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Immobilization of Manganese Peroxidase from *Lentinula edodes* on Azlactone-Functional Polymers and Generation of Mn^{3+} by the Enzyme–Polymer Complex

ANTHONY C. GRABSKI,*[,1] PATRICK L. COLEMAN,[2] GARY J. DRTINA,[3] AND RICHARD R. BURGESS[1]

[1]Protein Purification Facility, University of Wisconsin Biotechnology Center, Madison, WI 53705; [2]Biosciences Laboratory, Building 270-3S-06; and [3]Technology Development Laboratory, Building 201-2N-20; 3M Center, St. Paul, MN 55144

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ABSTRACT

Manganese peroxidase (MnP) purified from *Lentinula edodes* was covalently immobilized on 3M's azlactone-functional copolymer, 3M Emphaze™ AB1 Biosupport Medium. Tethered MnP is capable of generating Mn^{3+} from Mn^{2+} and H_2O_2. Mn^{3+}, properly chelated, can be used as a nonspecific oxidant of organopollutants. A variety of conditions designed to maximize coupling efficiency while maintaining Mn^{3+}-generating catalytic activity were tested. Biochemical characteristics of the MnP enzyme, including amino acid composition, pH and temperature stability, and concentration of its Mn^{2+} substrate, influenced chemical conditions necessary for the coupling reaction. The physical parameters of immobilization reaction time, protein concentration, ionic conditions, pH, and temperature were examined. Results of these experiments indicated maximum coupling efficiency and enzyme activity were achieved by immobilizing at MnP concentrations <2 mg/mL for at least 2 h using pH 7.0 buffer containing 1.0*M* sodium sulfate and 1.0 m*M* Mn^{2+}. Increasing coupling reaction temperature also improved coupling efficiency. A synthesis of these optimized immobilizations yielded MnP coupling efficiencies of 40–50% with 35% of the coupled protein retaining enzymatic activity. Results of

*Author to whom all correspondence and reprint requests should be addressed.

MnP immobilizations on nonporous azlactone-functional dispersion polymers more hydrophobic than Emphaze are also reported, and coupling efficiencies >65% with 100% of the coupled enzyme active have been measured.

Index Entries: Enzyme immobilization; manganese peroxidase; Mn^{3+}; azlactone polymers; Emphaze; biocatalyst.

INTRODUCTION

Immobilized proteins have been used in a wide variety of applications, including production and bioconversion of new antibiotics (*1*), production of specialty chemicals, such as the dipeptide sweetener aspartame, stereoselective synthesis (*2*), coagulation of milk (*3*), and separation of racemic drugs (*4*). More recently, immobilized proteins have gained attention for use as biocatalysts in the degradation of toxic organic compounds. The review by Wiseman (*5*) summarizes the potential for immobilized enzymes in water and air purification. He states that only inexpensive enzymes could be used in the soluble form, since treatment processes would require large quantities of enzyme that would be lost in the effluent. Another disadvantage to using free enzyme to detoxify waste streams is denaturation under extreme conditions. Immobilization of the protein on a solid support can eliminate these disadvantages (*6*). Therefore, the reuse of degradative enzymes immobilized on a solid support presents an attractive possibility for treatment of pumpable toxic waste streams.

Microbial oxidative enzymes play an important role in maintaining the global carbon cycle, and are instrumental in modification and degradation of the ever-increasing amounts of manmade chemicals constantly released into our enviroment (*7*). Oxidative enzymes from white-rot fungi, such as manganese peroxidases, ligninases, and laccases, are diverse biocatalysts with tremendous industrial potential for transformations and mineralizations of recalcitrant xenobiotics (*8*). The reactive diversity of MnP from *Phanerochaete chrysosporium* has been demonstrated through its ability to oxidize and/or decolorize a variety of phenols, amines, organic dyes (*9*), methoxybenzenes (*10*), and complex mixtures of chlorinated lignin (*11*). In order to take full advantage of MnP as a biocatalyst for organopollutant degradation and minimize the disadvantages of employing the free soluble enzyme, MnP must be immobilized.

MnP is secreted by white-rot fungi and participates in the degradation of lignin by these organisms. MnP from *L. edodes* is a monomeric, heme-containing protein with an apparent molecular weight of 44,600 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and an isoelectric point of 3.2 (*12*). Manganese peroxidases use H_2O_2 as an oxidant to oxidize Mn^{II} to Mn^{III} as the manganic ion Mn^{3+} (*13*). The Mn^{3+}, stabilized by chelation, is a highly reactive nonspecific oxidant (*9,14–16*) capable of oxidizing not only lignin, but also a variety of organic compounds (*10,17–23*). Since lack

of specificity and nonstereoselective bond cleavage are necessary for effective organopollutant degradation (*24*), the immobilized MnP/Mn^{3+}-chelate system may be ideally suited for this immobilized enzyme application.

