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Immobilization of Manganese Peroxidase from *Lentinula edodes* on Alkylaminated Emphaze™ AB 1 Polymer for Generation of Mn^{3+} as an Oxidizing Agent

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ABSTRACT

Manganese peroxidase (MnP) is secreted by white-rot fungi and participates in the degradation of lignin by these organisms. MnP uses H_2O_2 as an oxidant to oxidize Mn^{II} to Mn^{III} as the manganic ion Mn^{3+}. The Mn^{3+} stabilized by chelation, is a highly reactive nonspecific oxidant capable of oxidizing a variety of toxic organic compounds. Previous attempts at immobilization of MnP, purified from *Lentinula edodes* through reactive amino groups, have been hindered by the protein's low lysine content of only 1% and its instability above pH 6.0. As an alternative to amine coupling, the enzyme has now been covalently immobilized through its carboxyl groups, using an azlactone-functional copolymer derivatized with ethylenediamine and 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) as a coupling reagent. The immobilization reaction was performed under acidic (pH 5.25) conditions, and 90% coupling efficiency was achieved within 2 h. Net immobilization efficiencies, expressed as the product of protein coupling efficiency and enzyme activity, have been measured at > 95% within 4 h. The MnP-NH-polymer and the free soluble protein were characterized and compared for their pH, temperature, and storage stabilities, as well as their H_2O_2 dependence and kinetics. The tethered

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MnP, employed in an immobilized enzyme bioreactor for generation of chelated Mn^{3+} may have industrial applications as a nonspecific oxidant of organopollutants.

Index Entries: Enzyme immobilization; white-rot fungi; manganese peroxidase; Mn^{3+}; azlactone; Emphaze Biosupport Medium AB 1; EEDQ; biocatalyst.

INTRODUCTION

The use of enzymes in industrial processes is limited because they are proteins. Proteins are expensive to produce and purify and are sensitive to denaturation or inactivation by, among other things, pH and temperature extremes, organic solvents, detergents, and proteases. Although native enzymes in batch-stirred and similar reactors have been used for toxic waste stream treatment (*1*), only the cheapest of enzymes could be used in this soluble form, since these treatment processes would require large quantities of enzyme that would be lost in the effluent (*2*). For these reasons, immobilized enzymes have gained attention for use as biocatalysts in the degradation of toxic organic compounds from water, air, and soil (*2,3*). Immobilization allows reuse of the catalyst (*4,5*), and may improve stability against one or more of the denaturing extremes encountered by the enzyme during catalysis (*3,6,7*).

Oxidative enzymes from wood-degrading 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*). Manganese peroxidases use H_2O_2 as an oxidant to oxidize Mn^{II} to Mn^{III} as the manganic ion Mn^{3+} (*9*). Enzyme-generated Mn^{3+}-chelate has been postulated to be the primary agent responsible for ligninolysis (*10*). MnP is a particularly interesting enzyme because lignin, the most recalcitrant component of wood, is oxidized at a considerable distance from the protein by a diffusible reactive intermediate, MnP-generated Mn^{3+}-chelate. Mn^{3+}-chelate, is a highly-reactive nonspecific oxidant (*11–14*) capable of oxidizing not only lignin, but also a variety of organic compounds (*15–22*). Therefore, MnP is an attractive prospect for an immobilized-enzyme bioreactor that generates Mn^{3+}-chelate as an organopollutant oxidizing agent.

The commercially-grown white-rot basidiomycete, *Lentinula edodes* (edible shiitake mushroom) serves as an excellent source of MnP. It is the dominant cultivated mushroom of the eastern hemisphere (*23–25*) and is readily available in the United States. MnP is the major extracellular peroxidase detected in *L. edodes* cultures grown on a commercial wood substrate (*26*). MnP from *L. edodes* has been purified and characterized as 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 (*27*).

In recent years, many support matrices and coupling chemistries have been developed and made commercially available for use in protein immobilization (*28*), but each has inherent advantages and disadvantages. Selecting and efficiently employing an immobilization support is influenced not only by the biochemical characteristics of the protein but also by chemical, physical, and economic attributes of the matrix itself. We previously reported immobilization of MnP on Emphaze Biosupport Medium AB 1, optimizing immobilization conditions for maximum protein coupling efficiency and enzyme activity (*29*). However, the protein's biochemical characteristics, low lysine content and alkali-lability, were not ideally suitable for the coupling chemistry of that support. Net immobilization efficiencies (product of protein coupling efficiency and enzyme activity) were limited to only 35%. Through the methods reported in this article, net immobilization efficiencies for MnP have been increased to >95%, using alkylaminated Emphaze Biosupport Medium AB 1 (NH_2-Emphaze) and 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) as a coupling reagent. The MnP-NH-Emphaze polymer and the free soluble protein have been biochemically-characterized and compared. These results significantly advance our efforts toward the development of an immobilized MnP bioreactor for generation of Mn^{3+} as an oxidizing agent.

