Available online at www.sciencedirect.com
ScienceDirect
Journal of Molecular Catalysis B: Enzymatic 42 (2006) 78–84
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Effects of calcium on the thermal stability, stability in organic solvents and resistance to hydrogen peroxide of artichoke (*Cynara scolymus* L.) peroxidase: A potential method of enzyme control

Lara Sidrach, Alexander N.P. Hiner, Soledad Chazarra, José Tudela, Francisco García-Cánovas, José Neptuno Rodríguez-López *

GENZ: Grupo de Investigacíon de Enzimología, Departamento de Bioquímica y Biología Molecular-A, Facultad de Biología, Universidad de Murcia, E-30100 Espinardo, Murcia, Spain

Received 2 February 2006; received in revised form 22 June 2006; accepted 13 July 2006
Available online 15 August 2006

Abstract

The effects of calcium ions (Ca^{2+}) on the stability of artichoke (*Cynara scolymus* L.) peroxidase (AKPC) have been studied. The thermal stability of AKPC was improved by the addition of Ca^{2+}; the melting temperature increased by 20 °C and the deactivation energy by 26 kJ mol^{-1}. AKPC was stable in a selection of organic solvents but was less active with 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) than under aqueous conditions. Ca^{2+}-free AKPC retained more activity in the presence of organic solvents due to its better maintenance of the rate of compound I formation with hydrogen peroxide (H_2O_2) compared to AKPC-Ca^{2+}. AKPC retained at least 75% activity over 24 h in the pH range 3.0–10.5 and about 50% over 1 month at pH 7.0 or 5.5, irrespective of the Ca^{2+} content. AKPC-Ca^{2+} was considerably more resistant to inactivation by H_2O_2 than Ca^{2+}-free AKPC suggesting that the presence of Ca^{2+} boosts turnover under oxidizing conditions. AKPC has been applied as an alternative to horseradish peroxidase (HRP) in glucose concentration assays; the presence of Ca^{2+} or of the Ca^{2+} chelating agent ethylenediaminetetraacetic acid made no difference to the final result. The possibility is discussed that addition and removal of a labile Ca^{2+} from AKPC could be used to control enzyme activity both *in vivo* and *in vitro*.

Keywords: Artichoke; Peroxidase; Calcium; Heat stability; Organic solvent; Hydrogen peroxide; Glucose

1. Introduction

Haem peroxidases are ubiquitous oxidative enzymes that are usually isolated from plants, fungi, and bacteria (plant peroxidase super family) [1], but also increasingly from mammalian sources (animal peroxidase super family) [1]. The plant super family has been divided phylogenetically into three classes [2]; class I being of prokaryotic origin, class II from fungi and class III comprising the extra cellular plant enzymes. Peroxidases have been implicated in diverse biological processes that include both cell wall synthesis and degradation, stress response, signaling during oxidative stress and removal of xenobiotics.

Peroxidase catalysis is traditionally described by a three-step mechanism (Eqs. (1)–(3)). Following the binding of hydrogen peroxide (H_2O_2) to the high spin (HS) ferric haem iron of the resting enzyme, the peroxide oxygen–oxygen bond undergoes heterolytic cleavage [3]. A water molecule is released during this reaction with the concomitant two-electron oxidation of the haem to form an intermediate (compound I) comprising an oxyferryl species (Fe(IV)=O) and a porphyrin π-cation radical (a protein radical is observed in a few cases [4,5]).

$$\text{peroxidase} + H_2O_2 \rightarrow \text{compound I} + H_2O \quad (1)$$

with most substrates, compound I is then converted back to the resting enzyme via two successive single electron transfers from separate reducing substrate molecules. The first reduction, of the porphyrin π-cation radical, yields a second enzyme

* Corresponding author. Tel.: +34 968 398284; fax: +34 968 364147/3963.
E-mail address: neptuno@um.es (J.N. Rodríguez-López).
URL: http://www.um.es/genz.

1381-1177/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2006.07.005

intermediate, compound II, which retains the iron in the oxyferryl state [6].

$$\text{compound I} + AH_2 \rightarrow \text{compound II} + AH^{\bullet} \quad (2)$$

$$\text{compound II} + AH_2 \rightarrow \text{peroxidase} + AH^{\bullet} + H_2O \quad (3)$$

Peroxidases have long been of interest in research and are also used commercially as catalysts in phenolic resin synthesis, indicators for reactive oxygen species formed during food processing, and components in kits for research, medical diagnosis and bioremediation [7–10]. At present the major commercial source of peroxidase is horseradish root (HRP), but extensive investigations of several peroxidases of alternative origins have been reported in recent years [11–14]. We have examined the peroxidase activity of several local Spanish plants, among them the artichoke (*Cynara scolymus* L.) which is widely grown in Mediterranean countries.