Immobilized enzymes have been used in the degradation of pesticides (*5*) and 4-chlorophenol (*25*), and coimmobilized cell mass and enzymes from *P. chrysosporium* have been used to degrade toxic organic compounds (*26*). Immobilized lignin peroxidase (LP) from *Chrysonilia sitophila* decolorizes phenolic Kraft effluent from pulp mills (*27*). However, the use of MnP for similar immobilized enzyme applications is not well documented. Our goal is to employ the unique catalytic properties of MnP and the inherent advantages of protein immobilization through an immobilized MnP bioreactor that generates Mn^{3+}-chelate as an organopollutant oxidizing agent. Achieving this goal requires optimized coupling and reaction conditions for maximum enzyme-catalyzed Mn^{3+} generation by the immobilized MnP. The potential value of the MnP/Mn^{3+}-chelate system is realized through MnP's catalytic oxidation of Mn^{II} to Mn^{III}, allowing continuous generation of the nonspecific oxidant Mn^{3+}. Although Mn^{III} is available commercially as Mn^{III} acetate dihydrate, enzyme-generated Mn^{III} can be produced from Mn^{II} chloride tetrahydrate or Mn^{II} sulfate monohydrate. These MnP substrates are approx 40 times less expensive than Mn^{III} acetate dihydrate (Aldrich Chemical Co., Milwaukee, WI). The Mn^{III} generated by the system could oxidize a target compound, pentachlorophenol for example, be reduced back to Mn^{II}, and the Mn^{II} recycled dependent on the feasibility of such a step.

Recently, an activated bead composed of vinyldimethyl azlactone (oxazolone) and methylene-bis-acrylamide polymerized in various ratios has been described (*28*). The azlactone functional group of the polymer underwent nucleophilic attack by amines, thiols, and alcohols. The ring-opening reaction by such functional groups in proteins resulted in covalent attachment to the support. The reaction was rapid, successful at pH ranges from 4 to 9, and enhanced by the addition of high concentrations of polyanionic salts. Protein-coupling densities of 200–400 mg protein/g (1.0 g swells to ~8.5 mL) of dry support were achieved. In this article 3M's commercially available azlactone-functional polymer beads, Emphaze AB1, and several other azlactone copolymers are evaluated for their ability to couple MnP covalently. Immobilization conditions for maximum coupling efficiency while maintaining Mn^{3+}-generating catalytic activity are reported.

MATERIALS AND METHODS

MnP Source

Commercial *L. edodes* cultures of heterodikaryon strain MS-20 were purchased from L. F. Lambert Spawn Co., Inc. (Coatesville, PA). The cultures achieved maximum peroxidase production between 35 and 50 d

after inoculation. MnP was extracted, purified, and stored as described previously (*12*). Briefly, cultures were crumbled and extracted with pH 4.0 HCl-acidified distilled water. Coarse particulates were removed by sieving with 40-mesh stainless-steel screen, and the filtrate was clarified by polyethyleneimine (PEI; Sigma Chemical Co., St. Louis, MO) precipitation and centrifugation. PEI-clarified filtrate was concentrated 50-fold and diafiltered by ultrafiltration (30,000-Dalton cutoff membrane) into 10 m*M*, pH 7.0, sodium phosphate buffer. MnP was purified from the concentrated diafiltered extract by successive DEAE anion-exchange and Phenyl 5PW hydrophobic interaction chromatography steps.

MnP Amino Acid Analysis

MnP amino acid analysis was performed at the Medical College of Wisconsin (Milwaukee, WI). Purified MnP (~200 pmol) in 10 m*M* sodium phosphate buffer, pH 7.0, was lyophilized and redissolved in 200 μL of 5.7*N* HCl (distilled 3×) containing 0.02% 2-mercaptoethanol. The sample was transferred to a new borosilicate glass tube (disposable culture tube, 10 × 75 mm), flushed with nitrogen, sealed under vacuum, and hydrolyzed for 20 h at 110°C. The MnP hydrolysate was dried over NaOH under vacuum and then redissolved in 50 μL of 0.2*M* sodium citrate pH 2.4. The redissolved sample was applied to the Beckman 6300 amino acid analyzer and analyzed employing methods supplied by the manufacturer (Beckman Instruments, Inc., Palo Alta, CA) as adapted from (*29*).

MnP pH Stability

MnP (300 μg/mL) free in solution was incubated at 22°C in 100-m*M* buffers over a pH range of 3.0–9.0. Glycine buffer was used for pH 3.0 and 3.5, sodium acetate for 4.0–5.0, sodium phosphate for 6.0–8.0, and TAPS for 8.0–9.0. At the indicated time intervals (5 min to 6 h), an aliquot of MnP was removed and peroxidase activity determined under the standard MnP/vanillylacetone assay conditions described below.

Azlactone Polymers

The Emphaze polymer was prepared by an inverse-phase polymerization process (*30*). The majority of experiments described here employ 3M Emphaze™ AB1 Biosupport Medium (hydrophilic, macroporous, azlactone-functional polymer beads 50–80 μ in diameter) (Pierce Chemical Co., Rockford, IL). The nonporous, particulate, dispersion polymers used in the immobilization experiments (Table 2 data) were experimental materials synthesized by a dispersion polymerization process (*30*) and provided by Gary J. Drtina of the 3M Company. The following abbreviations are used for monomeric components of the dispersion polymers: Vinyldimethyl azlactone (VDM), 2-hydroxyethyl methacrylate (HEMA), methylene-bis-acrylamide (MBA), methacrylamide (MAM), trimethylol-

propane trimethacrylate (TMPTMA). The monomers and organic reagents necessary for polymer synthesis were purchased from Aldrich Chemical Co. (Milwaukee, WI).

