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Full Length Research Paper

Production and purification of horseradish peroxidase in Pakistan

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Horseradish peroxidase is heme containing enzyme belonging to oxidoreductases and is involved in degradation of certain recalcitrant organic compounds like phenol and substituted phenol via free radical polymerization. Peroxidase was extracted and purified from horseradish by $(NH_4)_2SO_4$ precipitation, dialysis and gel filtration chromatography. Spectrophotometer was used for the assay of horseradish peroxidase in this present project. Horseradish peroxidase increased activity from 6.3027 to 9.9452 U/ml by purification. It gained 45.77 folds purification at this final step. It is helpful in determination of glucose in blood and compounds in it kill bacterial strains.

Key words: Horseradish, peroxidase, heme, oxidoreductase, activity.

INTRODUCTION

Horseradish peroxidase (HRP,EC.1.11.1.7) is a heme-containing enzyme that oxidizes a variety of organic and inorganic compounds using hydrogen peroxide and it belongs to oxidoreductases. Horseradish *(Armoracia rusticana)* has medicinal characteristics to the taste of meals. It is widely used in plants and has thick pulpy yellow roots, spicy taste and antibiotic and anti-inflammatory ability. It is helpful in the determination of glucose in blood (Hames and Hooper, 2001). It is originated in South-eastern Europe and Western Asia. It is also resistant in low temperature and droughts. It grows in shaded places and also in warm places. But it does not grow in sandy soils. Argillaceous alkaline soils rich in humus are ideal for horseradish. It has length of 2 m. It has large edible leaves that are branched (Gajhede et al., 1997).

Horseradish has been cultivated since antiquity. According to Greek mythology, the Delphic Oracle told Apollo that the horseradish was worth its weight in gold. Horseradish was known in Egypt in 1500BC and has been used by Jews from Eastern Europe (Michael et al., 1992). Horseradish is probably the plant mentioned by Pliny, the elder in his natural history under the name of *Amoracia*, and is recommended by him for its medicinal qualities, and possibly the wild radish, or *Raphanos agrios* of the Greeks. Both root and leaves were used as a medicine during the middle ages and the root was used as a condiment on meats in Germany, Scandinavia and Britain. It was taken to North America during colonial times (Barbara, 2003). In recent years, horseradish peroxidase has become a tool in marking neurons with it.

Horseradish peroxidase is one of the two most widely used enzyme labels in medical diagnostics and it researches applications (Handley et al., 1998). Horseradish peroxidase is most commonly used in enzyme labeled antibody in rapid, sensitive and specific enzyme immunoassays e.g. ELISA test used for labeling antibody (Folks and Wardaman, 2001; Ramadan et al., 2011) and also, the produced antibody has many application in research, clinic and education. This poly-clonal antibody can be used for diagnosis and monitoring of free light chain producing diseases (Abdolalizadeh et al., 2008).

Bronchitis, sinusitis, paradontosis, rheumatism, anemia, flu and stomatitis can be treated by using horseradish. It is also used in many anti-cancerous drugs (Wardaman, 2002; Tupper et al., 2010). Horseradish root has several self-healing properties and is best to promote body's natural healing ability. The root contains a high content of mustard oil and is favorable in aromatherapy

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Table 1. Total protein versus absorbance.

Concentration (mg/ml)	Absorbance (OD)
0.00	0.10
0.05	0.20
0.10	0.30
0.15	0.40
0.20	0.50
0.25	0.60
0.30	0.70

