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CORALLINA OFFICINALIS BROMOPEROXIDASE IMMOBILIZED ON AGAROSE

DEBORAH J. SHEFFIELD, TIM R. HARRY,* ARNOLD J. SMITH and LYNDON J. ROGERS

Institute of Biological Sciences, University of Wales, Aberystwyth, Dyfed SY23 3DD, U.K.; *Sigma Chemical Co., Fancy Road, Poole, Dorset BH17 7NH, U.K.

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Abstract—Vanadium bromoperoxidase (VBrPO) purified from the macroalga *Corallina officinalis* was immobilized by covalent binding to cyanogen-bromide-activated Sepharose 4B and by hydrophobic binding to an agarose–$NH(CH_2)_{11}Me$ matrix. In both cases the enzyme bound to the support showed a lower specific activity than soluble VBrPO. The immobilized enzyme showed a somewhat lower pH optimum than soluble VBrPO and high thermal stability, although in contrast to soluble enzyme the activity lost on heating was not recovered on re-equilibration to 20°. Columns packed with either resin could be used repeatedly for substrate bromination. The VBrPO bound to Sepharose showed tolerance to organic solvents, similar to the soluble enzyme. The VBrPO bound to dodecyl-agarose was tolerant of acetone, ethanol and methanol at 10%(v/v) solvent, but at higher concentrations of solvent, and particularly at 30%(v/v) acetone, there was enhanced activity that appeared to be a result of the enzyme dissociating from the support.

INTRODUCTION

Bromoperoxidases possess a halide-dependent catalase activity but preferentially will catalyse the addition of bromide to an appropriate substrate. They have a range of potential uses in synthetic organic chemistry, derived from their ability to react with a wide range of substrates, including aliphatic and aromatic hydrocarbons, phenols, β-diketones and nitrogen- or sulphur-containing heterocycles [1, 2]. The industrial application of these and other haloperoxidases would benefit from the availability of immobilized forms of the enzymes, in particular in the prospect of repeated use and recovery of active enzyme [3].

The vanadium bromoperoxidase (VBrPO) from *Corallina officinalis* is an oligomeric protein of M_r 740 000, comprised of probably 12 identical subunits, each with a vanadium as cofactor [4]; some other bromoperoxidases are haem proteins or apparently lack a cofactor or prosthetic group. Kinetic studies at the pH optimum of 6.5 with monochlorodimedone (MCD) as the substrate gave the following: $K_m^{Br^-}$, 1.0 mM; $K_m^{H_2O_2}$, 60 μM and K_m^{MCD}, 21 μM. The purified enzyme was thermostable, retaining appreciable activity at 60–70° and recovering the original activity on re-equilibrium to 25°, even on repeated cycles of this temperature regime or heating to 90° [5]. The exploitation of this VBrPO [6] and the similar enzyme from *Corallina pulilifera* [1] in biotransformations has been reported.

Enzyme immobilization methods can be broadly divided into four categories: adsorption, ionic binding, covalent-binding, and matrix-entrapment. For other haloperoxidases, chloroperoxidase has been immobilized on aminopropyl glass [7, 8] while the iodoperoxidase from *Armoracia rusticana* (horseradish peroxidase) was immobilized by covalent coupling in a poly(acryloyl morpholine) gel [9]. Of the bromoperoxidases, lactoperoxidase has been immobilized on cyanogen bromide (CNBr)-activated Sepharose 4B and concanavalin-A Sepharose 4B [10], and VBrPO from the brown algae *Ascophyllum nodosum* and *Laminaria digitata* on DEAE-cellulose membranes [11], while DEAE-Cellulofine and a resin polymer ENT-2000 proved the most useful supports for immobilization of the VBrPO from the red alga *C. pilulifera* [12]. Immobilization often enhances the stability of an enzyme, e.g. lactoperoxidase [10] and horseradish peroxidase [9], though not chloroperoxidase [7], and may increase tolerance to organic solvents [9].

We have previously successfully immobilized *C. officinalis* VBrPO through ionic-binding to a cellulose acetate support and entrapment by cross-linking with glutaraldehyde [13]. The bound enzyme retained high thermostability and retained its effectiveness on repeated use. It also showed tolerance to organic solvents similar

to that of the soluble VBrPO. Here we report the properties of VBrPO immobilized by covalent binding to CNBr-activated Sepharose 4B and by adsorption through hydrophobic interaction to agarose beads with alkyl side-chains of various carbon number.