MnP Iodination

MnP was radiolabeled with ^{125}I (Dupont NEN, Billerica, MA) by the chloramine-T reaction using Iodo-beads™ (Pierce Chem. Co., Rockford, IL) (23). Typically, 0.5 mg MnP, 100 μCi of ^{125}I-labeled sodium iodide and and 2 Iodo-beads were combined in 100 m*M* sodium phosphate buffer plus 100 m*M* NaCl (pH 7.5, total reaction volume was 500 μL). Labeling was allowed to proceed for 30–60 min and was terminated by removal of the solution from the beads. Labeled protein was separated from free ^{125}I with a PD-10 column (Pharmacia, Piscataway, NJ).

MnP Immobilizations

A typical protein coupling reaction consisted of 10 mg (hydrated volume ~85 μL) of polymer suspended in 200–400 μL of 100 m*M* sodium phosphate buffer, pH 7.0, containing 1.0*M* sodium sulfate and a variable amount of MnP (50 μg to 2.4 mg) in a 2-mL tube. The reaction mixture was vortexed for 1 min to facilitate complete mixing and hydration of the dry polymer, and then rocked for 60 min at ambient temperature. Coupling reactions were terminated by the addition of quenching reagent (1.0 mL of 1.0*M* ethanolamine in 250 m*M* sodium phosphate titrated to pH 7.0 with HCl). The suspension was rocked for 5 min with quenching reagent, centrifuged (10,000*g* for 3 min), supernatant solution removed carefully by pipeting, and reacted for an additional 60 min with 1.0 mL fresh quenching reagent. The blocked coupled polymer was centrifuged and supernatant removed as above. Finally, the polymer was washed four times (15–30 min each) with 10 m*M* sodium acetate buffer, pH 4.0 (buffer A), buffer A + 1.0*M* NaCl, and two times with buffer A. Amounts of support and solutions can be increased proportionally and adjustments made for different polymer types. Protein-free coupling buffer replaced protein-containing buffer in the zero protein control reactions. The controls were used to determine the influence of the polymer itself in protein determinations and MnP activity assays. Any polymer effects above baseline were subtracted from the MnP-polymer reaction results.

Variations of the standard protein coupling conditions were tested in order to optimize MnP coupling to the support while maintaining catalytic activity. Coupling reactions were performed over a pH range of 4–9 to test the pH dependency for the reaction of MnP with Emphaze in the presence of 1.0*M* sodium sulfate. The buffering agents in each experiment were 50 m*M* of sodium acetate (pH 4.0 and 5.0), sodium phosphate (pH 6.0 and 7.0), and sodium pyrophosphate (pH 8.0 and 9.0). MnP concentration was 2.5 mg/mL in 300 μL test coupling buffer added to 10 mg

Emphaze polymer. No $MnCl_2$ was added to the mixture. Coupling reaction time was 1 h before addition of ethanolamine quenching reagent. Standard wash procedures were used prior to protein estimations of coupling efficiencies.

Coupling reactions were performed over a temperature range of 0–60°C. Time-course of coupling was performed under standard coupling conditions for 5 min to 18 h. The MnP concentration dependency for the reaction of MnP with Emphaze was tested. The effects of high ionic strength on MnP immobilization were determined since these conditions have been shown to increase the amount of other proteins coupled to activated polymeric supports (*31–34*). Coupling MnP in the presence of substrate (Mn^{2+}) was tested to determine if higher activity could be obtained by saturating the enzyme-active site. Specific conditions for each of these experiments are noted in the Results and Discussion section. MnP immobilization reactions involving the more hydrophobic HEMA, MAM, or TMPTMA-containing dispersion polymers included 0.4% (v/v) Pluronic L31 (BASF Corp., Parsippany, NJ) nonionic detergent as a wetting agent.

Activity of Free and Immobilized MnP

Peroxidase activity was determined with vanillylacetone [4-(4-hydroxy-3-methoxyphenyl)-3-butene-2-one] (Aldrich Chem. Co., Milwaukee, WI) using final concentrations of 0.10 m*M* vanillylacetone, 10 m*M* $MnCl_2 \cdot 4H_2O$, 10 m*M* oxalate (pH adjusted to 4.0), and 1.0 m*M* H_2O_2 all in 100 m*M* sodium acetate buffer, pH 4.0. Reaction volume was 1.0 mL, and assays were performed at 37°C measuring decreased absorbance at 336 nm (ϵ = 18,300) (*35*).

In order to overcome the problem of measuring the hydrated polymers for protein determinations and activity assays, each coupling reaction was normalized by diluting with buffer to the desired percentage of polymer (% m/v, mg dry polymer/μL buffer). Accurate measurement of the hydrated MnP polymer or control polymer was achieved by preparing a polymer suspension employing the following procedure:

1. The pelleted washed polymer (supernatant removed by pipeting) was weighed in its reaction tube;
2. The mass of an empty reaction tube was subtracted for tare correction; and
3. The pellet was diluted with 10 m*M* sodium acetate buffer, pH 4, to the desired final volume assuming the polymer solution density equaled that of water (1.0 g/mL).

