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Spring cabbage peroxidases – Potential tool in biocatalysis and bioelectrocatalysis

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Abstract

Two fractions of peroxidase activity, cationic Px-cat and anionic Px-ani, were isolated and partially purified (143.5- and 5.49-fold, respectively) from homogenate of spring cabbage heads. Optimum pH for both fractions is 6.0; however, Px-cat is almost equally active at neutral pH (7.0) while Px-ani reveals high activity in more acidic pHs (with 60% of maximum activity at pH 3.0). Optimal temperature for both fractions was 40 °C. Px-ani possessed much higher thermal stability at 40–50 °C (60% of remaining activity after 144 h of incubation) than Px-cat. The peroxidases remained fully active during 4 weeks of storage at 4 °C. Kinetic studies revealed that Px-cat and Px-ani had lower apparent Km values for ABTS (0.0377 and 0.0625 mM) and *o*-dianisidine (0.357 and 0.286 mM) than for guaiacol (6.41 and 13.89 mM). The best substrate for Px-cat was pyrogallol and for Px-ani–*o*-dianisidine. Px-cat immobilized on polyanionic PyBA-modified carbon electrode was found to produce linear repetitive signals upon consecutive additions of hydrogen peroxide during at least 1-week period and to work effectively under buffered and non-buffered conditions. These properties were comparable with those of commercially available horseradish peroxidase. Stability of the hybrid bioelectrocatalytic film and low costs of extraction and partial purification of Px-cat make it a highly promising enzyme for practical applications, including construction of bioelectrodes.

Keywords: Spring cabbage peroxidase; Enzyme stability; Electrodes; Carbon nanotubes; Hydrogen peroxide reduction

1. Introduction

Peroxidases belong to hemoprotein enzymes catalyzing the one electron oxidation of many organic and inorganic substrates using hydrogen peroxide oxygen as a hydrogen acceptor. They are present in plant cells in soluble, ionically or covalently bound to cell wall components forms (Kevers and Gaspar, 1985). These enzymes are widely distributed in higher plants (Van Huystee and Cairns, 1982) where they are involved in various processes e.g. cell elongation by cell wall-bound peroxidase (Lee and Lin, 1995), lignification process (Cai et al., 2006) and plant defense mechanisms (Saravanakumar et al., 2007; Morkunas and Gmerek, 2007). Therefore, peroxidases has often served for many scientists as a parameter of metabolism activity during growth alterations, environmental stress conditions, rigidification of cell walls by influence on lignin formation and other processes. However, due to a variety of reactions they catalyze and a large number of isoenzymes, the functions *in vivo* of particular isozymes were not yet precisely defined.

Aside of pure cognitive values, peroxidases are known to possess some practical applications. Porphyrins and metalloporphyrin-containing enzymes (including heme peroxidases) reveal optical and electrochemical properties which enable their use in creation of reusable catalytic coatings for biosensors and other biomedical devices (Heller, 1990). They were applied in fluorescent sensors for e.g. pH, DNA, zinc, copper and other metals determination (Purello et al., 1999). Among plant peroxidases, the most studied enzymes are native or recombinant horseradish peroxidases (HRP), widely used as enzyme labels in

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immunoassay kits (Shrivastav, 2003; Tuuminen et al., 1990; Tiirola et al., 2006), for organic synthesis, biotransformation of organic compounds, bioremediation of polluted waters (Veitch, 2004) and for construction of biosensors for H_2O_2 detection (Tatsuma et al., 1998; Lindgren et al., 1999; Ferapontova et al., 2001). Roots of horseradish serve at present as the major source of commercially available peroxidase; however, the researchers still investigate for new peroxidases of elevated stability and properties suitable for different biotechnological processes. Peroxidase from leaves of royal palm tree, with extreme heat stability (a decrease of its activity was registered only at 90 °C) may serve as an example of such enzymes of unusual properties, potentially useful in industry (Sakharov et al., 2001). Moreover, some novel plant peroxidases (from tobacco and peanut) were successfully immobilized on graphite electrodes and afterwards used for measurements of current of hydrogen peroxide reduction (Lindgren et al., 2000), thus confirming the necessity of further peroxidase studies for biotechnological applications.

Spring cabbage peroxidase is a relatively poorly studied enzyme. It is known that this heterogeneous heme protein presents quite significant heat stability, optimum activity at neutral (6–8) pH values and is suitable for effective immobilization (Chmielnicka, 1966; McLellan and Robinson, 1981,1987; Wójcik et al., 1990). These properties make cabbage peroxidases highly promising for biotechnological applications, specially for constructions of stable and highly effective bioelectrocatalytic systems for oxygen reduction, with respect to e.g. potential applications in biofuel cells, as shown for other peroxidases (Bullen et al., 2006; Kulesza et al., 2007). Moreover, cabbage heads are abundant and very cheap enzymatic source. However, it still lacks more detailed study, specially concerning storage stability or optical (e.g. UV–vis absorption spectra) and electrochemical (e.g. voltammetric measurements with electrode-immobilized enzyme) properties of these oxidases. Our research presented in this article were focused on augmentation of knowledge on spring cabbage peroxidases physiological features and possibility of their practical application.

2. Results and discussion

2.1. Partial purification of spring cabbage peroxidase and polygalacturonic acid binding test

Whole peroxidase activity from the heads homogenate was precipitated with 40–75% $(NH_4)_2SO_4$ saturation. White powdery precipitate appeared during dialysis before lyophilization, suggesting that cabbage heads extract was rich in globulin proteins. The fractionation procedure separated the protein from the majority of green pigment and resulting precipitate was light brownish.