RESULTS

Immobilization of VBrPO

Three methods of immobilization were attempted; previously the enzyme had also been immobilized on glutaraldehyde-fixed cellulose acetate [13]. An attempt at entrapment in polyacrylamide gel beads by a method described elsewhere [14] proved discouraging and no significant activity could be demonstrated; these data are not presented. Use of two agarose supports was more successful. Hydrophobic binding to agarose beads with alkyl side-chains of varying length showed that the VBrPO bound most effectively to agarose–$NH(CH_2)_{11}Me$, although there was also some immobilization on the pentyl and hexyl side-chains (Fig. 1). The dodecyl derivative was used in all subsequent work. The specific activity of the VBrPO bound to the support was some 10% of that of the applied enzyme as assayed at pH 6.5 in phosphate buffer, the optimum pH of the soluble enzyme. With covalent binding to the Sepharose support the specific activity of the immobilized enzyme was about 12% of that of the soluble VBrPO applied to the resin.

Characteristics of the immobilized VBrPO

The behaviour of the immobilized VBrPO compared with the soluble enzyme [4] and that immobilized on a cellulose acetate support [13] was studied in terms of pH optimum and stability in relation to time and temperature. The activities of the immobilized VBrPO were compared from MCD bromination at pH values over the range 3–10.5; the pH of the reaction mixtures did not change over the course of the reaction. The pH optimum of the agarose–VBrPO complex (Fig. 2) was lower than that found for the soluble enzyme (pH 5.5 cf. 6.5). Moreover, the response to the buffers used differed, with the bound enzyme being considerably more active in Tris–sulphate buffer; at pH 7 the VBrPO was several-fold more active in this than in K-Pi buffer. This did not appear to be due to release of enzyme from the support and a consequent increase in specific activity since the agarose–VBrPO complex had been stored in Tris–sulphate buffer, pH 8.3. The cellulose-acetate-bound enzyme had also proved more active in Tris–sulphate buffer than in K-Pi buffer, both at pH 7.5, though the difference was not so marked. For the Sepharose–VBrPO conjugate the pH activity profile corresponded more closely to that for the soluble enzyme although the pH optimum was 0.5 pH unit lower; here the activities in Tris–sulphate and K-Pi buffers at the same pH were similar.

The VBrPO immobilized on agarose was stable on storage, losing only some 10% of its activity over 6 weeks at 4°. The enzyme activity was increased some 25% after exposure at 40° for 10 min and was stable at 60° for this time although at 70° activity was lost. The Sepharose–VBrPO conjugate lost *ca* 25% of its activity over 3 weeks, although activity thereafter was stable. This immobilized form of the enzyme was more thermostable with slight loss of activity at 50° and thereafter a progressive loss as the temperature was increased; even after incubation at 80° some 40% of the activity was retained. In both cases enzyme had not apparently dissociated from the support on heating since on equilibrium subsequently to 25° activity was not regained; this had proved a characteristic of the soluble enzyme [4]. Also, no enzyme activity was detected in the buffer if the sample was centrifuged to pellet the complex.

Immobilized enzyme in repeated use

Substrate (Phenol Red) in K-Pi buffer pH 6.5 was applied to small columns containing 1 ml of either of the immobilized VBrPO preparations and elution with 10 ml of Tris–Sulphate buffer was carried out. In both cases the substrate was completely converted to Bromophenol Blue, and eluted in the first 2 ml of the eluate. This also occurred on three further additions of the substrate.

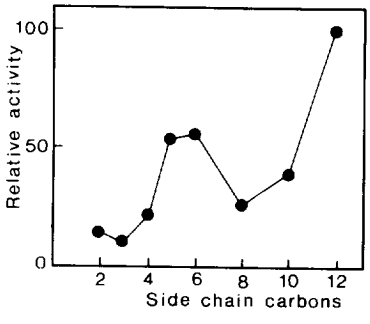

Fig. 1. Binding of *C. officinalis* VBrPO to agarose–$NH(CH_2)_nMe$. Relative activity is by comparison to the amount of enzyme bound to agarose with an alkyl side-chain $n = 11$.

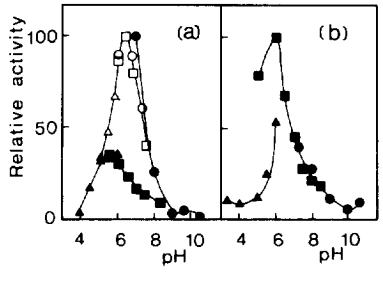

Fig. 2. pH Optima of *C. officinalis* VBrPO immobilized on (a) agarose–$NH(CH_2)_{11}Me$ and (b) CNBr-activated Sepharose 4B. The buffers used were: ○ ●, Tris–sulphate; □ ■, K-Pi; △ ▲, sodium acetate. The open symbols in (a) are data for the soluble enzyme taken from [4].