Artichoke peroxidase C (AKPC) is a basic enzyme (pI > 9) which has been purified to homogeneity from artichoke flowers [15]. AKPC, as obtained from the plant, has been characterized as a class III peroxidase with good activity at neutral to acid pH towards phenolic substrates. The UV–visible spectrum of native AKPC exhibited a rather high Soret (404 nm) molar absorptivity and the peak was unusually sharp. The haem iron in HRP and most other class III peroxidases has been characterized as a penta-coordinate high spin (5cHS) ferric species. However, the resonance Raman spectrum of AKPC indicated a majority 6-aquo hexa-coordinate high spin (6cHS) ferric iron in addition to the more usual 5cHS [15]. The effects of calcium ions (Ca^{2+}) on AKPC have recently been examined by our research group [16]. Ca^{2+} was found to modulate the haem iron coordination state (favouring 5cHS) and reactivity, particularly during compound I formation (rate constant, k_1, increased 30,000-fold). In light of these data, in the present study the effects of Ca^{2+} on the stability of AKPC versus temperature, pH, organic solvents and H_2O_2 have been examined. Previously we demonstrated the application of extracts from artichoke containing AKPC and other components for the removal of phenolic compounds from aqueous solution [17]. A comparison of AKPC and HRP in glucose concentration determinations (a common application of peroxidase) is now presented.

2. Materials and methods

2.1. Chemicals

All chemicals are commercially available and of reagent grade. H_2O_2 (30%, v/v) was obtained from BDH-Merck (Poole, UK) and its concentration was determined spectrophotometrically using $\varepsilon_{240\,nm} = 43.7\ M^{-1}\ cm^{-1}$. A 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) was from Sigma (Madrid, Spain). Other chemicals were obtained from Sigma or Aldrich (Madrid, Spain). Solutions were prepared using water drawn from a Milli-Q system (Millipore). Phosphate buffers were generally avoided since a precipitate may form in the presence of Ca^{2+}.

2.2. Enzymes

AKPC was purified from fresh artichoke (*C. scolymus* L.) flowers grown in Murcia (S.E., Spain), using the published procedure [15]. The enzyme was extensively dialyzed against water, lyophilized and stored at −20 °C. Purified AKPC was homogeneous by SDS-PAGE stained with silver, was found to consist of a single isoenzyme with a pI > 9 using isoelectric focusing, and exhibited an R_z ($A_{404\,nm}/A_{280\,nm}$) of 3.3–3.8 depending on the presence of Ca^{2+} (as $CaCl_2$) or ethylenediaminetetraacetic acid (EDTA). Artichokes are commercially cultivated in Murcia from November to June; purifications and studies carried out using material from different seasons and years revealed no evidence of variations in the enzyme. HRPC (type 4B) was purchased from Biozyme Ltd. (Blaenavon, Gwent, UK) and used without further purification. Glucose oxidase was obtained from Sigma and used without further purification.

2.3. Peroxidase assay

Unless otherwise stated, peroxidase activity was determined with ABTS as the reducing substrate in a reaction mixture (1 mL) containing 50 mM sodium citrate buffer, pH 4.5, 1 mM ABTS and 0.5 mM H_2O_2. The oxidation of ABTS, to the metastable radical, $ABTS^{\bullet+}$, was followed by observing the increase in absorbance at 414 nm ($\Delta\varepsilon_{414\,nm} = 31.1\ mM^{-1}\ cm^{-1}$). Spectrophotometric measurements were done on a computer controlled Perkin-Elmer Lambda-2 UV–visible spectrophotometer.

2.4. Thermal stability

The thermal stability of AKPC in the presence of Ca^{2+} or EDTA was studied at neutral pH (50 mM tris(hydroxymethyl)aminomethane–HCl (Tris–HCl) buffer, pH 7.0) by incubation at different temperatures. After equilibrating 450 μL of buffer solution at the desired temperature for 10 min, 50 μL of AKPC were added and the resultant solution (320 nM) was incubated at the same temperature in stoppered vials, to prevent changes of sample volume and, hence, enzyme concentration due to evaporation. At different times, aliquots (10 μL) of the enzyme solution were removed and placed in an ice bath for 5 min to stop the deactivation reaction. Then 5 μL of this solution were assayed as described above. Plots of the residual activities ($A_R = A_t/A_0$, where A_t and A_0 are enzyme activity at time t and at the start of the reaction, respectively) versus incubation time (t) were fitted by non-linear regression to obtain the observed rates of deactivation ($k_{deact,obs}$). The activation energies were calculated from linear fits to Arrhenius plots of $\ln k_{deact,obs}$ against the reciprocal of the absolute temperature ($1/T$).

The melting curves of AKPC with Ca^{2+} and EDTA were determined from the changes in circular dichroism (CD) ellipticity at 222 nm recorded on a Pi-Star 180 CD spectrometer (Applied Photophysics Ltd., Leatherhead, UK) fitted with a thermoelectric (Peltier) heater/cooler unit (Melcor, Trenton, NJ, USA). Experiments were carried out in a stirred and stoppered 1 cm path length cuvette in a total volume of 2.5 mL, 50 mM Tris–HCl, pH 7.0. The AKPC concentration was 2.1 or 2.26 μM

with 200 μM $CaCl_2$ or EDTA, respectively. The temperature range 30–90 °C was scanned at 1 °C min^{-1}.

