Soybean peroxidase-catalysed removal of benzidines from water

Bahaa Malik Altahir

Department of Chemistry, University of Al-Mustansiryah, Baghdad, Iraq;
Department of Civil and Environmental Engineering, University of Windsor,
Windsor, ON, Canada

Wei Feng

Department of Chemistry and Biochemistry, University of Windsor,
Windsor, ON, Canada

Hadi Hassan Jasim BSc, MSc, PhD

Assistant Professor, Department of Chemistry, University of
Al-Mustansiryah, Baghdad, Iraq

Keith E. Taylor

Department of Chemistry and Biochemistry, University of Windsor,
Windsor, ON, Canada (corresponding author: taylor@uwindsor.ca)

Niharendu Biswas

Department of Civil and Environmental Engineering, University of Windsor,
Windsor, ON, Canada

Jatinder K. Bewtra

Department of Civil and Environmental Engineering, University of Windsor,
Windsor, ON, Canada

Sabah A. A. Jassim

Department of Civil and Environmental Engineering, University of Windsor,
Windsor, ON, Canada; Applied Bio Research Inc., Windsor, ON, Canada

Crude soybean peroxidase (SBP), isolated from soybean seed coats (hulls) at unusually low concentrations, catalyses the oxidative polymerisation of hazardous aqueous benzidine and its 3,3'-dichloro, 3,3'-dimethyl and 3,3'-dimethoxy derivatives in the presence of hydrogen peroxide. The optimum operating conditions for oxidation of 0-10 mM benzidine were investigated. At pH 5, the hydrogen peroxide-to-substrate concentration ratio was 1.5 and the minimum SBP concentration required to achieve at least 95% conversion of the benzidine in synthetic wastewater was 0.43 mU/ml. Progress curves were established for the conversion of the four substrates, and apparent first-order rate constants were derived. Enzyme-catalysed polymerisation with SBP and subsequent removal of the polymeric products generated can provide an alternative means to conventional methods for treating many aromatic wastewater pollutants, including the benzidines studied here.

Introduction

Benzidine (BZ) is a moderately persistent pollutant in the environment, and the exposure of populations to disposed BZ waste is a matter of great concern with regard to human health (Karim *et al.*, 2012). BZ derivatives, as xenobiotic organic pollutants, could be generated from tannery wastewater containing large amounts of dyes and pigments after photolysis, heating or biological treatment (Saeed, 2014). These compounds are highly toxic, carcinogenic, mutagenic, teratogenic, stable in water, almost non-biodegradable and irremovable with conventional water treatment technology; thus, they are internationally recognised as priority pollutants (Azim, 2000; Lakshmi *et al.*, 2003). BZ-based azo dyes are widely used in dye manufacturing, textile dyeing, coloured paper printing and leather industries (Powell *et al.*, 1979). In 1980s, the US National Institute for Occupational Safety and Health published a survey of data on the carcinogenicity of BZ-based dyes from tests with experimental animals and epidemiological studies of workers exposed to the dyes, due to dye biotransformation to BZ (Chung and Stevens, 1993; Karim *et al.*, 2012). Many of these dyes find their way into the environment by way of wastewater facilities (Zougar *et al.*, 2014). They also exhibit a high resistance to microbial degradation in wastewater treatment systems (Bayramoglu and Yakup, 2007).

It has been shown that commercially used dyes based on BZ and its derivatives can be metabolised in the human body, releasing free aminobiphenyls (Cerniglia *et al.*, 1982). At the same time, BZ is a strong carcinogen that causes cancer in the bladder, the liver and mammary glands and also results in the development of carcinoma in the thyroid glands in animals (Sentchouk and Grintsevich, 2004). Aromatic amines, such as BZ, initiate bladder cancer in humans (Lakshmi *et al.*, 2003). Without adequate treatment, these dyes can remain in the environment for a long period of time (Hao *et al.*, 2000). BZ and other aromatic amines, such as *o*-tolidine and *o*-dianisidine, were found to be liver, mammary gland and intestine carcinogens (Beland and Kadlubar, 1985). BZ has been found in at least 27 of the 1430 National Priorities List sites identified by the US Environmental Protection Agency (Muneer *et al.*, 2002).

