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Cloning, Heterologous Expression and Properties of a Recombinant Active Turnip Peroxidase

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ABSTRACT: Turnip (*Brassica napus*) roots peroxidase isoforms have been used in diagnostic kits and can also efficiently polymerize phenolic compounds from wastewaters. Heterologous expression of a turnip acidic peroxidase (BnPA) was investigated to increase availability of this widely used enzyme. The mature *BnPA* was ligated into the pET28a(+) vector and used to transform *Escherichia coli* Rosetta 2. Recombinant BnPA peroxidase was overexpressed and accumulated in inclusion bodies from which it was purified to homogeneity by immobilized metal affinity chromatography under denaturing conditions. Peroxidase activity was observed after a refolding process under oxidative conditions. The yield of pure recombinant BnPA was 29 mg L^{-1} of culture with a specific activity of 981 ± 20 ABTS units mg^{-1} at optimal conditions (pH 6, 45 °C). Recombinant BnPA showed similar kinetic properties compared to native turnip peroxidase, and its secondary structure evaluated by circular dichroism comprised 20% α-helix, 32% β-sheet and 48% random structure. Recombinant BnPA showed high yield and good kinetic properties which are key steps for future structure−function studies and biotechnological applications.

KEYWORDS: recombinant turnip peroxidase, overexpression, refolding, kinetic properties

INTRODUCTION

Peroxidases are oxidoreductases that catalyze the reaction between H_2O_2 and various reducing compounds and most are heme proteins that contain iron(III) protoporphyrin IX, as prosthetic group. Class III plant peroxidases (EC 1.11.1.7) participate in lignification, the defense mechanism of physically damaged or infected tissues,[1] indole acetic acid degradation and stress response[2] among other physiological processes.

Since peroxidase is relatively stable at high temperature it is used as blanching indicator of processed vegetable products.[3] In addition, its easily measured activity has allowed its use as a model enzyme in the study of protein structure and enzyme reactions and functions.[4] Horseradish (*Armoracia rusticana*) roots are the major source of peroxidase, and the most abundant isoenzyme (HRP-C) is widely used in organic synthesis, coupled enzyme analysis,[5] chemiluminescent assays[6] and cancer therapy.[7] However, there is a demand for more stable new peroxidases specific for specialized applications. A good alternative source of peroxidases is turnip (*Brassica napus*) roots, because some isoforms are able to efficiently polymerize phenolic compounds from foodstuffs and industrial effluents[8,9] or may be used in diagnostic kits.[10]

Classical purification methods involve tedious and slow processes with low yields, in addition to the difficult task of isoenzymes separation.[11] Alternative methods such as molecular cloning might help to search peroxidases showing good catalytic properties similar to those of HRP-C. Many attempts have been reported for recombinant HRP-C production using a variety of hosts.[12,13] However, yields from prokaryotic organisms like *Escherichia coli,* are not higher than 16.7 mg L^{-1}.[14,15]

Expression of recombinant turnip peroxidase has not been previously reported while expression of significant amounts of this enzyme could be promising in the search of more economical, novel peroxidases. However, eukaryotic protein expression in *E. coli* is not always an easy task. The genome of certain organisms favors sequences using codons that occur infrequently in *E. coli* hosts. Forced high-level expression of rare codon-containing genes in *E. coli* depletes the endogenous pool of corresponding tRNAs. The deficit of tRNA molecules disrupts translation, leading to truncated proteins or no protein expression. There are however, some strains like Rosetta which supply tRNAs for codons rarely used in *E. coli.*[16] This strategy was used for the expression of a tobacco anionic peroxidase in *E. coli* BL21(DE3).[17]

Turnip peroxidase shows about 9% glycosylation,[18] and bacterial expression systems are not able to glycosylate recombinant proteins. However, a neutral turnip peroxidase was partially deglycosylated (88%) and about 40% activity was retained, with no effect on structural conformation.[19] We have previously isolated and cloned a turnip (*B. napus* L. var. purple top white globe) roots acidic isoperoxidase cDNA.[20] Thus, the objective of this work was to overexpress and purify from a bacterial system a recombinant turnip isoperoxidase and to investigate its refolding process and biochemical properties.