2.5. pH and storage stability

The pH stability of AKPC (1.1 μM) was studied in different buffers (50 mM): Tris–HCl, pH 7.0, sodium citrate or sodium formate, pH 3.0, and sodium carbonate, pH 10.5, in the presence or absence of Ca^{2+}. The peroxidase was incubated for 24 h at 25 °C; enzyme activity was assayed at the beginning and end of this period. Stability over longer periods (1 month) was also determined using AKPC (100 nM) in 50 mM Tris–HCl, pH 7.0, or sodium acetate, pH 5.5, at 4 °C.

2.6. Effects of organic media

To determine activity in aqueous/organic solvent mixtures (ethanol, methanol, dimethylsulphoxide (DMSO) and 1,4-dioxane), 10 μL HRPC or AKPC (with 100 mM $CaCl_2$ or EDTA) were placed in 490 μL double strength ABTS assay mixture. Five hundred microliters water/organic solvent mixture (to give the desired final proportion of organic solvent) were immediately added and the activity was determined. AKPC or HRPC were also incubated for 5–30 min in different proportions of water/organic solvent, 10 μL aliquots of the enzyme solution were then taken, and activity with ABTS was assayed. One hundred percent activity was taken, in each case, to be the value obtained in the absence of organic solvent.

Stopped-flow observations of the rate of AKPC compound I formation in buffer/ethanol mixtures were made on a Pi-Star 180 spectrometer with stopped-flow unit at 25 °C (Neslab RTE-7 circulating water bath). The apparatus was operated in single mixing absorbance mode using the 1 cm path length observation cell and demonstrated a dead time of approximately 1.1 ms. The reactions of AKPC-Ca^{2+} and AKPC + EDTA with H_2O_2 were observed under pseudo first-order conditions at 398 and 405 nm, respectively [16]. The enzyme and substrate solutions were prepared in buffer/ethanol mixtures (up to 25% ethanol). Observed rate constants (k_{obs}) were obtained from the kinetic data using the Pi-Star 180s non-linear regression curve fitting program.

2.7. Inactivation by H_2O_2

AKPC was incubated at 25 °C in 50 mM Tris–HCl buffer, pH 7.0, containing 1 mM $CaCl_2$ or 1 mM EDTA (500 μL total volume). Each incubation contained enzyme (100 nM) and H_2O_2 at the concentration required to give the desired [H_2O_2]/[AKPC] ratio. When the reaction was complete (≈8 h), AKPC activity with ABTS was measured. The residual enzyme activities (A_R) (expressed as percentage) were calculated as above.

2.8. Glucose determinations

Three different assay systems for glucose were used: 0.8 mM ampyrone (4-aminoantipyrine) with 5 mM phenol or 0.8 mM ampyrone with 10 mM *p*-hydroxybenzoic acid both determined at 510 nm in 50 mM sodium phosphate buffer, pH 7.0, 2 mM ABTS in 50 mM Tris–HCl, pH 7.0, or 50 mM sodium acetate, pH 5.5, measured at 414 or 600 nm. All assays contained glucose oxidase (≈100 μg mL^{-1} final concentration). HRP or AKPC were added to a final concentration of 5–10 μg mL^{-1}. In the case of AKPC 1 mM $CaCl_2$ or EDTA were also added. The D-glucose solution was allowed to stand at room temperature for at least 4 h prior to use. Progress curves of absorbance increases were observed spectrophotometrically to determine the approximate end points of the reactions.