Resulting fractionated lyophilisate (Px-FL) was subjected to ion-exchange chromatography on DEAE-Sepharose column (Fig. 1). Majority of peroxidase activity passed through the column without adsorption suggesting the cationic character of the protein (Px-cat). Very small activity of peroxidase – of anionic character – was adsorbed to the carrier and eluted with 95 mM NaCl (Px-ani). Similar elution profile was observed during purification of *Metroxylon sagu* peroxidase forms (Onsa et al., 2004). Native isoenzymes separated during this procedure differed in their ionic charge (Fig. 2).

Although no adsorption of Px-cat to the anionite was observed, the specific peroxidase activity in flow-through fraction was greatly enhanced in comparison with fractionated lyophilisate (Px-FL). At this purification step, Px-cat and Px-ani fractions were purified with 61.2% and 0.1% yield, respectively and their purification coefficients were 143.5 and 5.49, respectively (Table 1).

Attempts of further purification of obtained fractions resulted in a drastic loss of enzymatic activity, especially in a case of Px-ani. However, Px-cat has been highly purified during the ion-exchange chromatography step (143.5-fold; RZ = 0.86) and after lyophilization possessed

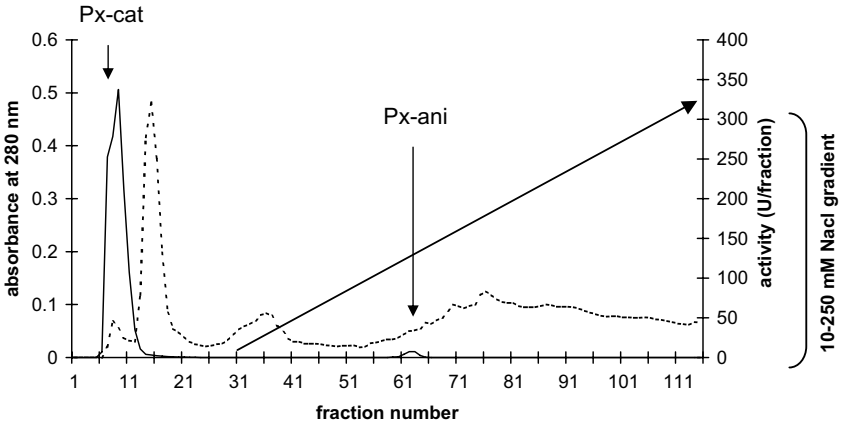

Fig. 1. Elution profile of the DEAE-Sepharose CL-6B chromatography of cabbage peroxidase 40–75% $(NH_4)_2SO_4$ liophylisate. Solid line – peroxidase activity; dotted line – absorbance at 280 nm. Peroxidase activity was measured using guaiacol as a substrate.

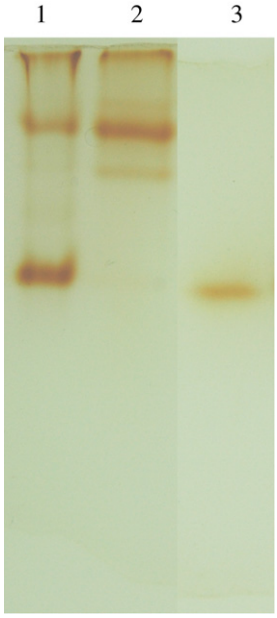

Fig. 2. Native PAGE of spring cabbage peroxidase isoenzymes: lane 1 – Px-FL (lyophilisate after 40–75% $(NH_4)_2SO_4$ crude extract fractionation); lane 2 – partially purified Px-cat; lane 3 – partially purified Px-ani. Gels were stained for peroxidase activity using *o*-dianisidine.

brownish tint suggesting that the enzyme contained ferriprotoporphyrin as a prosthetic group (Wong, 1995). In our strategy we gave priority to the yield over purification fold because we wanted to obtain highly active preparation suitable for practical application instead of homogeneous protein. Therefore, lyophilized Px-cat and Px-ani fractions were used for characterization and application study without further purification. The lyophilisates retained 100 ± 5% activity during 30 days of storage at −20 °C.

Ionically-bound peroxidases are often reported to be cationic proteins which adsorb to cationites (Moulding et al., 1989). Among these, some isoenzymes bind firmly to Ca^{2+}–polygalacturonate (Ca^{2+}–PGA), a specific labile structure found mainly in middle lamella and cell corners, as was reported for zucchini (Penel and Greppin, 1996) and horseradish (Penel et al., 1996). Ca^{2+}–polygalacturonate complex, a labile structure influenced by factors controlling growth and differentiation of plant cells, is supposed to affect the peroxidase localization within a cell and thus provide a spatial control for the function of this enzyme (Penel et al., 1999). In our binding test, Px-cat fraction was adsorbed completely to Ca^{2+}–PGA and Px-FL – at 96.7% (Table 2). Px-ani fraction was not adsorbed at all. However, treatment of obtained Px-cat–Ca^{2+}–PGA pellets with EGTA (Ca^{2+} chelator) did nor result in release of peroxidase from PGA in amount larger than 7% of total bound activity. Moreover, when Px-cat and Px-FL were mixed with PGA in absence of calcium ions, they also bound to the polysaccharide in the same proportions, as to Ca^{2+}–PGA (Table 2). In addition, when the mixture of Px-cat, PGA and Ca^{2+} was enriched with an excess of heparin, another negatively changed polyanion (25 mg heparin/1 mg PGA), the enzyme bound competitively to heparin rather than to PGA and remained in supernatant (Table 2). Therefore, Px-cat belongs to peroxidases ionically bound to cell wall components but does not possess the specific Ca^{2+}–PGA-binding properties. The results of binding experiment confirmed also the results obtained during ion-exchange chromatography – Px-cat is the major (about 97%) activity of Px-FL; Px-ani, anionic-type protein, constitutes only few percents of total peroxidase activity extracted from cabbage heads homogenate.