MATERIALS AND METHODS

Materials. NaCl, tris-HCl, imidazol, sodium phosphate, dithiothreitol (DTT), oxidized glutathione (GSSG), hemin, isopropyl-β-D-thiogalactopyranoside (IPTG), bovine serum albumin (BSA), and diammonium salt of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonate) (ABTS) were obtained from Sigma (St. Louis, MO). Electrophoresis chemicals [acrylamide, *N*,*N*′-methylenebisacrylamide, 1,2-bis(dimethylamino)-ethane (TEMED), sodium dodecyl sulfate (SDS), tris(hydroxymethyl)-aminomethane,

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ammonium persulfate] were purchased from BioRad (Hercules, CA). A vector containing a DNA fragment which codes for turnip roots (*Brassica napus* var. purple top white globe) mature peroxidase (BnPA) was obtained as previously reported.[20]

Construction of Expression Vector pET28-*BnPA*. BnPA was amplified by PCR using the plasmid pGEMT-*BnPA* as DNA template.[20] Gene-specific primers (Sigma) used to amplify the *BnPA* gene, were: 5′-TAT**GGATCC**CAGTTAAACCCAACG-TTTTACTC-3′ and 5′- AAT**AAGCTT**TTATTGTCCATTAA-CCACCTTACAG-3′ for sense and antisense, respectively. The primers contained *Bam*HI and *Hind*III restriction sites (bold) to allow directional cloning of amplified DNA. The PCR product was cloned into the pGEM-T Easy Vector (Promega, Madison, WI) and digested with *Bam*HI and *Hin*dIII (Fermentas, Ontario, Canada). After electrophoresis and gel band purification (GE Healthcare, Uppsala, Sweden), *BnPA* was ligated to the pET28a-(+) vector (Novagen, Madison, WI) previously digested with same restriction enzymes. Cloned genes in this vector are expressed as His_6-tagged fusion proteins. *E. coli* JM109 competent cells were used for cloning (Invitrogen, Carlsbad, CA), and positive clones were analyzed by PCR and restriction enzymes. The expression vector pET28-*BnPA* was used to transform *E. coli* Rosetta 2 (DE3) competent cells (Novagen) using heat shock.[21] Plasmidic DNA was isolated from transformants using the Qiagen DNA mini-prep (Hamburg, Germany) and confirmed by PCR and restriction analysis.

Expression of Recombinant BnPA Peroxidase. Transformed colonies were inoculated into 3 mL of Luria−Bertani (LB) broth containing 30 μg mL^{-1} kanamycin and 34 μg mL^{-1} chloramphenicol. The cultures were incubated overnight at 37 °C in an orbital shaker (Lab-line, Melrose Park, IL) at 200 rpm and used to inoculate two 1 L flasks, each containing 500 mL LB added with both antibiotics. Cultures were grown at 37 °C at 200 rpm to an OD_{600} of about 0.5, using a Genesys 10 UV spectrophotometer (Thermoscientific, Madison, WI). The recombinant protein was induced by addition of IPTG to a final concentration of 0.5 mM. Bacterial cells from 1 L cultures were grown another 5 h at 37 °C, or 12 h at 16 or 25 °C and harvested by centrifugation (Eppendorf 5804R, Hamburg, Germany) at 4000*g* for 10 min at 4 °C, and were either immediately used or stored at −20 °C.

Polyacrylamide Gel Electrophoresis (PAGE). The expression level of recombinant BnPA in cell extracts, in the soluble and insoluble fractions and purity of protein samples, were analyzed by SDS−PAGE[21] on a Mini Protean II unit (Bio-Rad) using a 10% polyacrylamide gel. Low molecular weight markers (GE Healthcare, Uppsala, Sweden) were used as reference and protein bands were stained using Coomassie blue G-250 dye (Bio-Rad).

Solubilization of Inclusion Bodies. The cell pellet was resuspended in lysis buffer (5 mL g^{-1} wet pellet) containing 2 M NaCl, 10 mM DTT, 10 mM tris−HCl buffer pH 8.0 and lysed by ultrasonic treatment (Vibra-cell, Sonics & Materials, Newtown, CT) at 20 kHz for 10 min, in an ice-bath. The disrupted mixture was incubated for 1.5 h in the ice-bath and sonication procedure was repeated.[15] The lysate was centrifuged at 10 000*g* for 25 min at 4 °C to separate the soluble and insoluble fractions. The pellet containing inclusion bodies was washed twice with 50 mM tris−HCl buffer (pH 8.0), centrifuged at 10,000*g* for 15 min and solubilized in 150 mL of 8 M urea containing 1 mM DTT at room temperature (25 ± 2 °C) following manufacturer instructions (Novagen).