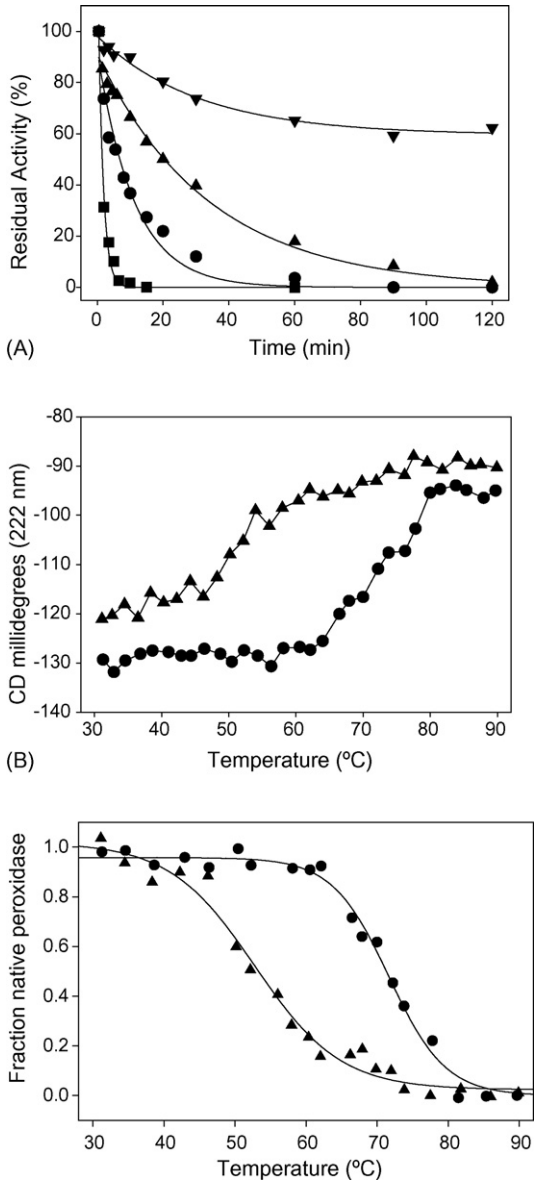

Fig. 1. Thermal inactivation of AKPC: (A) thermal inactivation curves for AKPC with EDTA (500 μM). Inactivation was measured as the enzyme activity with ABTS at 50 °C (▼), 55 °C (▲), 60 °C (●) and 70 °C (■); (B) thermal scans of AKPC with 200 μM Ca^{2+} (●) and 200 μM EDTA (▲) as depicted by CD spectroscopy at 222 nm. The temperature was raised from 30 to 90 °C at 1 °C min^{-1}; (C) thermal denaturation of AKPC with Ca^{2+} (●) and EDTA (▲), the data in (B) were replotted to show the fraction of native (folded) AKPC at each temperature.

3. Results and discussion

3.1. Thermal stability of AKPC

Several industrial applications of peroxidases (for instance wastewater treatment and phenolic resin synthesis) involve high temperatures, which increase the yields and economic viability of the processes. Thermostable peroxidases have recently been obtained from soybean [12] and African oil palm tree [13]. First-order deactivation curves (determined from the loss of activity with ABTS) of AKPC as extracted from the plant showed double exponential decreases (data not shown) in the range 60–90 °C from which the corresponding deactivation constants ($k^{fast}_{deact,obs}$ and $k^{slow}_{deact,obs}$) were calculated by non-linear regression. In order to test if this double exponential behaviour was related to the confirmed presence of two enzymatic forms (Ca^{2+}-5cHS and 6-aquo 6cHS [16]) in AKPC samples (as purified), thermal deactivation was also studied in the presence of excess Ca^{2+} (AKPC-Ca^{2+}) or EDTA (AKPC + EDTA), which, being a Ca^{2+} chelator, favours formation of Ca^{2+}-free 6-aquo 6cHS AKPC. AKPC-Ca^{2+} exhibited single exponential thermal deactivation (data not shown) with an observed rate ($k_{deact,obs}$) at each temperature of the same order as that of the slow process observed for AKPC as extracted from the plant. The addition of EDTA to AKPC had essentially the opposite effect; the enzyme was deactivated in a single process but at higher observed rates (Fig. 1A). The observed deactivation energies ($E_{a,obs}$) of AKPC-Ca^{2+} and AKPC + EDTA were calculated from Arrhenius plots (Table 1). The melting curves of AKPC with Ca^{2+} and EDTA (Fig. 1B) were determined from changes in the CD ellipticity at 222 nm giving an indication of the fraction of native (i.e. folded) AKPC present at each temperature (Fig. 1C). It was clear that the addition of Ca^{2+} had an important effect on thermal stability with the melting temperature of AKPC being approximately 52 and 72 °C with EDTA and Ca^{2+}, respectively. Despite this, at first sight, the values of $E_{a,obs}$ for AKPC-Ca^{2+} (112.6 kJ mol^{-1}) and AKPC + EDTA (150.4 kJ mol^{-1}) did not appear to indicate that the presence of Ca^{2+} resulted in higher thermal resistance. The thermal deactivation of proteins is often discussed in terms of a two-step mechanism (the Lumry–Eyring model [12,18]) in which a reversible unfolding equilibrium step (with equilibrium constant K) is followed by irreversible deactivation (with rate constant, k_{deact}), so that:

$$k_{deact,obs} = \frac{k_{deact}}{1 + K} \quad (4)$$

The value of K is the temperature dependent and can be calculated from the melting curves of the protein. However, we found little evidence that the thermal deactivation of AKPC was reversible and, in this case, a simple single step model with rate k_{deact} could be used [18]. Thus, for AKPC-Ca^{2+} the $E_{a,obs}$ takes a realistic value but the $E_{a,obs}$ for AKPC + EDTA was much higher than expected. This may be due to the presence of 5cHS and 6-aquo 6cHS forms of Ca^{2+}-free AKPC in a temperature dependent equilibrium (with constant K_{eq}). This effect was not considered in the case of AKPC-Ca^{2+} because in the presence of Ca^{2+} the enzyme has been shown previously to be present exclusively as a 5cHS species [16], thus no value of K_{eq} can be obtained. The absorption spectrum of Ca^{2+}-free AKPC was observed to change as the temperature was increased (data not shown) in a way that was consistent [16] with the loss of the 6-aquo ligand, the main difference being that the Soret absorbance peak fell significantly, red-shifted slightly and the peak broadened. However, this reaction was not fully reversible and haem loss was also observed. Nevertheless, estimates of K_{eq}, were obtained from the Soret absorbance changes (Table 1) and were applied in Eq. (4) to adjust $k_{deact,obs}$. The values of k_{deact} obtained were used to re-calculate E_a for AKPC + EDTA yielding 86.5 kJ mol^{-1}, a value more consistent with (and lower than) that calculated for AKPC-Ca^{2+}. The implications of these data for industrial applications are of some interest since they demonstrate a way of controlling peroxidase catalysis: the reaction, if performed at a suitable temperature in the presence of Ca^{2+}, can be stopped simply by adding EDTA.

