectrophotometrically using 1 m*M* BAEE as a substrate in 0.04 *M* Tris, pH 8.0, 10 m*M* calcium chloride at 25°C. One BAEE unit was defined as the amount of enzyme giving a A_{253} of 0.001/min using 1.0 mL reaction volume *(14)*.

Thiolation of Enzymes

One-milliliter aliquots of enzyme solutions containing 0.3–1.0 mg/mL protein were gel-filtered on PD-10 columns in the following buffers: 0.1 *M* sodium borate, pH 8.0, 5 m*M* calcium chloride for α-amylase and trypsin; 0.2 *M* sodium phosphate, pH 8.5, for alkaline phosphatase. The enzyme samples were incubated for 30 min with aliquots of 25 m*M* SPDP solution in methanol to give SPDP/protein molar ratios in the range 10–75. After gel filtration on PD-10 columns in the corresponding buffers used to determine enzymatic activity, the enzyme-bound 2-pyridyldithio groups were reduced to thiol groups by incubation with DTT for 30 min. The number of enzyme-bound thiol groups incorporated was estimated by determining the increase in absorbance at 343 nm owing to the released thiopyridone on DTT addition *(9)*. The reduced enzyme (Thiol-Enzyme) was gel filtered on a PD-10 column to separate it from low-mol-wt compounds.

Immobilization of Enzymes

α-Amylase was immobilized by reacting the introduced thiol groups with TSI-agarose. An aliquot of a pyridyldithio-α-amylase solution in 0.04 *M* Tris, 10 m*M* calcium chloride (containing an average of 5.8 pyridyldithio-moieties added per enzyme molecule, 0.45 mg protein and, 143 amylase units) was reduced by addition of DTT and incubation for 15 min. The resulting reduced enzyme solution was incubated for 1 h with 500 mg of filter dried TSI-agarose in the presence of DTT. The gel was filtered and the supernatant analyzed for protein and enzymatic activity. After exhaustive washing, the gel was suspended in 0.04 *M* Tris, pH 7.0, 10 m*M* calcium chloride, and stored at 4°C.

Alkaline phosphatase and trypsin were immobilized by reacting their amino groups with CNBr-activated Sepharose 4B. Alkaline phosphatase (0.6 mg, 200 phosphatase units) and trypsin (1.5 mg, 27300 BAEE units) were dissolved in 2.0 mL of 0.25 *M* sodium bicarbonate pH 8.3 (coupling buffer) and incubated at 25°C for 1.5 h under end-over-end mixing with 400 mg of filter-dried CNBr-Sepharose 4B. The derivatives were washed three times with 2.0 mL aliquots of coupling buffer. Excess of reactive groups was blocked by incubating the washed derivatives with 0.2 *M* glycine in coupling buffer. After exhaustive washing, the derivatives obtained were suspended in 1.5 mL of 0.04 *M* Tris pH 8.5 containing 1 m*M* magnesium chloride for alkaline phosphatase and 0.04 *M* Tris, pH 8.0, containing 10 m*M* calcium chloride for trypsin, and stored at 4°C. The amount of immobilized enzyme was calculated from the difference between the soluble enzyme activity applied and the activity recovered in the filtrate and washings. Control experiments were performed with non-activated Sepharose 4B. The activities of immobilized enzymes were assayed by incubating appropriate aliquots of the individual gel suspensions in 5 mL of substrate solutions under continuous agitation; 1.2-mL aliquots of the incubation mixtures were withdrawn at 1-min intervals, filtered by suction, and analyzed for products as described above for soluble enzymes. In order to check enzyme release during the assay, the filtrates were incubated for 30 min and afterward analyzed again for products.

DNA Manipulations and Transformation

Plasmid pUC 18 was digested to completion with *Eco* RI, as determined by agarose gel electrophoresis. After phenol/chloroform extraction and ethanol precipitation, the DNA was dissolved in 10 m*M* Tris-HCl, pH 7.9, 10 m*M* magnesium chloride, and 0.1 m*M* zinc chloride. The plasmid PHSG399 was digested to completion with *Hin*dIII. The linearized plasmid was extracted, precipitated as above, and dissolved in 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA.

Ligations were performed by incubating (1 h at room temperature) 100 ng of plasmid with 1 Weiss unit of T4 DNA ligase. The reaction volume was 50 μL in order to favor self-ligation over concatemer formation. *E. coli* was rendered competent using the rubidium chloride method of Hanahan *(15)*. The transformation was carried out by incubating the DNA samples with competent *E. coli* DHα5 cells at 42°C for 90 s. The samples were then spread on selective plates containing either ampicillin or chloramphenicol. In order to determine the number of plasmid molecules that had escaped digestion and could result in ligation-independent transformation, parallel transformations were performed with nonligated samples.