Recombinant BnPA Purification. Solubilized recombinant BnPA protein was purified by immobilized-metal affinity chromatography (IMAC) using a 1 mL Ni−NTA column (GE Healthcare, Uppsala, Sweden) previously equilibrated with binding buffer (20 mM sodium phosphate, 500 mM NaCl, 30 mM imidazole, 8 M urea, pH 7.4). After sample injection (1 mL) 15 column volumes of binding buffer were used for washing, followed by BnPA elution using five column volumes of elution buffer (binding buffer adjusted to 500 mM imidazole). Fractions of 0.5 mL were collected.

BnPA fractions were desalted by size exclusion chromatography (10DG column, Bio-Rad). Three mL of the purified and denatured BnPA was applied to the 10DG column pre-equilibrated with 50 mM tris-HCl buffer, pH 8.0, containing 4 M urea. Three mL of the partially desalted BnPA fractions were applied to another 10DG column pre-equilibrated with the same buffer containing 2 M urea. One mL fractions were collected and analyzed by SDS−PAGE. This two-step process allowed a 4-fold reduction in urea concentration and permitted adjustment of recombinant BnPA refolding conditions.

Refolding of Recombinant BnPA. BnPA (50 μg mL^{-1}) was added dropwise to the refolding buffer (2 M urea, 0.7 mM oxidized glutathione, 5 mM $CaCl_2$, 0.1 mM DTT, 5% glycerol in 50 mM tris-HCl buffer, pH 8) and incubated overnight at 4 °C, as described by Gazaryan et al.[15] Hemin (5 μM) was then added and the solution was incubated for 2 h at 4 °C, followed by activity determination of the refolded enzyme. Enhancement of recombinant BnPA refolding was attempted by independently varying urea and glutathione concentrations. Urea was varied from 1.5 to 3 M, while glutathione concentration ranged 0.1−1.5 mM, keeping the concentration of all other chemicals constant. Recombinant BnPA was concentrated by ultrafiltration using 10 kDa molecular weight cut off membranes (Millipore, Billerica, MA).

Activity Determination. Peroxidase activity was determined using ABTS as hydrogen donor. The reaction mixture consisted of 10 mM potassium phosphate buffer (pH 6.0), 2.5 mM ABTS, 50 μL of purified enzyme and 2 mM H_2O_2, in a total volume of 1.5 mL. The change in absorbance at 414 nm (ε_{414} = 36 mM^{-1} cm^{-1}; ref 22) was followed using a UV spectrophotometer (Thermoscientific). One unit of activity is defined as the μmoles of ABTS radical produced per min at pH 6.0 and 25 °C. Protein was quantified according to the dye-binding method of Bradford[23] using BSA as standard.

Recombinant BnPA Characterization. *Optimum pH.* The following buffers were used: 0.02 M citrate-phosphate buffer, pH 3−7; 0.02 M phosphate buffer, pH 8; and 0.02 M tris-HCl buffer, pH 9. Two mM H_2O_2, 2.5 mM ABTS, and 50 μL purified recombinant enzyme (50 μg mL^{-1}) were used.

Optimum Temperature. Peroxidase activity was measured between 25 and 70 °C with 5 °C increments at the optimum pH. Temperature control was achieved using a digital dry bath (Labnet, Woodbridge, NJ).

Kinetic Studies. Rate constants of ABTS oxidation reactions were determined from steady-state data by varying H_2O_2 concentration from 0.5 to 5 mM and ABTS concentration from 0.05 to 3 mM. Ionic strength, pH and temperature were kept constant in these series of experiments. K_m and V_{max} of ABTS were calculated using the Lineweaver−Burk graphic method for the two-substrate ping-pong mechanism followed by peroxidase.[11] The rate equations in double reciprocal form are given below [24]

$$\frac{1}{v_0} = \frac{K_a}{V_{max}A_0} + \frac{K_b}{V_{max}B_0} + \frac{1}{V_{max}} \quad (1)$$

Where, V_{max} = maximum velocity, K_a = K_m for substrate A

(hydrogen peroxide), $K_b = K_m$ for substrate B (ABTS), A_0 = substrate A concentration and B_0 = substrate B concentration. A plot of $1/v_o$ versus $1/B_0$ for different values of A_0 (eq 1) yields a family of parallel lines, characteristic of a ping-pong mechanism. Using this graph and from a replot of intercepts versus $1/A_0$ the parameters K_a, K_b, and V_{max} can be determined.[24]

Effect of Metal Ions and Salts. The purified and refolded recombinant peroxidase (50 μL) was incubated individually with 50 μL of 0.25 mM $AgNO_3$, 5 mM of $MgCl_2$, $CaCl_2$, NaCl, KCl, $ZnCl_2$, EDTA, and $MnSO_4$ or deionized water as control at 25 °C for 30 min. The enzyme activity was then measured and results were expressed as relative activity calculated as the ratio of refolded recombinant BnPA activity in the presence of salts to that without salts, expressed as percentage.