Recent studies of BZ and its derivatives, including azo dyes, involved many chemical, physical and biological treatments, including various chemical oxidation methods such as oxidation by reactive nitrogen oxygen species (Lakshmi *et al.*, 2003), chlorine dioxide oxidation (Cao *et al.*, 2007), dark Fenton reaction and solar driven photo-Fenton process (Hao *et al.*, 2000); various physicochemical methods such as photochemical degradation in

different halomethane solvents (Azim, 2000), photo-catalysed degradation (Muneer *et al.*, 2002) and oxidation on cationic clay surfaces in aqueous suspension (Do Nascimento *et al.*, 2006); and various biological approaches using organisms such as bacteria, fungi and helminths (Chung and Stevens, 1993) as well as native and heat-treated white rot fungus (Bayramoglu and Yakup, 2007). These methods suffer from various drawbacks such as high cost, incomplete removal, formation of hazardous by-products, low efficiency, high energy requirements and/or applicability only in a low concentration range (Nicell *et al.*, 1993).

The long-term goal of this study is to determine whether, and if so, under what circumstances, enzymatic treatment with a peroxidase may overcome these drawbacks in a cost-effective manner (Steevensz *et al.*, 2009). Peroxidases catalyse the oxidation of phenols (Al-Kassim *et al.*, 1994; Caza *et al.*, 1999; Wilberg *et al.*, 2002; Yu *et al.*, 1994) and anilines (Saha *et al.*, 2008) to form aromatic radicals in the presence of hydrogen peroxide (H_2O_2). These radicals diffuse from the active site of the enzyme into the solution, where they couple non-enzymatically to form dimers. If the dimers are soluble and still phenolic or anilino, they become substrates for another enzymatic cycle, forming higher oligomers. The cycle continues until the polymer generated reaches its solubility limit and precipitates out of solution, later to be removed by filtration or sedimentation (Al-Ansari *et al.*, 2009; Dunford, 1999).

Oxidation by hydrogen peroxide catalysed by thyroid peroxidase (TP) has been found to be effective in the removal of BZ from wastewater with 95% or higher efficiency (Sentchouk and Grintsevich, 2004). The main drawback in using TP is its unavailability in large quantity at a price appropriate for waste treatment (Sentchouk and Grintsevich, 2004). However, bitter gourd peroxidase (BGP) has been investigated for its ability to oxidise BZ, but a high concentration of this enzyme was needed to oxidise more than 90% of BZ (Karim *et al.*, 2012). An immobilised laccase has also been investigated because of its ability to oxidise BZs, analogous to the oxidative ability of peroxidases (Karagoz *et al.*, 2011).

Soybean peroxidase (SBP), an oxidoreductase extracted from the soybean seed coat (Sessa and Anderson, 1981; Dunford, 1999), has several features that recommend it for wastewater treatment. Above all, SBP can be certainly cheaper than TP, since it is easily extracted from soybean seed coats, which are a waste product of the soybean processing industry (Wilberg *et al.*, 2002). A crude SBP extract has been found to be more efficient than the purified one (Flock *et al.*, 1999), as are the peroxidases cited earlier, and it is active over a broad range of pH (3.5–8.0) (Caza *et al.*, 1999; Kamal and Behere, 2003; Wright and Nicell, 1999). SBP has a higher thermal stability (being active at 70°C) than other peroxidases (McEldoon and Dordick, 1996), and it is less susceptible to irreversible inactivation by peroxide. In keeping with the long-term goal given at the outset, the hypothesis to be tested here is that SBP is an efficient catalyst for treatment of the target compounds. Preliminary optimisation of

SBP-catalysed polymerisation of aqueous BZ and its 3,3'-dichloro (DCB), 3,3'-dimethyl (DMB) and 3,3'-dimethoxy (DMXB) derivatives has been carried out.