Methods for the Determination of Enzymatic Activities on High-mol-wt Substrates and Selective Removal of the Enzymes

Starch Liquefaction by α-Amylase

A volume of 2.0 mL of soluble thiolated α-amylase (Thiol-Enzyme, after removal of DTT by gel filtration; 0.2 mg protein, and 99 amylase units), containing an average of 5.8 thiol groups/enzyme molecule, was incubated with 40.0 mL of 10% (w/v) starch dissolved in 0.04 *M* Tris, pH 7.0, buffer containing 10 m*M* calcium chloride. Immobilized enzyme derivatives with an equivalent amount of bound activity in 2.0 mL gel suspension were incubated with starch in the same conditions. Control experiments were performed with a gel suspension containing nonactivated Sepharose and 2.0 mL of buffer, without enzyme. At regular time intervals, samples were taken and analyzed for reducing products with DNS, and viscosity at constant shear rate in a Bohlin Visco 88, BV viscometer. Starch degradation was allowed to proceed until the concentration of reducing products reached a plateau. Then, TSI-agarose was added to the mixture (50–150 mg of filter dry gel/mL) and rotated end-over-end for 5–30 min. The solid phase was separated by centrifugation for 15 min and the supernatant analyzed for residual amylase activity by the Phadebas test. A control experiment with nonactivated Sepharose was performed. The percentage of activity remaining in the reaction mixture was calculated from the difference between the number of enzyme units in the supernatants after incubation with TSI-agarose and the control. The experiments were performed in triplicate.

Alkaline Phosphatase–DNA Incubation—Enzyme Removal or Inactivation

The hydrolysis of 5′-phosphate groups from linearized plasmid DNA renders it unable to ligate and thereby to transform competent *E. coli* cells. Plasmid pUC 18 carrying ampicillin resistance was used, and the activity of the alkaline phosphatase enzyme was monitored in ligation and transfor-

mation experiments as the reduction of the number of ampicillin-resistant colonies obtained on ampicillin-containing plates.

Aliquots (5 μL) of solutions of soluble alkaline phosphatase and suspensions of immobilized alkaline phosphatase (0.15–150 mPU) in 0.1 *M* Tris, pH 8.5, containing 1 m*M* magnesium chloride, 0.2 m*M* zinc chloride, and 1 m*M* DTT, were combined with 35 μL of linearized pUC 18 (0.7 μg DNA in 10 m*M* Tris, pH 7.9, 10 m*M* magnesium chloride, 0.1 m*M* zinc chloride) and incubated for 10 min at 37°C. After the incubation period, the reaction was interrupted by different procedures depending on the form of alkaline phosphatase used:

1. In the case of unmodified, soluble alkaline phosphatase, thermal treatment for 7 min at 80°C was performed.
2. When thiolated soluble alkaline phosphatase was used, the enzyme was adsorbed on TSI-agarose by incubation with 50 mg filter-dried TSI gel at room temperature for 15 min. The mixture with the gel was then transferred to Microspin filters (Pharmacia Biotech, Uppsala) fitted in Eppendorf tubes, by the aid of 15 μL buffer, and centrifuged (600*g*, 2 min).
3. The reaction utilizing immobilized enzyme was interrupted by removing the enzyme bound to the solid phase, using low-speed centrifugation through Microspin filters. Samples were kept on ice after treatment, and their integrity examined by agarose-gel electrophoresis. After ligation and transformation, the samples were spread on ampicillin plates and incubated overnight at 37°C. The experiments were done in duplicate.

Carryover of Alkaline Phosphatase After the Different Inactivation Procedures

To evaluate the efficiency of the inactivation procedures, the presence of remaining alkaline phosphatase activity in the pUC 18 plasmid solutions was assayed in a second ligation/transformation experiment. Two hundred-nanogram samples of linearized plasmid PHSG399 (which confers chloramphenicol resistance to *E. coli* cells) were incubated for 30 min at 37°C with each of the pUC 18 solutions in which the phosphatase enzyme had been inactivated (or removed). The samples were then subjected to ligase treatment and used to transform *E. coli*. The number of colonies obtained on chloramphenicol plates was compared with that obtained in the following control experiments:

- Control 1: pHSG399 was incubated with pUC 18 containing the 5′-phosphate groups (plasmid not treated with alkaline phosphatase).

- Control 2: pHSG399 was incubated for 10 min at 37°C with pUC 18 treated with soluble alkaline phosphatase without an inactivation step.

These experiments were performed in duplicate.