MATERIALS AND METHODS

MnP Source

Commercial *L. edodes* cultures of heterodikaryon strain MS-20 were purchased from L. F. Lambert Spawn (Coatesville, PA). The cultures achieved maximum peroxidase production between 35 and 50 d after inoculation. MnP was extracted, and stored as described previously (*27*). (*Note:* The purification procedure was simplified and yield improved by decreasing the pH of the diafiltration and chromatography buffer from pH 7.0 sodium phosphate (10 m*M*) to pH 5.5 sodium acetate (10 m*M*) and by substituting an ammonium sulfate precipitation step in place of the DEAE anion exchange chromatography step.) 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, St. Louis, MO) precipitation and centrifugation. PEI-clarified filtrate was concentrated 50-fold and diafiltered by ultrafiltration (30,000 Da cut-off membrane) into the acetate buffer. MnP was purified from the concentrated diafiltered extract by ammonium sulfate precipitation (45–65% pellet contained ~75% of the MnP activity). The acetate buffer was added to the pellet until the ammonium sulfate concentration equaled 1.5*M* as determined by conductivity measurement. The pellet was allowed to dissolve with stirring (2 h, 4°C), centrifuged to remove particulates (15,000*g*, 30

min), and the supernatant proteins separated by Phenyl 5PW hydrophobic interaction chromatography as described previously (*27*).

Alkylaminated Emphaze Biosupport Medium AB 1

The Emphaze polymer was prepared by an inverse-phase polymerization process (*30*). The experiments described here employ Emphaze Biosupport Medium AB 1 (hydrophilic, macroporous, azlactone-functional polymer beads 50–80 μ in diameter), (Pierce, Rockford, IL) modified by alkylamination. Physical characterization and chromatographic performance of Emphaze beads has been performed by the manufacturer (*31*). The azlactone functionality was modified by addition of free amino groups through the reaction of 1.2 g Emphaze AB 1 polymer with 20 mL 1.0*M* ethylenediamine (Aldrich, Milwaukee, WI). The reaction mixture was vortexed for 1 min to facilitate complete mixing and hydration of the dry polymer and then rocked for 120 min at ambient temperature. The ethylenediamine modified polymer was centrifuged (5000*g* for 3 min), supernatant solution removed carefully by pipetting, washed repeatedly (15–30 min each) with 30 mL Milli-Q H_2O until supernatant was of neutral pH, and supernatant removed as above. The washed NH_2-Emphaze polymer was stored at 4°C (50% v/v slurry in 20% ethanol).

MnP Immobilizations

Stored NH_2-Emphaze polymer was washed three times with 100 m*M* MES buffer, pH 5.25, containing 1.0*M* sodium sulfate, 1.0 m*M* $MnCl_2$ (coupling buffer). The buffer volume for each wash was five times the polymer volume. A typical protein-coupling reaction consisted of 150 μL of washed polymer suspended in 200 μL of coupling buffer, and the desired amount of MnP (350 to 1050 μg optimal) in a 2 mL tube. The reaction mixture was vortexed for 1 min to facilitate complete mixing and rocked for 15 min at ambient temperature. Coupling reagent 0.5*M* 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) (Aldrich) in ethanol (*Note*: 0.5*M* EEDQ has limited solubility in ethanol and this stock solution must be warmed slightly above ambient temperature in order for it to dissolve) was added to a final concentration 10.0 m*M*; the reaction was quickly vortexed for 30 s, and rocked for at least 4 h at ambient temperature. Alternatively, 1.0*M* 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (Aldrich) in water was added to a final concentration 10.0 m*M* in some experiments in order to compare it to EEDQ as a coupling reagent for MnP. The MnP-coupled polymer was centrifuged (5000*g* for 3 min), supernatant solution removed carefully by pipetting, and washed 4 times (15–30 min each) with 100 m*M* sodium acetate buffer, pH 5.0 (buffer A), buffer A + 1.0 M NaCl, and 2 times with 10 m*M* buffer A. Amounts of support and solutions can be increased proportionally. 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.

Time-course of coupling was performed under typical coupling conditions, except reaction time was varied from 5 min to 18 h. The MnP concentration dependency for the reaction of MnP with the NH_2-Emphaze polymer was tested by adjusting the final concentration of MnP in each reaction from 0.5 mg/mL to 15 mg/mL and reacting in the usual manner. The effect of MnP load on peroxidase activity was determined under the standard MnP/vanillylacetone assay conditions described below.

Activity of Free and Immobilized MnP

Peroxidase activity was determined with vanillylacetone [4-(4-hydroxy-3-methoxyphenyl)-3-butene-2-one] (Aldrich) 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 0.10 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 40°C, measuring decreased absorbance at 336 nm ($\in$ = 18,300) (*32*). MnP activity for the MnP-polymer was expressed as the percentage of coupled MnP retaining its enzyme activity calculated by: [Total International Units (IU) coupled to the polymer as measured by the vanillylacetone method above / Total IU added to the original coupling reaction × %CE for that reaction] × 100 = % oxidase activity.

