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Research Article

Extraction and Purification of Horseradish Peroxidase Enzyme From Radish Roots (*Raphanus sativus*)

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ABSTRACT

Horseradish peroxidase (HRP; EC 1.11.1.7) is the most widely used enzyme to label antibodies. It is 44,173.9 dalton glycoprotein with four lysine residue for conjugation to a labeled molecule. HRP is a single chain polypeptide containing four disulfide bridges. It is a glycoprotein containing 18% carbohydrate. The horseradish peroxidase was extracted from the radish roots with 0.1M K_2HPO_4 was isolated by ammonium sulphate precipitation to precipitate out the protein present in extract. The desired protein was dialysed to remove the salt present along with the protein. Then it was passed through DEAE Cellulose column, Sephadex G-100 column, DEAE carboxy methyl cellulose column for further purification of enzyme. The quantification of enzyme was done by spectrophotometer. The measurement of enzyme activity was determined by pyragallol assay.

KEY WORDS: HRP, antibodies, radish roots, precipitation, purification, enzyme activity

Introduction

Peroxidase (E.C. 1.11.1.7) is a heme-containing enzyme, which catalyses the oxidation of a wide variety of organic and inorganic substrates using hydrogen peroxide as the electron acceptor (L.Banci, 1997; A. Yemenicioglu, 1998). Peroxidases are widely distributed in living organisms including microorganisms, plants, and animals (J.N. Rodriguez-Lopez et al., 1996). It is mainly located in the cell wall (E. L. Chen et al., 2002), and it is one of the key enzymes controlling plant growth and development. It is involved in various cellular processes including construction, rigidification, and eventual lignifications of cell walls (M. Quiroga et al., 2000), suberization (M. A. Bernards et al., 1999), organogenesis (D. J. Lee et al., 2002), phenol oxidation (L. M. Lagrimini, 1991), crosslinking of cell wall proteins (L. S. Schnabelrauch, 1996).

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Material and Methods

Collection of Radish Roots

In the present study, the radish roots of radish plant were collected from Ambala Cantt, Haryana, India.

Extracting crude from Radish Roots

First of all 5 Kg of radish roots were washed thoroughly. They were cut into the cubes. The cubes with their peroxidase rich skin were minced in blender with equal volume of 0.1 M K_2HPO_4. This homogenate was filtered with cheese cloth and collected in jar. To make it pure from fibers and heterogeneous substance the centrifugation of juice

was done at 10,000 rpm and the supernatant was collected.

Precipitation of Peroxidase by Ammonium Sulfate Fractionation (Wilkinson et al., 1985)

An aliquot of enzyme crude extract was saturated with ammonium sulfate under gentle stirring and let the mixture stand overnight at 4oC after the salt was completely diluted. To separate the supernatant from the precipitate, the mixture was centrifuged at 12000 rpm, 4oC for 20 minutes. The supernatant was then used as the enzyme crude extract and treated for further fractionation, while the precipitate was diluted with 0.01 M phosphate buffer pH 6.0 for measurements of enzyme activity.

Dialysis (Wilkinson et al., 1985)

The precipitate resulted from ammonium sulphate fractionation with 30-80% saturated was diluted with 0.01 M phosphate buffer pH 6.0 and dialysed in cellofan tube under gentle stirring in 0.01 M phosphate buffer pH 6.0 at 4oC until all salt was removed from the solution.

Ion-Exchange Column Chromatography – DEAE Cellulose (Voet, 1990)

Column of 30cm×3cm (height: diameter) was packed with DEAE cellulose and mixed with 0.05M tris phosphate, pH8.0. The column was left with 1cm layer of buffer at the top of packing so as to prevent the development of cracks after the evaporation of buffer. The clamp of the stopcock was opened and allowed the buffer to flow through the column. An even packing was essential and top of the column is reached, leave1.5-2inches empty at the top. The column was then washed approximately with 200ml of the same buffer. The sample (50ml) was layered over the resin with a Pasteur pipette, after draining of buffer. The sample was allowed to flow through the column and sides of the column were washed. A few millilitres of buffer were added and a constant flow rate of buffer was maintained and the eluant was collected in different test tubes. A gradually increasing concentration of gradient was applied. The DEAE cellulose column was then eluted with linear gradient of NaCl of 0.1M, 0.5M, 1M in Tris phosphate buffer and fractions (10ml each) were collected in test tube.