Trypsin Activity Toward Insoluble Collagen

Aliquots of native and thiolated soluble trypsin (100 μL containing approx 48 μg protein and 1200 BAEE units) were incubated with 14 mL of a 5 mg/mL Azocoll suspension in 0.04 *M* Tris, pH 8.0, 10 m*M* calcium chloride, and rotated end-over-end *(16)*. A gel suspension of immobilized-trypsin (100 μL) containing an equal number of BAEE units was incubated with Azocoll under the same conditions. At regular time intervals, 1.2-mL samples were centrifuged in Eppendorf tubes for 10 min at 15,000*g*, and the absorbance at 520 nm measured. After 30 min of incubation, three 1.2-mL aliquots of the suspension were taken and mixed with 10 mg of dry TSI-agarose. The reaction mixture containing the solid substrate and the added TSI-agarose was incubated for 15, 30, and 90 min. Following centrifugation, the absorbance at 520 nm was determined. The experiments were performed in triplicate.

RESULTS

Activity of Soluble and Immobilized Enzymes Toward Macromolecular or Solid Substrates

In order to characterize the performance of immobilized enzymes on the macromolecular or insoluble substrates selected, the three model enzymes were bound to agarose, and the activities were first measured with the standard activity assays. The α-amylase activity measured as the production of reducing sugars on a low-viscosity starch solution was only 18% of the soluble applied activity, whereas the activities with the low-mol-wt substrates of alkaline phosphatase and trypsin were 59% and 45% of the originally applied native activity respectively (Table 1).

α-Amylase/Highly Viscous Starch Solution

The activity of both soluble and immobilized α-amylase toward a viscous starch solution was determined both from the production of reducing products and by viscosity measurements. As estimated from the graph shown in Fig. 1, the time required for a 50% reduction of the initial viscosity was about 1 h for the soluble enzyme and about 24 h for the immobilized derivative. The formation of reducing products also showed large differences between soluble and immobilized enzyme. The initial rate of formation of reducing products was six times faster for the soluble enzyme

Table 1
Activity of the Immobilized Enzyme Derivatives

Enzyme	Substrate	Activity %[a]
Alkaline-phosphatase	pNPP	59
	DNA (plasmid)	2
α-Amylase	Soluble starch[b]	18
	Phadebas-starch	0
Trypsin	BAEE	45
	Azocoll	0

[a]The percentage refers to the soluble native activity applied to the solid phase. The values reported are the mean of three experiments; the coefficient of variation was in all cases <10%.

[b]Activity measured by the production of reducing sugars.

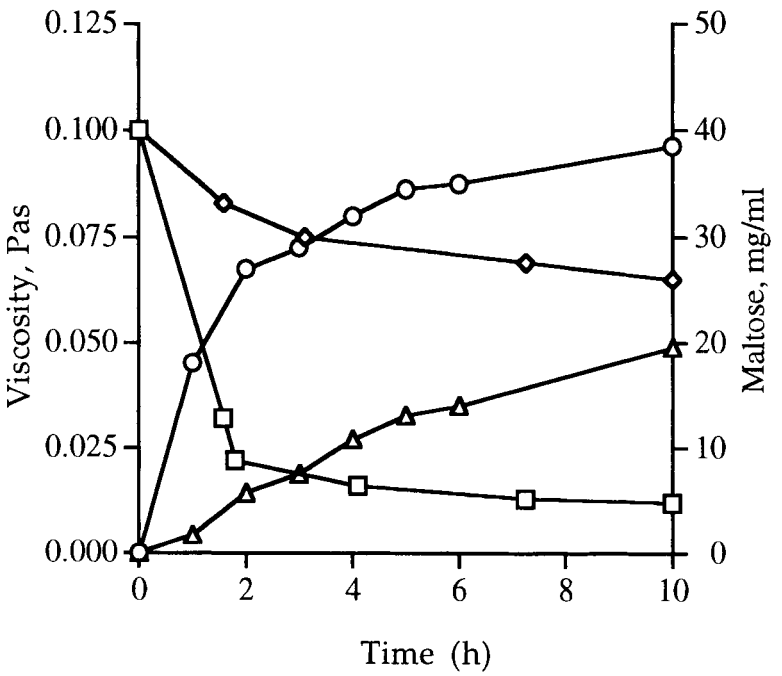

Fig. 1. A typical time course of α-amylase activity on 10% starch followed by viscosity and measurement of reducing products. Squares and rhombuses show viscosity data (Pas) for soluble (Thiol-enzyme) and immobilized enzyme, respectively.

Circles and triangles represent reducing products (as maltose in mg/mL) for soluble (Thiol-enzyme) and immobilized enzyme, respectively.

than for the immobilized enzyme. The Phadebas assay correlated well with the method of reducing sugars for the soluble enzyme, but as shown in Table 1, the immobilized α-amylase was not active on insoluble starch (Phadebas blue starch).