Materials and methods

Materials

Crude dry solid SBP (Enzyme Commission (EC) number 1.11.7, industrial grade lot number 18541NX, Reinheitszahl value of 0.75 ± 0.10) was obtained from Organic Technologies (Coshocton, OH, USA). Dry solid bovine liver catalase (EC 1.11.1.6, lot number 120H7060, 19 900 U/mg solid) was purchased from Sigma Chemical Company Inc. (St. Louis, MO, USA). The BZs (all with a purity of $\geq 98.0\%$) were obtained from Aldrich Chemical Corporation (Milwaukee, WI, USA). All other chemicals were of analytical grade and purchased from either Sigma Chemical Company Inc., Aldrich Chemical Corporation or BDH Inc. (Toronto, ON, Canada).

Analytical equipment

BZ concentrations were analysed with a high-performance liquid chromatography system with ultraviolet detection (HPLC-UV) obtained from Waters Corporation (Milford, MA, USA). The system consisted of a binary HPLC pump, an auto sampler, a dual absorbance detector and a C18 reverse-phase column (5 μm , 4.6 mm $\times$ 150 mm) operated by Breeze software. Elutions were isocratic (detection at 280 nm) with a mobile phase consisting of methanol and water with a ratio of 50:50 and a flow rate of 1 ml/min for BZ alone and a ratio of 60:40 and a flow rate of 0.8 ml/min for the mixture (BZ, DCB, DMB and DMXB). The injection volume was 10 μL .

A Hewlett Packard diode array spectrometer (model 8453), with a wavelength range between 190 and 820 nm and a 2 nm resolution and controlled by a Hewlett Packard Vectra ES/12 computer, was used to measure sample absorbance. Quartz semi-microspectrometer cells with a 1 cm optical path length were supplied by Hellma Canada Limited (Concord, ON, Canada).

The laboratory filtration system, which was an all-glass filter holder assembly with a glass funnel, a fritted base, a cap, a clamp, a 47 mm membrane filter, a vacuum connector and a receiving flask connected by ground glass outer and inner joints, was supplied by Merck Millipore.

Analytical methods

Colorimetric assay for SBP activity

Enzyme catalytic activity in units (U) is defined as the number of micromoles of hydrogen peroxide converted per minute at pH 7.4 and 23°C in a standard, 'Trinder-type' assay described previously (Wu *et al.*, 1997). One unit by this assay corresponds to 8 U by the standard guaiacol activity assay (Sessa and Anderson, 1981). A colorimetric assay was used for measuring free SBP activities. The reaction mixture contained 40 mmol/l phosphate buffer (pH = 7.4), 10 mmol/l phenol and 0.2 mmol/l hydrogen peroxide and 2.4 mmol/l,

4-aminoantipyrine (AAP) in a total volume of 950 μl . The reaction was then started by adding 50 μl of diluted enzyme sample, and the initial rate of colour formation at 510 nm was monitored for 30 s. The sample dilution was adjusted according to the expected enzyme activity (to have about 0.2 absorbance change in 30 s) (Feng *et al.*, 2013; Steevensz *et al.*, 2009).

Colorimetric assay for hydrogen peroxide

This assay determined the concentration of hydrogen peroxide present in the solution by measuring the colour intensity resulting from oxidative coupling of phenol and AAP in the presence of hydrogen peroxide at pH 7.4 with *Arthromyces ramosus* peroxidase as the enzymatic catalyst. The product of such reaction is a pink quinoneimine chromophore, identical to that in the preceding enzyme assay, with an absorption maximum at 510 nm (Steevensz *et al.*, 2009).

Buffer preparation

The buffers used were prepared according to Gomori (1955). An acetic acid-sodium acetate buffer was used in the pH range 3.0–5.8, and a monobasic-dibasic sodium phosphate buffer was used in the pH range 5.6–8.0; a pH higher than 9.0 was achieved with a sodium bicarbonate-sodium carbonate buffer.