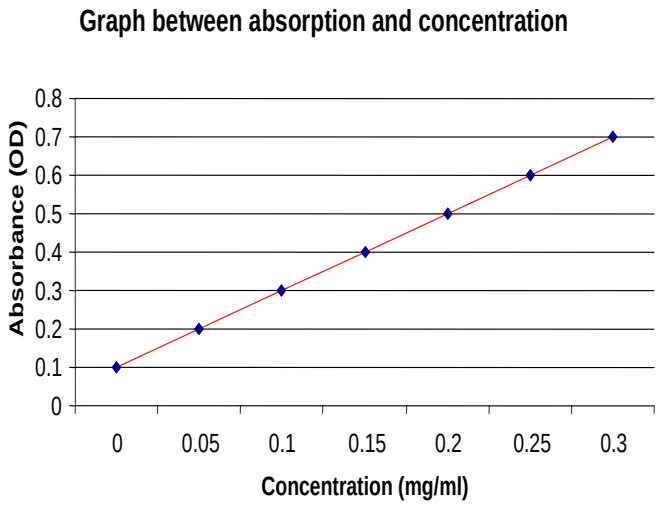

Figure 1. Standard curve of protein.

due to the natural healing ability. Mustard oil is made from grating or cutting the horseradish root. Many antibacterial agents that are good for preventing infections are found in horseradish (Tognolli et al., 2002; Welinder et al., 2002). Compounds found in horseradish have been found to kill some bacterial strains and also used in recombinant enzyme production (Vitch and Smith, 2001).

Recently, immobilized horseradish peroxidase possesses excellent catalytic ability and well-retained activity for the fabrication of a H_2O_2 biosensor based on nanostructured γ-Al_2O_3 (Xiaojuan et al., 2011), and is also used in azo dye decolourization (Metin, 2011). Hence, development of purification method related to diagnosis of different diseases is of great importance. The aim of this study is to explore the indigenous natural resources for remedy of different diseases like bronchitis, sinusitis, periodontitis, anemia and cancer specifically.

MATERIALS AND METHODS

Horseradish of about 100 g was obtained from local market. All other chemicals and reagents were of analytical grade.

Production of peroxidase

Horseradish peroxidase production involves the following steps:

Enzyme extraction

About 100 g of horseradish was thoroughly washed with water and was cut down into small pieces and was homogenized in blender with 500 ml distilled water. It was centrifuged at 10,000 rpm for 5 min at 4°C. The filtrate was heated in water bath at 65°C for 3 min to inactivate catalase and cooled quickly in iced water. Enzyme assay was performed for this extract (Civello et al., 1995; Zia et al., 2001) (Table 1 and Figure 1).

Enzyme assay

The activity of peroxidase enzyme was determined by the method described by Faizyme Laboratory Manual and Zia (2001).

Unit definition

One unit of enzyme activity is defined as the amount of enzyme which catalyses the conversion of one micromole of hydrogen peroxide per minute at 25°C under assay conditions.

Preparation of buffer substrate

Buffer substrate solution was prepared as follows:

1) Phosphate buffer (pH 6.5) of 46.6 ml
2) H_2O_2 (30%) of 0.32 ml
3) Guaiacol of 1.00 ml

The reagents were mixed and completely covered for a whole day. In case of blank solution preparation, H_2O_2 was not added. Guaiacol (1 ml) and phosphate buffer (46.6) were mixed in agitator and used as blank. Spectrophotometer was set to zero at 470 nm wavelength after setting blank solution in it. Then, in ultraviolet (UV) cell 1 ml of buffered substrate solution was taken along with 0.02 ml of enzyme extract and was kept in a spectrophotometer and absorbance was recorded after 3 min.

Calculations

$$\text{Activity (U/ml)} = \frac{A \times 4 \times Vt \times \text{Dilution factor}}{\varepsilon \times Vs}$$

where Vt is the final volume of reaction mixture (ml) = 3.00, Vs is the sample volume (ml) = 0.1 and ε is the micromolar extinction co-efficient 25.5 (cm/micromole). The formula used is as follows:

Activity (U/ml) = $\Delta A \times 4.7$

$$\text{Specific activity} = \frac{\text{units/ml}}{\text{mg total protein/ml}}$$

Protein estimation method and reagents

Proteins were estimated according to the method of Moss and Bond (1957). The reagents used are as follows: solution A = 40 g

Table 2. Crude enzyme activity.