However, the column retained some of the product as evidenced by a blue tinge imparted to the agarose supports.

Stability to organic solvents

The agarose–VBrPO complex proved to be tolerant to exposure to ethanol and methanol at 10% (v/v) for 24 hr. At higher solvent concentrations (20 and 30%, v/v) activity was increased, maximally by some 50% (Fig. 3). This behaviour was more exaggerated in the case of acetone. Even at 10% (v/v) solvent, activity after 24 hr was increased by some 50%, and at 20 and 30% (v/v) solvent the increases were about three- and seven-fold, respectively. In these cases the presence of soluble enzyme in the resin suspension could be demonstrated, indicating that protein had been dissociated from the agarose support.

For the Sepharose–VBrPO the results (Fig. 4) showed a quite different pattern with retention of near total activity at 10% (v/v) of the three solvents, and of *ca* 75% activity at 30% (v/v) solvent. In all cases any activity loss occurred during the first hour of exposure to the solvent and thereafter the activity remained stable, or in the case of 10% (v/v) methanol increased by some 20%; propan-1-ol gave similar results to methanol (data not shown). Enzyme activity was not released into solution during incubation of the resin with the organic solvents.

DISCUSSION

The properties of the VBrPO immobilized on agarose supports can be compared with those of the soluble enzyme [4] and enzyme immobilized on cellulose acetate [13]. The failure to immobilize VBrPO in polyacrylamide beads was unexpected since the similar enzyme from *C. pilulifera* was successfully immobilized by entrapment in some other gel types [12]; possibly free radical generation during polymerization of the polyacrylamide gel had an adverse effect on the VBrPO. The pattern of binding to the agarose-alkyl side-chain series was unexpected since the anticipated profile would be effective binding at low carbon number, declining at some point as the alkyl side-chain was extended. The large size and subunit arrangement (M_r of $12 \times 64\,000$) of the *C. officinalis* VBrPO presumably has some bearing on the binding pattern observed, and it may be that interaction of the VBrPO with the pentyl and hexyl alkyl derivatives differs from that with the larger (dodecyl) alkyl side-chain. The pH optimum of the agarose–VBrPO complex was at somewhat lower pH in K-Pi buffer than the soluble enzyme (pH 5.5 *cf.* 6.5). A shift of 0.5–1.0 unit in optimum pH is commonly observed for polymer-bound enzymes compared with the soluble protein [10] although *Caldariomyces fumago* chloroperoxidase immobilized on aminopropyl glass had the same pH optimum as the soluble enzyme [8], as did immobilized lactoperoxidase [10].

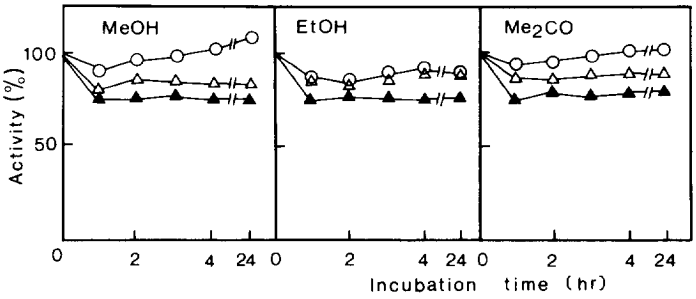

Fig. 3. Effect of organic solvents on *C. officinalis* VBrPO immobilized on agarose–$NH(CH_2)_{11}Me$. The bound enzyme was exposed for the time shown to solvent at: ○ 10% (v/v); △, 20% (v/v); ▲, 30% (v/v). The activities shown are given as a percentage of the original activity at zero time.

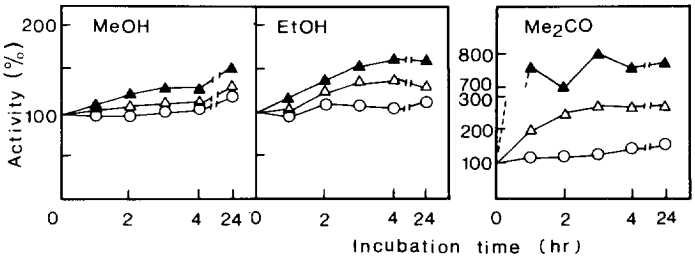

Fig. 4. Effect of organic solvents on *C. officinalis* VBrPO immobilized on CNBr-activated Sepharose 4B. The bound enzyme was exposed for the time shown to solvent at: ○, 10% (v/v); △, 20% (v/v); ▲, 30% (v/v). The activities shown are given as a percentage of the original activity at zero time.