Experimental protocol

Batch reactors consisted of 20 ml of a buffered mixture of a BZ, hydrogen peroxide and SBP. Generally, the reactors were stirred using magnetic stirrers and Teflon-coated stir bars at a room temperature of about 21°C. The components of the sample mixture were added in the following order: water (distilled or tap water),

40 mM acetate, phosphate or carbonate buffer, a BZ as aqueous stock solution, SBP to appropriate concentration and hydrogen peroxide to appropriate concentration to initiate the reaction. The batch reactors were stirred gently for 3 h open to the atmosphere, after which the samples were quenched with excess catalase to a concentration of 62.5 U/ml to quickly consume any residual hydrogen peroxide, microfiltered and then tested for the residual concentration by using a HPLC-UV (Mazloun, 2014). All reactions were carried out in triplicate. The study was designed to achieve at least 95% conversion of aromatic substrate by optimising the following parameters: pH, SBP concentration and hydrogen peroxide-to-substrate molar concentration ratio.

Experiments were also conducted to monitor the substrate and hydrogen peroxide consumption over time. The experiments were conducted under the optimum enzymatic conditions to achieve at least 95% conversion of aromatic substrates (Feng *et al.*, 2013; Steevensz *et al.*, 2009).

Results and discussion

The following sections present the optimisation of the enzymatic conversion of the BZ, DCB, DMB and DMXB monomers into polymeric products.

Optimum pH

The optimum pH for the enzymatic conversion of BZ was investigated in defined buffers over the pH range 3.8–10.6. SBP is active over a wide range of pH (2.0–10.0) (Mazloun, 2014). Batch reactors were run for 3 h with initial substrate concentrations of 0.1 and 0.1 mM hydrogen peroxide with an enzyme activity of

Figure 1. Effect of pH on removal of 0.1 mM BZ by 1 mU/ml SBP with 0.1 mM hydrogen peroxide, 3 h reaction time

Figure 2. SBP optimisation for the removal of 0.1 mM BZ in the presence of 0.15 mM hydrogen peroxide at pH 5.0, 3 h reaction time

1 mU/ml. The experiments were designed to test the effect of pH in the presence of limited SBP concentration. Thus, stringent conditions existed (insufficient SBP for complete conversion of the BZ) in order to clearly determine pH effect (Figure 1). The optimum pH for the enzymatic conversion of BZ was in the slightly acidic region, 4.5–5.6. The optimum pH was 5.0, which is close to the pK_a of the monoprotonated substrate (4.3, monoprotonated; 3.3, deprotonated; Zierath *et al.*, 1980). A sharp reduction in the percentage conversion of BZ was observed in the basic region when compared to the acidic region, which is possibly due to a change in the ionisation of catalytic residues of the enzyme at high pH (Al-Ansari *et al.*, 2009). These results are consistent with those found by others (Karim *et al.*, 2012; Sentchouk and Grintsevich, 2004) by using BGP and TP respectively. It was concluded that the optimum pH depends not only on the proper ionisation of the catalytic residues of SBP during the enzymatic reaction but also on the type of the aromatic substrate (Al-Ansari *et al.*, 2009).

Minimum SBP concentrations

Experiments were conducted for 3 h at the previously established optimal pH of 5.0 by varying SBP concentrations to determine the minimum SBP required for $\geq 95\%$ conversion of BZ. All other variables were held constant, hydrogen peroxide at 0.15 mM and BZ at 0.1 mM. The results in Figure 2 show that BZ required 0.43 mU/ml of SBP for 95% removal. A significant change was observed in the range 0.1–0.43 mU/ml of enzyme, and no significant change occurred from 0.43 to 1 mU/ml. This value of 0.43 mU/ml of SBP for 0.1 mM BZ may be qualitatively compared to 0.6 U/ml for 0.5 mM BZ with BGP (but note the different unit definitions (Karim and Husain, 2011)) and quantitatively compared to 0.6 U/ml for 1 mM aniline with SBP (same unit definition (Mazloum, 2014)). Relative reactivities are inferred from the inverse radical stabilities, which are estimated from the corresponding homolytic bond dissociation energies, N–H for anilines (Jonsson *et al.*, 1994). However, the higher the bond