Enzyme fraction	Absorbance (OD)	Activity (U/ml)	Protein contents (mg/ml)	Specific activity (U/ml)
Crude enzyme	1.341	6.3027	7.339	0.8586

sodium carbonate in 500 ml distilled water; solution B = 0.38 g $CuSO_4 \cdot 5H_2O$ and 0.6 g potassium-sodium tartarate in 500 ml of distilled water; solution C = 1 part Folin reagent; solution D = 1 N NaOH.

Standard bovine serum albumin solution (500 µg/ml)

5.0 mg of bovine serum albumin (BSA) was dissolved in 10 ml of distilled water. The serum albumin dissolved very slowly. The standard was made two days before use and was kept at 4°C.

Procedure

0.5 ml NaOH, 1 N (solution D) was added in each tube containing protein sample (250 to 750 µg) and were shaken in water bath at 37°C for 30 min. After cooling, 2.6 ml of solution A and 2.5 ml of solution B were added in each as well as in standard tube. The solution in each tube was thoroughly mixed and incubated 37°C for 30 min. Then, 0.5 ml of solution C was added, mixed well and kept for 20 min at room temperature. Standard BSA (0.05 to 1.5 ml) was simultaneously used in the experiment. The optical density was measured at 661 nm on spectrophotometer and a standard calibration curve was drawn and with its help proteins were estimated.

Purification of peroxidase

Purification of peroxidase involve following steps:

Purification of peroxidase by $(NH_4)_2SO_4$

The enzyme was precipitated by ammonium sulphate for partial purification as described by the method of Bentely (1962). Solid ammonium sulphate is added to 100 ml of peroxidase at the concentration of 30% (w/v). The suspension was stirred for half an hour at 4°C. After sufficient shaking, the precipitates were collected by centrifugation at 10,000 rpm for 30 min. Enzyme activity was determined for each concentration and precipitates were collected for further purification. Enzyme solution was then treated with 40, 50, 60, 70 and finally 80% (w/v) $(NH_4)_2SO_4$ (Bentely, 1962).

Dialysis

Before dialysis procedure, the dialysis bag was boiled with 0.1 M sodium carbonate solution for about 1 h and then retained for overnight. By this procedure, the dialysis bag was opened. The precipitates obtained by ammonium sulphate precipitate were dialyzed in dialysis bag with 0.2 M phosphate buffer (pH 6.5) with constant stirring on magnetic stirrer for 2 h. The precipitates were subjected to enzyme assay and protein estimation.

Gel filtration chromatography using Sephadex G-75

The reagents used were 0.2 M phosphate buffer (pH 6.5), Sephadex G-75 and 0.5% dextrin blue. Sephadex G-75 was soaked in 500 ml phosphate buffer (pH 6.5) containing 0.1 g of sodium azide and was incubated at room temperature for 24 h. After soaking, the gel was deaerated by direct drive rotary vacuum pump and then was poured in a 0.96 × 60 cm column. The packed column was washed with 0.2 M phosphate buffer (pH 6.5). Dextrin blue (0.5% w/v) was washed for the determination of its void volume. Dialysed extract was applied on the column and fractions each of 3 ml were collected. Each fraction was then assayed for enzyme activity and total protein (Jakoby, 1971).

RESULTS

Protein estimation

Protein contents were determined by the method of Moss and Bond (1957). Standard curve was obtained by the help of proteins estimated.

Horseradish peroxidase activity in crude extract

The crude sample was subjected to spectrophotometric analysis at 470 nm wavelength for determination of enzyme activity, which is given in Table 2.