However, the complex showed a significantly higher activity in Tris–sulphate buffer at pH 7.5 than at the pH optimum seen using sodium acetate and K-Pi buffers at lower pH value. A consequence of this is that, whereas only 10% of the VBrPO (as measured at pH 6.5 in K-Pi buffer by the standard assay) was apparently bound to the agarose support, the calculation of units at pH 7.5 in Tris–sulphate buffer suggests that specific activity of the immobilized enzyme was about 40% of that of the soluble protein. Depending on the carrier and method of immobilization a lower specific activity than for the soluble enzyme may result through, e.g. inactivation by the interaction with the support; inaccessibility of the active sites; or limitation of access to the substrate [10].

The behaviour in these respects of the Sepharose–VBrPO conjugate was more similar to that of the soluble enzyme with a pH optimum only slightly lower. In this case some 20% of the applied activity was evident. The VBrPO has a p*I* of 3.9 (experimental data not given) and may not readily participate in immobilization coupling reactions that involve amino groups. However, since VBrPO was not apparently present in free solution after mixture with the resin and was not released from the resin on washing, this suggested that a considerable amount of the VBrPO had been bound in an unfavourable conformation for catalytic activity. This could arise since the basis of enzyme binding is formation through CNBr activation of imidocarbonate derivatives of Sepharose that react with protein -NH_2 groups; some of these may be in the vicinity of the active site of the VBrPO. Thus immobilized enzyme had a considerably lower specific activity than soluble VBrPO, as did similarly immobilized lactoperoxidase [10]. In both immobilization procedures the loading of enzyme to the support may not have been optimal for retention of specific activity compared with the soluble VBrPO; for chloroperoxidase the best percentage retention was obtained by using a low enzyme to support ratio [8].

The soluble VBrPO was stable on storage in Tris–sulphate buffer, pH 8.3, with retention of full activity over 14 days at 20°, 28 days at 4° and 12 months at − 20°. The immobilized enzymes were similarly stable except for the Sepharose–VBrPO conjugate where there was some 25% loss in activity over the first 3 weeks at 4°; the remaining activity was stable. Stability over a range of pH values was not investigated; the chloroperoxidase from *Caldariomyces fumago* proved most stable if stored at somewhat acid pH, but in repeated use retained activity best at near neutral pH [8].

With regard to thermostability, the soluble enzyme had been stable at 50° and retained 60% of its activity at 70°; the loss in activity occurred over the first few min of heating and thereafter the enzyme activity stabilized at the new lower value. The VBrPO immobilized on agarose beads was stable to 60° but lost its activity entirely at higher temperature. The VBrPO immobilized on cellulose acetate behaved similarly but retained 60% activity at 70° and required exposure to 80° to lose activity entirely. In contrast, the Sepharose–VBrPO conjugate behaved similarly to the soluble enzyme and retained some 30% activity even at 80°. All forms of immobilized VBrPO differed in one characteristic compared to soluble enzyme. This was a lack of ability to regain activity lost on heating by restoration to 25° and re-equilibration at the lower temperature. It is speculated that this may be a consequence of changes in protein conformation on heating, with formation of new or additional interactions with the supporting matrix that decrease specific activity of the VBrPO and are irreversible on cooling.

For other haloperoxidases, immobilization did not affect the thermostability of chloroperoxidase [7, 8] but increased that of lactoperoxidase [10]. The type of support may be a significant factor, and horseradish peroxidase bound to poly(acryloyl morpholine) was more thermostable [9] and that bound to aminoporous silica was less thermostable [15] than the soluble enzyme.

