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PURIFICATION OF PEROXIDASE ISOENZYMES FROM TURNIP ROOTS

RAGAA R. HAMED,* TAHANY M. MAHAREM,† MOHAMED M. ABDEL FATAH† and FARID SH. ATAYA

Molecular Biology Department, National Research Centre, Dokki, Cairo, Egypt; † Biochemistry Department, Faculty of Science, Ain Shams University, Abbasia, Cairo, Egypt

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Abstract—Simple reproducible procedures for purification of the main soluble (S) and ionically bound (IB) cationic peroxidase isoenzymes from turnip roots were established. The procedures included ammonium sulfate precipitation of the isoenzymes, chromatographic separation of the main isoenzymes using cellulose phosphate columns and purification to homogeneity by hydrophobic interaction chromatography on phenyl Sepharose columns. The specific activity of the phenyl Sepharose purified S and IB isoenzymes were 2760 and 896 units/mg protein with 140 and 4.8 fold increase over the crude extract and 38 and 13% recovery. The pH maxima and K_m for phenol and H_2O_2 of purified S and IB were determined.

INTRODUCTION

Peroxidases (EC 1.11.1.7) are ubiquitous and have been found in a wide range of plant species, in which it has multiple molecular forms and a broad subcellular distribution. It is found not only in cytoplasm, and cell organelles, but is also associated with cell walls [1, 2]. The isoenzyme composition of free and bound peroxidases appears to differ [3]. Cationic peroxidase may not be extracted by a low ionic strength medium, while anionic and neutral peroxidases can readily be eluted by this medium. This observation was also reported by other authors [2, 4].

Seven isoperoxidases were resolved from horseradish and from turnip roots. Horseradish peroxidases (HRPs) were designated A_1, A_2, A_3 (acidic proteins), B (neutral), C, D and E (basic proteins) [5], while turnip peroxidases (TPs) were designated TP_1, TP_2, TP_3 (acidic), TP_4 (neutral), TP_5, TP_6, TP_7 (basic proteins) [6]. A complete amino acid sequence was carried out on the horseradish peroxidase c (HRPc) [7] which was then compared with turnip peroxidase 7 [8], and cytochrome-c peroxidase from yeast [9]. A computer analysis of the amino acid sequences of HRPc, TP_7 and cytochrome-c peroxidase, together with knowledge of the crystallographic structure of cytochrome-c peroxidase [10], identified the structure of HRPc.

The only source of commercial production of peroxidase is from horseradish roots which are cultivated in relatively cool climates but not in Egypt. Economic sources of enzymes, including a limited number of plants and animals and a few species of micro-organisms, have been found. Peroxidase has been widely used clinically in the enzymatic determination of glucose, uric acid, cholesterol and triglycerides. Although sources other than horseradish are rich in peroxidases and locally available in Egypt, production of peroxidase from these sources has not been studied. The aim of this work is to establish the purification of peroxidase isoenzymes from turnip in a form suitable for preparation of enzymatic diagnostic kits.

RESULTS AND DISCUSSION

Purification of peroxidase isoenzymes from turnip roots

Two forms of peroxidase isoenzymes were extracted from turnip roots, the soluble (S) and ionically bound (IB) isoenzymes. Seven isoenzymes were separated by PAGE, three anionic and four cationic from both extracts. This pattern is in accordance with the literature [2–4]. The anionic isoenzymes represented the majority of the S extract. In contrast to the anionic isoenzymes, the cationic isoenzymes represented the majority of the IB extract.

Different reasons for the formation of peroxidase with different electrophoretic mobilities in crude extracts of plant tissues have been suggested, including interaction with phenolics and proteolytic digestion.

* Author to whom correspondence should be addressed.

The degree of glycosylation could be another reason. Although these observations could be found in some cases, the different amino acid sequences of four isoenzymes of turnip roots *B. napus* suggested the presence of at least four separate genes for peroxidase [8, 11]. Also petunia isoenzymes were found to be under the control of three separate genes [12]. In wheat, the nature of peroxidase isoenzymes is controlled by different genomes and the growing conditions have no effect in the isoenzyme pattern [13].