Far-UV Circular Dichroism (CD) Spectra. The CD spectrum in the far-UV region (190 to 240 nm) was recorded using a 0.1 cm path length quartz cell under constant nitrogen flush using protein concentration of 0.1 mg mL^{-1} in 0.01 M phosphate buffer at pH 6.0. CD data were expressed as molar ellipticity $[\theta]$ (degrees cm^2 $dmol^{-1}$), based on an assumed mean amino acid residue weight (MRW) of 110 Da. The molar ellipticity was determined as $[\theta] = (\theta \times 100\ MRW)\ (c\ l)^{-1}$, where c is protein concentration (mg mL^{-1}), l is light path length (cm), and θ is the measured ellipticity (millidegrees). CD spectra were reported as the average of three scans corrected by subtracting the adequate blank runs on BnPA-free buffer. The fractions of secondary structure (α-helix, β-sheet and random) were calculated using the SELCON3 program.[25] Thermodynamic properties for thermal unfolding of recombinant BnPA were calculated from θ_{222} data by heating from 25 to 90 °C at constant rate of 2 °C/min.

RESULTS AND DISCUSSION

Construction of the Expression Vector pET28-*BnPA*. The full-length *BnPA* comprises 1077 base pairs (bp) and encodes a predicted protein of 358 amino acids.[20] However, only 921 bp were amplified to encode the mature BnPA. *BnPA* fragment was amplified successfully from the pGEMT-*BnPA* vector (Figure 1A, lane 2), and ligated to yield the expression plasmid pET28-*BnPA* which was verified by *Bam*HI and *Hind*III digestion (Figure 1B, lane 2).

Expression of Recombinant BnPA. *BnPA* contains 5 Ile (ATA), 23 Arg (AGA and CGG), 10 Leu (CTA), 9 Pro (CCC), and 13 Gly (GGA) codons which are rare for prokaryotes. However, this problem was overcome by using *E. coli* Rosetta 2 (DE3) which supplies the genes of the corresponding tRNAs. Attempts to obtain BnPA in the soluble fraction by using lower expression temperatures (16 and 25 °C) were unsuccessful (data not shown).

Cell extracts without IPTG addition (Figure 2, lane 2) did not exhibit a differential expression band characteristic of those after induction which showed a molecular weight of about 37 kDa (Figure 2, lane 3). This band corresponds to the expected size of the His_6-tagged recombinant BnPA, and represents about 20% of the total protein produced by *E. coli*. From SDS−PAGE analysis of soluble and insoluble fractions (Figure 2, lanes 4 and 5, respectively) most induced protein was found as insoluble, suggesting that the His-tagged BnPA was present in inclusion bodies. According to lane 5, recombinant BnPA represents about 50% of the insoluble cellular protein. Thus, like other peroxidases BnPA was expressed within inclusion bodies.[14,17]

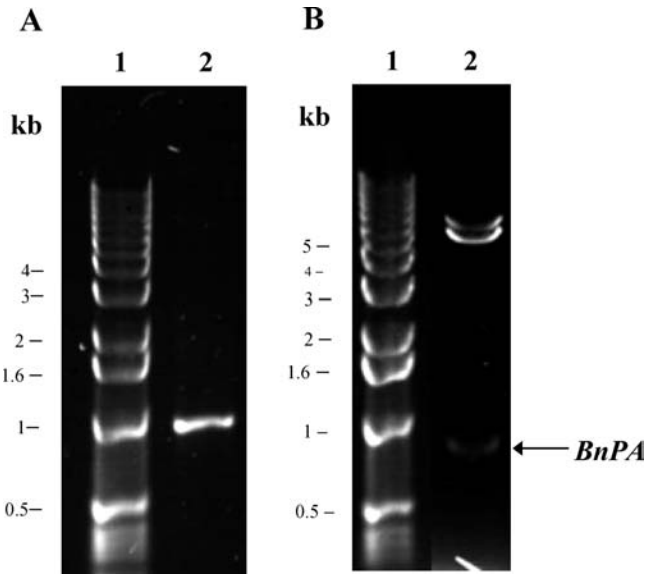

Figure 1. Construction of recombinant vector pET28-*BnPA*. A) Lane 1, 1 kb DNA ladder; lane 2, PCR product of *BnPA*. B) Lane 1, 1 kb DNA ladder; lane 2, transformant clone containing the recombinant vector pET28-*BnPA* digested with *Bam*HI and *Hind*III.