Alkaline Phosphatase/Linearized Plasmid DNA

The activity of alkaline-phosphatase on the terminal phosphate groups of linearized plasmid DNA was followed in terms of the reduction of the DNA's ability to circularize by ligation, transform efficiently into *E. coli* cells, and give rise to antibiotic-resistant colonies on plates. The number of transformants obtained reflects the number of plasmid molecules in which one or both of the 5'-phosphate groups remained after phosphatase treatment. The immobilized enzyme is significantly less efficient than the native soluble enzyme in catalyzing DNA dephosphorylation (Table 1).

Trypsin/Insoluble Collagen

As expected, the immobilized trypsin showed no activity at all when assayed with insoluble collagen (Table 1).

Modification of Enzymes with SPDP

The thiolation is a two-step procedure. The first step carried out by reaction with SPDP leads to the attachment of 2-dithiopyridine moieties to the amino-terminal group or to the ϵ-amino groups of exposed lysine side chains on the proteins. The enzyme-bound dithiopyridine moieties are subsequently converted to free thiols by reaction with DTT (Fig. 2A).

Both alkaline phosphatase and α-amylase were thiolated with 8–9 groups/protein molecule using SPDP/protein ratios of about 75 (Table 2). The use of lower SPDP/protein ratios (in the range 35–50) led to the introduction of 6 dithiopyridine groups/enzyme molecule without significant loss of enzymatic activity. Reduction of the 2-dithiopyridine groups of alkaline phosphatase to thiols with DTT changed the enzymatic activity only to a very small extent. α-Amylase, on the other hand, which was less active in the 2-dithiopyridine form, recovered full activity after reduction with DTT. Trypsin could only be provided with a maximum dithiopyridine content of about 6 groups/molecule, which occurred at a SPDP/protein ratio of 18, whereas larger excess of reagent led to precipitation of the enzyme. The activity of dithiopyridine-trypsin derivatives towards the low-mol-wt substrate BAEE was considerably higher (30–40%) than that of the native unmodified enzyme. The thiol-trypsin derivative formed as a result of the treatment with DTT showed 80–85% of the activity of the native enzyme (corresponding to about 60% of the activity of dithiopyridine-trypsin).

The thiolation of both α-amylase and trypsin did not affect their enzymatic activities towards their respective solid substrates (Phadebas blue starch and Azocoll). However, in the case of alkaline phosphatase, an interesting activation effect was observed for the thiolated enzyme acting on the linearized plasmid DNA. This is clearly shown in Fig. 3, where the influence of the enzyme concentration is studied at a fixed incubation time of 10 min. The same number of phosphatase units of the different enzyme

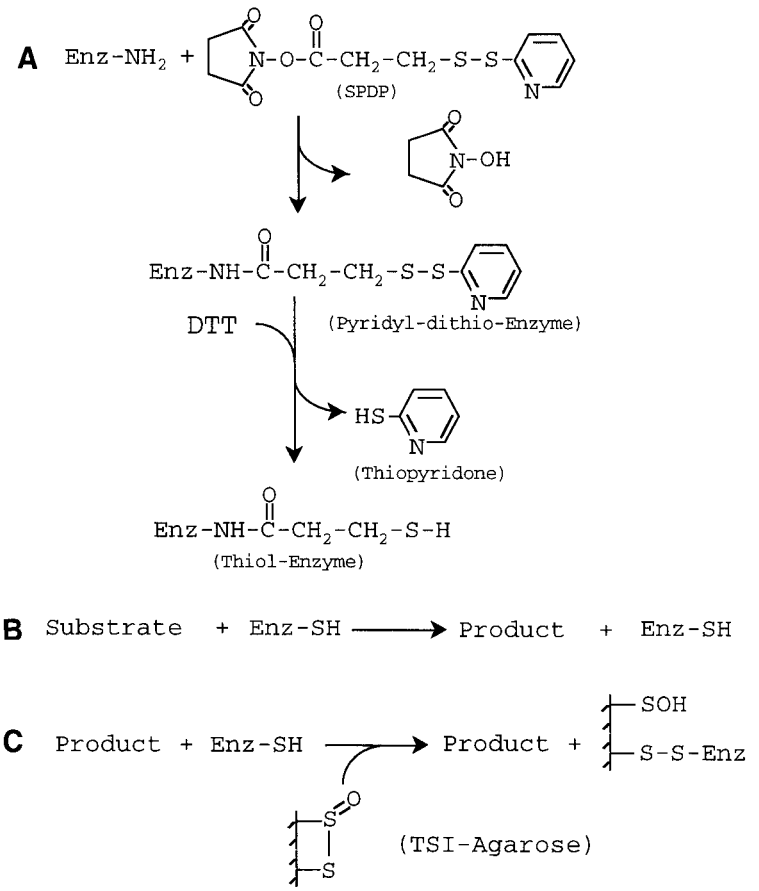

Fig. 2. Enzyme thiolation and subsequent adsorption as a tool for reaction control. (A) Introduction of 2-pyridyldithio-propionamide moieties in the protein by reaction with SPDP and reduction of *de novo* introduced 2-pyridyldithio-groups to thiols with DTT; (B) enzymatic reaction catalyzed by the thiolated enzyme; (C) selective immobilization of thiolated enzyme to TSI-agarose.

