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Full Length Research Paper

Comparative study of peroxidase purification from apple and orange seeds

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This paper reports the isolation and purification of peroxidase from low cost material; moreover, no significant work has been done on the isolation and purification of peroxidase from such cost effective sources (apple and orange seeds). Peroxidases had attracted considerable interest in recent years because of their activities towards a wide variety of chromogenic substances. Peroxidase activity in crude extract of apple and orange seeds was measured by recording a spectrophotometric value. Partial purification of crude enzyme extract was done by ammonium sulfate precipitation and ion exchange chromatography. It was observed that after partial purification, the enzyme activity was increased as compared to crude enzyme extract. Peroxidase from orange seed was purified up to 17.17 fold with specific activity of 10.17 U/mg and that from apple seed was 6.82 fold with specific activity of 7.53 U/mg after diethyl amino ethyl (DEAE) cellulose chromatography. It was shown that orange seed peroxidase had more activity than apple seed peroxidase in crude extract and each step of purification. Further purification was obtained through gel filtration chromatography by using sephadex-G-75 column. Peroxidase from orange and apple seeds got purified up to 30.64 and 8.34 fold with their specific activity of 18.16 and 9.20 U/mg, respectively. It is more evident that peroxidase is the most heat stable enzyme; therefore, it is concluded that it may be potentially useful for industrial purposes.

Key words: Apple and orange seeds, extraction, peroxidase, purification.

INTRODUCTION

Peroxidase is a ubiquitous enzyme which belongs to the oxidoreductase class of enzyme and generally catalyze a reaction between H_2O_2 as electron acceptor and many kinds of substrates by means of O_2 liberation from H_2O_2 (Brill, 1971). Peroxidases are divided into three classes which differ in molecular weight and in absorption spectra including: (i) Ferriprotoporphyrin peroxidases which are brown in nature and their main sources are plants, animals and microorganisms, (ii) Verdoperoxidases which are green in nature and are gotten from animals and (iii) flavoprotein peroxidases having FAD as prosthetic group and found in animals and microorganisms (Burnette, 1977). Plants are the rich sources of peroxidases and primarily found in roots and sprouts of higher plants. The rich plant sources of peroxidases are potato tuber, horse radish and beet, while it is also present in higher plants like turnip, sweet potato, tomato, sour lime, soybean, carrot, wheat, pears, apricot, bananas, date and sap of fig tree (Reed, 1975).

The production of peroxidase from microorganisms has also attracted research interest (Tsujimura et al., 1994; Yao et al., 1995), and *Coprinus cinereus* (Morita et al., 1988) and *Coprinus macrorhizus* (Kjalke et al., 1992) are known to produce peroxidase. Since these basidiomycetes are known to be sensitive to shear stress (Tsujimura et al., 1994), the production in a stirred tank fermentor is not suitable. Peroxidase is widely used in the health sciences, food industry and for diagnostic

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Abbreviations: IAA, Indoleacetic acid; **DEAE,** diethyl amino ethyl.

purposes (Kawak et al., 1995). Chitinase and peroxidase which were isolated from soybean were considered to be involved in the defense of plant against pathogens (Staehelin et al., 1992). Being the most heat resistant enzyme, it is used in food industry as an index of blanching procedures (Reed, 1975). There is an empirical relationship between residual peroxidase activity and the development of flavors and odors in foods (Burnette, 1977). The enzyme peroxidase has acquired popularity with plant scientists partly because its activity is inversely related to growth rate, perhaps as a consequence of the enzyme's indoleacetic acid (IAA) oxidase activity, and partly because its "isozymes" vary with the developmental state of the tissue studied (Van and Cairns, 2008). This paper reports the novel results of a study carried out to isolate peroxidase enzyme from apple and orange seeds extracts as the sources mentioned will explore a new idea about such sources of peroxidase.

MATERIALS AND METHODS

Enzyme extraction

Peroxidase was extracted from apple and orange seeds by the method of Geng et al. (2001) with minor modifications. Apple and orange seeds were separated from fruits, dried and soaked in 200 ml of 0.1 M phosphate buffer of pH 6.0 over night and thoroughly homogenized by blending for 15 to 20 min. The contents were centrifuged at 10,000 g for 15 min to remove cell debris. The supernatant was removed carefully from the sediments and filtered through Whatman No 1 filter paper to get more clarity of the crude enzyme extracted.

Thermal treatment

To selectively inactivate the contaminating traces of the catalase moieties, crude enzyme extract was heated at 65°C for 3 min in a water bath and cooled promptly by placing it in ice bucket for 30 min (Wang et al., 1999). After thermal inactivation, the final extract was preserved at 4°C until further use.