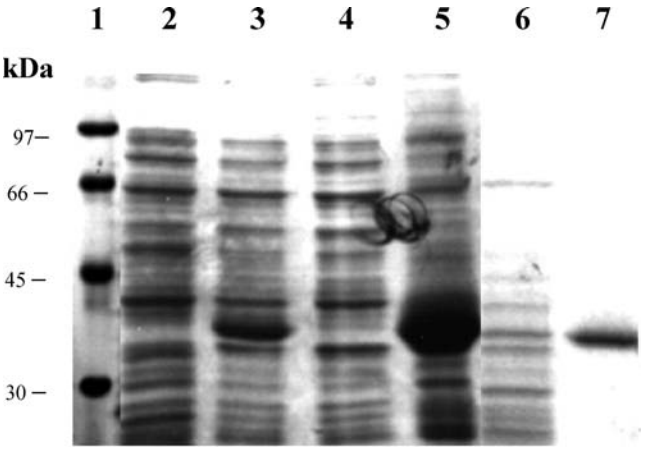

Figure 2. SDS−PAGE analysis of recombinant BnPA. Lane 1, low range molecular weight markers; lane 2, cell extract from *E. coli* Rosetta harboring the pET28a-*BnPA* construct before induction; lane 3, cell extract after IPTG induction; lane 4, soluble fraction; lane 5, insoluble fraction; lane 6, first washing from Ni−NTA column; lane 7, BnPA elution from Ni−NTA column.

Purification of Recombinant BnPA by IMAC. BnPA was solubilized from inclusion bodies using 8 M urea, and purified by IMAC under denaturing conditions. After extensive washing of nonretained protein (Figure 2, lane 6) BnPA was eluted with 0.5 M imidazole (Figure 2, lane 7). At this point, recombinant BnPA was purified to homogeneity, since no other protein bands were observed.

Protein concentration after two-step desalting by gel filtration was approximately 483.6 μg mL^{-1} (Table 1) with a yield of 29 mg of purified BnPA L^{-1} of cell culture. Recombinant HRP from *E. coli* achieved yields of 16.7 mg L^{-1},[15] and 0.086 mg L^{-1},[26] which are about 1.7 and 340 times lower than that obtained here, respectively. Using the same system a recombinant HRP-conjugate

Table 1. Purification of Recombinant BnPA (Mean of Three Replicates with Standard Error Within 5% of the Mean)

sample	protein concentration (μg mL^{-1})	total protein (mg)	protein yield (%)
total cell lysate[a]	3819.4	76.4	100
insoluble fraction[b]	1710.2	34.2	45.0
IMAC	711.2	6.4	8.4
gel-filtration	483.6	5.8	7.6

[a] A total of 3 g wet weight of cells from 250 mL culture were lysed. [b] Inclusion bodies were solubilized in 8 M urea and loaded into the Ni−NTA column.

Table 2. Effect of Urea and Oxidized Glutathione on Recombinant BnPA Refolding

urea (M)	relative activity[a] (%)	oxidized glutathione (mM)	relative activity[a] (%)
1.5	65 ± 2.4	0.5	61 ± 2.3
2.0	100 ± 1.1	0.7	100 ± 1.2
2.4	63 ± 2.1	1.2	92 ± 1.8
3.0	21 ± 0.5	1.5	87 ± 1.4

[a] Ratio of actual to maximum activity expressed as percentage. Data are expressed as mean ± standard deviation of three replicates. Initial conditions were 2 M urea, 0.7 mM GSSG, 5 mM $CaCl_2$, 0.1 mM DTT, 5 μM hemin, in 50 mM tris−HCl buffer pH 8.0. Urea and glutathione concentrations were independently varied.

yield was 12 mg L^{-1},[27] which is less than half our value. On the other hand recombinant glycosylated HRP-C was expressed in insect cells culture with added hemin and the yield of active peroxidase was 28 mg L^{-1},[13] which is similar to the one obtained here. However, recombinant protein expression using tissue culture is time-consuming and involves high costs.[28] Using insect larva as expression hosts, which is a more cost-effective strategy than tissue culture, the yield of HRP-C was 480 mg kg^{-1} after optimization of infection conditions.[29]

