JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

PURIFICATION AND CHARACTERIZATION OF HIGH POTENTIAL TYROSINASE FROM MACROFUNGI AND ITS APPLIANCE IN FOOD ENGINEERING

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doi: 10.15414/jmbfs.2015/16.5.3.203-206

ARTICLE INFO

Received 10. 10. 2013
Revised 14. 7. 2014
Accepted 14. 8. 2015
Published 1. 12. 2015

Regular article

OPEN ACCESS

ABSTRACT

Tyrosinase (EC1.14.18.1) was extracted from oyster mushroom, *Pleurotus ostreatus*, using 100 mM potassium phosphate buffer (pH 5.8) containing 1 mM of ethylenediaminetetra acetic acid. The enzyme was purified by ammonium sulfate precipitation, followed by Sephadex G-100 and diethylaminoethyl chromatography. The purified enzyme showed a specific activity of 46.4 U/mg with 20.3 % yield. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis showed single peptide chain with a molecular weight of 75 kDa. The enzyme has optimum activity on pH 6.0 at 35˚ C. The kinetics parameter with L-DOPA (3,4-dihydroxy-L-phenylalanine), with a K_M of 0.119 mM and V_{max} of 2.97 mM. Thus, purified tyrosinase from *P.ostreatus* showed similarities with other tyrosinase sources. The results indicate that ***P.ostreatus*** can be a novel and better source of tyrosinase extraction due to its higher specific activity. The information offered here should help food industry in developing and using potential tyrosinase desirable efficacy and safety, and for improving food quality.

Keywords: *Pleurotus ostreatus*, Tyrosinase, Purification, Specific activity, Charaterization, Crosslinking enzyme, Extraction

INTRODUCTION

Food industries are in search for new biopolymers with special properties, as emulsifying, thickening agents, with low-calorie and low-fat foods. The uses of cross-linking enzyme represent the novel approach to improvement of the structure and texture of food by increasing the number of covalent bonds between its polymeric components, i.e. carbohydrates or proteins. Cross-linking enzyme such as tyrosinase, have been investigated in cereal, dairy, meat and fish processing **(Buchert *et al.*, 2010; Zaidi *et al.*, 2013; Zaidi *et al.*, 2014 a,b)**. The modification of food proteins via crosslinking affects not only the texture of food but also their digestibility **(Monogioudi *et al.*, 2011).** Cross-linking has also been reported to decrease the allergenicity of certain proteins (**Stanic *et al.*, 2010; Tantoush *et al.*, 2011)**.

Different enzymes are used as hydrolyzing food biopolymers to improve product characteristics. Tyrosinase (EC1.14.18.1) is a oxidoreductase, catecholase, diphenol oxidase with bifunctional copper containing enzyme which performs a variety of functions ranging from pigmentation, defense to sclerotization and it has potential for use for application in the construction of a biosensor for detection of benzoate in food and beverages **(Lee 1997; Ali et al 2011;Vania *et al.*, 2013)**. Biosensors are devices that combine the specificity of biological reactions with suitable transducers electronics **(Van Dorst *et al.*, 2010)**. It has advantages over the traditional techniques of analysis such as: portable, fast, selectivity, cost effective, detection without extensive sample pretreatment for greater applicability in food industries (**Kotanen *et al.*, 2012)**.

Selinheimo 2007, reported the use of tyrosinase, to generate food biopolymers with added functionalities or novel food structures from diverse raw materials. On reviewing the literature, it becomes evident that many species of mushrooms such as *Lentinula edodes* **(Kanda *et al.*, 1996)**, *Amanita muscaria* **(Muller *et al.*, 1996)**, *Pycnoporus sanguineus* **(Halaouli *et al.*, 2005)** and *Lentinula boryana* **(Faria *et al.*, 2007)** have been used to extract tyrosinase. The present study focused on purification and characterization of tyrosinase from *P. ostretus* for the first time. For the best of our knowledge the purified tyrosinase showed very high similarities to the other sources of tyrosinase.