Solvent effects on enzyme activity are similarly unpredictable since they are a consequence of the interaction of a number of factors, including alterations in protein conformation generally or specifically in the environment of the active site, and changes in chemical reactivity of the substrate [16]. Immobilization can have a significant influence on behaviour of the enzyme with respect to solvent. Although soluble horseradish peroxidase was unstable in the presence of organic solvents the immobilized enzyme was appreciably more stable to ethanol [15] and methanol [9]. The VBrPOs immobilized on agarose beads and by covalent linkage to Sepharose differed in their tolerance on exposure to organic solvents. The latter behaved similarly to the soluble enzyme but was somewhat more stable. A notable feature of the response of the agarose–VBrPO complex was an appreciable increase in activity at the higher solvent concentrations, particularly in the case of exposure to acetone. For acetone treatment this was attributed to release of the enzyme from the support since, after removal of the resin enzyme, was present in soluble form in the supernatant. The large increase in activity would be a consequence of the much lower specific activity of immobilized compared with soluble VBrPO, as noted earlier. This may also explain the apparent increase in activity in the other solvents, though with the enzyme immobilized on cellulose acetate [13] a three-fold increase in activity in 30% (v/v) ethanol could not be attributed to loss of enzyme from the support into solution.

An advantage of immobilized enzymes for industrial application is in a potential for repeated operation or continuous use. Immobilized chloroperoxidase was effective in the former but not the latter application [1, 17]. All forms of immobilized *C. officinalis* VBrPO studied ([13] and present work) have been used successfully in repeated operation and this was also feasible with *C. pulilifera* VBrPO immobilized on DEAE-Cellulofine and ENT-2000 [12], although product retention would necessitate extensive washing before any change in process application. Product retention was also evident in repeated use of immobilized lactoperoxidase [15]. Given the stability of immobilized enzyme, continuous operation as a packed-bed bioreactor would appear to be feasible.

EXPERIMENTAL

Supports. Agarose with alkyl ligands of carbon number 2 to 12 (Agarose–NH[CH_2]$_n$Me) and CNBr-activated Sepharose 4B were supplied by Sigma.

VBrPO purification. VBrPO was isolated from *C. officinalis* by the methods described previously [4], except that to facilitate scale-up of the procedure the final stage of purification by FPLC on a mono-Q HR5 column was omitted. Consequently, the VBrPO used contained a small amount of phycoerythrin and less than 1% of a chloroperoxidase. The final enzyme preparation, at 33 mg ml^{-1} of K-Pi buffer, pH 6.5, had a specific activity of 126 μmol of MCD formed min^{-1} mg^{-1} protein.

Enzyme assay. Based either on the conversion of MCD by following the decrease in *A*290 nm on conversion to monobromomonochlorodimedone and spontaneous breakdown of this product [4], or the conversion of Phenol Red to Bromophenol Blue [3]. For the pH optimum determination, K-Pi in the standard assay [4] was replaced by NaOAc or Tris at equivalent concn.

In studies with VBrPO exposed to solvent, the standard assay using a molar absorption coefficient for MCD of 20.2 mM^{-1} cm^{-1} at 290 nm was used. EtOH and MeOH transferred with the immobilized enzyme to the assay mixture had no significant effect on this; however, acetone itself absorbs strongly at 290 nm and should be present at the same v/v ratio in the assay and reference cuvettes.

*Immobilization on agarose–*NH(CH_2)$_n$Me. Agarose beads with ethyl, propyl, butyl, pentyl, hexyl, octyl, decyl and dodecyl ligands were used. Each gel suspension (2.5 ml) was washed 4 times with 1 ml of 50 mM K-Pi buffer, pH 7, before incubation with 7.25 mg VBrPO in 2.5 ml of buffer for 30 min at 20°. The agarose beads were recovered by centrifugation at 13000 rpm for 1 min and the supernatant assayed for VBrPO. Assays of the supernatants from successive washes with 1 ml buffer enabled the enzyme units bound to the support to be calculated from a balance sheet of enzyme units applied originally and those in the supernatants after the centrifugations. The agarose–NH(CH_2)$_{11}$Me–VBrPO complex used in subsequent studies was stored at 4° in 1 ml of 50 mM Tris–sulphate buffer, pH 8.3.

Linkage to CNBr-activated Sepharose 4B. Resin (10 g) was washed with 1 l of 0.1 M $NaHCO_3$, pH 9.5, by vacuum filtration on a sinter funnel. The resin was added to 5 ml of 50 mM K-Pi, pH 7, containing 10 mg of VBrPO. After stirring for 16 hr at 4° the remaining binding sites on the resin were blocked for 3 hr by the further addition of 1.5 g glycine. The resin recovered by vacuum filtration was successively washed with 200 ml each of H_2O; 0.1 M $NaHCO_3$; 1 mM HCl; 0.5 M NaCl and finally H_2O before storing at 4° in 2 vol. of 50 mM Tris–sulphate, pH 8.3.

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