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 wet polymer/μL buffer). Accurate measurement of the hydrated MnP-polymer or control polymer was achieved by preparing a polymer suspension employing the procedure described in (*29*). The MnP-polymer suspension (10 to 50 μL appropriately diluted) was added to the cuvet first, and the reaction was started by addition of preheated (40°C) reaction mixture described above (stir at 300 rpm). A Shimadzu UV 160 spectrophotometer equipped with a stirring motor (Instech Labs., Plymouth Meeting, PA) was employed.

Oxidation of Mn^{2+} to Mn^{3+} oxalate was measured in a modified assay system (*29*) based on that described by Glenn and Gold (*33*).

Protein Determinations

The amount of coupled protein was measured by direct protein determination of the MnP-polymer complex by the bicinchoninic acid method (*34,35,29*). Briefly, polymer suspension (50 μL appropriately diluted) was dispensed into 2 mL tubes and 1.0 mL BCA working reagent (Pierce) added to each tube. The tubes were capped and rocked for 2 h at room temperature or incubated (30 min at 37°C or 60°C, depending on the desired sensitivity). Tubes were rocked to suspend the protein-polymer matrix and obtain good color development. Samples were cooled to room

temperature and suspended material was removed by centrifugation (16,000*g*, 5 min). Supernatant absorbance was measured at 562 nm and values interpolated from a standard curve of bovine serum albumin, Cohn fraction V (Sigma, St. Louis, MO). Zero protein control reactions of the test polymer was prepared as above and their absorbance value subtracted from the protein-containing reactions. Protein-coupling efficiency (CE) was expressed as a percentage calculated by: [Total μg of protein coupled to the polymer as measured by the direct BCA method above / Total μg of protein added to the original coupling reaction as measured by the BCA method] × 100 = % CE.

Biochemical Characteristics of Free and Immobilized MnP

pH Stability

MnP (50–80 μg/mL) free in solution or immobilized 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 4.0–5.5, Tris-maleate for 6.0–7.5, and TAPS for 8.0–9.0. After incubation at the test pH for 1 h, a 50 μL aliquot of MnP was removed and peroxidase activity determined under the standard MnP/vanillylacetone assay conditions described above.

Temperature Effect and 60°C Thermal Stability

MnP (50–80 μg/mL) free in solution or immobilized was incubated in 10 m*M* buffer A over a temperature range of 0–60°C. At the indicated time intervals (5 min–2 h), an aliquot of MnP was removed and peroxidase activity determined under the standard MnP/vanillylacetone assay conditions described above.

Storage Stability

MnP (50–80 μg/mL) free in solution or immobilized was stored (4°C) in 10 m*M* buffer A. At the indicated time intervals (7–90 d), an aliquot of MnP was removed and peroxidase activity determined under the standard MnP/vanillylacetone assay conditions described above.

H_2O_2 Dependence

The H_2O_2 dependence was determined by MnP/vanillylacetone assay with 4.5 μg of free or immobilized MnP in each 1.0 mL assay and an increasing H_2O_2 concentration of 0 to 500 μ*M*.

K_m and V_{max}

The K_m and V_{max} were determined by MnP/vanillylacetone assay with 2.5 μg of free or immobilized MnP in each 1.0 mL assay and an increasing substrate concentration of 0.10 to 10 m*M* $MnCl_2$. Data were plotted by the method of Lineweaver-Burk (*36*).

RESULTS AND DISCUSSION

Immobilization of MnP

In our previous studies (*29*), two properties of MnP that influenced optimization of the immobilization conditions with the azlactone polymers were lysine content and pH stability of the protein. An important reaction in the chemistry of azlactones is the ring-opening addition, which occurs with nucleophiles such as amines (*37*). Lysyl residues are the most probable residues for immobilization (*38*), where amide bond formation can occur between a primary amine, such as the ∈-amino of L-lysine, and the azlactone (*37*). Lysine comprises only 1% of MnP's amino acid composition, 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% when attempting to immobilize MnP to Emphaze AB 1 polymer through reactive amines. In contrast, Asp and Glu residues constitute 12.1% and 8.7% of MnP's amino acid composition, respectively (*29*). This is well above the average protein's Asp and Glu content of 4.8% and 3.8%, respectively (*39*). Since these acidic residues usually represent the majority of surface functional groups in proteins (*40*), their reactive carboxy groups were likely candidates for immobilization with an aminated support.