MATERIAL AND METHODS

Purification of tyrosinase from *Pleurotus ostreatus* mushroom extracts

The extraction of tyrosinase from ***P. ostreatus*** was performed by the method of **Haghbeen *et al.* (2004),** with minor modifications. 300 grams of the fresh mushroom was homogenized with 300 ml of potassium phosphate buffer (100 mM, pH 5.8) containing 1 mM of EDTA in a warring blender. The suspension was stirred for 30 min at room temperature and filtered through a muslin cloth. This step was repeated twice and the filtrates were pooled together and centrifuged at 5000 rpm for 30 min at 4˚C. The resulting supernatant was subjected to ammonium sulfate precipitation. Finely powdered ammonium sulfate was added to 75% saturation. The mixture was left for 2 hr at 4˚C, followed by centrifugation at 1500×g rpm for 20 min at 4˚C. The supernatant was discarded; the resulting precipitate was dissolved in potassium phosphate buffer (100 mM, pH 5.8) and dialyzed overnight against the same buffer at 4˚C. The dialyzed ammonium sulfate fraction was applied to a Sephadex G-100 column that was preequilibrated with a 100 mM potassium phosphate buffer pH 6.0. The protein elution was done with the same buffer at a flow rate of 5 ml/min. The fractions were collected at 4˚C and were assayed for protein content at 280 nm as well as for enzyme activity. The active fractions were pooled against the 100 mM potassium phosphate buffer pH 6.0, and concentrated. The concentrated enzyme solution was applied to the column of diethylaminoethyl (DEAE) cellulose that was preequilibrated with a 100 mM potassium phosphate buffer, pH 6.0. It was eluted with the NaCl gradient (0.1-0.5 M) and 0.1 M borate buffer, pH 6.0. The active fractions were collected, dialyzed and concentrated. The quality of the purified tyrosinase was evaluated by tyrosinase assay and SDS-PAGE.

Assay for tyrosinase activity

The tyrosinase activity was performed as reported by **(Sung *et al.*, 1992),** in which the formation of the final dopachrome product was monitored spectrophotometrically at 475 nm. The assay mixture containing tyrosinase was incubated for 5 min at 35˚C. At time zero, 1 ml of L-DOPA (3,4-dihydroxy-L-phenylalanine, Sigma) concentration of 4mg/ml spectrophotometrically, measuring conversion of L-DOPA to red colored oxidation product dopachrome. After incubation for additional 5 min, the mixture was shaken again and a second reading was measured for 3 minutes. The change in absorbance was proportional

to enzyme concentration. One unit of enzyme corresponded to the amount which catalyzed the transformation of 1µmol of substrate to product per min under above conditions and produced 1.35 changes in absorbance. Specific activity was expressed as enzyme unit per milligram of protein. The protein content of the enzyme was determined by the method of **(Lowry *et al.*, 1951)** with bovine serum albumin as standard.

Molecular weight determination

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a 3-mm slab gel of 15% acrylamide in a Tris-HCl buffer pH 8.3 containing 0.1% SDS. Molecular mass markers from 14.4 to 200 kDa were used. The gels were stained with 0.025 Coomassie brilliant blue R-250.

RESULTS AND DISCUSSION

Characterization of the crude extract

The effects of pH on L-DOPA oxidation by the tyrosinase are shown in (Figure.1). The optimum activity of the enzyme on L-DOPA was observed at pH 6.0 whereas from pH 4.0 to 5.0, the enzyme had 20 to 30% activity. A similar result has been observed in *Lentinula edodes***(Kanda et al., 1996)** and *Lentinula boryana* **(Faria *et al.*, 2007)**. On the other hand, optimum pH near neutral has been reported for tyrosinase of Portabella mushrooms **(Fan *et al.*, 2004)** *Agaricus bisporus* **(Gouzi *et al.*, 2007)** and *Pycnoporus sanguineus* **(Halaouli *et al.*, 2005) .** The tyrosinase isolated sunflower plant remained fully active between pH4.8 to 7.9 after 20-hour exposure to buffers of different pH at 4˚C. **Sang *et al.* (2005)**, have reported that in case of recombinant human tyrosinase, the optimum pH was 7.5 which as compared to that of presently studied *Pleurotus ostreatus*, is quite low, being 6.0.

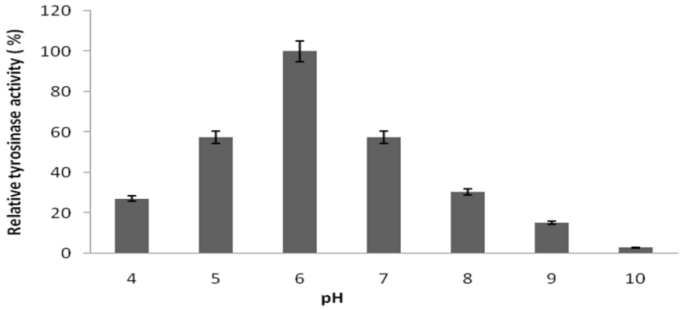

Figure 1 Effect of pH on the tyrosinase activity of the crude extract of *P.**ostreatus***. Data were obtained as mean value of optical density. Assays were done at 35°C, the activity of the sample was incubated in 100mM acetate buffer at (4.0-5.0 pH), 100mM phosphate buffer at (6.0-8.0 pH) and 100mM Tris-HCl buffer at (9.0-10 pH). The optimum activity of the sample at pH 6.0 was taken as 100%