Purification of soluble isoenzymes

The soluble peroxidase isoenzymes (S) were prepared from 5 kg of turnip roots. The initial specific activity was 19.7 units/mg protein (Table 1). The ammonium sulfate precipitation increased the specific activity 2.1-fold. The dialysed preparation was subjected to cation exchange chromatography on cellulose phosphate column. The anionic fraction (P_1) did not exchange with the column material and the cationic fractions were eluted from the column with 0.05 (P_2), 0.1 (P_3) and 0.3 M NaCl (P_4). The specific activities of P_1, P_2, P_3, and P_4 were 101, 428, 104 and 66.7, respectively, and 48% of the total peroxidase activity was recovered in P_2.

The soluble cationic cellulose phosphate (SP_2) was subjected to hydrophobic interaction chromatography on phenyl Sepharose CL-4B. Most of the peroxidase activity was bound to the matrix and eluted by buffer lacking $(NH_4)_2SO_4$. The specific activity was increased to 2760 units/mg protein with 140-fold increase in the purification and 38.0% of the activity was recovered.

Purification of ionically bound isoenzymes

The initial specific activity of the IB isoenzymes was 187 units/mg protein (Table 2). The ammonium sulfate precipitation increased the specific activity to 322 units/mg protein. The dialysed preparation was subjected to cation exchange chromatography on cellulose phosphate column. The anionic fraction (P_1) did not exchange with the column matrix and the cationic fractions were eluted with 0.05 (P_2), 0.1 (P_3) and 0.3 (P_4) M NaCl. The specific activities of P_1, P_2, P_3 and P_4 were 450, 698, 655 and 245 units/mg protein, respectively. 20.4% of the peroxidase activity was recovered in P_4.

When the IBP_4 was subjected to chromatography on phenyl Sepharose CL-4B, the enzyme activity was detected in the unadsorbed fraction, in contrast to SP_2. The specific activity was increased to 896 units/mg protein.

The behaviours of SP_2 and IBP_4 on phenyl Sepharose CL-4B column are different. Although SP_2 is tightly bound to the matrix at 1 M ammonium sulfate and could be eluted with decreasing ammonium sulfate concentration, IBP_4 did not, indicating that the cationic isoenzymes SP_2 is much more hydrophobic than IBP_4 under the test conditions.

Effect of pH

The effect of pH on SP_2 and IBP_4 was examined between pH 4 and 9 using 0.05 M buffers of sodium acetate for pH values ranging from 4–6, phosphate buffer for pH ranging from 6.5–8 and glycine–NaOH buffer for pH ranging from 8–9. The pH optima were found to be 5.5 and 6 for SF_2 and IBP_4, respectively.

Michaelis constants (K_m)

The double reciprocal plots of the initial velocities at pH 5.6 as a function of H_2O_2 and phenol for SP_2 and IBP_4 exhibited typical Michaelian behaviour. The K_m values for H_2O_2 were found to be 2×10^{-4} and 2.5×10^{-5} while the K_m for phenol was 5×10^{-4} and 8.3×10^{-3} for SP_2 and IBP_4, respectively.

For large scale production, SP_2, but not IBP_4, was found to be economically more suitable for the following reasons:

(a) Most of the peroxidase activity was extracted by juicing (6000 units/kg turnip roots) compared with the NaCl extract (2000 unit/kg turnip roots) which means that the ionically bound isoenzymes represent 25% of the total activity.

(b) The procedures for purification of SP_2 and IBP_4 include the same steps; however the recovery of the cellulose phosphate fraction SP_2 (48%) is higher than that of IBP_4 (20.4%).

(c) The specific activity of the cellulose phosphate fraction, SP_2 is higher than that of IBP_4.