Resulting polymer suspensions of 300–1000 mg (300–1000 μL) from an initial 10 mg dry Emphaze sample were commonly used for both enzyme

activity and protein estimations. The MnP-polymer suspension (5–50 μL measured using a 200-μL pipet tip with about 5 mm cut off the end) was added to the stirred cuvett (Hellma Cells, Inc., Forest Hills, NY) and the reaction started by addition of preheated (37°C), stirred (300 rpm) reaction mixture described below. A Hewlett Packard HP 8452A spectrophotometer equipped with a Peltier temperature controller and stirring motor was employed.

Oxidation of Mn^{2+} to Mn^{3+}-oxalate was measured in an assay system based on that described by Glenn and Gold (*36*), using a 1.0-mL reaction mixture containing 1–5 μg MnP, 50 m*M* oxalate, 0.10–10 m*M* $MnCl_2 \cdot 4H_2O$, and 1.0 m*M* H_2O_2 in 50 m*M* sodium acetate, pH 4.0. Absorbance at 256 nm was monitored for Mn^{3+}-oxalate complex formation (ϵ = 6012 at pH 4.0). Catalytic generation of Mn^{3+}-oxalate paralleled catalytic oxidation of vanillylacetone. Therefore, vanillyacetone oxidation was used as an indirect measure of Mn^{3+}-oxalate formation owing to the increased accuracy and sensitivity of the former.

Protein Determinations

The amount of bound radiolabeled protein was determined in a Pharmacia/Wallac 1470 γ scintillation counter. The specific radioactivity of the MnP to be coupled was adjusted prior to each experiment by addition of unlabeled MnP to ~500 cpm/μg protein. After the initial determination of bound radiolabeled protein, covalently linked MnP was measured following incubation of the MnP-polymer complex with 1.0 mL 1% sodium dodecyl sulfate (SDS) for 4–16 h at 37°C, followed by centrifugation, removal of the supernatant, and washing with SDS three times for 15–30 min each.

In experiments performed without radiolabeled MnP, coupled protein was measured by direct protein determination of the MnP-polymer complex by the bicinchoninic acid (BCA) method (*37,38*). Polymer suspension was prepared and measured as described above. Polymer suspension (50 μL appropriately diluted) was dispensed into 2-mL tubes and 1.0 mL BCA working reagent (Pierce Chem. Co., Rockford, IL) added to each tube. The tubes were capped and rocked for 2 h at room temperature or incubated (30 min at 37 or 60°C depending on the desired sensitivity). Tubes must be rocked to suspend the protein-polymer matrix and obtain good color development. Samples were cooled to room temperature and suspended material removed by centrifugation. Supernatant absorbance was measured at 562 nm and values interpolated from a standard curve of bovine serum albumin, Cohn fraction V (Sigma Chemical Co.). Zero protein control reactions of the test polymer were prepared as above and their absorbance value subtracted from the protein-containing reactions.

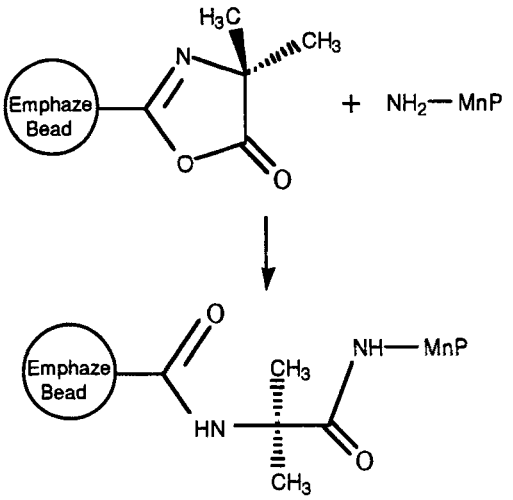

Fig. 1. The ring-opening reaction of Emphaze dimethyl azlactone functional polymer with an amine of MnP.

RESULTS AND DISCUSSION

Amino Acid Analysis, pH Stability, and pH Effect on Immobilization

Two properties of MnP influencing optimization of the immobilization conditions with the azlactone polymers were the lysine content and pH stability of the protein. An important reaction in the chemistry of azlactones is the ring-operation addition that occurs with various nucleophiles: RNH_2, RSH, and ROH (Fig. 1). In this reaction, amide bond formation can occur between a primary amine, such as the lysyl group of a protein, and the azlactone (*28*). Although the thiol of L-cysteine is considered to be a more reactive nucleophile than the ϵ-amino of L-lysine, thioesters are much less stable than substituted amides, and furthermore, the average protein is comprised of 3.4% cysteine vs 7% lysine (*39*). Therefore, lysyl residues are the most probable residues for immobilization (*40*). Unfortunately, lysine comprises only 1% of MnP's amino acid composition (Table 1), well below the average protein's lysine content of 7%. The 1% lysine content of MnP is one explanation for the relatively low azlactone polymer coupling efficiency (CE), ~40–50%. In contrast, azlactone polymer CEs of > 90% have been reported for recombinant protein A, which contains 12% lysine (*28*).