Temperature profile showed that the enzyme had optimum activity at 35˚C (Figure. 2) which is similar to those reported earlier. The optimum temperature for tyrosinase from *Pycnoporus sanguineus* has been reported to be 25˚C, and 25 to 40˚C for *Agaricus bisporus* **(Xu *et al.*, 2011)**. In contrast to the data of the present study, it has been found that the optimum temperature of tyrosinase from *Solenum melongena* was high as 65˚C **(Lee *et al.*, 1997)**.

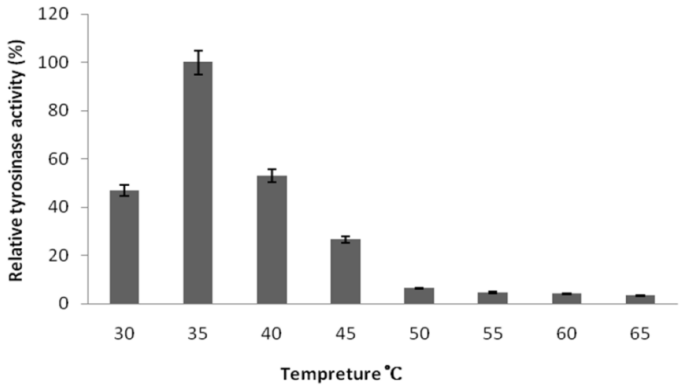

Figure 2 Effect of temperature on the tyrosinase activity of the crude extract prepared from *P. **ostreatus.*** Data were obtained as mean value of optical density. Assays were done in potassium phosphate buffer (100 mM, pH=6.0). The optimum activity of the sample incubated at 35°C was taken as 100 %

Based on the Lineweaver-Burk analysis, the *Km* and *Vmax* values of tyrosinase from *P. ostreatus* were 0.119 mM and 2.97 mg, respectively (Figure.3). This indicates the high affinity of the enzyme to the substrate. Tyrosinase of different sources has different substrate affinities and probably plays different physiological roles in the enzyme activity. Higher *Km* values (1.9 mM and 0.9 mM) for tyrosinase from *L. boryana* and Pycnoporus species, respectively, have been reported **(Faria *et al.*, 2007; Halaouli *et al.*, 2005)**. On the other hand, a lower *Km* value (0.35 mM) was obtained for tyrosinase from *Bacilus megaterium* **(Shuster *et al.*, 2009).**

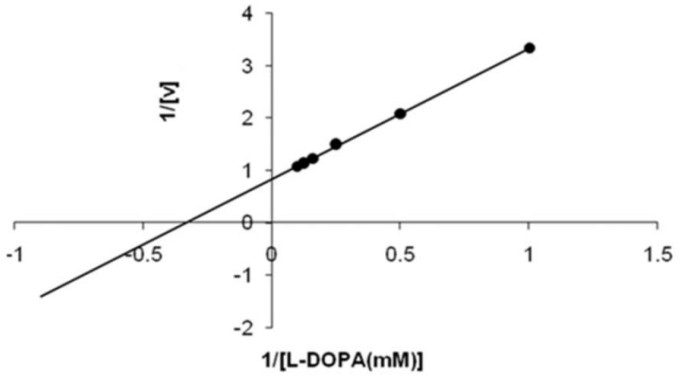

Figure 3 Lineweaver-Burk plot of *P. ostreatus* tyrosinase. Data were obtained as mean value of 1/ [V], inverse of the increaser of optical density at 475 nm per min. (OD475/min/mg), of three independent tests with different concentrations of L-DOPA as a substrate