forms (as determined by the low-mol-wt substrate) had different levels of activity on the macromolecular substrate (Fig. 3). In order to obtain 100% dephosphorylation, approx 30 mPU of soluble native enzyme were required, and only 3 mPU of thiolated-enzyme.

The Stability of the Modified Enzymes

The dithiopyridine-forms of both alkaline phosphatase and α-amylase were stable at 4°C for at least 1 mo without noticeable decrease in activity. The activity of the dithiopyridine-form of trypsin declined at a rate of 1%/d when stored at +4°C in 0.1 *M* Tris pH 8.0. The thiolated enzymes, after reduction with DTT (Thiol-enzymes), were in general less stable during storage than native enzymes. However, in all cases, the

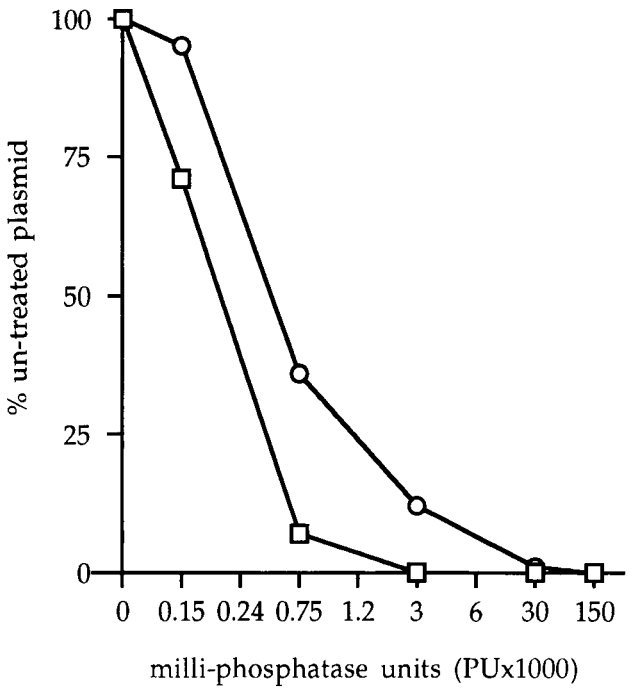

Fig. 3. The effect of the amount of native soluble and thiolated soluble alkaline phosphatase added, on the dephosphorylation of linearized plasmid DNA. Circles, native soluble enzyme; squares, thiolated soluble enzyme.

reduced forms were stable for several hours at 25 degree C in the absence of DTT. Although both thiolated alkaline phosphatase and α-amylase could be stored over long periods of time in the reduced form in the presence of DTT, trypsin was 80% inactivated by 1 mM DTT at pH 8.0 within 24 h. The modified enzymes were thus stored in the protected form (i.e., dithiopyridine-enzyme derivatives) and reduced just before use.

Studies of the Removal of the Soluble Modified Enzymes from Their Reaction Mixtures

Capturing α-Amylase After Starch Hydrolysis

The α-amylase was incubated with a 10% starch solution, and when the degradation was completed, aliquots of the reaction mixture containing soluble thiolated enzyme were incubated with different amounts of TSI-agarose. The percentage of enzyme adsorbed at pH 7.0 was strongly dependent on the amount of gel added, especially at short reaction times of about 5 min. In order to obtain more than 99 ± 1% immobilization of the enzyme in 30 min, it was necessary to use 150 mg of filter-dried gel/mL of reaction mixture. However, after 3 h of incubation time at pH 7.0, it was

enough with only 50 mg of filter-dried gel/mL of mixture to attain undetectable levels of activity (<0.1%) in the supernatant. At pH 8.0, using 50 mg of TSI-agarose/mL reaction mixture, <0.1% of the applied activity was left in the supernatant after 20 min. Thus, the kinetics of immobilization of α-amylase to TSI-agarose is a function of both pH and the amount of gel added. The binding of thiol-amylase to the gel was slower in the presence of the reaction mixture than in buffer of the same pH (data not shown).