Table 1
Thermal inactivation constants of AKPC with Ca^{2+} and with EDTA

	T (°C)		
	65	70	80
AKPC-Ca^{2+}			
$k_{deact,obs}$ (min^{-1})	0.041	0.073	0.223
$E_{a,obs}$ (kJ mol^{-1})[a]	112.6		
	T (°C)		
	50	55	60
AKPC + EDTA			
$k_{deact,obs}$ (min^{-1})	0.022	0.029	0.090
$E_{a,obs}$ (kJ mol^{-1})[a]	150.4		
K_{eq}	1.78	1.24	0.80
k_{deact} (min^{-1})	0.062	0.066	0.162
E_a (kJ mol^{-1})[b]	86.5		

[a] Activation energy obtained from the Arrhenius plot ($\ln k_{deact}$ against $1/T$ (K^{-1}), where the slope of the line fitted to the data = $-E_a/R$, $R = 8.314$ J K^{-1} mol^{-1}). $E_{a,obs}$ were calculated using $k_{deact,obs}$.
[b] Value calculated from Arrhenius plots of k_{deact}, obtained using Eq. (4) in main text.

3.2. Stability of AKPC in organic solvents

Several peroxidase applications offer better performance in organic solvents [7]. Peroxidase-catalyzed asymmetric sulfoxidations are an attractive procedure [19]; however, they suffer from relatively low reaction rates due to poor substrate solubility in water and from appreciable spontaneous substrate oxidation with hydrogen peroxide. Both problems can be alleviated using an organic solvent as the reaction medium [19]. As another example, HRP and milk lactoperoxidase, while inactive toward lignin in water, can vigorously de-polymerize both synthetic and natural lignins in organic media [20]. Additionally, organic solvents are often used for chemical modifications of electrodes or for the solublization of polymers in which peroxidase will be entrapped [21]. However, many organic solvents are strong proteins denaturants; therefore, we have carried out a comparative study of the effect of four organic solvents on the activity/stability of AKPC and HRPC.

Both HRPC and AKPC were essentially stable when incubated in up to 90% (v/v, with water) ethanol, methanol or 1,4-dioxane for between 5 and 30 min measuring the residual activities (in 10 μL aliquots) under aqueous conditions. On the other hand, after incubation in DMSO (60% or more) for 5 min at least 90% peroxidase activity had been lost (data not shown). These results contrasted with those in Table 2 which shows the activities with ABTS of HRPC, AKPC-Ca^{2+} and AKPC + EDTA in ethanol, methanol, DMSO and 1,4-dioxane/aqueous mixtures containing 0–50% organic solvent. It is clear that in all cases the presence of organic solvent reduced peroxidase activity and that with 50% organic only 0–5% activity remained. With the exception of DMSO the activity of AKPC-Ca^{2+} appeared to be as high as or higher than that of HRPC. It is also worth noting that AKPC + EDTA was often more active in both percentage and absolute terms than either AKPC-Ca^{2+} or HRPC although it exhibited lower activity (<50%) in control experiments in aqueous solution.

As a whole, the data show that whilst peroxidases are generally quite stable in organic solvents, they may not actually be that active towards substrates under such conditions and in some cases, such as in DMSO, can undergo irreversible inactivation. The data for AKPC + EDTA suggest that the subtle conformational differences between AKPC with and without Ca^{2+} help to maintain enzyme activity in organic solvents in the case of the Ca^{2+}-free enzyme. This effect could be due to stabilization of the active site or other structural features of the enzyme, for instance, leading to increased protein hydrophobicity. Stopped-flow experiments (data not shown) indicated that the rate of compound I formation (Eq. (1)) in ethanol/buffer mixtures fell more rapidly in the case of AKPC-Ca^{2+} (in 25% ethanol k_{+1} was approximately 65% of the value in aqueous buffer) than for AKPC + EDTA (k_{+1} was approximately 90% in 25% ethanol). It is possible that the rate constants for the ABTS oxidation steps (Eqs. (2) and (3)) are similarly affected. Therefore, although the cause of stabilization is not clear, it appears that Ca^{2+} chelation by EDTA is a potential way to maintain catalysis by AKPC in organic media. This represents a potential advantage in peroxidase-catalyzed transformations under such conditions.

Table 2
Activity with ABTS[a] of AKPC-Ca^{2+}, AKPC + EDTA and HRPC in aqueous/organic media

Solvent (%)[b]	HRP	AKPC-Ca^{2+}	AKPC + EDTA
Ethanol			
0	100	100	100
10	52.91	53.22	77.01
20	15.10	27.16	47.47
30	4.69	12.36	23.97
40	4.07	4.35	19.4
50	1.06	1.77	4.31
Methanol			
0	100	100	100
10	48.60	58.63	76.86
20	28.67	31.38	51.34
30	10.56	22.83	36.9
40	5.02	7.13	14.58
50	3.64	3.53	5.66
DMSO			
0	100	100	100
10	83.19	56.83	81.6
20	41.00	29.87	55.49
30	14.53	14.52	28.65
40	6.43	5.44	1.25
50	3.98	1.59	0
1,4-Dioxane			
0	100	100	100
10	35.71	41.26	64.69
20	9.06	13.27	33.84
30	0.33	1.70	7.28
40	0	0.69	0.61
50	0	0	0.21

[a] Enzyme activity (%) was determined by placing a 10 μL aliquot of enzyme in 490 μL assay medium (2 mM ABTS and 1 mM H_2O_2 in 50 mM Na citrate buffer, pH 4.5) and then adding 500 μL water/organic solvent mixture. The activity determined in aqueous medium was taken to be 100%.
[b] Organic solvent content (%) expressed v/v with water.