Partial purification of Tyrosinase

The partial purification of tyrosinase crude extracts of *P.**ostreatus*** was achieved by 75% ammonium sulfate precipitation. The total protein decreased from 274 to 50.0 mg in ammonium sulfate precipitation steps, whereas the specific activity of tyrosinase **was measured to** 46.4 U/mg (Table.1). The data reported by other researchers regarding the specific activity of tyrosinase, isolated from different species of mushrooms was found to be highly variable, *Trifolium pretense* as 5.94 U/mg **(Schmitz *et al.*, 2008)**, *Crocus sativus* as 27 U/mg **(Saiedian *et al.*, 2007)**, *Agaricus bisporus* as 30 U/mg **(Shi *et al.*, 2002)**, *Pycnoporus sanguineus* as 30.2 U/mg **(Halaouli *et al.*, 2005)** and *Aeromonas media* as 34U/mg **(Wan *et al.*, 2009)**. The present study indicated that the specific activity of tyrosinase from ***P.**ostreatus*, was found to be higher than the earlier reports. Mushroom contains a considerable amount of various phenolic compounds, which are readily oxidized during the homogenizing process. By foremost oxidation and successive polymerization of the phenolic content of the mushroom extract, macromolecules of melanin are formed. Separating the unwanted melanin from the protein content of the extract is the first, or probably the most, important task during the mushroom tyrosinase purification. Though an organic solvent can prevent the melanin formation to a great amount, but increases the proteins denaturation risk. It is required to reduce the salt content of a protein mixture before loading it onto an ion-exchange column. Therefore, the collected proteins by 75% saturated ammonium sulfate were chromatographed on a Sephadex G-100 column. Doing so, not only ammonium sulfate was replaced by buffer, but also large amount of phenolic compounds were washed down.

Table 1 Purification of tyrosinase from extracted mushroom

Fractions	Volume mL	Total Protein (mg)	Activity (Units)	Total activity (Units)	Specific activity U/mg	Purification Fold	Yield (%)
Crude extract	300	274	1.48	777	2.83	1	100
Ammonium sulphate precipitate	100	50.8	2.03	503	9.90	3.49	64.7
Dialysis	80	25	3.55	284	11.3	3.99	36.5
Gel filtration G100 Column	45	8.5	4.31	194	22.8	8.05	24.9
DEAE-Cellulose Column	20	3.4	7.9	158	46.4	16.39	20.3

DEAE Diethylaminoethyle

The Sephadex G-100 gel filtration column chromatography showed that the profile fractions contained different protein molecules, although only one peak showed activity for tyrosinase from *P.**ostreatus*** **(Figure.4)**. Also, the purification of the enzyme rich fractions of the Sephadex G-100 gel filtration on the DEAE cellulose column is shown in (Figure.5). A sharp distinctive peak of tyrosinase activity, indicates the purity of extraction with only one protein peak was obtained. It is possible to omit both the phenolics and melanin impurities from the protein mixture at this stage. Since a matter of fact, melanin shows very high affinity for DEAE cellulose polymer. Hence, the mixture of protein and melanin is loaded on the DEAE cellulose column and successively washed by molar solution of NaCl. Here, tyrosinase is the only protein which is released on elution.

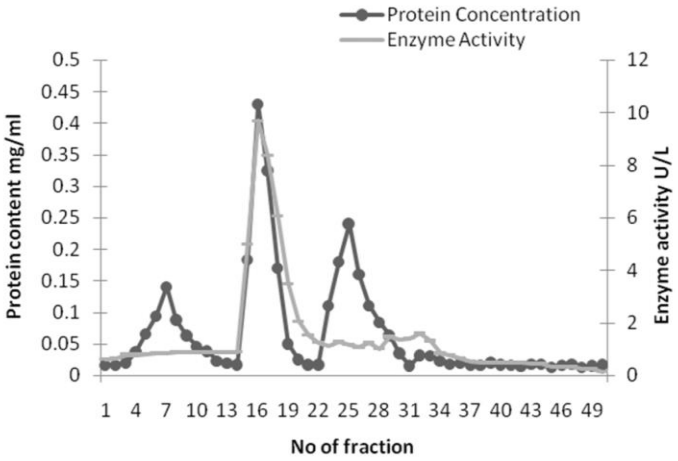

Figure 4 First gel filtration chromatography of tyrosinase. The dialyzed ammonium sulfate precipitate was chromatographed on Sephadex G-100. Total protein was monitored at 280 nm. The fractions were assayed for the tyrosinase activity

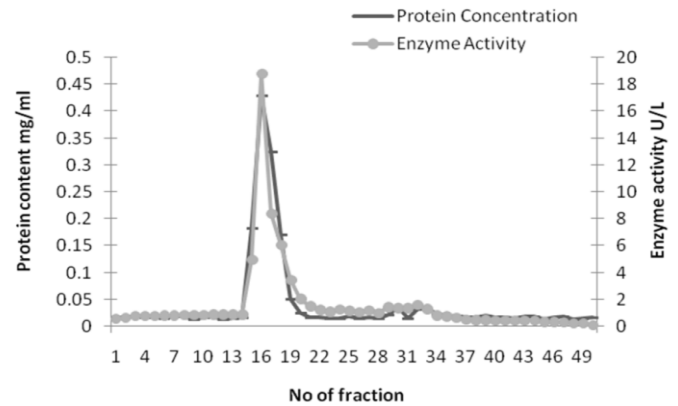

Figure 5 Second DEAE-Cellulose chromatography of tyrosinase. The first gel filtration G-100 collected fraction was applied to DEAE-Cellulose. Total protein was monitored at 280 nm and the fractions were assayed for tyrosinase activity