Removal or Inactivation of Alkaline Phosphatase After DNA Dephosphorylation

In cloning experiments, when alkaline phosphatase is used to dephosphorylate vector DNA, it is critically important that the enzyme activity be eliminated before the vector is used for ligation. In order to compare the efficiency of the covalent capturing procedure onto TSI-agarose with thermal inactivation and with the use of the immobilized enzyme (which can be separated from the reaction mixture by filtration), a second set of ligation and transformation experiments were performed. This was done by incubating the dephosphorylated pUC 18 solutions (after the inactivation or removal treatment), with a second plasmid (pHSG399), which confers chloramphenicol resistance. In the absence of a removal treatment, alkaline phosphatase completely blocked the ligation and transformation of the pHSG399 plasmid DNA. We have found that the three different removal procedures tested: heat inactivation, filtration of immobilized enzyme, and adsorption to TSI-agarose, were equally effective, since none of them caused a significant reduction in the ligation/transformation efficiency of pHSG399, as compared to the control where no enzyme had been present.

It was confirmed by agarose-gel electrophoresis that the three removal procedures preserved the integrity of the pUC plasmid DNA.

Interruption of the Trypsin/Catalyzed Hydrolysis of Azocoll

The time-course of the activity of soluble dithiopyridine-trypsin on Azocoll is shown in Fig. 4. The dithiopyridine-enzyme derivative was used because of its higher activity and stability (*see* Table 2). The time-course of the reaction was followed by absorbance measurements at 520 nm. At the time indicated by the arrow, TSI-agarose was added to the mixture, and the reaction was interrupted. The immobilization of dithiopyridine-trypsin to TSI-agarose relies on the existence of residual thiol groups in the TSI-agarose. In order to avoid dilution of the product, the gel was used in dry form. The binding of dithiopyridine-trypsin to TSI-agarose was as efficient as the binding of the reduced enzyme (Thiol-enzyme). In a control experiment with native trypsin carried out under the same conditions it was found that <10% of the enzyme was adsorbed to TSI-agarose.

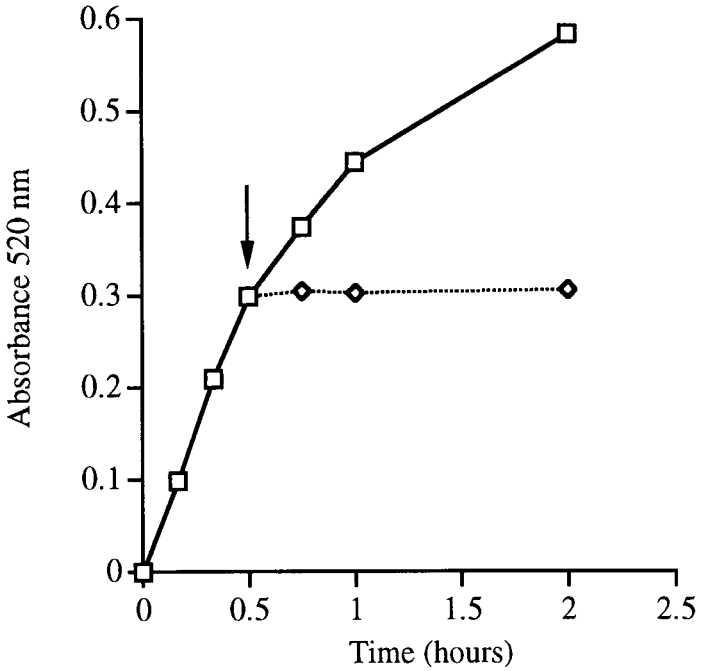

Fig. 4. Interruption of Azocoll degradation by adsorption to TSI-agarose. The time-course of the reaction of soluble pyridyl-dithio-trypsin was followed by absorbance measurements at 520 nm (squares). At the time indicated by the arrow, an aliquot of the reaction mixture was incubated with 10 mg dry TSI-agarose/mL, and the absorbance was measured after 15, 30, and 90 min (rhomboids). The results are from a representative experiment and the variation between experiments was 10%.