The stability of MnP has been determined over a pH range of 3.0–9.0, and the protein remains stable for at least 6 h between pH 3.0–6.0, retaining > 95% of its activity. However, at neutral and alkaline pH, a rapid and permanent loss of peroxidase activity was observed (*29*). Given MnP's poor availability of reactive surface amino groups, apparent abundance of reactive carboxyl groups, and instability at pH > 6.0, the Emphaze AB 1 polymer was derivatized by alkylamination with excess ethylenediamine. Covalent attachment was achieved through the carboxyl groups of MnP, employing EEDQ as a coupling reagent (Fig. 1). In this reaction, ethylenediamine reacts with the azlactone functionality, adding a two-carbon spacer arm and amino group. The resultant alkylaminated polymer reacts with MnP's free carboxyl groups under acidic conditions (pH 5.25). Acidic coupling conditions not only prevent alkaline-associated activity losses in MnP, but increase carboxyl group activation efficiency by EEDQ (*41*).

Carboxyl group activation is commonly achieved through condensing reagents, such as water-soluble EDC (*38*). Our initial experiments compared EDC and EEDQ as coupling reagents for immobilization of MnP to the NH_2-Emphaze polymer, and efficient coupling was achieved with both reagents (> 96% in 4 h). However, the reactions employing EDC as a coupling reagent yielded only 46% of the initial MnP activity vs > 90% for the EEDQ-coupled preparation. EDC may be inactivating the MnP through conversion of carboxyl groups into stable isoureas, e.g. tyrosine modification (*42*). Therefore, EEDQ was the reagent of choice for carboxyl activation in MnP.

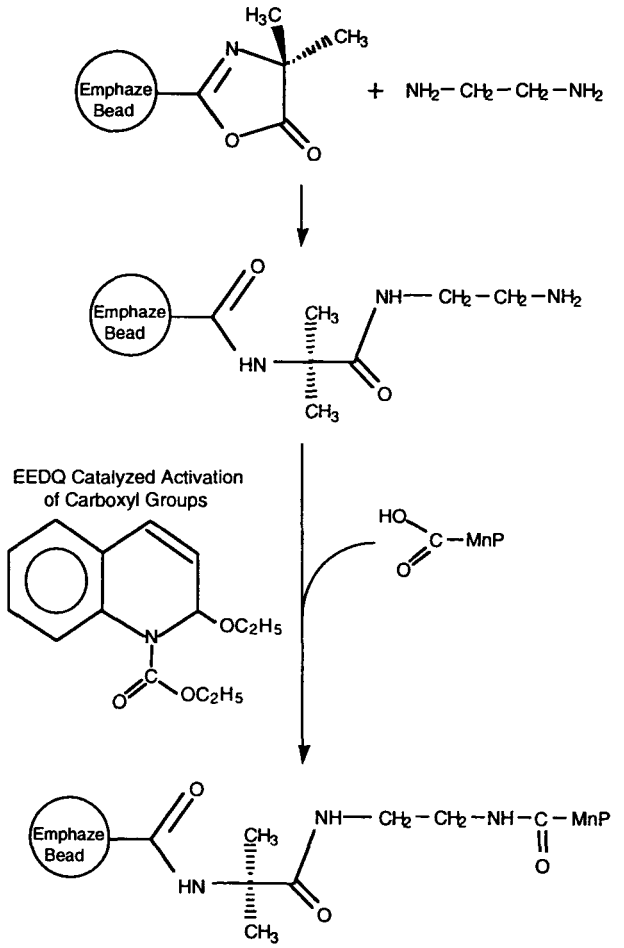

Fig. 1. The ring-opening reaction of Emphaze AB 1 dimethyl azlactone functional polymer with ethylenediamine and subsequent coupling of MnP through its EEDQ-activated carboxyl groups.

EEDQ has been used in peptide synthesis to activate carboxyl groups, yielding a mixed carbonic anhydride intermediate, which subsequently reacts with free amines to form amide bonds (*43*). Several enzymes, including horseradish peroxidase (*44*) and trypsin (*41*), have been immobilized on carboxyl-containing polymers activated with EEDQ. We have found that EEDQ's activation of carboxyl groups can be employed to activate MnP, a protein poor in surface amino groups, allowing extremely efficient attachment to an amino-containing polymer.

Time-Course of Coupling

The time-course for MnP coupling (Fig. 2) showed that the reaction is rapid, with 90% of the final coupling achieved within the first 2 h. The reaction was complete after only 4 h, and CE was frequently measured at 100%. The MnP activity for bound MnP parallels CE, with greater than 95% of the bound protein retaining the ability to oxidize vanillylacetone.