3.3. pH and storage stability of AKPC

The spectrum of AKPC (with or without Ca^{2+}) was unaltered between pH 4 and 10, but it has previously been shown that AKPC-Ca^{2+} underwent a reversible alkaline transition between pH 10 and 11 that was suppressed in the presence of EDTA [16]. Incubation of native AKPC (no added Ca^{2+} or EDTA) at pH 10.5 over 24 h showed that over 90% of the activity originally present was retained, similar results being obtained at pH 7.0. At pH 3.0 the spectrum of AKPC indicated that Ca^{2+} had been lost. In HRP, leaching of Ca^{2+} at pH 3.0 over 10–30 min has also been described and found to be dependent on the buffer anion present [22], probably as a result of different rates of Ca^{2+}–buffer complex formation. The loss of Ca^{2+} from AKPC at pH 3.0 was considerably more rapid, being complete in less than the time required to obtain a spectrum (≈1 min). No difference was detected over this time scale when sodium citrate and sodium formate buffers were used. However, despite the loss of Ca^{2+}, over a period of 24 h at pH 3.0 AKPC retained more than 75%

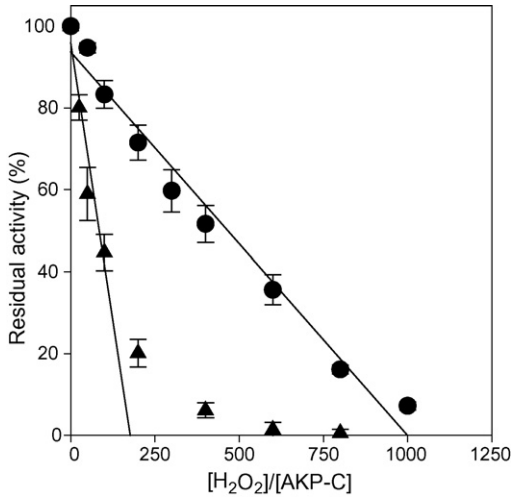

Fig. 2. AKPC catalase activity and inactivation by H_2O_2. Plots of percent residual activity with ABTS in incubations of H_2O_2 with AKPC-Ca^{2+} (●) and AKPC + EDTA (▲).

of the original activity present, albeit of the less active Ca^{2+}-free form of the enzyme. AKPC in dilute solution (100 nM) at pH 7.0 or 5.5 and 4 °C retained some 50% of its activity over 1 month independent of the presence of added Ca^{2+} or EDTA. Thus, AKPC appears to be a stable enzyme during storage in solution, this being an important consideration for its use in applications such as clinical analysis (see Section 3.5).

3.4. AKPC inactivation by hydrogen peroxide

Peroxidase can be inactivated by several mechanisms: (i) irreversible reactions between the enzyme and phenyl or phenoxy radicals formed by one-electron oxidation of phenolic substrates during the catalytic cycle [23,24]; (ii) adsorption of polymerized phenols on the peroxidase resulting in steric hindrance of substrate access to the enzyme's active site [25]; (iii) reactions between H_2O_2 or associated radical species and intermediates of the enzyme's catalytic cycle [23,26,27]. Such mechanism-based or suicide inactivation by peroxides is a characteristic aspect of the peroxidase mechanism [26,28] occurring at the level of the complex between compound I and H_2O_2 whence a partition occurs leading to either enzyme turnover or inactivation [26,29]. Turnover of the enzyme with H_2O_2 in the absence of another suitable reducing substrate (AH_2 in Eqs. (2) and (3)) is mainly due to peroxidase-catalyzed oxygen gas production (the catalase reaction) [30]. The formation of compound II and a further enzyme intermediate known as compound III in a series of reactions which generate superoxide radical anion are a minor but still influential mode of turnover [30].

Incubations in which residual peroxidase activity with ABTS (A_R) was measured at the end of reaction at known $[H_2O_2]/[AKPC]$ ratios (Fig. 2) are a simple way of determining sensitivity to suicide inactivation. Using these data the partition ratios (r, the number of turnovers with H_2O_2 before the enzyme is inactivated) were calculated by inserting the values of $[H_2O_2]/[AKPC]$ at which $A_R = 0$ into Eq. (5) [31]:

$$A_R = 1 - \frac{1}{2r+2}\frac{[H_2O_2]_0}{[AKPC]_0} \quad (5)$$

In the case of AKPC-Ca^{2+} the partition ratio (r) was 500 whereas with AKPC + EDTA it was 90. The r of AKPC-Ca^{2+} is comparable to that of HRPC [31]. Under conditions where excess H_2O_2 is likely to be present (e.g. in biosensors for peroxide) maintaining an adequate level of Ca^{2+} will be important to maximize both AKPC reactivity and lifetime. Equally, the greater sensitivity of Ca^{2+}-free AKPC could potentially be a way to purposely slow or stop a reaction by adding EDTA. The presence of reducing substrate protects peroxidase from inactivation [32] and it is often more effective to add H_2O_2 continuously at low concentrations to avoid oxidizing/reducing substrate imbalances that can promote inactivation [17].