(d) Affinity of SP_2 to H_2O_2 and phenol (2×10^{-4} M

Table 1. Summary of the purification of the main soluble peroxidase isoenzyme SP_2

Sample	Activity (units)	Protein (mg)	Specific activity	Recovery (%)	Fold purification
Crude S enzyme	30,000	1520	19.7	100	—
$(NH_4)_2SO_4$	22,800	550	41.4	75.8	2.1
Cellulose phosphate SP_2 (0.05)	14,400	33.6	428	48.0	21.8
Phenyl sepharose	11,300	4.1	2760	38.0	140

Table 2. Summary of the purification of the main ionically bound peroxidase isoenzyme IBP_4

Sample	Activity (units)	Protein (mg)	Specific activity	Recovery (%)	Fold purification
Crude IB enzyme	60,000	320	187	100.0	1.0
$(NH_4)_2SO_4$	43,500	135	322	72.6	1.7
Cellulose phosphate IBP_4 (0.3)	12,300	49.9	245	20.4	1.3
Phenyl sepharose	7800	8.7	896	13.0	4.8

and 5×10^{-4} M, respectively) is comparable. IBP_4 has higher affinity to H_2O_2 (2.5×10^{-5} M) compared with phenol (8.3×10^{-3} M).

Two grades of the soluble peroxidase-namely ammonium sulfate fraction (A) and cellulose phosphate SP_2 (B) were examined for their efficiency as a component of the enzymatic diagnostic kits for determination of cholesterol and glucose in serum. The two grades were prepared in solid salt-free form by lyophilization of the dialysed fractions.

Enzymatic determination of glucose and cholesterol

The optimal peroxidase concentrations which give linear response with time and increasing glucose or cholesterol concentration was determined. Glucose and cholesterol were estimated in seven and eight serum samples using the prepared kits containing either soluble peroxidase ammonium sulfate fraction or the major cellulose phosphate fraction (SP_2). The results indicated the efficiency of both kits with respect to sensitivity, clearance of the reagents, colorless blank and the rate of color formation with efficiency of $102 \pm 3\%$ and $98.6 \pm 2.3\%$ for A and B for glucose and $98.9 \pm 7.3\%$ and $101 \pm 4\%$ for A and B for cholesterol determination.

HRP C with Rz around 1.0 was routinely used for preparation of enzymatic diagnostic kits. The enzymatic diagnostic kits for determination of glucose and cholesterol prepared using either turnip peroxidase A or B were found to be efficient compared with the diagnostic kits prepared using HRP (product of BDH) and international diagnostic kits (Boehringer). Moreover, the blank reagents (containing all the reagents except glucose or cholesterol) are clear and colorless, and the rate of color appearance due to cholesterol or glucose at 500 nm is equal to that of the reference kits. The cost of the two products was reduced mainly because of the use of locally available materials, turnip roots, ammonium sulfate and the chromatographic matrix (cellulose phosphate).

EXPERIMENTAL

Materials

Turnip roots (*Brassica rapa*), were purchased from a local market; phenyl Sepharose CL-4B from Pharmacia; cholesterol oxidase (cholesterol: oxygen oxidoreductase: EC 1.1.3.6, from *Pseudomonas fluorescens*, 36 units/mg protein); and cholesterol esterase (sterol-ester acylhydrolase: EC 3.1.1.13, from porcine pancreas, 1400 units/g protein), from Sigma; glucose oxidase (β-D-glucose: oxygen 1-oxidoreductase; EC 1.1.3.4, from *Aspergillus niger* grade I); cholesterol determination kit from Boehringer; and horseradish peroxidase (donor: H_2O_2, oxidoreductase; EC 1.11.1.7, from horseradish, 820 EU/mg) from BDH. Cellulose phosphate was prepared according to the method of [14]. Other general chemicals were of the highest purity commercially available.

Enzyme extraction

Unless otherwise stated, all purification procedures were carried out at 4–7°. Turnip roots were cut into cubes and juiced. The homogenate was filtered and the clear filtrate was designated crude soluble enzyme (S). The cell debris was washed with 0.05 M NH_4–P_i buffer, pH 6.8 and filtered to exclude any soluble enzyme traces. The washed cell debris was soaked in 0.1 M NH_4–P_i buffer, pH 6.8 containing 2 M NaCl for 24 h and filtered. The filtrate was designated crude ionically bound (IB) enzyme.