2.2. Optimal pH, temperature and thermal stability of cabbage peroxidase

pH is known to alter the activity of enzymes as it affects ionization state of side chains of enzymatic proteins. Optimum pH of all cabbage peroxidases was 6.0 (Fig. 3a). However, it should be noted that at pH 7.0 the activity of Px-cat fraction exceeded 97% of maximal value, while within a range of 4.0–8.0 – 60%. Similar pattern was

Table 2
Peroxidase–PGA binding test

Sample	Activity remaining in supernatant (%)		
	Px-cat	Px-ani	Px-FL
Control with Ca^{2+}	100 ± 0.3	100 ± 0.2	100 ± 0.6
Peroxidase with PGA with Ca^{2+}	0.04 ± 0.006	99.8 ± 0.4	3.4 ± 2.07
Supernatant after EGTA-pellet treatment	3.9 ± 0.03	–	6.7 ± 0.4
Peroxidase with PGA with Ca^{2+} with 25 mg heparin	102.9 ± 1,5	–	–
Control without Ca^{2+}	100 ± 0.25	100 ± 0.11	100 ± 0.25
Peroxidase with PGA without Ca^{2+}	0.07 ± 0.09	99.1 ± 0.9	4.1 ± 1.4

–, Not estimated.

Table 1
Partial purification of peroxidase isoenzymes (Px-cat and Px-ani) from spring cabbage heads

Step		Total activity (U)	Specific activity (U/mg protein)	Purification factor	Yield (%)
Crude extract		61,163	7.47	1	100
Lyophilisate after 40–75% $(NH_4)_2SO_4$ fractionation		44,034	63.7	8.5	72
DEAE-Sepharose CL-6B	Px-cat	37,470	1064.9	143.5	61.2
	Px-ani	542.8	40.98	5.49	0.9

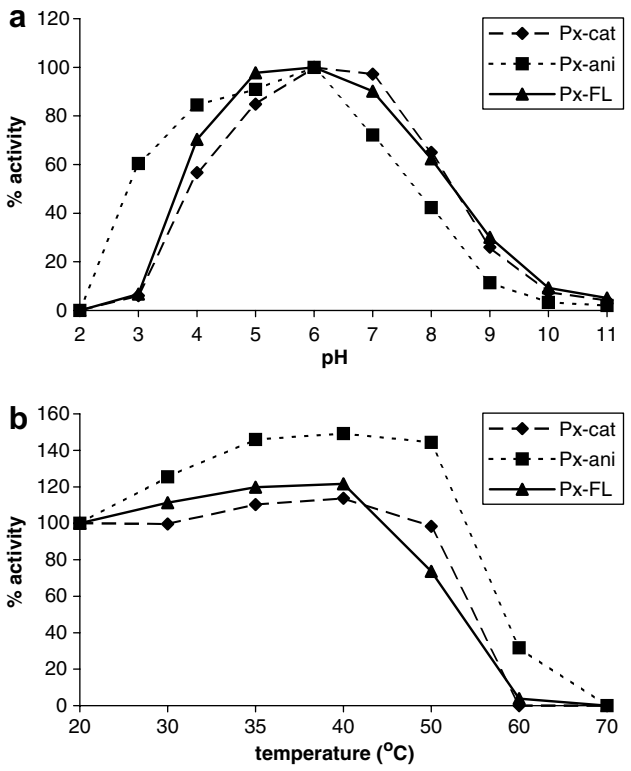

Fig. 3. Effect of pH (a) and temperature (b) on activity of peroxidase isozymes from spring cabbage. Peroxidase samples were incubated at each pH for 1 h or at each temperature for 20 min before the measurements. Peroxidase activity was measured using guaiacol as a substrate.

observed for Px-FL peroxidase (this is obvious for the high content of Px-cat activity in Px-FL lyophilisate). Therefore, Px-cat fraction is highly active within a wide range of pH with its optimum at neutral values. This is a promising property of Px-cat for all types of practical application requiring neutral pH as a best environmental parameter. Many of plant peroxidases possess the optimal pH at acidic values and relatively low activity at pH 7.0: sweet potato peroxidase (optimal pH 5.5 against guaiacol; Leon et al., 2002), isoenzymes of *Aloe barbadensis* (Esteban-Carrasco et al., 2002), from oil palm (optimal pH 5.0 against guaiacol; Deepa and Arumughan, 2002), from royal palm tree (optimal pH 5.0 against guaiacol; Sakharov et al., 2001), peroxidases from Chinese cabbage roots (optimal pH 5.0; Wang et al., 1999). Therefore, Px-cat fraction from spring cabbage with optimum activity and positive charge within pH range of 6.0–7.0 may be – in contrast to many other plant peroxidases – highly promising for practical applications e.g. for electrodes preparation via adsorption and ionic binding as immobilization techniques. Such properties of horseradish peroxidase were found to be useful in construction of an electrode for dioxygen reduction, based on cationic enzyme deposition (together with Co-protoporphyrin) on a glassy carbon substrate covered with the polyanion ($PMo_{12}O_{40}^{3-}$)-modified carbon nanotubes (Kulesza et al., 2007). The great advantage of using adsorption or ionic binding as the immobilization techniques is that usually no additional reagents are required and only a minimum of activation or clean up need be done.