DISCUSSION

The Activity of Immobilized Enzymes on High-Mol-Wt or Solid Substrates

Many potentially useful properties of immobilized enzymes have been reported, such as increased stability toward denaturing conditions and increased shelf-life. One of the more important and potentially useful properties of immobilized enzymes is the ease of separation from their substrates and products by a simple filtration. When the substrates are of polymeric type and especially if they are insoluble, steric and diffusion effects prevent the interaction between the immobilized enzyme and its substrate. The immobilization may also in some cases change the characteristic pattern of products derived from the macromolecular substrate. A well-characterized example of this problem is α-amylase, which in free state catalyzes the random splitting of internal glycosidic linkages of α-1,4 glucans. In immobilized form, the mode of action of the enzyme is changed from a typical endo- to an exo-glucanhydrolase mode: the production of higher mol-wt dextrins diminishes, and the amount of glucose

Table 2
Thiolation of Model Enzymes with SPDP and DTT[a]

Enzyme	SPDP/ protein ratio	SH/protein mol/mol	Activity, %[b] Py-S-S-enzyme	Activity, %[b] SH-enzyme
Alkaline-phosphatase	18	2.9	96	84
	36	6.3	89	80
	72	8.9	82	73
α-Amylase	25	3.5	100	100
	50	5.8	88	99
	75	7.5	58	82
Trypsin	10	3.4	133	83
	18	6.3	140	85
	>22	Precipitated	—	—

[a]The values reported are the mean of at least three experiments; the coefficient of variation was within 12%.

[b]% of native specific activity in each case, analyzed using the reducing sugars technique for α-amylase and the low-mol-wt substrates for trypsin and alkaline phosphatase.

and other low-mol-wt malto-oligosaccharides increases *(14,17)*. Thus, we have found that the rate of formation of reducing sugars by soluble α-amylase acting on starch was 6 times faster than that of the immobilized form of the enzyme, and when the amylolytic activity was measured by following the decrease in viscosity, the soluble enzyme was 24 times faster than the immobilized enzyme (Fig. 1). When insoluble starch labeled with cibachrome blue (Phadebas amylase test) was used, the immobilized amylase showed no activity at all (Table 1).

As a result of the factors discussed above, immobilized enzymes acting on macromolecular substrates usually show a decrease in the rate of reactions as compared to soluble enzymes, and with solid substrates, no catalysis at all occurs *(3,4)*. This is also clearly demonstrated in this article with the chosen model enzymes (Table 1). Alkaline phosphatase immobilized to beaded agarose, although showing a reasonably high activity toward the low-mol-wt substrate PNPP, was much less efficient than the native enzyme on plasmid DNA. Immobilized pancreatic trypsin showed 45% of the activity of native enzyme when assayed with BAEE (the low-mol-wt substrate), but showed no activity at all when assayed with the insoluble substrate Azocoll.

The problems of the steric and diffusion limitations of immobilized enzymes have been extensively studied elsewhere, and many attempts have been made to improve the situation *(4,6,15)*. However, it seems that

the transition from homogenous to heterogeneous catalysis is a major step, and so far no immobilized derivative has been found to behave as its native soluble counterpart.

The Selective Capture Approach

In this article, we have tried to demonstrate a way to combine the properties of free, soluble, sterically unrestricted enzymes, with the separation advantages of enzyme immobilization. The native enzyme is first provided with a number of small reactive groups in a way that does not significantly change its activity and specificity. After catalyzing the transformation of a high-mol-wt or insoluble substrate into the corresponding product in a manner similar to the native enzyme, the soluble modified enzyme is tied up by reaction of the introduced substituent with a suitable group on the solid phase. The enzyme bound to the solid phase can then be separated from the reaction mixture by filtration or centrifugation.

In this article, we propose the use of an aliphatic thiol as enzyme substituent, and beaded agarose containing thiolsulfinate groups as the reactive solid phase (Fig. 2). The thiol groups are introduced in a two-step procedure in which the enzyme is first substituted with 2-dithiopyridine groups. The enzyme derivative thus formed is, after conversion of the dithiopyridine groups to thiol groups, used to catalyze the conversion of the substrate. At the appropriate time, the modified enzyme is removed from the reaction mixture by adding the reactive solid phase, TSI-agarose. The thiol-containing enzyme reacts with the gel-bound thiolsulfinate groups and becomes immobilized to the agarose via disulfide bonds. An important characteristic of this reaction is that it proceeds by formation of a solid-phase bound sulfenic acid group and thus does not give rise to any soluble by product *(8)*.

As an alternative, it is also possible to use the 2-dithiopyridine-substituted enzymes without prior reduction, since the TSI-agarose derivative also contains a number of free thiol groups. These remaining thiol groups from TSI-agarose react with the 2-dithiopyridine groups of the enzyme in a typical thiol-disulfide exchange reaction. The result is the immobilization of the enzyme through disulfide bridges and the formation of thiopyridone. The 2-thiopyridone is, in turn, efficiently bound to thiolsulfinate groups under formation of a gel-bound dithiopyridine group *(8,18)*. Thus, both possible reactions with TSI-agarose proceed without contaminating the solution.