The pH optimization experiments allowed us to define a suitable pH that would not destroy the enzyme's catalytic activity while maximizing the reactivity of the available nucleophiles. The stability of MnP was determined over a pH range of 3–9, and the reactivity of available nucleophiles measured through the CE over a pH range of 4–9. MnP remains stable for

Table 1
Amino Acid Composition of MnP vs Average Protein
(Reactive Residues Only)

Residue	MnP[a], %	Average[b], %
Ser	7.4	7.8
Lys	1.1	7.0
Thr	7.6	6.5
Asp	12.1	4.8
Glu	8.7	4.8
Arg	3.1	3.8
Tyr	ND	3.4
Cys	2.5	3.4
His	1.8	2.2
Met	1.6	1.6
Trp	ND	1.2

[a]Purified from *L. edodes* commercial cultures.
[b]Source (*39*).

at least 6 h between pH 3 and 6, retaining >95% of its activity. However at pH 7 and 7.5, the peroxidase activity decreases by 20–30% and is not recoverable when assayed within the optimum pH range. A dramatic and permanent loss of activity occurs above pH 7.5 with 55, 65, and 75% lost in 1 h and 65, 75, and 90% lost after 6 h of room temperature exposure to pH 8, 8.5, and 9, respectively. These results were significant, since CEs are enhanced by alkaline pH in the presence of sulfate (*28*). CE responded in a sigmoidal fashion as the pH of the coupling reaction was increased. CE was very low at pH < 6, increased exponentially from pH 6 to 8, and began to level off above pH 8. Considering the data in Table 1 and the pH effects, and taking into account the desired catalytic activity with reasonably high CE, a compromise of activity loss and CE gain was achieved by selecting pH 7 as time optimum pH for immobilizing MnP on the azlactone polymers.

Time-Course of Coupling

The time-course for MnP coupling and percentage of coupled MnP that is covalently attached (resistant to SDS incubation) after immobilization on Emphaze (Fig. 2) showed that the reaction is very rapid with 45% of the final binding density achieved within the first 5 min. However, a substantial amount of coupling occurs up to 2 h, when the reaction reached 90% of its final CE of 53%. No increase in CE was obtained when the coupling reaction was allowed to proceed beyond 6 h. The amount of MnP covalently linked to the polymer and not simply adsorbed or trapped in the matrix is indicated by the SDS resistance curve. The CE for covalently

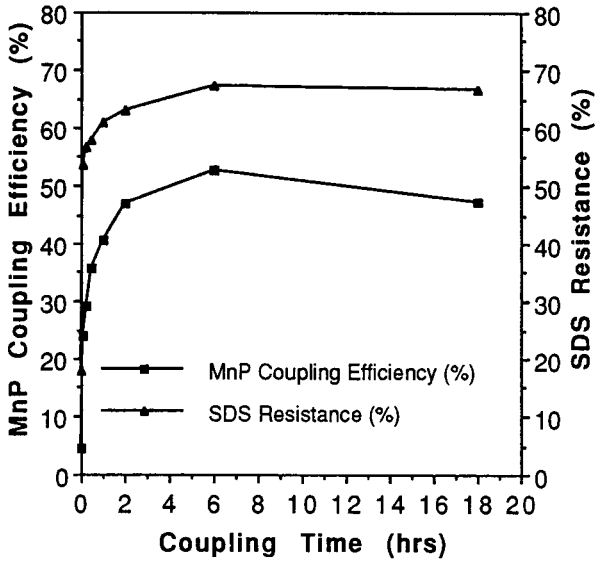

Fig. 2. The time-course for the reaction of MnP with Emphaze in the presence of 1.0*M* sodium sulfate and 1.0 m*M* $MnCl_2$. The buffering agent in each experiment was 100 m*M* sodium phosphate (pH 7.0). MnP concentration was 1.0 mg/mL in 200 μL coupling buffer added to 10 mg Emphaze polymer. Coupling reactions were stopped by addition of ethanolamine quenching reagent. Standard wash procedures were used prior to protein estimations of coupling efficiency using ^{125}I-labeled MnP tracer at 500 cpm/μg MnP. Zero time-point was determined by addition of MnP-^{125}I MnP coupling mixture to a prequenched and washed sample of Emphaze (10 mg Emphaze reacted 4 h with ethanolamine followed by the standard wash procedure).

bound MnP parallels overall CE with >60% of the bound protein covalently coupled to the support. From these results, coupling times for MnP with the azlactone polymers were extended to at least 2 h and typically were allowed to proceed 4 h prior to addition of the ethanolamine-quenching reagent.

Concentration Dependency

MnP was coupled under the standard conditions with a reaction time of 3 h and MnP concentrations of 0.25–12 mg/mL (Fig. 3). These experiments showed that MnP could be immobilized at 60 mg of enzyme/g (1.0 g swells to ~8.5 mL) of Emphaze by coupling at an initial concentration of 12 mg/mL. Maximum CEs (40–50%) were achieved at MnP coupling concentrations <1.5 mg/mL. MnP density increased almost linearly to 60 mg/g at a coupling concentration of 12 mg/mL. However, CE decreased at MnP concentrations above 1.0 mg/mL.