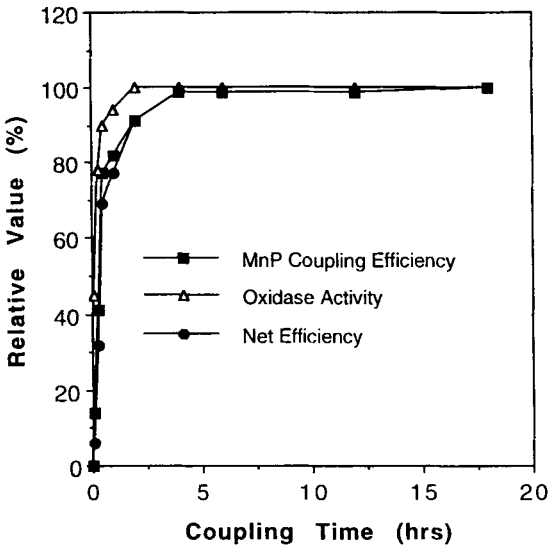

Fig. 2. The time-course for the reaction of MnP with NH_2-Emphaze in the presence of 1.0*M* sodium sulfate and 1.0 m*M* $MnCl_2$. The buffering agent in each experiment was 100 m*M* MES (pH 5.25). EEDQ (10 m*M* final concentration) was added as a coupling reagent. MnP concentration was 2.0 mg/mL in 350 μL coupling buffer containing 150 μL NH_2-Emphase polymer. Coupling reactions were stopped by centrifugation (5000*g* for 0.5 min) and removal of MnP containing supernatant. Standard wash procedures were used prior to protein and activity estimations as described in the text. Net efficiency = coupling efficiency × oxidase activity. Zero time point was determined by addition of MnP-coupling mixture to a prequenched and washed sample of Emphaze (150 μL NH_2-Emphaze reacted 18 h with acetic anhydride and EEDQ followed by the standard wash procedure).

Coupling reactions extended beyond 20 h resulted in 100% CE, but MnP activity losses of 10 to 30% were observed (data not shown). From these results, coupling times for MnP with the NH_2-Emphaze polymer were allowed to proceed 4 h prior to terminating the reaction through the wash procedure.

Concentration Dependency

MnP was coupled under the typical conditions with a reaction time of 4 h and MnP concentrations of 0.5–15 mg/mL (Fig. 3). These experiments showed that MnP could be immobilized at 319 mg of enzyme/g of NH_2-Emphaze by coupling at an initial concentration of 15 mg/mL (1.0 g polymer swells to ~8.5 mL). Maximum net efficiency (~90%) was achieved at MnP-coupling concentrations < 5 mg/mL. MnP density increased linearly from 11 mg/g–319 mg/g with 100% CE at coupling concentrations from 0.5 to 15 mg/mL, respectively. However, oxidase activity decreased at MnP concentrations above 2.0 mg/mL. Differences in the kinetic behavior of immobilized versus free enzymes have been previously attributed to diffusional and conformational limitations (*45*). It is possible that the

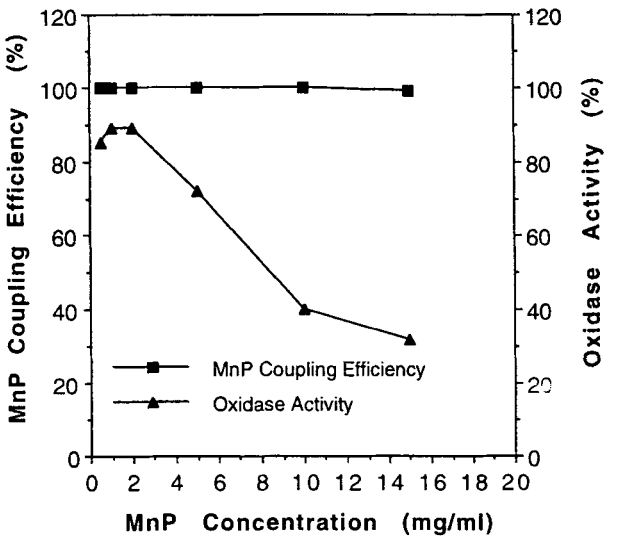

Fig. 3. The concentration dependency for the reaction of MnP with NH_2-Emphaze in the presence of 1.0*M* sodium sulfate and 1.0 m*M* $MnCl_2$. The buffering agent in each experiment was 100 m*M* MES (pH 5.25). EEDQ (10 m*M* final concentration) was added as a coupling reagent. MnP concentrations ranged from 0.5 to 15.0 mg/mL, and coupling reaction time was 6 h. Standard wash procedures were used prior to protein and activity estimations as described in the text.

substrate penetrates only a short distance into the MnP-NH-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 high number of available functional groups at the outer surface of the porous polymer beads may couple MnP rapidly in the low protein concentration coupling reactions, preventing the protein's penetration to the center of the bead. Therefore, MnP-NH-Emphaze polymer with low density MnP 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, functional groups at the outer surface of the porous polymer beads may couple MnP rapidly in the high protein concentration coupling reactions, and the resultant high surface MnP density sterically hinder substrate penetration and conversion to product. Excessive packing of the enzyme on the surface could block active site accessibility (*46*). Similarly, intermolecular steric hinderance of immunosorbent antigen binding has been hypothesized to occur between immobilized monoclonal antibodies (mAbs) and other proximally immobilized mAbs and antigen-mAb complexes (*47*). In this example, excess Emphaze polymer functional groups coupled mAb rapidly before it could penetrate by diffusion, and high surface density shell-type immobilization of mAb resulted. A modified coupling procedure yielded immunosorbent with distributed mAbs. Comparison of the shell-type and distributed immuno-