3.5. Glucose concentration determinations

The use of peroxidase in determinations of glucose in body fluids is long established and widely used in clinical settings. Commercial kits generally link the action of glucose oxidase that generates D-gluconic acid and H_2O_2 from β-D-glucose to that of peroxidase that utilizes the H_2O_2 to oxidize a substrate yielding a coloured product. The absorbance value obtained after a fixed time is compared to the value for a standard solution containing a known glucose concentration, simplifying the method for a number of reasons. For example, experimental conditions such as exact temperature, exact incubation time, loss of enzyme activity in stock solutions during storage, the stoichiometry and extinction coefficient of the coloured product, etc., can be largely ignored. Additionally, it has to be taken into account that glucose oxidase is highly specific for β-D-glucose but the α and β anomers are in equilibrium. This being the case, the true end point of the reaction may take many hours to be reached as once the available β anomer (50% of the total D-glucose present) has been exhausted (a few minutes) the rate of reaction becomes dependent on the much slower (several hours depending on temperature and pH) conversion of α to β.

To our knowledge the peroxidase used in commercial glucose determination kits is, without exception, HRP. In order to establish the validity of AKPC as an alternative we compared the two enzymes in three assay systems (see Section 2). In all cases HRP and AKPC yielded the same values of the glucose concentration. The assay using AKPC and ABTS was shown to be linear over at least the range 5–800 mg and 100 mL^{-1} glucose in the original stock solution (Fig. 3). The presence of Ca^{2+} or of EDTA (which may be present in some blood plasma samples) did not affect the results since we observed that the rate of chromophore production was limited more by the activity of glucose oxidase than that of peroxidase. It is clear that AKPC can be used as an alternative to HRP in clinical assays of glucose concentration.

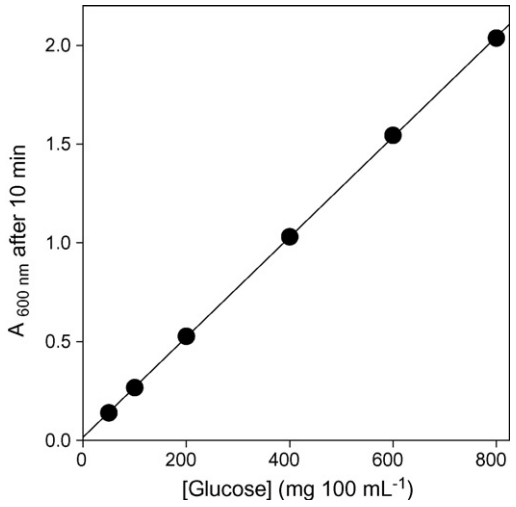

Fig. 3. Linearity of glucose concentration assay using AKPC. [Glucose] range shown 50–800 mg and 100 mL^{-1}. Determined using ABTS as chromophore, $A_{600\,nm}$ was measured after 10 min. Lower concentrations could be determined using $A_{414\,nm}$ [AKPC] ≈ 5 μg mL^{-1}, [glucose oxidase] ≈ 100 μg mL^{-1}, in 50 mM Tris–HCl buffer, pH 7.0 with 1 mM $CaCl_2$.

4. Conclusions

In this study we have examined some of the characteristics of AKPC including its activity in aqueous and organic media, thermal and pH stability, and resistance to inactivation by H_2O_2. Together with our previous work on this enzyme [15–17] we have found that AKPC is in many respects a typical class III plant peroxidase, but that in other ways it exhibits unusual characteristics that could be advantageous in certain circumstances. The Ca^{2+} concentration in particular has an important influence on both the activity and stability of AKPC and could potentially be used as a means to control the enzyme *in vitro*, tailoring to a certain extent its reaction profile to the specific requirements of a given application. We would suggest that such control could also play a role in the *in vivo* function of AKPC, although, as is the case with many peroxidases, this function still remains to be established. Therefore, AKPC, with its mixture of typical and atypical features, offers considerable scope for further research the results of which could be used to better understand peroxidase biochemistry in general.

Acknowledgements

This research was supported by grants from the Consejería de Educación y Cultura, Region de Murcia, Spain, project 07 BIO2005/01-6464, to F.G.-C. and J.N.R.-L., and the Fundación Seneca (Murcia, Spain), project 00672/PI/04 to J.T. and J.N.R.-L.L.S. has a grant from the Program Torres Quevedo, Ministerio de Educación y Cultura (Spain). S.C. and A.N.P.H. are supported by contracts with ArtBiochem S.L. (Murcia, Spain).