Enzyme activity and protein concentration

The activity of the enzyme was determined using a UV–Vis spectrophotometer at the wavelength of 470 nm according to the method of Rad et al. (2007) with minor modifications. A mixture of pyrocatechol (170 mM) and aniline (2.5 mM) was prepared in 0.2 M phosphate buffer solution of pH 6.5. To each blank and sample cuvette, 500 µl of the earlier mentioned mixture solution and 500 µl of hydrogen peroxide (35%) was pipette and incubated at 25°C for 3 to 4 min. Then, 50 µl of crude enzyme extract and 50 µl phosphate buffer solution was added to the sample and blank cuvettes, respectively. Increase in absorbance was recorded for 4 to 5 min interval. Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Partial purification and dialysis of peroxidase

Ammonium sulfate was added to the crude enzyme extract until it was 50% saturated and kept for 4 to 6 h at 4°C. The resulting precipitate was collected by centrifugation at 10,000 g for 15 min at 4°C. The pellet of precipitated proteins was discarded. In the supernatant, more crystals of ammonium sulfate were added to attain 85% saturation at 0°C. It was again kept for 4 to 6 h at 4°C and centrifuged as described previously. After centrifugation, the supernatant was kept separate and sediments were dissolved in small amount of buffer in which the enzyme was originally extracted. The solution was kept in a dialysis bag after sealing securely, and dialyzed against distilled water for a few hours with 4 regular change of the water after every 6 h.

Table 1. Peroxidase activities of apple and orange seeds extracts.

Sample	Peroxidase activity (U/ml)	
	Apple seed	Orange seed
Blended seeds	1.39	3.39
Sediments	0.81	0.76
Supernatant (crude)	3.90	4.20

Ion exchange chromatography

Further purification of partially purified peroxidase was carried out by ion exchange chromatography using diethyl amino ethyl (DEAE) cellulose column (Cooper, 1977). The column was packed to the height of 25 cm in a glass column with an internal diameter of 2.0 cm and equilibrated with phosphate buffer (pH 6.5) for 24 h. A total of 50 fractions of 2 ml each were collected at constant drop rate and both the enzyme activity as well as the protein content was determined for each separate fraction, as mentioned in the previous section.

Gel filtration chromatography

Purified peroxidase enzyme (0.5 ml) obtained from ion exchange chromatography was subjected to gel filtration chromatography using sephadex-G-75 column. The column was packed to the height of 16 cm in a glass column with an internal diameter of 1.5 cm. Sample was poured on top of the column and eluted with phosphate buffer of pH 6.5. A total of 50 fractions of 2 ml each were collected at constant drop rate and both the enzyme activity as well as the protein content was determined for each separate fraction, as mentioned in the previous section.

RESULTS AND DISCUSSION

Crude peroxidase was extracted from apple and orange seeds using distilled water as solvent at room temperature by blending the seeds for 15 min with short intervals and centrifuged at 10,000 g for 15 min at 4°C to remove particulate matter and any intact nuclei from solution. Peroxidase values obtained after enzyme assay of blended seeds, supernatant (crude enzyme) and sediments of orange and apple seeds are shown in Table 1. The intensity of the characteristic brown color produced during reaction expressed the amount of enzyme and the magnitude of enzyme reaction in the crude extract. Tohit (2000) investigated peroxidase enzyme activities during rooting in cuttings, for enzyme

Table 2. Peroxidase activities of apple and orange seeds extracts after ammonium sulfate precipitation.

Saturation with $(NH_4)_2SO_4$	Peroxidase activity (U/ml)	
	Apple seed	Orange seed
50% sediments	3.81	1.20
50% supernatant	1.77	1.71
85% supernatant	4.21	0.65
85% sediments	3.05	4.71
After dialysis	5.09	5.43

assay using guaiacol. Moreover, the amount of H_2O_2 required to convert the whole enzyme in new compound was equivalent to exactly one molecule of H_2O_2 for each atom of peroxidase iron (Valdir, 2002).