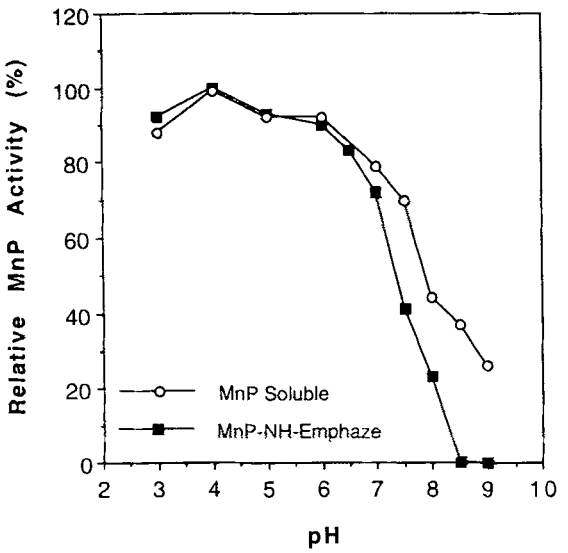

Fig. 4. The pH stability of soluble MnP vs MnP-NH-Emphaze. One h ambient temperature incubation in 100 m*M* buffers: Glycine-HCl pH 3.0 and 3.5; sodium acetate pH 4.0–5.5; Tris-maleate pH 6.0–7.5; and TAPS pH 8.0–9.0.

sorbents revealed increased antigen binding efficiency and usefulness for the later.

Biochemical Characteristics of Free and Immobilized MnP

pH Stability

The free and immobilized MnP exhibited similar pH stability between pH 3.0–7.0 (Fig. 4). However, from pH 7.5–9.0 the immobilized enzyme was less stable and activity yields averaged 25% lower than for the free protein. At pH 8.5, the free protein retained 35% of its initial activity, whereas the immobilized MnP was completely inactivated. This difference in behavior can be explained by the bulk properties of solution and the microenvironment near the enzyme. A polycationic support has a higher pH in the immediate vicinity of the immobilized protein due to a partitioning of protons away from the highly positively-charged surface (*48–50*). The immobilized protein is exposed to a higher pH than the free protein in bulk solution. Therefore, the activity of alkali-labile MnP displays a more rapid and complete decay when the enzyme is immobilized on alkylaminated Emphaze polymer.

Temperature Effect and 60°C Thermal Stability

The free and immobilized MnP remained stable up to 45°C and retained greater than 95% of their initial activity when incubated at that temperature for 2 h (data not shown). The immobilized MnP showed greater thermal stability at 60°C (Fig. 5). The relative activity loss for both immobilized and free protein was similar for the first 30 min of 60°C exposure, but

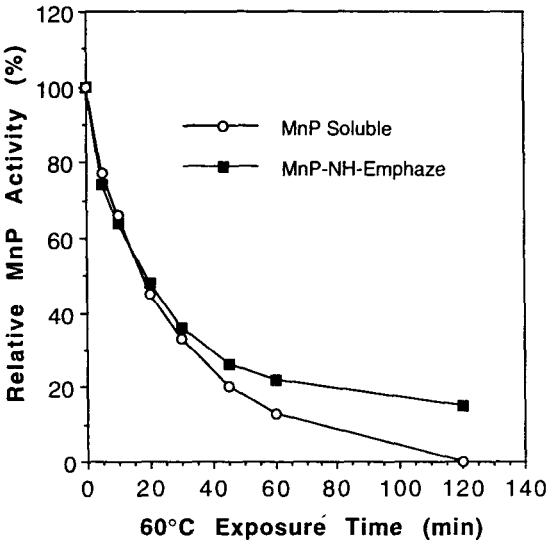

Fig. 5. Thermal inactivation (60°C) of soluble MnP vs MnP-NH-Emphaze incubated in 10 m*M* Buffer A.

as the incubation time increased to 2 h, the soluble protein was completely inactivated compared to 15% activity for the immobilized MnP. It is generally accepted that the first and crucial step in enzyme thermal inactivation is unfolding of the molecule. Increasing the rigidity of the native conformation through covalent multipoint attachment to a matrix results in a more rigid protein globule less likely to denature upon exposure to high temperature (*48,51*). Our results are in agreement with this explanation, since the immobilized MnP exhibits greater thermal stability than the free protein.