References

[1] H.B. Dunford, Heme Peroxidases, Wiley-VCH, New York, 1999.
[2] K.G. Welinder, Curr. Opin. Struct. Biol. 2 (1992) 388–393.
[3] J.N. Rodríguez-López, D.L. Lowe, J. Hernández-Ruiz, A.N.P. Hiner, F. García-Cánovas, R.N.F. Thorneley, J. Amer. Chem. Soc. 123 (2001) 11838–11847.
[4] M. Sivaraja, D.B. Goodin, M. Smith, B.M. Hoffman, Science 245 (1989) 738–740.
[5] A.N.P. Hiner, J.I. Martínez, M.B. Arnao, M. Acosta, D.D. Turner, E.L. Raven, J.N. Rodríguez-López, Eur. J. Biochem. 268 (2001) 3091–3098.
[6] H.B. Dunford, J.S. Stillman, Coord. Chem. Rev. 19 (1976) 187–251.
[7] S. Dordick, M.A. Marletta, A.M. Klibanov, Biotechnol. Bioeng. 30 (1987) 31–36.
[8] R.Q. Thompson, Anal. Chem. 59 (1987) 1119–1121.
[9] Z. Weng, M. Hendrickx, G. Maesmans, P. Tobback, J. Food Sci. 56 (1991) 567–570.
[10] W. Adam, M. Lazarus, C.R. Saha-Moller, O. Weichold, U. Hoch, P. Schreier, Adv. Biochem. Eng. 63 (1998) 73–108.
[11] M. Kvaratskhelia, C. Winkel, M.T. Naldrett, R.N.F. Thorneley, J. Plant. Physiol. 154 (1999) 273–282.
[12] J.P. McEldoon, J.S. Dordick, Biotechnol. Prog. 12 (1996) 555–558.
[13] I.Y. Sakharov, M.K. Vesga, I.Y. Galaev, I.V. Sakharova, O.Y. Pletjushkina, Plant Sci. 161 (2001) 853–860.
[14] I.Y. Sakharov, L. Castillo, J.C. Areza, I.Y. Galaev, Bioseparation 9 (2000) 125–132.
[15] D. López-Molina, H.A. Heering, G. Smulevich, J. Tudela, R.N.F. Thorneley, F. García-Cánovas, J.N. Rodríguez-López, J. Inorg. Biochem. 94 (2003) 243–254.
[16] A.N.P. Hiner, L. Sidrach, S. Chazarra, R. Varón, J. Tudela, F. García-Cánovas, J.N. Rodríguez-López, Biochemistry 42 (2003) 8799–8808.
[17] D. López-Molina, A.N.P. Hiner, J. Tudela, F. García-Cánovas, J.N. Rodríguez-López, Enz. Microb. Technol. 33 (2003) 738–742.
[18] D.G. Pina, A.V. Shnyrova, F. Gavilanes, A. Rodríguez, F. Leal, M.G. Roig, I.Y. Sakharov, G.G. Zhadan, E. Villar, V.L. Shnyrov, Eur. J. Biochem. 268 (2001) 120–126.
[19] L. Dai, A.M. Klibanov, Biotechnol. Bioeng. 70 (2000) 353–357.
[20] J.S. Dordick, M.A. Marletta, A.M. Klibanov, Proc. Nat. Acad. Sci. U.S.A. 83 (1986) 6255–6257.
[21] T. Ferri, A. Poscia, R. Santucci, Bioelectrochem. Bioenerg. 44 (1998) 177–181.
[22] P.J. Wright, A.M. English, J. Biol. Chem. 6 (2001) 348–358.
[23] K.J. Baynton, J.K. Bewtra, N. Biswas, K.E. Taylor, Biochim. Biophys. Acta 1206 (1994) 272–278.
[24] M.D. Aitken, P.E. Heck, Biotechnol. Prog. 14 (1998) 487–492.
[25] S. Nakamoto, N. Machida, Water Res. 26 (1992) 49–54.
[26] M.B. Arnao, M. Acosta, J.A. del Rio, R. Varón, F. García-Cánovas, Biochim. Biophys. Acta 1041 (1990) 43–47.
[27] A.N.P. Hiner, J. Hernández-Ruiz, F. García-Cánovas, A.T. Smith, M.B. Arnao, M. Acosta, Eur. J. Biochem. 234 (1995) 506–512.
[28] J.N. Rodríguez-López, J. Hernández-Ruiz, F. García-Cánovas, R.N.F. Thorneley, M. Acosta, M.B. Arnao, J. Biol. Chem. 272 (1997) 5469–5476.
[29] A.N.P. Hiner, J. Hernández-Ruiz, J.N. Rodríguez-López, M.B. Arnao, R. Varón, F. García-Cánovas, M. Acosta, J. Biol. Inorg. Chem. 6 (2001) 504–516.
[30] A.N.P. Hiner, J. Hernández-Ruiz, G.A. Williams, M.B. Arnao, F. García-Cánovas, M. Acosta, Arch. Biochem. Biophys. 392 (2001) 295–302.
[31] J. Hernández-Ruiz, M.B. Arnao, A.N.P. Hiner, F. García-Cánovas, M. Acosta, Biochem. J. 354 (2001) 107–114.
[32] M.B. Arnao, M. Acosta, J.A. del Rio, F. García-Cánovas, Biochim. Biophys. Acta 1038 (1990) 85–89.