Px-ani fraction was found to be more active at acidic pH than Px-cat and at pH 8.0 it retained only 42% of its maximum activity while at pH 3.0 – 60% of maximum activity. Similar phenomenon was observed for anionic POD II from *Metroxylon sagu* – it also presented 60% of maximum activity at pH 3.0 (Onsa et al., 2004).

Px-FL peroxidase was active at similar level at 20–40 °C; at higher temperatures it lost its activity with complete inactivation at 60 °C (Fig. 3b). Px-cat remained highly active up to 50 °C with a total activity loss at 60 °C. Px-ani fraction presented elevated (150% of the activity observed at 20 °C) activity up to 50 °C and even at 60 °C still remained active in 30% (Fig. 3b). The same tendency was observed when the fractions were stored at different temperatures. Px-cat and Px-FL were significantly stable during storage at 25 and 30 °C for 6 days (Fig. 4a and c). However, it was observed that Px-FL stored at 40 and 50 °C retained only 14.8% and 6.2% of activity, respectively (Fig. 4c) while Px-cat – 43.2% and 11.2%, respectively (Fig. 4a). It is therefore clear that partial purification of Px-cat fraction increased the thermal stability of the isoenzyme when compared with Px-FL. Px-ani fraction was much more stable than Px-cat – when stored at 25 and 30 °C it did not loose its activity during 6 days; when stored at 40 and 50 °C, it retained 64.9% and 60.2% of its initial activity, respectively (Fig. 4b). These results are in accordance with the data previously described for spring cabbage: cationic isoenzyme (p*I* 9.9) was reported to be readily inactivated by heat while the anionic one (p*I* 3.7) – relatively heat stable (McLellan and Robinson, 1987).

Presence of isozymes of significant heat stability is not an unusual phenomenon for plant peroxidases; it was observed for peroxidases from Chinese cabbage (Wang et al., 1999), oil palm (Deepa and Arumughan, 2002) or oranges (Clemente, 2002). Peroxidase isolated from royal palm tree was shown to be specially heat stable – partial inactivation of this enzyme was registered only at 90 °C (Sakharov et al., 2001). Some authors suggest that such a high thermal resistance may appear due to a protective carbohydrate domain in the enzymes (Triplett and Mellon, 1992) or a formation of more thermostable complexes between denatured enzyme and these particles that remain active (Vamos-Vigyazo, 1981). Also other enzymes seem to show similar properties: in our earlier study we reported that under some conditions invertase from *Candida utilis* consists of two isoenzymes: large, major and relatively heat labile and small, minor and active even at 100 °C, especially after immobilization (Belcarz et al., 2002).

When stored at 4 °C in 0.1 M phosphate buffer pH 7.0, both Px-cat and Px-ani fractions were found to be very stable. They retained 100% of initial activity during 4 weeks of storage; afterwards their activity gradually decreased (after 6 weeks they retained about 65% of their initial activities (data not shown).