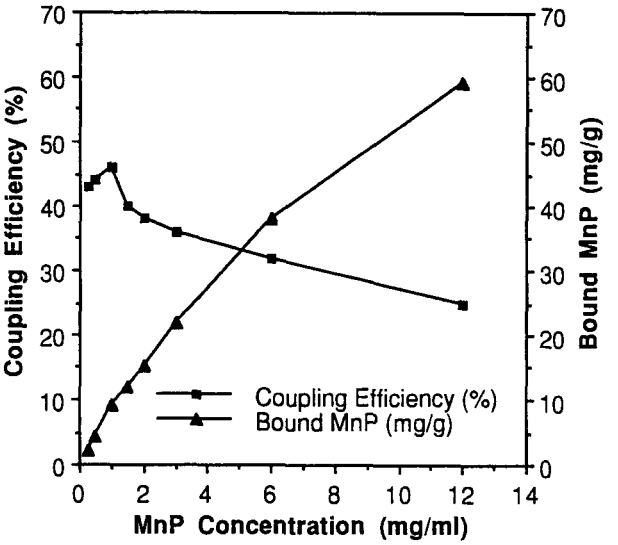

Fig. 3. The concentration dependency for the reaction of MnP with Emphaze in the presence of 1.0*M* sodium sulfate. The buffering agent in each experiment was 100 m*M* sodium phoshate (pH 7.0). MnP concentrations ranged from 0.25 to 12.0 mg/mL in 200 μL coupling buffer added to 10 mg Emphaze polymer. No $MnCl_2$ was added to the mixture. Coupling reaction time was 3 h before addition of ethanolamine quenching reagent. Standard wash procedures were used prior to protein estimations of coupling efficiency by the BCA method.

Enhancement by Highly Ionic Conditions

The coupling of MnP to the azlactone polymers is enhanced by ionic conditions induced by high concentrations of citrate, sulfate, or tartrate (Fig. 4). High sodium chloride concentrations had no significant effect on CE. Sodium sulfate was the most effective enhancer, increasing MnP CE from 1% when no enhancer was present to >40% when 1.0*M* sodium sulfate was included in the reaction mixture. Citrate and tartrate also dramatically increased CE to maximums of 38 and 36% CEs, respectively. Although tartrate and sulfate show linear increases in CE from 0.6 to 1.0*M* concentrations, CE decreases sharply at concentrations above 1.0*M* for these enhancers (data not shown). The decline in CE above 1.0*M* is attributed to salting out of MnP. From these results, we designated 1.0*M* sodium sulfate as the enhancer of choice for MnP immobilizations on the azlactone polymers.

The sulfate-enhanced immobilization of proteins to the azlactone-functional polymers has been previously reported (*28*). The mechanism of enhancement by highly ionic coupling conditions can be explained if covalent immobilization is considered as a two-step process. The first step

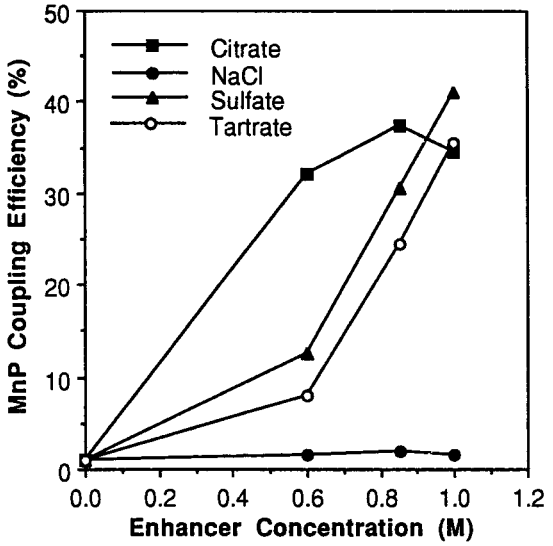

Fig. 4. The effects of ionic enhancers on the reaction of MnP with Emphaze. The buffering agent in each experiment was 250 m*M* MOPS adjusted to pH 7.5 after addition of enhancer. Enhancers were sodium citrate, sodium chloride, sodium sulfate, and sodium tartrate. MnP concentration was 2.0 mg/mL in 200 μL coupling buffer added to 10 mg Emphaze polymer. No $MnCl_2$ was added to the mixture. Coupling reaction time was 3 h before addition of ethanolamine quenching reagent. Standard wash procedures were used prior to protein estimations of coupling efficiency using ^{125}I-labeled MnP tracer at 500 cpm/μg MnP.

is adsorption and promotes the second step of covalent bond formation (*41*). Since adsorptive phenomena and all the noncovalent interactions can affect formation of the covalent bond, ions with a strong salting-out effect would inhibit ionic interactions, but promote hydrophobic interactions and subsequent covalent bond formation between the protein and support. At the salt concentration where the protein begins to precipitate from solution, the salting-out ions not only preserve enzymatic activity by stabilizing the α-helical structure, but also increase protein coupling efficiency through hydrophobic adsorption (*33,34*).

Temperature Effect

The temperature of the coupling reaction has a significant effect on coupling efficiency (Fig. 5). The coupling efficiency increases in direct relationship to higher temperature. The amount of MnP coupled was 60% higher at 60°C than it was at the standard coupling temperature of 22°C. Temperatures above 60°C were not tested owing to activity losses and protein denaturation at higher temperatures.