$(NH_4)_2SO_4$ precipitation

The extracted frs (S and IB) were precipitated by solid $(NH_4)_2SO_4$ up to 75% satn. After standing overnight, the ppt was collected by centrifugation on Beckman C-21 cooling centrifuge at 11,000 *g* for 15 min, redissolved in a minimal vol. of 0.05 M NaOAc buffer, pH 5.6 and dialysed against 0.02 M NaOAc buffer for 24 h. The ppt was removed by centrifugation at 11,000 *g* for 15 min.

Chromatography of peroxidase isoenzymes on cellulose phosphate column

The dialysed frs were applied on cellulose phosphate column previously equilibrated with 0.02 M NaOAc buffer, pH 5.6. After sample application, the column was washed by the equilibration buffer (EB) and the adsorbed proteins were eluted by stepwise NaCl gradient ranging from 0.05 M–0.5 M NaCl in

the same buffer. The fr. vol. was 5 ml and the absorbency of each fraction was read at 280 nm for protein detection. Fractions containing enzyme activity were pooled and designated P_1, P_2, P_3, and P_4 according to their elution order.

Chromatography on phenyl Sepharose CL-4B column

The enzyme sample in 0.05 M NaP_i buffer, pH 6.8 containing 1 M $(NH_4)_2SO_4$ was applied onto deaerated pre-swollen phenyl Sepharose CL-4B column, previously equilibrated with the same sample buffer and $(NH_4)_2SO_4$ (EB). The proteins bound to the hydrophobic resin were eluted by a stepwise decrease in $(NH_4)_2SO_4$ concn (0.8 EB, 0.6 EB, 0.2 EB and NaP_i buffer) followed by 50% ethylene glycol in H_2O. The fractions were collected in 2 ml vol. at a flow rate of 30 ml/h and A at 280 nm was recorded. The peroxidase activity was assayed and fractions containing activity were pooled.

Enzyme assay

Peroxidase activity using phenol and aminoantipyrine was carried out in a reaction mixture containing in 1 ml vol. 0.5 μmol H_2O_2, 10 μmol phenol, 0.4 μmol aminoantipyrine, 50 μmol NaOAc buffer, pH 5.6 and peroxidase concn which gave linear response over a period of 5 min. The change in A at 505 nm was followed at 1 min interval [14]. One unit of peroxidase activity was defined as the amount of enzyme which causes an increase of one A unit per min, at 25° under the assay conditions and sp. act. as units/mg protein.

Protein determination. Protein was determined by Coomassie brilliant blue G-250 [15] using BSA as a standard.

Enzymatic determination of glucose. Glucose was determined enzymatically using glucose oxidase-peroxidase in assay reaction mixture contained in 1 ml vol. 0.15 M NaP_i buffer, pH 7, 10 mM phenol, 0.4 mM aminoantipyrine, 26 units glucose oxidase (sp. act. 290 units/mg protein) and 0.62 unit of turnip peroxidase (sp. act. 28 units/mg protein). Horseradish peroxidase from BDH company was used as standard. The reaction was started by the addition of 10 μl of standard glucose. The change in A was measured at 505 nm after 10 min incubation at 37° against a blank containing 10 μl H_2O instead of glucose [16].

Enzymatic determination of cholesterol. Cholesterol was determined enzymatically using cholesterol oxidase, cholesterol esterase in an assay reaction mixture contained in 1 ml vol., 0.1 mmol tris-HCl buffer, pH 7.7, 15 μmol phenol, 0.5 μmol aminoantipyrine, >0.2 unit cholesterol oxidase, >0.125 unit cholesterol esterase, and 1 unit of turnip peroxidase [16]. Boehringer kit was used as reference for cholesterol determination. The reaction was started by the addition of 10 μl of cholesterol (Boehringer). The change in A was measured at 500 nm after 10 min at room temp. against a blank containing 10 μl H_2O instead of cholesterol.

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