In the present study, a sharp distinctive peak of tyrosinase activity, indicates the purity of extraction with only one protein peak was obtained. SDS PAGE of the enzyme preparation from different purification steps showed that the resolved electrophoretic bands progressively improved from the crude extract to the final steps of the DEAE Cellulose column with a distinctive band of 75 kDa and several minor proteins smaller than 45 kDa were observed but not clearly visible in the scanned gels. (Figure.6).The molecular weight of tyrosinase was found to be highly variable with various studies conducted by other researchers. Similar results have been reported from the fungal tyrosinase of *Aspergilus orizae* being 67 kDa **(Ichishima *et al.*, 1984),** of *Lentinula edodes* being 70 kDa**(Kanda *et al.*, 1996)** and of the Protabella mushroomas70 kDa **(Fan and Flurkey, 2004)**. Comparative assessment on the molecular weight of some plant tyrosinases lies approximately between 40 to 65 kDa as in *Brassica oleracea* **(Gawlik *et al.*, 2007)** and *Trifolium pretense* **(Schmitz *et al.*, 2008).** These reports have simiarity with the present study.The purification scheme removed most of the major proteins with lower molecular weight found in the crude extracts (Figure. 6 lane A). One major stained banks of protein were present in the final sample along with a protein with less staining intensity (Figure 6 lane E). The estimated size of the major bands was approximately 75 kDa. The minor protein bands were estimated to have a size of 45 and 50 kDa. Several minor proteins smaller than 45 kDa were present but not readily visible in the scanned gels. We assume that the bands in the 42 kDa are the active proteolyzed forms of tyrosinase that many other investigators have observed **(Wicherse *et al.*, 1996; Gerritsen *et al.*, 1994; Zhang *et al.*, 1999)**. The minor band 45 and 50 kDa could be the latent tyrosinase that is known to have a higher molecular weight **(Espin and Wichers, 1999; Espin et al., 2000)**. The tyrosinase extracted from *P. ostreatus* was observed to have higher activity at a alkaline pH, make it extremely valuable for different therapeutic and industrial applications. In the further experimentation, development of cross-linked biopolymers would be done to modify the structural properties of the food matrix.

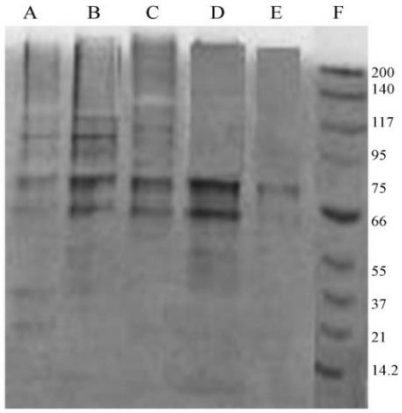

Figure 6 Polyacrylamide gel electrophoresis of tyrosinase from *P. **ostreatus,*** lane A, crude extract; lane B, ammonium sulfate fraction; lane C Dialysis, lane D Sephadex G-100 gel filtration fraction; lane E, DEAE Cellulose fraction; lane F standard protein of different molecular weight. Arrow indicates location of tyrosinase approximately ~75 kDa.

CONCLUSION

There is an increasing demand for various natural enzymes in industries, in spite of their wide acceptance in the global market. Different enzymes are used for hydrolyzing food biopolymers so as to improve product characteristics.

Tyrosinase constitutes one of the most important groups of commercial enzymes due to its ability to utilize a wide variety of mono- and di-phenolic compounds. In the present work, an initial step is taken to evaluate the industrial potential of a preliminary characterized tyrosinase enzyme of *P. ostreatus*. It is concluded that the purified and characterized tyrosinase from *P. ostreatus* has comparatively better enzyme properties than reported earlier in other sources. The tyrosinase extracted from *P. ostreatus* was observed to have higher activity at a alkaline pH, create an important and different applications in food industry. The enzyme can also be used to produce cross-linked proteins, allowing enzyme biocatalysts to recycle easily and to improve the consistency and texture of proteins. The tyrosinase from *P.ostreatus* has economic advantage over the commercialy synthesized tyrosinase.

Acknowledgments: The authors are thankful to Peoples University, People's Group, Bhopal for providing laboratory facilities and for granting financial assistance to carry out present research work. and to the Principal and Secretary, Saifia College of Science, Bhopal for encouragement.

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