On the Chemical Modification of Proteins by Reaction with SPDP

The modification of proteins with SPDP is a well-established procedure (Fig. 2). This reagent has been successfully used to modify a rather large number of amino groups in other starch-degrading enzymes, while

Table 3
The Variation of the Number of Unmodified Enzyme Molecules, Calculated According to the Poisson Distribution for Different Average Numbers of Modifications per Enzyme Molecule

Average number of SH/ enzyme molecule	% of Enzyme molecules without modification[a]
2	13.53
4	1.83
6	0.25
8	0.03
10	0.005

[a]Data calculated according to the following formula derived from the Poisson distribution: $P(0) = e \times p^{-m}$.
m = Average number of modifications per molecule;
P(0) = fraction of molecules with zero modifications/ molecule.

preserving the enzymatic activity *(19,20)*. The three model enzymes of this study could also be modified to a great extent, with a very small loss in activity (Table 2).

The degree of modification achieved in the reaction with SPDP can be determined by incubating a known amount of the dithiopyridine-enzyme with DTT. The number of thiol groups incorporated per protein molecule can then be calculated from the absorbance at 343 nm, which corresponds to the released 2-thiopyridone. This number represents an average for the whole population of enzyme molecules. Assuming that the thiolation of proteins is a random process, the population of modified proteins could be described by the Poisson distribution. Then, for an average number of 4 modifications/ molecule, 1.8% molecules still have no added thiol (Table 3). This consideration seems to limit the application of the technique mainly to those enzymes that can tolerate extensive modification, in order to avoid the problem of having a substantial fraction of nonmodified molecules. It is noteworthy that we have found that proteins are adsorbed to thiol-reactive adsorbents to a greater extent than would be expected if this assumption was valid. This observation indicates that the modification of proteins may not necessarily be a random process, e.g., some groups, such as the amino-terminal, may be more reactive than the ϵ-amino groups, owing to a lower pK_a.

Removal of Modified Enzymes from the Reaction Mixtures

The kinetics of adsorption of α-amylase to TSI-agarose were found to be a function of both pH and amount of gel added. The case of α-amylase

represents a particularly difficult situation from the point of view of kinetics. Thus, the adsorption of the enzyme to the gel is slower in the presence of the reaction mixture than in buffer alone (data not shown). Nevertheless, the use of an excess of TSI-agarose allowed practically complete adsorption in short times. For α-amylase, enzyme reuse is possible because the adsorbed enzyme could be eluted quantitatively by DTT treatment.

The capture of alkaline phosphatase from the reaction mixture containing the DNA substrate and DTT demonstrated that the adsorption system is also effective in the presence of a competing thiol, provided an excess of the TSI-gel is added. Interestingly, under the conditions described, the thiolated soluble enzyme was more efficient at promoting DNA dephosphorylation than the native alkaline phosphatase. This is probably owing to the decrease in the number of positive charges in the modified enzyme, which avoids nonspecific binding to the negatively charged DNA molecule.

Trypsin was selected to demonstrate the enzyme-capturing system in the presence of a solid substrate. In this case the reverse-phase approach was used, i.e., the thiol groups are on the gel and the thiol-reactive group is on the protein. The addition of dry gel allowed a rapid interruption of the reaction without dilution of the reaction mixture.

In this work, agarose was used as a model solid phase. However, the approach should be possible to perform also with other cheaper porous (cellulose, and so forth), or nonporous supports. A high capacity is not a requirement for this application owing to the very small amounts of enzymes used in the reaction mixtures. The use of nonporous supports might in fact improve the kinetics of immobilization and allow the use of lower amounts of reactive support. The "capture technology" should be particularly interesting for industrial applications in reactors, such as expanded beds, which are compatible with the presence of solids.

Concluding Remarks

To our knowledge, the enzyme-capture method described here has not been used before. However, a similar approach has been used in an immunoassay technique. In this case, dithiopyridyl-agarose was used as a thiol reactive solid phase for the separation of an immunocomplex (antigen-thiolated antibody) from unconjugated antigen (*21*). In principle, it should be possible to extend the "enzyme-capturing approach" to interactions, such as ion-exchange or hydrophobic bonding. However, for such systems, it would be very difficult to find optimal conditions for a complete binding of the enzymes as well as to avoid unspecific binding of substrate and products. Other more specific approaches would be biotinylation of the enzyme for binding to an avidin solid phase or substi-

tution of the enzyme with an oligohistidine chain for adsorption to a solid phase-bound metal chelate. With the high binding constant of biotin/avidin, it is doubtful that the trapped enzyme could be removed and reused, unless a soft binding mutant of avidin was employed. The advantages of using thiolation/binding to TSI-agarose are that it seems to be a rather general procedure, no product is released or leaked during the adsorption, and the system is simple. The possibility of performing the reaction with the dithiopyridyl form of the enzyme adds further to the versatility of the approach.

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