Partial purification of crude extract

For the partial purification of peroxidase, crude extract was precipitated by using solid ammonium sulfate that was added gradually to the extract until they were 85% saturated. During precipitation of peroxidase by ammonium sulfate, the absorbance values of 50% sediments, 50% supernatant, 85% sediments and 85% supernatant recorded in Table 2 showed that the sediments of 85% saturation were the richest source of peroxidase. So, this rich form of peroxidase was subjected to dialysis to remove an extra salt and dialyzed against distilled water for several times. Bruemmer et al. (1976) partially purified the peroxidase from fresh orange juices by using ammonium sulfate precipitation which increases activities to different fold in each variety. Kermasha and Metche, (1988) purifid apple seed peroxidase with solid ammonium sulfate which increased its activity by a factor of 3. Civello et al. (1995) also used ammonium sulfate precipitation method for the partial purification of peroxidase from strawberry fruit. The purification factor of peroxidase was found to be 2.37. From the crude extract of apple seeds, a major peroxidase isoenzyme was purified by ammonium sulfate precipitation which gave 12.8% increase in total yield (Hui et al., 2006). While in this present study, after ammonium sulfate precipitation, 1.16 and 4.14 fold purification was achieved in apple and orange seeds, respectively. Dogan et al. (2007) also purified the peroxidase enzyme from different saliva species by using ammonium sulfate. Mohamed et al. (2008) reported the peroxidase activities from citrus species and cultivators and purified peroxidase by ammonium sulfate precipitation.

Ion exchange chromatography

After ammonium sulfate precipitation, the sample was passed through DEAE cellulose column and was eluted with phosphate buffer of pH 6.5. Up to 50 fractions of 2 ml each were subjected to protein estimation and enzyme assay and 6.82 and 17.17 fold purification with 42.89 and 45.64% recovery was achieved for apple and orange seed peroxidase, respectively (Tables 3 and 4). According to Civello et al. (1995), strawberry peroxidase showed 0.95 fold increases in the specific activity of peroxidase and then degree of purification in strawberry fruit was 34.87, after DEAE cellulose chromatography. Chilaka et al. (2002) used DEAE-cellulose ion exchange chromatography for the purification of peroxidase and polyphenol oxidase from white yam (*Dioscorea rotundata*). Soluble peroxidase was extracted from oranges (*Citrus sinenses* (L.) Osbeck) by Clemente (2004) who used DEAE cellulose chromatography for the partial purification of peroxidase which increased its specific activity up to 1.76 fold. Opstal et al. (2006) and Mohamed et al. (2008) also used ion exchange chromatography by DEAE cellulose in their research studies.

Gel filtration chromatography

After ion exchange chromatography, fractions having maximum activity were loaded on sephadex-G-75 column. After gel filtration chromatography, 8.34 and 30.64 fold purification with 39.99 and 33.70% recovery was achieved for apple and orange seed peroxidase, respectively (Tables 3 and 4). An increase in specific activity was observed after gel filtration chromatography by Moulding et al. (1988) who isolated four peroxidase isoenzymes from extracts of orange seed using gel filtration and ion-exchange chromatography. Three peroxidase isoenzymes were isolated from extracts of apple pulp using gel filtration and ion-exchange chromatography (Moulding et al., 1988). Clemente (2004) reported that small amounts of purified anionic and cationic isoperoxidases have been obtained by gel filtration and ion-exchange chromatography.

Conclusion

In the present study, apple and orange seeds were used as low-cost residues for peroxidase extraction. Moreover, no significant work has been done on the isolation and purification of peroxidase from such cost effective sources (apple and orange seeds). After purification, the peroxidase activity of orange and apple seed extracts were increased as compared to crude enzyme extract up to 30.64 and 8.34 fold with their specific activity of 18.16 and 9.20 U/mg, respectively. It was shown that orange seed extract gave more activity of peroxidase than apple seed peroxidase; hence, the present study showed that orange seed is a rich natural source of peroxidase as compared to apple seeds. However, the suitability of peroxidase for biotechnological applications can be investigated through its kinetic characterization.

Table 3. Purification summary of peroxidase extracted from apple seeds.

Purification Step	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude extract	3.91	3.54	1.10	1	100
$(NH_4)_2SO_4$ Precipitation	5.09	3.90	1.28	1.16	76.84
DEAE cellulose	9.11	1.21	7.53	6.82	42.89
Sephadex-G-75	9.77	1.06	9.20	8.34	39.99

Table 4. Purification summary of peroxidase extracted from orange seeds.

Purification step	Activity (U/ml)	Protein (mg/ml)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude extract	4.20	7.08	0.59	1	100
$(NH_4)_2SO_4$ Precipitation	5.43	2.21	2.45	4.14	77.34
DEAE Cellulose	9.20	0.90	10.17	17.17	45.64
Sephadex-G-75	12.46	0.69	18.16	30.64	33.70

REFERENCES

Brill AS (1971). Peroxidase and Catalase. In: Florkin and Stotz, Editors, (3rd ed.), Comp. Biochem. 14: 447-479.

Bruemmer JH, Roe B, Bowen ER (1976). Peroxidase Reaction and Orange Juice Quality. J. Food Sci. 41: 186-189.