Purification of horseradish peroxidase by ammonium sulphate precipitation technique

Precipitation of proteins from cell free supernatant by the addition of different concentrations of ammonium sulphate is as shown in Table 3. The maximum amount of total proteins could be precipitated by the addition of 80% $(NH4)_2SO_4$. Hence, 80% $(NH_4)_2SO_4$ was found suitable for precipitation of horseradish peroxidase. Activity was 6.6928 U/ml in 80% ammonium sulphate concentration which indicates the presence of enzyme (Figure 2).

Dialysis

Dialysis was done after ammonium sulphate precipitation. The results are as shown in Table 4.

Gel filtration chromatography of horseradish peroxidase

The enzyme fraction after dialysis was subjected to

Table 3. Horseradish peroxidase precipitation by $(NH_4)_2SO_4$.

Ammonium sulphate concentration (%)	Absorbance (OD)	Activity (U/ml)	Protein contents (mg/ml)	Specific activity (U/mg)
20	1.011	4.7517	0.737	6.447
40	1.213	5.7011	1.480	3.8520
60	1.329	6.2463	0.350	17.84
80	1.424	6.6928	0.524	12.77

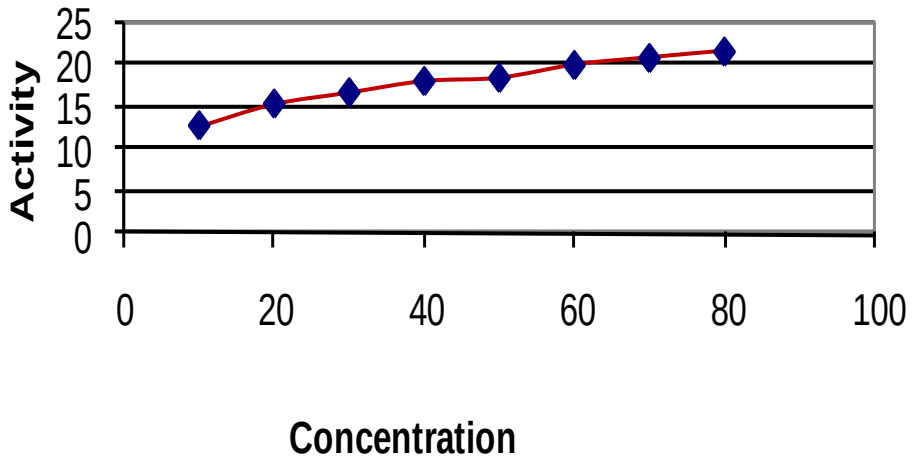

Figure 2. Graph between ammonium sulphate concentration and activity.

Table 4. Dialysis.

Enzyme fraction	Absorbance (OD)	Activity (U/ml)	Protein contents (mg/ml)	Specific activity (U/mg)
Dialysis	1.542	7.247	0.445	16.213

Sephadex G-75 column for gel filtration chromatography out of a total of 20 fractions, 4 have the maximum activity of 9.9452 U/ml. The results are as shown in Tables 5 and 6 and Figures 3 to 5.

DISCUSSION

Horseradish peroxidase has long attracted intense research interest and is used in many biotechnological fields, including diagnostics, biosensors and biocatalysis. The indigenous natural resources provide fresh and cheaper raw material for large scale peroxidase extraction and purification. Till now in Pakistan, horseradish plant is not cultivated on large scale and it is not used in any renowned allopathic, homeopathic or eastern medicine or commercial health product for the treatment of diseases.

CONCLUSIONS AND RECOMMENDATIONS

The highest activity of enzyme is confined in horseradish root. Purification of horseradish peroxidase has been reported from horseradish root. From the calculated results, the activity of crude horseradish peroxidase extract was 6.3027 U/ml and specific activity of 0.8586 U/mg. It was shown that the activity was increased to 6.6928 U/ml and 12.77 U/mg specific activities by $(NH_4)_2SO_4$ precipitation.