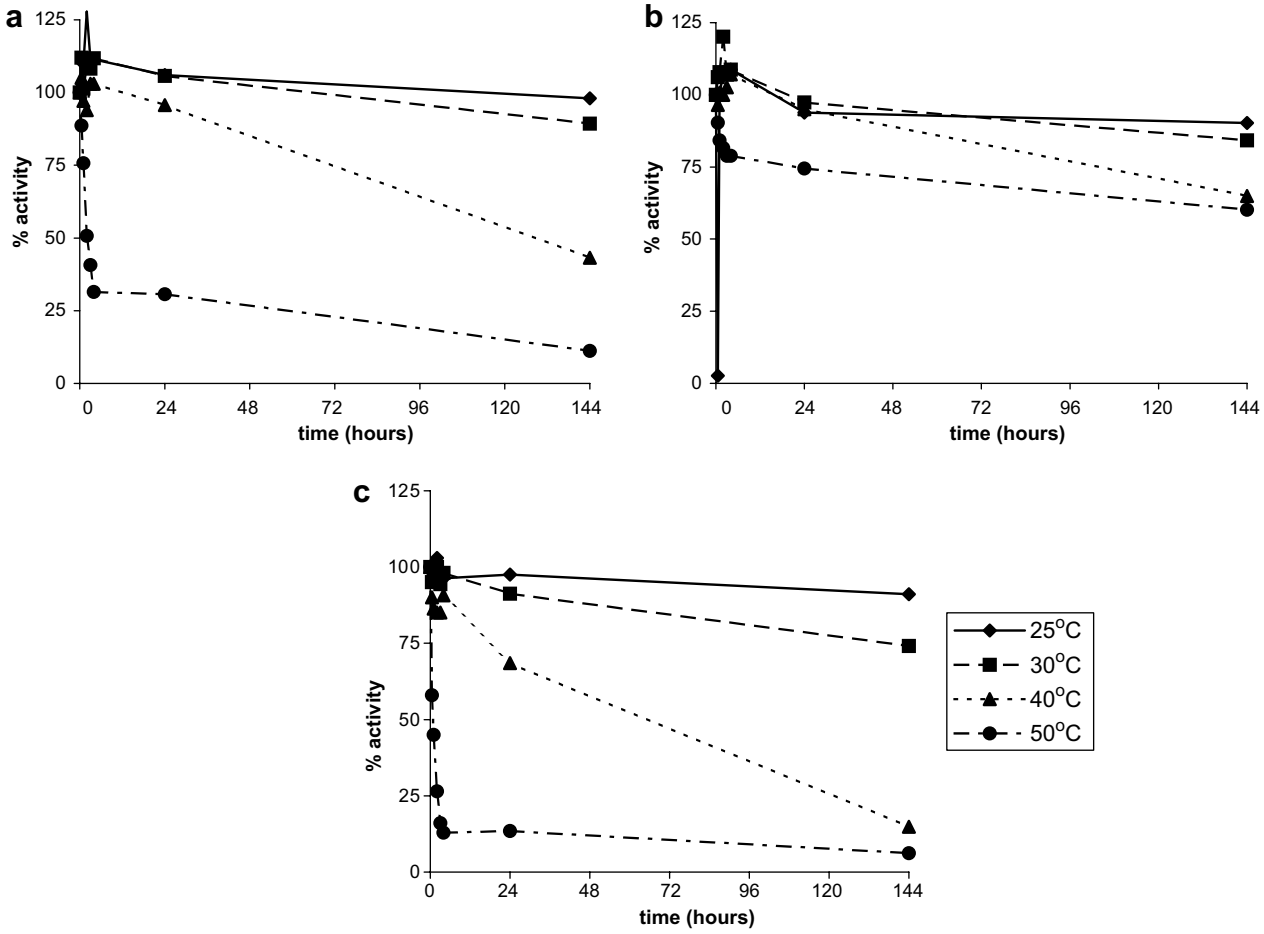

Fig. 4. Thermal stability of cabbage peroxidase (25–50 °C). (a) Px-cat; (b) Px-ani; (c) Px-FL. Peroxidase activity was measured using guaiacol as a substrate.

2.3. Substrate specificity and apparent Km values

The substrate specificity of Px-cat and Px-ani fractions has been examined using four well known peroxidases substrates: ABTS, *o*-dianisidine, pyrogallol and guaiacol. Conditions for each substrate were optimized for both fractions (Table 3). According to our results, in case of ABTS, the optimum pH was different for both fractions (for Px-ani – 3.0 and for Px-cat – 3.5). Px-cat revealed similar specific activities against ABTS and *o*-dianisidine; the best substrate for this isozyme was pyrogallol (Table 4). The best Px-ani substrate was *o*-dianisidine (14-fold better than ABTS and four-fold better that pyrogallol (Table 4). Guaiacol was found to be the less attractive substrate for both cabbage isoenzymes.

Apparent Km values for Px-cat fraction were: 0.357 mM (for *o*-dianisidine), 6.41 mM (for guaiacol), 0.0377 mM (for ABTS) and 0.121 mM (for H_2O_2 with ABTS). The same values in a case of Px-ani fraction were: 0.286 mM (for *o*-dianisidine), 13.89 mM (for guaiacol), 0.0625 mM (for ABTS) and 0.078 mM (for H_2O_2 with ABTS). Therefore, for cationic and anionic peroxidase fractions, apparent Km differs at last two-fold for each substrate. Affinity of Px-cat and Px-ani to phenolic guaiacol is quite low and the highest – for ABTS. However, the affinity of both isoenzymes against H_2O_2 is high – 10-fold higher than for e.g. *Vitis pseudereticulata* peroxidase (Liao et al., 1999), suggesting than spring cabbage peroxidases are effective in hydrogen peroxide removal from cabbage cells. Vmax for Px-cat fraction were (the value for guaiacol were taken for 100%): 100% (guaiacol), 14.2% (for ABTS) and 13.6% (for *o*-dianisidine) while for Px-ani fractions were: 100% (guaiacol), 21% (for ABTS) and 13.4% (for *o*-dianisidine).

2.4. Absorption spectra

The Px-cat fraction spectrum showed the maxima at 404 nm (Soret band) and 498 and 644 nm (visible region) which is typical for plant peroxidases (Leon et al., 2002). Px-ani fraction was not purified enough to show absorbance peaks typical for peroxidases.

Addition of H_2O_2 (0.1 mM) to ferric Px-cat (Fig. 5, insert, line a) showed the quick shift of maximum adsorption from 404 to 420 nm (Fig. 5, insert, line b). This suggested the formation of oxidative state of Px-cat. Addition of guaiacol to a produced oxidative state of

Table 3
Experimental conditions for the determination of cabbage peroxidase substrate specificity

Substrate (AH)	λ (nm)	ε (M^{-1} cm^{-1})	[AH] (mM)	[H_2O_2] (mM)	pH	Britton–Robinson buffer (mM)
ABTS	414	31,000	0.006	0.3	3.0 Px-ani 3.5 Px-cat	50
o-Dianisidine	420	30,000	0.17	9.0	6.0	100
Pyrogallol	420	2640	5.5	4.0	6.0	100
Guaiacol	470	5200	4.0	2.0	6.0	100

Table 4
Substrate specificity of Px-cat and Px-ani peroxidase

Substrate	Px-cat		Px-ani	
	Specific activity (U/mg protein)	Relative activity (%)	Specific activity (U/mg protein)	Relative activity (%)
ABTS	464.51	100	6.33	100
o-Dianisidine	468.7	100.1	89.55	1414.69
Pyrogallol	875.6	188.5	20.36	321.64
Guaiacol	66.67	14.35	1.98	31.28

Standard deviations ($n = 3$) were less than 1.8%.