Refolding of Recombinant BnPA. From refolding enhancing experiments 2.0 M urea concentration gave the maximum peroxidase activity (Table 2). Disulfide bonds formation plays a significant role in peroxidase refolding and recombinant BnPA showed 61% relative activity at 0.5 mM of oxidized glutathione, reached a maximum at 0.7 mM and steadily decreased with increasing concentrations (Table 2). These conditions were similar to those reported for HRP-C[14] and lignin peroxidase,[30] but slightly different (1.8 M urea and 0.5 mM oxidized glutathione) from those found for recombinant tobacco anionic peroxidase.[17]

Recombinant BnPA showed a Reinheitszahl (RZ) value of 3.6, which is higher than that found for recombinant nonglycosylated HRP-C (2.11[14]) and glycosylated HRP-C produced in insect cell culture (3.2[13]). Purified native acidic turnip peroxidase showed a RZ value of 3.1,[18] indicating similar heme group environment of recombinant BnPA.

Specific activity of the refolded recombinant BnPA was 981 ± 20 U mg^{-1} under the best conditions which is lower than that shown by recombinant tobacco anionic peroxidase (1100 U mg^{-1}; [17]), slightly higher than a HRP-conjugate (920 U mg^{-1}; [27]) and much higher than that of HRP (0.58 U mg^{-1}; [26]), all three expressed in *E. coli*. BnPA showed about the same activity as native partially deglycosylated turnip peroxidase,[19] indicating an unclear effect of the carbohydrate moiety on turnip peroxidase activity.

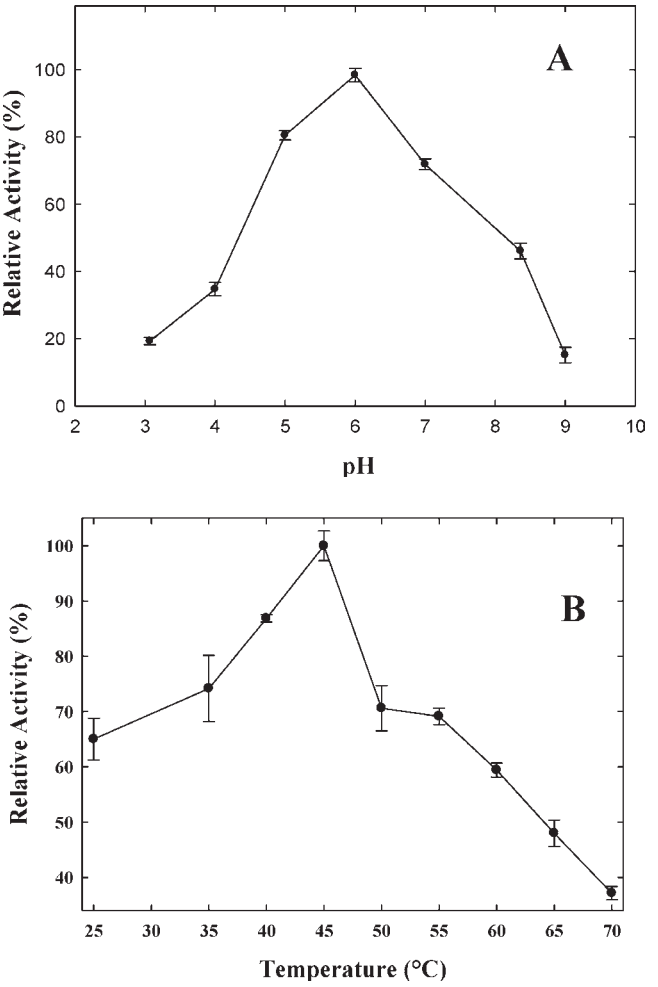

Figure 3. Effect of pH (A) and temperature (B) on recombinant BnPA activity, using ABTS as H donor. The ordinate represents relative activity, that is, the ratio of activity to the maximum activity expressed as percentage. Vertical bars indicate standard deviation.