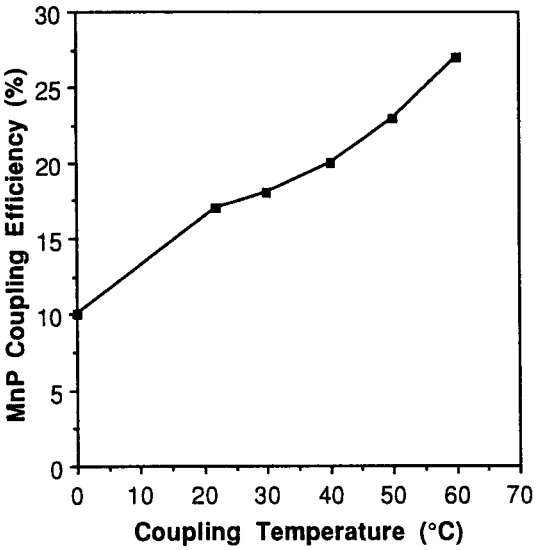

Fig. 5. The effect of temperature on the reaction of MnP with Emphaze in the presence of 1.0*M* sodium sulfate. The buffering agent in each experiment was 250 m*M* MOPS, pH 7.5. MnP concentration was 2.0 mg/mL in 200 μL coupling buffer added to 10 mg Emphaze polymer. No $MnCl_2$ was added to the mixture. All coupling reactions were vortexed at 10-min standard room temperature reactions. Coupling reaction time was 1 h before addition of ethanolamine quenching reagent. Standard wash procedures were used prior to protein estimations of coupling efficiency using ^{125}I-labeled MnP tracer at 500 cpm/μg MnP.

Activity of Immobilized MnP

Initial measurements of MnP activity using MnP polymer prepared by the coupling efficiency optimized procedures with the exception of temperature (4-h reaction time, pH 7, coupling buffer + 1*M* sodium sulfate, room temperature) showed coupled MnP only exhibited 10–15% of the expected activity (data not shown) as compared to the 100% value obtained by assaying a comparable amount of the soluble protein. In order to increase the oxidase activity of the tethered MnP, coupling conditions were altered by the addition of various amounts of the substrate, Mn^{2+}. The resuls of this experiment (Fig. 6) show the catalytic Mn^{3+}-generating activity of the MnP polymer was increased dramatically when Mn^{2+} was included in the coupling buffer during the immobilization reaction. A sharp increase in oxidase activity was observed up to 1 m*M* Mn^{2+} followed by progressively lower activity measurements up to 20 m*M* Mn^{2+}. All Mn^{2+} concentrations tested increased the activity of the final MnP polymer as compared to the control immobilization without Mn^{2+}. In this experiment, Mn^{2+} concentration was optimal at 1.0 m*M*, resulting in a

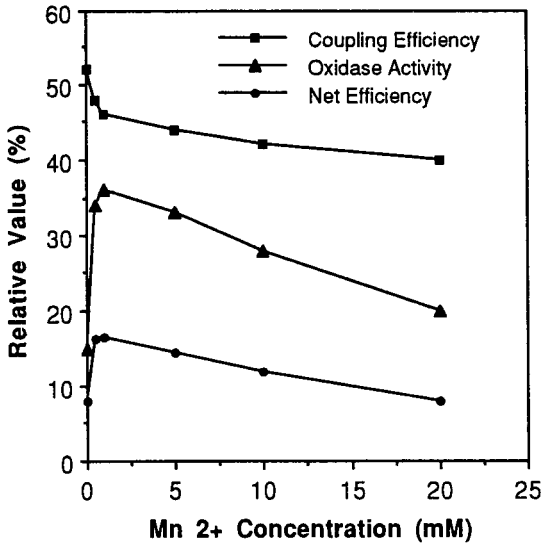

Fig. 6. The effect of $MnCl_2$ (substrate) concentration on the reaction of MnP with Emphaze and oxidase activity of the MnP-Emphaze polymer. The buffering agent in each experiment was 100 m*M* sodium phosphate (pH 7.0) containing 1.0*M* sodium sulfate. MnP concentration was 1.0 mg/mL in 200 μL coupling buffer added to 10 mg Emphaze polymer. $MnCl_2$ concentrations ranged from 0 to 20 m*M*. Coupling reaction time was 16 h before addition of ethanolamine quenching reagent. Standard wash procedures were used prior to protein estimations of coupling efficiency using ^{125}I-labeled MnP tracer at 500 cpm/μg MnP. MnP activity was measured as described in the text. Net efficiency = coupling efficiency × oxidase activity.

2.5-fold increase in activity. However, coupling efficiency does decrease slightly as a result of Mn^{2+} inclusion, but net efficiency (coupling efficiency × oxidase activity) still more than doubles going from 7.8% without Mn^{2+} to 16.4% at 1 m*M* Mn^{2+}.

A possible explanation for the increased activity after coupling MnP to the polymer in the presence of Mn^{2+} is that Mn^{2+} occupies the enzyme active site during the immobilization reaction, thereby promoting the correct conformation of the MnP on the polymer and protecting essential amino acid residues. The resultant MnP-polymer complex active sites are not conformationally restricted and allow substrate-enzyme complex formation. Correct enzyme conformation and the ability of the enzyme to change this conformation during catalysis are generally considered the primary factors governing the ability of an enzyme to act as a specific catalyst (*42*). In addition to substrate protection of essential amino acid residues during covalent immobilization (*43*), reversible covalently linked enzyme-inhibitor complexes (*41*), a chemically modified soluble enzyme whose covalent linkage to matrix is achieved by newly incorporated residues (*44*), and a zymogen precursor (*45*) have also been used to increase the final activity of some immobilized enzymes.