dissociation energy, the more reactive is the radical and the more damaging it is expected to be to the enzyme (Al-Ansari *et al.*, 2009). Free radicals are stabilised by resonance with the aryl ring, and the elongation of conjugation increases the free radical stability (Al-Ansari *et al.*, 2009). The authors speculate that this may be the reason that the enzyme requirement is so low with BZ, relative to aniline for example. Radical stability will be further discussed in ‘Time course with a mixture of BZ, DCB, DMB and DMXB’.

Optimum hydrogen peroxide-to-substrate concentration ratio

The molar concentrations of hydrogen peroxide studied were between 0.1 and 0.2 mM for chemical (the control) and enzymatic reactions. In the chemical reaction the results shows that the BZ is

Figure 3. Effect of hydrogen peroxide concentration on the removal of 0.1 mM BZ by chemical oxidation (a) and enzymatic oxidation with 0.050 (b), 0.25 (c) and 0.75 mU/ml SBP (d) at pH 5.0, 3 h reaction time

Figure 4. The hydrogen peroxide-to-substrate concentration ratio for 0.1 mM BZ with SBP at optimum enzyme activity, 0.43 mU/ml, and pH 5, 3 h reaction time

slightly sensitive to oxidation by hydrogen peroxide (Figure 3, curve a). In enzymatic oxidation of BZ with enzyme activities of 0.05, 0.25 and 0.75 mU/ml, the results revealed little change in percentage remaining for peroxide concentration beyond 0.15 mM (Figure 3, curves b–d).

At optimum conditions, the previously established optimal enzyme activity of 0.43 mU/ml and pH 5, the requirement of hydrogen peroxide was also studied in detail for enzymatic reaction with 0.1 mM BZ for 95% conversion in 3 h (Figure 4).

The hydrogen peroxide demand was comparable with what is expected theoretically, 0.15 mM of hydrogen peroxide for 0.2 mM of the aromatic functional group, consistent with further polymerisation (Al-Ansari *et al.*, 2010; Yu *et al.*, 1994). Since the soluble dimers produced become substrates of the enzyme (Dunford, 1999), further cycles of polymerisation require additional peroxide. This is consistent with observations for other anilino compounds (Al-Ansari *et al.*, 2009).

Effect of reaction time

Benzidine

The time course for reaction to achieve 95% removal of BZ was studied over 3 h. In 250 ml batch reactors under optimal conditions, 0.1 mM substrate was treated at pH 5, enzymatic activity of 0.43 mU/ml, and 0.15 mM hydrogen peroxide concentration, and aliquots were withdrawn at various time intervals, quenched with catalase then microfiltered and analysed by HPLC (Figure 5). SBP's catalytic reaction with substrate proceeded rapidly at the start and then slowed down after 30 min. It is evident that a 3 h reaction time is sufficient to determine the influence of different parameters on achieving at least 95% removal. Conversion of the substrate continued at a slower rate after the first 30 min, possibly due to competitive conversion of dimers and higher oligomers and/or due to enzyme inactivation caused by the reactive radicals or end-product oligomers (Mazloun, 2014). The rate of reaction should be directly proportional to the enzyme concentration (Dunford, 1999); thus, the reaction would progressively slow down with loss of enzyme activity over the time course. For quantitative analysis, the data were fitted to a pseudo-first-order model, resulting