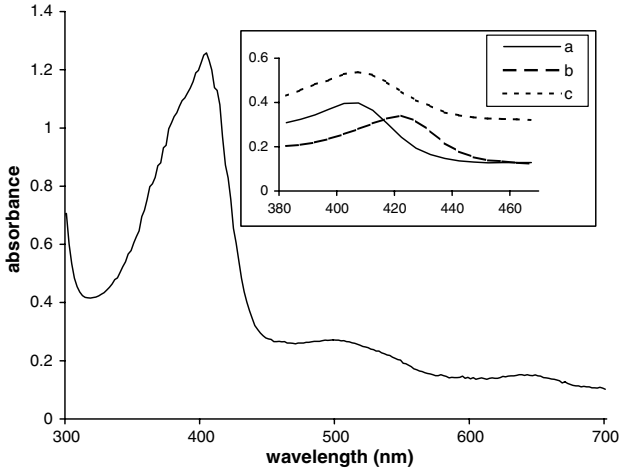

Fig. 5. Absorption spectrum of Px-cat (0.2 mg/ml). In the inset – Soret absorption spectra of: (a) native Px-cat; (b) Px-cat 30 s after addition of 0.1 mM H_2O_2; (c) Px-cat and H_2O_2 mixture 30 s after addition of 0.05 mM guaiacol.

Px-cat resulted in the shift of absorption peak to 404 nm (Fig. 5, insert, line c), suggesting the conversion back to ferric enzyme. This phenomenon is similar to that observed for HRP peroxidase (Chen et al., 2000).

2.5. Diagnostic electrochemical experiments

To comment on the electrocatalytic activity of cabbage peroxidase (in cationic form) towards reduction of hydrogen peroxide, diagnostic cyclic voltammetric experiments have been performed. The enzyme was deposited together with multi-walled carbon nanotubes (CNTs) onto the glassy carbon electrode surface. We utilize here CNTs modified with 4(pyrrole-1-yl)-benzoic acid, PyBA. Thus it is reasonable to expect that functional carboxylate groups originating from the PyBA would either form stable covalent amide bindings with amine groups from the enzyme (Koncki and Wolfbeis, 1998; Derwinska et al., 2003) or they would interact (attract electrostatically) with positively charged sites of the enzyme. The presence of CNTs within the film is likely to improve charge distribution (electron transfers) to catalytic sites (cabbage peroxidase) with the bioelectrocatalytic film.

The representative voltammetric responses in the presence and absence (background currents) of hydrogen peroxide recorded at 5 mV s^{-1} in 0.1 M KCl at are shown in Fig. 6. For comparison, the responses recorded using non purified cabbage peroxidase or conventional (commercially available) HRP are provided. All three peroxidase systems considered exhibited comparable electrocatalytic reactivities towards reduction of hydrogen peroxide at potentials lower than 0.3 V (vs. Ag/AgCl).

Because the reduction of H_2O_2 in neutral media would require involvement of protons (to produce H_2O), local alkalization of the reaction medium in the vicinity of the electrode surface may occur. Therefore, our diagnostic voltammetric experiments (recorded in the presence of H_2O_2) were repeated in the solution of controlled pH: 0.01 M citrate buffer (+0.1 M KCl) at pH 6 (Fig. 7). The resulting cyclic voltammetric responses are similar to those recorded in KCl. The observed increase of voltammetric currents during positive scans at potentials higher than 0.35 V shall be understood in terms the of system's ability not only to reduce but also to oxidize hydrogen peroxide. Some differences in voltammetric current densities and reduction potentials reflect both possible pH changes and differences in the enzymes' activity in the presences of different anions (citrates, chlorides).

Some applications, including electrochemical detection in a flow system, would require measurements to be done in the controlled potential electrolysis mode. Fig. 8 shows the amperometric responses recorded upon consecutive

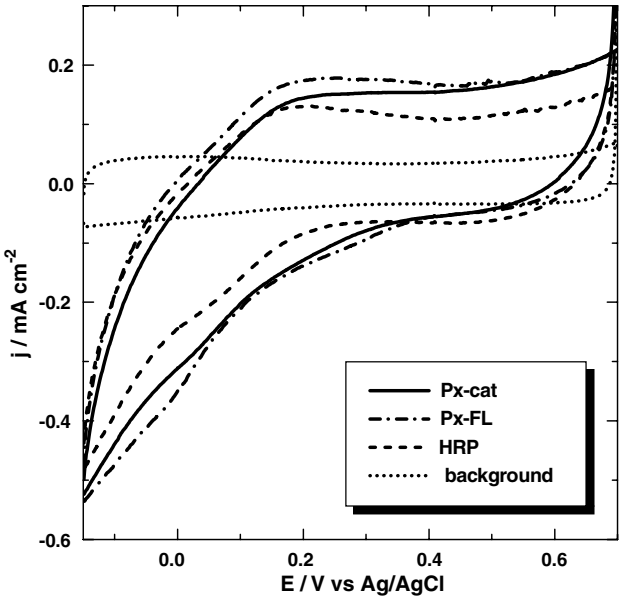

Fig. 6. Voltammetric responses of hydrogen peroxide (5 mM) recorded at glassy carbon electrode covered with purified cabbage peroxidase (Px-cat), unpurified cabbage peroxidase (Px-FL) and conventional HRP that were supported onto PyBA-modified CNTs. Electrolyte: 0.1 M KCl. Scan rate: 5 mV s^{-1}. Background current refers to Px-cat based system in the absence of H_2O_2 (following purging of argon to remove oxygen).