Recombinant BnPA Characterization. *Optimum pH.* Recombinant BnPA showed above 70% relative activity (i.e., ratio of actual to maximum activity) in a narrow pH range (5−7) with a maximum at pH 6.0 (Figure 3A). The optimum pH was about the same as that reported for a recombinant ascorbate peroxidase from soybean[31] and slightly higher than those of two native turnip roots isozymes (neutral and acidic) ranging between 5.0 and 5.5.[11,32]

Optimum Temperature. Under the conditions used here maximum activity of purified recombinant BnPA was observed at 45 °C (Figure 3B). At 50 °C relative peroxidase activity showed a sharp decrease reaching a value of about 70%, and increasingly higher temperatures resulted in a steady decrease of relative peroxidase activity, reaching a value of about 37% at 70 °C. The optimum temperature found here is similar to those of three native turnip peroxidases whose optimum temperatures ranged from 40 to 55 °C.[32]

Kinetic Studies. Initial velocities (v_0) were determined as a function of both substrates (A_0, H_2O_2, and B_0, ATBS). Double-reciprocal plots according to eq 1 (results not shown) showed

Table 3. Kinetic and Thermodynamic Parameters of Native and Recombinant Turnip Peroxidase. [a]

peroxidase	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})	Tm (°C)	$\Delta H_u^{\circ b}$(kJ mol^{-1})	$\Delta G_u^{\circ b}$(kJ mol^{-1})	$\Delta S_u^{\circ b}$(kJ mol^{-1})
recombinant BnPA	0.33 ± 0.04	1722 ± 37	5218 ± 85	57.4 ± 0.2	91.2 ± 1.9	183 ± 2.1	0.27 ± 0.03
native turnip[c]	0.56 ± 0.03	33000 ± 55	59000 ± 1700	66.4 ± 0.3	128.75 ± 3.2	257.5 ± 2.3	0.38 ± 0.02

[a] Mean of three replicates ± standard deviation. [b] ΔH_u°, ΔG_u°, ΔS_u° are the standard state change of enthalpy, Gibbs free energy and entropy of unfolding, respectively. Parameters calculated by thermal unfolding profile using CD at $\theta_{222\ nm}$. [c] Kinetic parameters were obtained from ref 8, while thermodynamic data were calculated from ref 18.

that recombinant BnPA kinetics follows a ping-pong mechanism, which has also been reported for tobacco anionic peroxidase[17] and a neutral turnip peroxidase.[11]

Using graphical analysis[24] kinetic parameters were calculated (Table 3). The ABTS K_m value was lower than those reported for native turnip peroxidase and a partially deglycosylated turnip peroxidase (0.9 mM[19]). In comparison to reported recombinant enzymes, our K_m value was 3.5 higher than that of HRP (0.093 mM[26]) and about twice that of tobacco anionic peroxidase (0.17 mM[17]). These results suggest that recombinant nonglycosylated peroxidase may offer better substrate accessibility to its binding site as compared to the native enzyme, leading to increased substrate affinity. However, more experiments are needed to confirm this hypothesis. Recombinant BnPA showed a k_{cat} similar to that reported for a single mutant (K232N) recombinant HRP (1849 s^{-1},[26]), but significantly lower than that of native turnip peroxidase (Table 3). The recombinant BnPA catalytic efficiency (k_{cat} K_m^{-1}) was similar to that of recombinant tobacco peroxidase (6×10^6 M^{-1} s^{-1},[17]) and slightly higher than native peroxidase from broccoli stems (3.4×10^6 $M^{-1}s^{-1}$,[33]). However, native or chemically modified peroxidase from turnip (~6×10^7 $M^{-1}s^{-1}$,[8]) showed about 11 times higher catalytic efficiency. These findings suggest that recombinant BnPA may show a similar performance as native or chemically modified turnip peroxidase in environmental applications, but tests under real conditions should be conducted to clarify this assumption.

Effect of Metal Ions and Salts. Salts concentrations of 0.25 mM $AgNO_3$, 5 mM of NaCl, KCl, $ZnCl_2$, EDTA, and $MnSO_4$ resulted in peroxidase activity inhibition, while 5 mM $MgCl_2$ and $CaCl_2$ enhanced peroxidase activity (Table 4). These results are in agreement with those reported for native turnip peroxidase.[34] Ca^{2+} and Mg^{2+} may interact through electrostatic forces with side chains of specific amino acids of the protein possibly resulting in subtle changes in the heme environment leading to enhanced affinity between recombinant BnPA and its substrate.