Table 2
Comparison of Coupling Efficiency and Activity of MnP-Azlactone Polymers

Polymer	C.E., %[a]	Activity, %[b]	Net, %[c]
HEMA 10	57	62	35
HEMA 30	55	63	35
TMPTMA:VDM (80:20)	56	35	20
MAM 10	64	94	60
MAM 20	63	79	50
MAM 30	63	100	63
MAM 40	50	16	8
Emphaze	48	22	11

[a]Relative coupling efficiency (C.E.) measured by ^{125}I MnP bound/^{125}I MnP added × 100 (pH 7.0, 1.0 m*M* Mn^{2+}, 1.0*M* Na_2SO_4, and 16 h coupling reaction).
[b]Relative vanillylacetone oxidation by bound MnP with soluble MnP incubated at pH 7.0 (6 h) as 100% value.
[c]Net efficiency determined by C.E. × activity.

Comparisons of MnP Coupling Efficiency and Enzyme Activity on Other Azlactone Polymers

MnP CE and enzyme activity were significantly affected by support matrix properties in addition to the physical and biochemical influences previously discussed. MnP was immobilized on several azlactone polymers more hydrophobic than Emphaze. The irregular particulate matrix of these nonporous polymers also differed from the uniform 50–80 μm spherical Emphaze. MnP CEs were slightly higher for the particulate dispersion polymers than for Emphaze, and the coupled enzyme was >90% active on two of the MAM supports (Table 2). The net efficiency (CE × activity) for MnP utilization ranged from two- to sixfold higher than Emphaze with these polymers, and net efficiency only decreased in the case of MAM 40.

Although the differences in MnP's activity and CE on the particulate polymers vs Emphaze are still under investigation in our laboratory, several possible explanations for these differences exist. Emphaze is the most hydrophilic of the polymers tested in Table 2. Hydrophilicity of the carrier is an important matrix property, since enzyme inactivation has been observed when some proteins adsorb to highly hydrophobic surfaces (*46,47*). This inactivation is perhaps owing to unfolding and inversion of hydrophobic regions of the protein, which then interact with the hydrophobic surface of the carrier (*48*).

In contrast to the hydrophobically induced denaturation reported for some proteins (*47,48*), MnP appears to respond favorably to hydrophobic carriers. The particulate dispersion polymers are more hydrophobic than Emphaze, and their hydrophobicity results in higher CE possibly because

of the high sulfate concentration in the coupling mixture and enhanced hydrophobic interaction prior to amide bond formation. The higher activity of MnP coupled to the more hydrophobic dispersion polymers could be explained if the native environment of MnP is considered. MnP is secreted by the fungal hyphae as they grow throughout their natural wood substrate. The ultimate target of MnP is the lignin component of wood. Lignin is degraded in order to permit fungal cellulases and hemicellulases to access and utilize the cellulose and hemicellulose as carbon and energy sources. Since wood is comprised of 20–30% lignin, a hydrophobic polymer of phenylpropane units (*49,50*), MnP may be accustomed to the more hydrophobic chemistry of the dispersion polymers and therefore exhibit higher activity when coupled to surfaces of greater hydrophobicity.

Other possible explanations for lower CE and MnP activity for Emphaze compared to the dispersion polymers are differences in the MnP-polymer microenvironment caused by diffusional limitations and conformational restraint of the enzyme. Differences in the kinetic behavior of immobilized vs free proteins have been previously attributed to diffusional and conformational limitations (*51*). MnP immobilized throughout the porous Emphaze polymer may exhibit lower activity owing to diffusional limitations. It is possible that the substrate penetrates only a short distance into the MnP-Emphaze polymer before it is completely converted to product. In this instance, the MnP at the center of the particle will not take part in the reaction even if it is active. The nonporous MnP-particulate polymers would not exhibit this diffusional limitation, since all of the immobilized protein is available for surface interaction with the substrate as long as essential active site amino acids are not involved in the coupling reaction. Likewise, the number of available functional groups at the outer surface of the porous polymer beads may couple MnP rapidly, and the immobilized protein sterically hinder penetration and subsequent immobilization of free MnP remaining in solution. Multipoint attachment of MnP to Emphaze is also more likely the result of the dimensionality of the porous polymer. Multipoint attachment is thought to increase thermal and pH stability by rigidification of the protein preventing denaturation (*52*). However, rigidification can also restrict conformational changes in the enzyme, decreasing activity by limiting allosteric interaction with the substrate (*42,51*).

CONCLUSION

Selection and use of an immobilization support is influenced by a multiplicity of factors, including the chemical characteristics and composition of the enzyme; the properties of substrates and products; the capacity of the matrix, its chemistry, stability, and flow properties; and the expense and difficulty of the coupling procedures. Ultimately, the final application intended for the biosupport system dictates selection of a

simple, cost-effective procedure that utilizes enzyme and produces product as efficiently as possible. Preparation of a catalytically active MnP-azlactone polymer complex was achieved by considering several of these factors. Our results presented here show how the biochemical characteristics of MnP influence physical and chemical parameters necessary for maximum coupling efficiency, while maintaining the catalytic activity of the manganese peroxidase purified from *L. edodes*.

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