Burnette FS (1977). Peroxidases and its relationship to food flavour and quality: A review. J. Food Sci. 42(1): 1-6.

Chilaka FC, Eze S, Anyadiegwu C, Uvere PO (2002). Browning in processed yams: peroxidase or polyphenol oxidase. J. Sci. Food Agric. 82: 899-903

Civello PM, Arting GA, Chaves AR, Anan MC (1995). Peroxidase from strawberry fruite by partial purification and determination of some properties. J. Agri. Food Chem. 43(10): 2596-2601.

Cooper TG (1977). Tools of biochemistry, university of Pittsbrough. New York, Chickester, Brisbance Sinapore, pp. 50-54.

Dogan S, Turan P, Doğan M, Arslan O, Alkan M (2007). Variations of peroxidase activity among Salvia species. J. Food Eng. 79: 375-382.

Geng Z, Rao J, Bassi AS, Gijzen M, Krishnamoorthy N (2001). Investigation of biocatalytic properties of soyabean seed hull peroxidase. Catal. Today, 64: 233.

Hui LY, Jian HX, Wen YL, Guo QL (2006). Identification, purification and characterization of peroxidase from apple seed. Enzyme Microb. Technol. 40: 354-361.

Kawak SS, Kim SK, Lee MS, Jung KH, Park IH, Liu JR (1995). Acidic peroxidases from suspension culture of sweet potato. Pytochemistry, 39(5): 981-984.

Kermasha S, Metche M (1988). Studies on apple Seed Peroxidase. J. Food Sci. 53: 247-247.

Kjalke M, Anderson MB, Schneider P, Christensen B, Schulein M, Welinder KG (1992). Comparison of structure and activities of peroxidases from Coprinus cinereus, Coprinus macrorhizus and Arthromyces ramosus. Biochim. Biophys. Acta. 1120: 248-256.

Mohamed SA, El-Badry MO, Drees EA, Fahmy AS (2008). Properties of a Cationic Peroxidase from citrus jambhiri cv. Adalia. Appl. Biochem. Biotechnol. 150: 127-137.

Morita Y, Yamashita H, Mikami B, Iwamoto H, Aibara S, Terada M, Minami J (1988). Purification, crystallization and characterization of peroxidase from *Coprinus cinereus*. J. Biochem. 103: 693-699.

Moulding PH, Singleton DE, McLellan KM, Robinson DS (1988). Purification and heat stability of Cox's apple pulp peroxidase isoenzymes. Int. J. Food Sci. Tech. 23: 343-351.

Opstal V, Bagamboula CF, Theys T, Vanmuysen SCM, Michiels CW (2006). Inactivation of Escherichia coli and Shigella in acidic fruit and vegetable juices by peroxidase systems. J. Appl. Microb. 10: 242-250.

Rad AM, Ghourchian H, Moosavi-Movahedi AA, Hong J, Nazari K (2007). Spectrophotometric assay for horseradish peroxidase activity based on pyrocatechol–aniline coupling hydrogen donor. Anal. Biochem. 362: 38-43.

Reed G (1975). Oxidoreductase. In enzymes in food processing. Acadmixc press, New York, p. 216.

Staehelin CM, Mellor RB, Weiemken A, Boller T (1992). Chitinase and peroxidase in the effective (fix+) and ineffective (fix-) soybean nodules. Planta, 187(3): 295-300.

Tohit G (2000). Peroxidase and IAA-oxidase activities during rooting in cuttings of three poplar species. Turk. J. Bot. 24: 97-101.

Tsujimura H, Takaya M, Katano K, Matsumoto N, Park YS, Okabe M (1994). Scaleup of peroxidase production by rthromyces ramosus based on analysis of fluid velocity distribution. J. Ferment. Bioeng.77: 650-654.

Clemente E, Valderrama P (2004). Isolation and thermostability of peroxidase isoenzymes from apple cultivars Gala and Fuji. Food Chem. 87: 601-606.

Valdir AN (2002). Ionically Bound Peroxidase from apple Fruit. J. Bra. Arch. Bio. Tech. 45: 7-16.

Van Huystee RB, Cairns WL (2008). Appraisal of studies on induction of peroxidase and associated porphyrin metabolism. J. Bot. Rev. 429-446.

Wang MH, Lee SY, Rhee HI (1999). Properties of anionic peroxidases purified from Chinese cabbage roots. Plant Physiol. Biochem. 37: 459.

Yao PX, Matsumoto N, Tsujimura H, Takaya M, Toda K (1995). Enhanced production of peroxidase in continuous culture of *Arthoromyces ramosus* by selective bleeding of mycelium. J. Ferment. Bioeng. 79: 307-312.