The protein contents were decreased from 7.339 mg/ml of crude extract to 0.524 mg/ml which indicate that unwanted proteins have been removed. The enzyme fraction having highest activity after dialysis was passed through Sephadex G-75 column for gel filtration chromatography. The maximum activity of 9.9452 U/ml was obtained in the 4th fraction during the experiment, with 0253 mg/ml of protein contents and 39.30 U/mg of

Table 5. Analysis of gel filtration chromatography for activity of horseradish peroxidase.

Enzyme fraction	Absorbance (OD)	Activity (U/ml)	Protein contents (mg/ml)	Specific activity (U/mg)
1	0.000	0.000	0.009	0.000
2	0.000	0.000	0.025	0.000
3	1..542	7.2474	0.279	25.97
4	2.116	9.9452	0.253	39.30
5	2.041	9.5927	0.255	37.61
6	0.495	2.3265	0.109	21.34
7	0.174	0.8178	0.094	8.79
8	0.116	0.4552	0.078	1.080
9	0.085	0.3995	0.079	5.056
10	0.041	0.1927	0.044	4.37
11	0.037	0.1739	0.064	2.71
12	0.034	0.1598	0.061	2.61
13	0.032	0.1504	0.061	2.465
14	0.021	0.0987	0.046	2.14
15	0.018	0.0846	0.038	2.22
16	0.014	0.0658	0.035	1.88
17	0.011	0.0517	0.045	1.14
18	0.004	0.0188	0.031	0.606
19	0.002	0.0094	0.056	0.167
20	0.001	0.0047	0.016	0.293

Table 6. Summary of horseradish peroxidase purification.

Sample	Activity (U/ml)	Protein contents (mg/ml)	Specific activity (U/mg)	Purification fold
Crude	6.3027	7.339	0.8586	1.00
Ammonium sulphate precipitate	6.6928	0.524	12.77	14.8
Dialysis	7.247	0.445	16.285	18.96
After gel filtration chromatography	9.9452	0.253	39.30	45.77

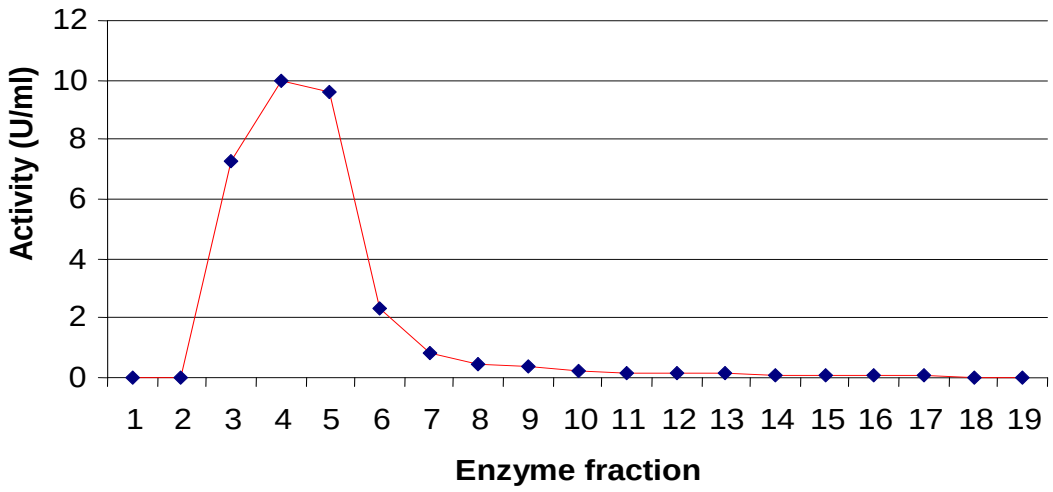

Figure 3. Graph between enzyme fraction and activity.