Figure 5. BZ removal with respect to time under optimal conditions: 0.1 mM BZ, pH 5, 0.15 mM hydrogen peroxide, 0.43 mU/ml SBP and 3 h reaction time. Lines drawn are from direct fitting by non-linear regression to the first-order decay model, percentage remaining = (initial percentage) e^{-kt} , where the fitted value of the initial

percentage should closely approximate 100% and k is the apparent first-order rate constant. Solid line parameters: k , $(3.92 \pm 0.16) \times 10^{-2} \text{ min}^{-1}$; initial percentage, $100.0 \pm 1.6\%$; R^2 , 0.9916. Dashed line parameters: k , $(3.51 \pm 0.25) \times 10^{-2} \text{ min}^{-1}$; initial percentage, $97.1 \pm 2.6\%$; R^2 , 0.9759

in the lines drawn in Figure 5. As seen from the fitting statistics in the caption, SBP-catalysed removal of BZ can be well represented as a first-order process over nearly two half-lives of conversion (to 30 min; apparent rate constant k , 0.039 min^{-1} ; half-life: 18 min). Even fitting over the whole time course was only slightly less precise, giving a rate constant about 9% lower with a greater standard deviation. Thus, for comparison purposes in the following section, kinetic analysis was limited to the first 30 min of the progress curves.

Time course with a mixture of BZ, DCB, DMB and DMXB

Under the optimum conditions for BZ removal, the time course for reaction of a mixture of BZ, DCB, DMB and DMXB was determined by HPLC to show the relative removal of the four compounds. The combined mixture at 0.16 mM (0.04 mM of each), 0.24 mM hydrogen peroxide and 0.68 mU/ml SBP (peroxide and enzyme concentrations were prorated from the optimal values for 0.10 mM BZ) and reacted for 3 h (Figure 6). It is seen that 65–85% of the BZs were converted during the initial 20 min of the reaction, with DCB and DMB showing slightly higher rates. Kinetic analysis carried out as detailed in the preceding section yielded apparent first-order rate constants given in the caption and ranked as follows: $\text{DCB} > \text{DMB} \geq \text{BZ} > \text{DMXB}$, where the ratio of the fastest to the slowest is only around 2. While this range of relative rate constants is not large, it does parallel what would be expected of the corresponding phenoxy radical reactivities, dichloro being the fastest, dimethoxy being the slowest (i.e. the most stabilised, due to the electron-donating methoxyl substituents), because the removal mechanism consists of enzyme-catalysed cycles of radical coupling as outlined in the ‘Introduction’.

Figure 6. Removal of BZ and its derivatives DCB, DMB and DMXB under prorated optimal conditions for BZ removal with respect to time. Initial conditions: 0.16 mM of combined substrates (0.04 mM of each one), pH 5, 0.24 mM hydrogen peroxide and 0.680 mU/ml SBP, 3 h reaction time. Kinetic analysis carried out on the 0–30 min data for each progress curve as described in Figure 5 caption yielded the following apparent first-order rate constants: BZ, $(3.90 \pm 0.26) \times 10^{-2} \text{ min}^{-1}$; DMXB, $(2.99 \pm 0.01) \times 10^{-2} \text{ min}^{-1}$; DCB, $(5.62 \pm 0.18) \times 10^{-2} \text{ min}^{-1}$; DMB, $(4.01 \pm 0.17) \times 10^{-2} \text{ min}^{-1}$

From Figure 6, it is also apparent that the optimum conditions for 95% conversion of BZ alone can work fairly well with its derivatives, but in the 3 h reaction time chosen, their conversion is less complete: 92.1% for dichloro-, 93% for dimethyl- and 83.8% for dimethoxy-BZs. Even BZ itself suffered a little in the mutual competition, showing 93.87% conversion. To increase their conversion to at least 95%, a longer time and/or a slightly higher concentration of SBP and/or peroxide would be needed.

Conclusions

Crude SBP has been shown to be a suitable enzyme for the peroxidase-catalysed polymerisation of BZ and three of its substituted derivatives; the maximum conversion of these substrates was dependent on the optimal conditions, which were substrate specific. Most notably, the SBP activity required for the complete removal of the compounds was two to three orders of magnitude lower than reported with SBP and simple anilines or another peroxidase and BZ. Thus, SBP-catalysed conversion could, in future work, form the basis of a cost-effective alternative to conventional processes for removal of these four compounds tested from industrial waste and process water.

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