Storage Stability

Immobilized MnP was significantly more stable than the soluble protein when stored at 4°C for extended periods. The soluble enzyme lost 50% of its initial activity after approximately 30 d, but the immobilized enzyme remained 50% active after 90 d (Fig. 6). After 60 d at 4°C, soluble MnP only retained 30% of its initial activity, compared to 80% for MnP-NH-Emphaze. In addition to the stabilization effects resulting from increased rigidity of the tethered protein discussed above, stability may also be influenced by decreased water activity and increased hydrophobic interactions within the microenvironment of the MnP-polymer complex. The water activity for the soluble protein would be comparatively higher and hydrophobic interactions lower than that of the MnP coupled to the polymer. Dilute protein solutions are generally less stable than concentrated solutions because of greater water activity and decreased hydrophobic interactions among nonpolar amino acid residues (*51,52*). Polyols, sugars, and salts have demonstrated stabilizing effects by decreasing the water activity and increasing hydrophobic interactions (*51–54*). Similar stabiliz-

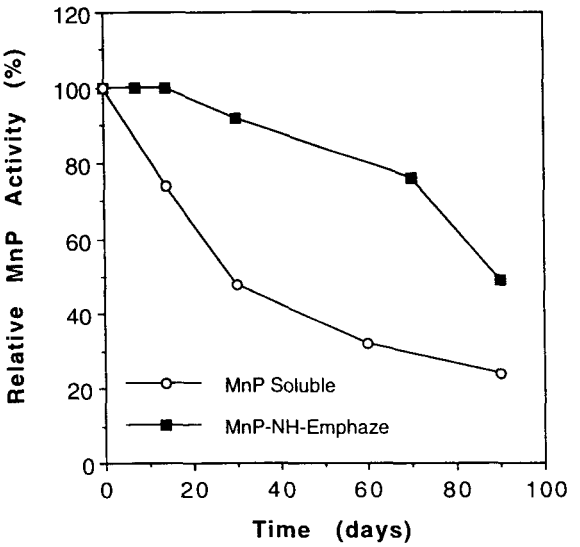

Fig. 6. Storage stability (4°C) of soluble MnP vs MnP-NH-Emphaze in 10 m*M* Buffer A.

ing effects may be realized when the local concentration of MnP is increased by attachment to the polymer matrix promoting hydrophobic interactions and excluding water from the beads.

H_2O_2 Dependence

The catalytic cycles for horseradish peroxidase (HRP (*55*), *Phanerochaete chrysosporium's* lignin peroxidase (LiP) (*56*), and MnP from *P. chrysosporium* have been characterized (*57*). The iron protoporphyrin IX heme prosthetic group of these peroxidases is oxidized by H_2O_2 to initiate the catalytic cycle: Native enzyme → compound I → compound II → native enzyme. The H_2O_2-oxidized intermediate compound I undergoes two one-electron reductions by a reducing substrate in its return to native state. MnP compound I is reduced to native enzyme by oxidizing two Mn^{II} and generating two Mn^{III}. Excess H_2O_2 and limiting concentrations of reducing substrate can result in the three-electron oxidized state, compound III. Compound III is not involved in the normal catalytic cycle and represents an inactive form of the enzyme (*57,58*).

The MnP from *L. edodes* catalyzes similar H_2O_2-dependent oxidations of Mn^{II} (*27*), and we have spectrophotometrically identified (data not shown) compounds I, II, and III described in (*57*). Exogenous H_2O_2 is used as the primary oxidant for compound I formation, although oxalate is present. In addition to chelating the Mn^{II} and generated Mn^{III}, oxalate may also serve as an additional source of H_2O_2 (*27,59,60*). Peroxide is essential for MnP catalysis and can be responsible for enzyme inactivation in the event of compound III formation. Therefore, H_2O_2-dependence of soluble and immobilized MnP was determined (Fig. 7). Immobilized MnP exhibited enhanced stability upon exposure to increasing concen-

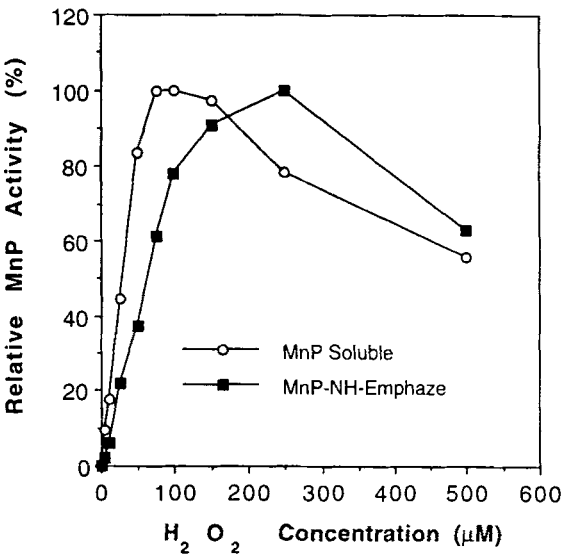

Fig. 7. H_2O_2 dependence of soluble MnP vs MnP-NH-Emphaze. Activity was determined by MnP/vanillylacetone assay with 4.5 μg of free or immobilized MnP in each 1.0 mL assay and an increasing H_2O_2 concentration of 0 to 500 μ*M*.