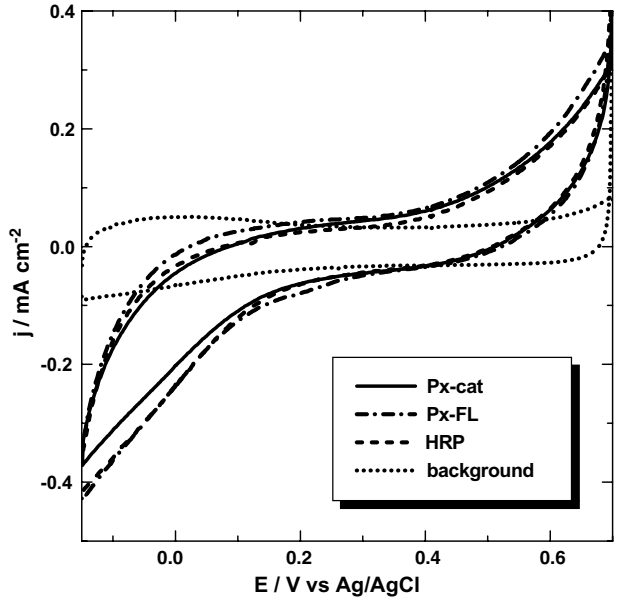

Fig. 7. Voltammetric responses of hydrogen peroxide recorded as for Fig. 6 except that 0.01 M citrate buffer (+0.1 M KCl) at pH 6 was used as supporting electrolyte.

additions of hydrogen peroxide (in time). The enzyme (cabbage Px-cat fraction) modified electrode responded very rapidly and produced steady-state signal within less than 2–3 s. A working curve is presented in the inset. At concentrations of H_2O_2 higher than 0.6 mM, some saturation effect is observed and the current responses start to increase not linearly. The hybrid bioelectrocatalytic film was very

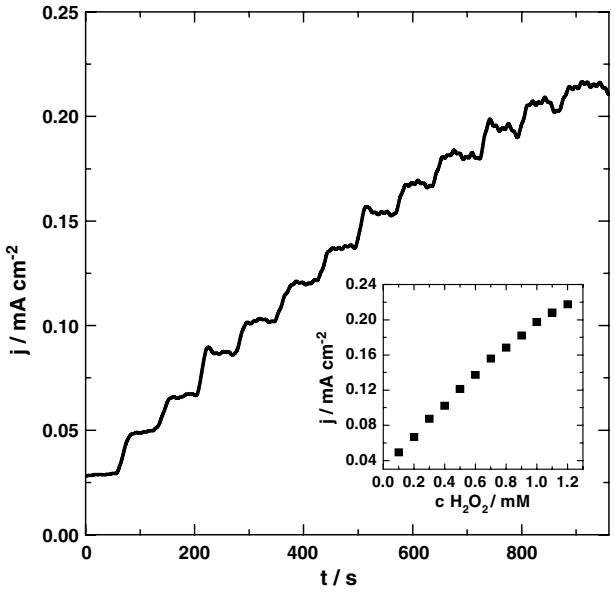

Fig. 8. Amperometric (current–time) responses recorded upon consecutive additions of hydrogen peroxide to 0.01 M citrate buffer (+0.1 M KCl) at pH 6. Potential applied: −0.1 V. Inset shows dependence of the amperometric current on the concentration of H_2O_2. Glassy carbon electrode was covered with Px-cat supported onto PyBA-modified NCTs.

stable, and there was no significant change in its catalytic activity during repetitive measurements over a period of at least a week (after which the modified surface was intentionally destroyed).

3. Concluding remarks

Spring cabbage peroxidase is a relatively poorly studied enzyme, in contrast to well-known horseradish peroxidase. However, our studies revealed that Px-cat peroxidase fraction isolated from cabbage heads possesses highly promising properties: affinity to various substrates, great stability at ambient temperatures and at 4 °C, optimum activity at neutral pH: 6–7, ability to bind to polyanionic substrates. Moreover, Px-cat immobilized on PyBA-modified carbon nanotubes is effective at H_2O_2 reduction and this bioelectrocatalytic system is stable during at least 1-week period. These properties, comparable with those of commercially available horseradish peroxidase and low costs of extraction and partial purification of Px-cat make it a highly promising biological material for practical applications, including construction of bioelectrodes.

4. Experimental

4.1. Partial purification of peroxidase

Spring cabbage (*Brassica oleracea* var. *capitata*) heads were collected from autumn (2005) harvest from a field near Lublin, Poland. The heads were homogenized in

Pro300 homogeniser (Bioeko) for 3 min at 3000 rpm, with 0.1 M phosphate buffer pH 7.0 (100 ml/1 kg leaves). Extract from head homogenate (specific activity: 7.47 U/mg of protein), containing particles of cabbage leaves, was subjected to 40–75% ammonium sulphate precipitation (each step performed overnight) at 4 °C, with subsequent exhausting dialysis (five times against 10 mM phosphate buffer pH 7.0 and finally against water) and centrifugation (twice; K6 15 Sigma centrifuge) in order to remove undesired precipitates. Resulting solution was lyophilized Freezone Freeze Dry System (both from Labconco) and stored at −20 °C as fractioned lyophilisate (Px-FL).

The lyophilisate was dissolved in SB (starting buffer: 25 mM Tris/HCl buffer pH 6.8 with 1 mM PMSF) and applied to a DEAE-Sepharose CL-6B (2.4 × 11 cm, Pharmacia Fine Chemicals) equilibrated with S buffer with 5 mM NaCl. After washing with SB with 5 mM NaCl, the bound proteins were eluted with linear 300 ml gradient of 5–250 mM of NaCl in S buffer at a flow rate 1 ml/min. Active fractions (6 ml) were desalted, concentrated at Amicon 8050 with 10 kDa ultrafiltration membranes (Millipore) and lyophilized.

4.2. Assay of in vitro peroxidase activity and substrate specificity

Peroxidase activity was assayed using guaiacol as a according to Lobarzewski et al. (1990). One unit of enzymatic activity was defined as amount of enzyme that oxidises 1 μmol min^{-1} of guaiacol under assay conditions (25 °C).