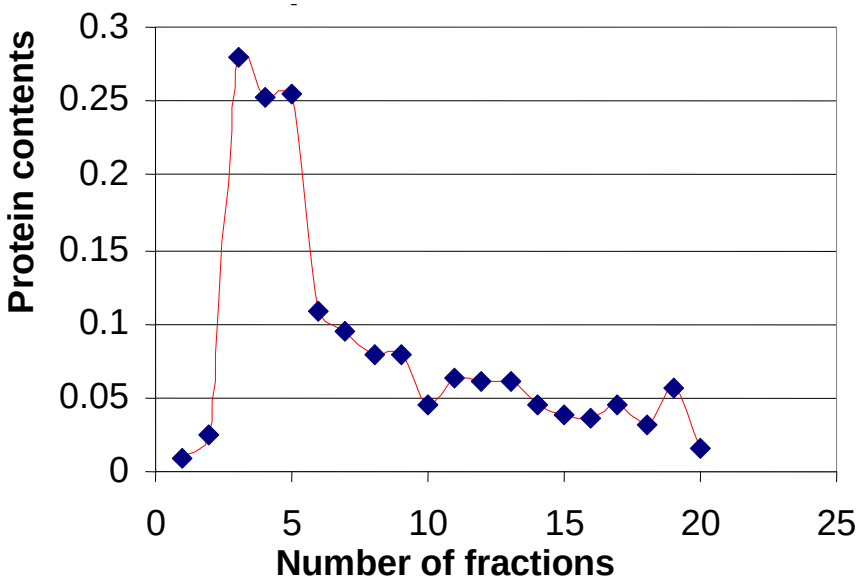

Figure 4. Graph between enzyme fraction and protein contents.

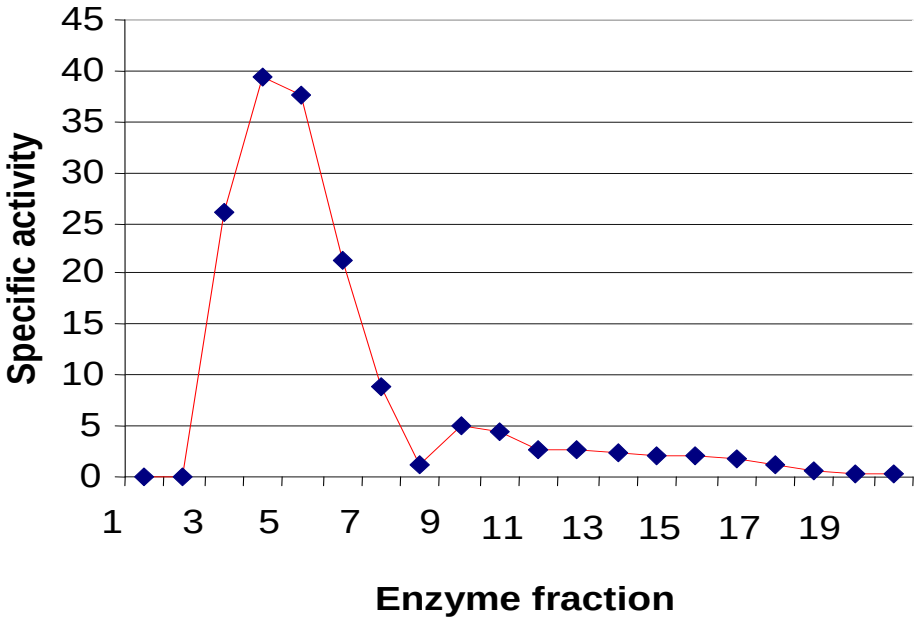

Figure 5. Graph between enzyme concentration and specific activity.

specific activity. So, the horseradish peroxidase enzyme was purified up to 45.77 fold which are much more than that previously purified by Zia et al. (2001, 2011).

It is recommended that activities and fold purification of horseradish peroxidase can be increased by much improved techniques of purification like ion exchange chromatography or FPLC; hence, enhancing the production of this enzyme. Also, a vast field of research is also available for formulation of different medicines and preparation of more sensitive and accurate diagnostic kits with the help of horseradish peroxidase.

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