trations of H_2O_2. Not only did the immobilized enzyme activity peak at a higher H_2O_2 concentration, 250 μ*M* vs 75 μ*M* for the soluble enzyme, but the immobilized MnP showed a broader range of tolerance for peroxide than the soluble form as it retained ~80% of its initial activity between 100–375 μ*M* H_2O_2. In contrast, the soluble enzyme gave similar activity yields between 40 and 240 μ*M* H_2O_2. These differences in activity may be the result of electrostatic partitioning (discussed below) not experienced by the soluble MnP.

K_m and V_{max}

The double reciprocal Lineweaver-Burk plots of vanillylacetone oxidation vs Mn^{II} concentration for both soluble and immobilized MnP were linear (Fig. 8) allowing K_m and V_{max} calculations. The K_m for Mn^{II} was 91 μ*M* for soluble MnP, and MnP-NH-Emphaze had an apparent K_m of 440 μ*M*. Vanillylacetone was oxidized at a rate of 7.3 and 6.1 μ*M*min/mg MnP for soluble and immobilized MnP, respectively. Deviations in the kinetic constants are expected, since immobilization creates differences between bulk properties of solution and the microenvironment near the enzyme by imposing conformational and steric changes, in addition to diffusional limitations. These differences can affect electrostatic partitioning, diffusion, and mass transfer of substrates and products. The review by Kokufuta (*61*) summarizes these variables and provides references discussing each of them in greater detail. Given the importance of electron transfer in the redox cycle described above, electrostatic partitioning effects may have particular significance in the immobilized MnP reaction. The charges of

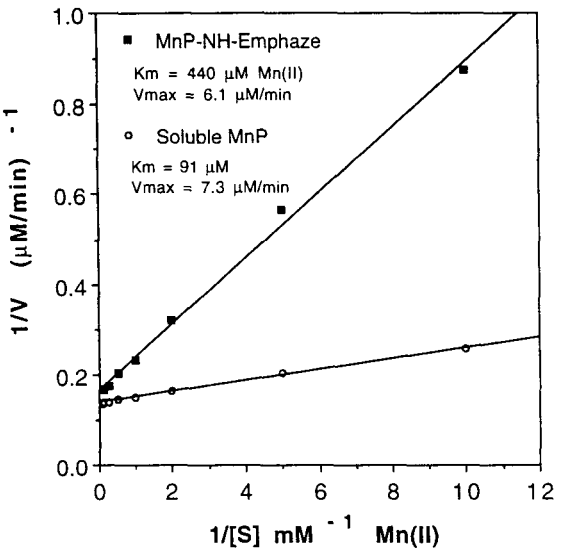

Fig. 8. Soluble MnP and MnP-NH-Emphaze Lineweaver-Burk plots for Mn(II). K_m and V_{max} were determined by MnP/vanillylacetone assay with 2.5 µg of free or immobilized MnP in each 1.0 mL assay and an increasing substrate concentration of 0.10 to 10 m*M* $MnCl_2$.

polymer, MnP enzyme, substrate (Mn^{II}), and product (Mn^{III}) could each have an influence on the catalytic cycle of immobilized MnP.

CONCLUSION

Reuse and stabilization of degradative enzymes immobilized on solid supports is an attractive possibility for treatment of pumpable toxic waste streams. Immobilization of active MnP is an essential step in realizing this possibility for an immobilized MnP bioreactor that generates chelated Mn^{3+} as an oxidant of organopollutants. Efficient immobilization of MnP through EEDQ-activated carboxyl groups to alkylaminated Emphaze Biosupport Medium AB 1 was achieved and optimized through the experiments presented here. The MnP-NH-Emphaze polymer exhibits improved stability over the soluble protein and will allow us to establish maximum Mn^{3+}-chelate generating conditions by reusing the tethered enzyme in a steady state flow system. Alkylamination extends the utility of Emphaze AB 1 polymer, converting the amine-coupling azlactone functionality to a carboxyl-coupling amino functionality. EEDQ has proved to be an extremely useful reagent in the coupling of MnP, an alkaline-labile protein with only 1% lysine. Although water-soluble EDC is more commonly used for carboxyl activation, it was deleterious to MnP activity in our coupling reactions, in comparison to EEDQ. Finally, the use of EEDQ as a coupling reagent to activate carboxyls of proteins deficient in free amino groups

and sensitive to alkaline pH may have general application in covalent immobilization of such proteins to aminated supports.

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