Substrate specificity of cabbage peroxidase was determined under conditions optimized for each substrate for Px-FL (Table 3). Each measurement was made in triplicate.

4.3. Electrophoretical procedures

Native PAGE of peroxidases was performed in 12% gel with discontinuous buffer system (Laemmli, 1970). Activity staining was performed at 25 °C, in 0.1 M phosphate buffer pH 7.0 with 1% *o*-dianisidine and 0.3% H_2O_2.

4.4. Determination of optimal pH, optimal temperature and thermal stability of cabbage peroxidase isozymes

Enzyme activity was determined with guaiacol in a range of pH 2.0–11.0 (2.0–3.0 in 0.1 M glycine–HCl buffer; 4.0–11.0 in freshly prepared 0.1 M Britton–Robinson (BR) buffer) at 25 °C, in triplicate, after 1-h incubation at each pH before the measurement.

Enzyme activity was determined at 20–70 °C using guaiacol as a substrate. Reaction mixtures (in 0.1 M phosphate buffer pH 7.0) were pre-incubated at each temperature for 20 min prior to activity measurement. The measurements were made in triplicate.

Thermal stability of peroxidase was investigated at 25–50 °C; with 144 h incubation period (in 0.1 M phosphate buffer pH 7.0). The samples were collected at 0.5, 1, 2, 3, 4, 24 and 144 h and subjected to standard peroxidase activity measurement. The measurements were made in triplicate.

4.5. Km values

Km values were measured at 25 °C for: ABTS (0.005–0.04 mM, 0.5 mM H_2O_2, 414 nm, 0.05 M BR buffer pH 3.0 for Px-ani/3.5 for Px-cat), *o*-dianisidine (0085–0.64 mM, 8 mM H_2O_2, 420 nm, 0.1 M BR buffer pH 6), guaiacol (3.125–25 mM, 1 mM H_2O_2, 470 nm, 0.1 M BR buffer pH 6.0), H_2O_2 (0.125–1 mM, 0.02 mM ABTS, 414 nm, 0.1 M BR buffer pH 6.0) and calculated from Lineweaver–Burke plots.

4.6. Polygalacturonic acid binding test

The test was based on experiments proposed by Penel and Greppin (1996). One milligrams of orange polygalacturonic acid (PGA; Sigma) was suspended in 2 ml of 10 mM HEPES buffer pH 7.0 with or without 2 mM $CaCl_2$ and incubated for 2 h at R/T. Then, 20 U of Px-cat, Px-ani or Px-FL were mixed with preincubated PGA suspensions and incubated for 1 h at 25 °C on a hematologic rotator (5 rpm). Controls did not contain PGA. For Px-cat activity, the additional competition test with heparin was performed (25 mg heparin (Polfa, Poland) per 1 mg PGA). After the incubation, samples were centrifuged 5 min, at 10,000 rpm (Minispin, Eppendorf) and peroxidase activity was assayed in supernatants. Pellets were resuspended in 2 ml of 10 mM HEPES buffer with 1 mM EGTA, incubated for 1 h at 25 °C on a hematologic rotator (5 rpm), centrifuged as before. Released peroxidase activity was assayed in supernatants.

4.7. Absorption spectra

Soret–vis absorption spectrum was performed with Px-cat solution (0.2 mg/ml) in 0.05 M phosphate buffer pH 7.0. The scans were performed using VISIONlite Scan program on Genesys 6 Spectrophotometer.

For compound I formation, ferric enzyme was mixed with 0.1 mM H_2O_2 and monitored at Soret band after 30 s of reaction. Resulting mixture was treated with 0.05 mM guaiacol and monitored after 30 s of reaction.

4.8. Electrochemical experiments

Electrochemical measurements were done with CH Instruments (Model CHI 660B) workstation (Austin, USA). A standard three-electrode cell was used for all experiments. The working electrode was glassy carbon of 3 mm diameter. Before modification, a glassy carbon electrode was activated by polishing with fine grade aqueous alumina slurries (grain size, 5–0.5 μm) on a polishing piece of cloth. The counter electrode was made from Pt wire. All

potentials were expressed versus the saturated (KCl) Ag/AgCl electrode. Argon was used to de-aerate solutions.

All chemicals were of analytical grade purity, and they were used as received. Multi-walled carbon nanotubes and 4-(pyrrole-1-yl) benzoic acid (PyBA) were from Aldrich. Horseradish peroxidase (HRP, EC 1.11.1.7) was from Fluka. Solutions were prepared using doubly-distilled and subsequently-deionized (Millipore Milli-Q) water. Experiments were carried out at room temperature (25 °C).

To produce PyBA protected (stabilized) carbon nanotubes (CNTs), the carbon suspension was formed by dispersing ca. 50 mg of CNTs (length, 1–10 m; outer diameter, 10–30 nm; inner diameter, 3–10 nm) in 5 ml aqueous PyBA solution (50 mg/l). The suspension was sonicated for 12 h. Subsequently, it was centrifuged, and the supernatant solution was removed and replaced with fresh PyBA solution. The centrifuging procedures were typically repeated three to four times. Later, the PyBA solution was decanted. The procedures of washing out and centrifuging were done with water and repeated at least twice. Thus, stable colloidal solution of PyBA-stabilized nanostructured carbon was obtained.

To form bioelectrocatalytic layer, first 0.5 ml of the aqueous horseradish peroxidase or cabbage peroxidase (250 U/ml) was introduced onto the glassy carbon electrode surface modified with the PyBA-covered multi-walled carbon nanotubes (fabricated by dropping the respective colloidal suspension stabilized with Nafion). This step was followed by overnight drying and rinsing with water.

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