Far-UV CD Spectra. The percentage secondary structure of refolded recombinant BnPA was investigated by far-UV CD. A purified turnip peroxidase sample was kindly supplied by Dr. Quintanilla-Guerrero (UAQ, Mexico) and its spectrum showed a characteristic α-helix structure, such as negative bands at 208 and 222 nm (Figure 4) in agreement with Quintanilla-Guerrero et al.[8] In contrast, CD spectrum of recombinant BnPA showed significant changes such as lower negative ellipticities and is summarized in Table 5. Helical structure decreased from 28% to 20%, while β-sheet increased from 27% to 32% for native turnip peroxidase and BnPA, respectively. From previous studies on native and partially deglycosylated turnip peroxidase, no significant changes in secondary structure were detected.[19] Therefore, it may be concluded that changes observed in the CD spectrum of BnPA may be attributed to a partial refolding process of the enzyme molecule which may also explain the lower specific

Table 4. Effect of Metal Ions and Salts on Recombinant BnPA Activity

chemical	concentration (mM)	relative activity[a] (%)
none		100 ± 1.2
$MgCl_2$	5	111.49 ± 2.3
$CaCl_2$	5	124.36 ± 1.8
NaCl	5	53.6 ± 0.8
KCl	5	59.78 ± 1.1
$ZnCl_2$	5	45.97 ± 1.5
EDTA	5	60.55 ± 2.1
$AgNO_3$	0.25	39.11 ± 0.7
$MnSO_4$	5	41.77 ± 1.3

[a] Relative activity = ratio of activity in presence of salt to that without salt. Data are expressed as mean ± standard deviation of three replicates.

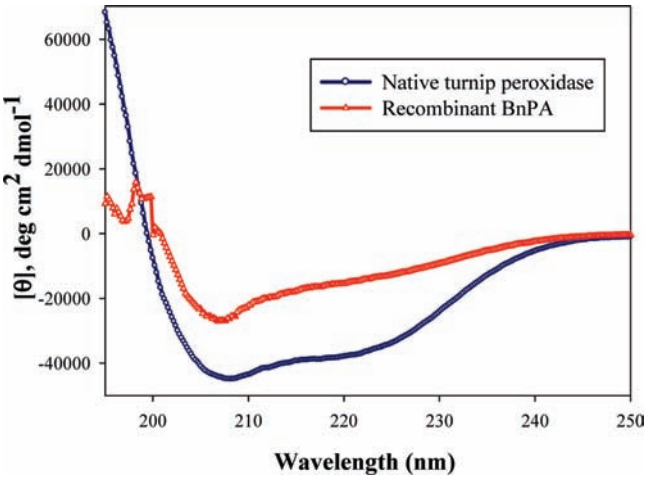

Figure 4. Far-UV CD spectra of native turnip peroxidase and recombinant BnPA. Protein concentration was 100 μg mL^{-1} in 10 mM phosphate buffer, pH 6.0.

activity of recombinant BnPA (981 U mg^{-1}) as compared to purified native acidic turnip peroxidase (1810 U mg^{-1},[18]).

Based on protein structural properties thermal denaturation of BnPA was monitored by following ellipticity changes at 222 nm (results not shown). A thermal transition plot was used to calculate the corresponding standard state thermodynamic properties for unfolding (ΔH_u°, ΔG_u°, ΔS_u°). The recombinant BnPA showed a lower thermal stability than native turnip peroxidase, as indicated by their thermodynamic properties (Table 3), probably associated to a lack of carbohydrate moiety.

Melting temperature (T_m, midpoint of the transition from helical to random structure) was lower than the T_m value of native turnip peroxidase (Table 3). Onset temperature (T_0,

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Table 5. Secondary Structure of Native Turnip Peroxidase and Recombinant BnPA[a]

enzyme	α-helix (%)	β-sheet (%)	random (%)
recombinant BnPA	20 ± 0.3	32 ± 0.4	48 ± 1.3
native turnip peroxidase	28 ± 0.4	27 ± 0.4	45 ± 0.9

[a] Calculated from CD spectra using SELCON3 software (mean of three replicates ± standard deviation).

where the loss in ellipticity signal reaches 5% of the original) for BnPA was 37 °C, while native turnip peroxidase showed a T_0 of 35 °C.[8] These values indicate that despite being initially slightly more stable, the partially folded recombinant BnPA showed lower thermal stability.

This investigation resulted in the successful heterologous expression of a plant peroxidase. Enhanced refolding conditions of the purified recombinant BnPA, solubilization from inclusion bodies and overnight incubation with hemin allowed us to obtain an active peroxidase. Structural characteristics and thermal stability of recombinant BnPA were different from those found for native peroxidase, probably associated to partial refolding and lack of carbohydrate moiety.

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