Gel filtration chromatography

Column of 25cm×1.5cm (height: diameter) was packed with Sephadex-G75 and mixed with 0.05M tris phosphate, pH8.4. The column was left with 1cm layer of buffer at the top of packing so as to prevent the development of cracks after the evaporation of buffer. The clamp of the stopcock was opened and allowed the buffer to flow through the column. An even packing was essential and top of the column is reached, leave1.5-2inches empty at the top. The packing was done at room temperature. The column was then washed approximately with 200 ml of the same buffer. The sample (2 ml) was layered over the resin with a Pasteur pipette, after draining of buffer. The sample was allowed to flow through the column and sides of the column were washed. A few milliliters of buffer were added and a constant flow rate of buffer was maintained and the eluant was collected in different test tubes.

Ion-Exchange Column Chromatography - CMC (carboxy methyl cellulose)

Column of 25cm×1.5cm (height: diameter) was packed with CMC (carboxy methyl cellulose) and mixed with 0.05M acetate buffer, pH4.4. The column was left with 1cm layer of buffer at the top of packing so as to prevent the development of cracks after the evaporation of buffer. The clamp of the stopcock was opened and allowed the buffer to flow through the column. An even packing was essential and top of the column is reached, leave1.5-2inches empty at the top. The packing was done at room temperature. The column was then washed approximately with 200ml of the same buffer. The sample (2 ml) was layered over the resin with a Pasteur pipette, after draining of buffer. The sample was allowed to flow through the column and sides of the column were washed. A few milliliters of buffer were added and a constant flow rate of buffer was maintained and the eluant was collected in different test tubes. The carboxy methyl cellulose column was then eluted with gradient of 0.005, 0.10, 0.25 buffer and fractions (5ml each) were collected in test tube.

Quantification of enzyme

The quantification of enzyme of different collected fractions were measured by taking O.D through spectrophotometer at 280 and 403 nm.

Measurement of Enzyme Activity

Peroxidase activity was determined by spectrophotometry method using pyrogallol as hydrogen donor (Saunders et al., 1974). Pyrogallol was converted by peroxidase in the presence of H2O2 at 20oC and pH 6.0 to purpurogallin. The orange color of purpurogallin was measured at 420 nm. Concentration of purpurogallin was calculated from calibration curve of purpurogallin standard. One unit of enzyme activity was defined as mg purpurogallin formed from pyrogallol at 20 C, pH 6.0 in five minutes.

Results and Discussion

O.D. of fractions collected after DEAE chromatography are shown in Table 1.

Table 1. After DEAE chromatography the O.D of different fractions are collected at 280 nm and 403 nm

Effluent	280 nm (10ml each)	403nm (10ml) each
1	0.156	0.019
2	0.124	0.003
3	0.117	0.015
4	0.102	0.013
5	0.094	0.010
6	0.291	0.089
7	0.694	0.152
8	0.674	0.158
9	0.767	0.164
10	0.835	0.171
11	0.845	0.172
12	0.847	0.169

Table 2. After DEAE chromatography the O.D of different fractions are collected with Tris phosphate buffer (pH-8) at 280nm and 403nm.

Effluent	280nm (15ml each)	403nm (15ml each)
13	0.818	0.172
14	0.827	0.170
15	0.838	0.170
16	0.856	0.175
17	0.676	0.099
18	0.204	0.014
20	0.088	0.003
21	0.056	0.003

Table 3. After DEAE chromatography the O.D of different fractions are collected with 0.1 M NaCl at 280nm and 403nm.

Effluent	280nm (15ml each)	403nm (15mleach)
21	0.063	0.024
22	0.053	0.005
23	0.046	0.008
24	0.043	0.005
25	0.044	0.001

Table 4. After DEAE chromatography the O.D of different fractions are collected with 0.5 M NaCl at 280nm and 403nm.

Effluent	280nm	403nm
26	0.055	0.011
27	0.125	0.019
28	0.397	0.019
29	0.260	0.019
30	0.172	0.013
31	0.147	0.015

Table 5. After DEAE chromatography the O.D of different fractions are collected with 1M NaCl at 280nm and 403nm.

Effluent	280nm (15ml each)	403nm (15mleach)
32	0.174	0.021
33	1.910	0.151
34	1.501	0.125
35	0.430	0.061
36	0.261	0.047
37	0.204	0.039
38	0.181	0.037

Table 6. After Gel filtration chromatography the O.D of different fractions are collected with 0.25M acetate buffer at 280nm and 403nm.

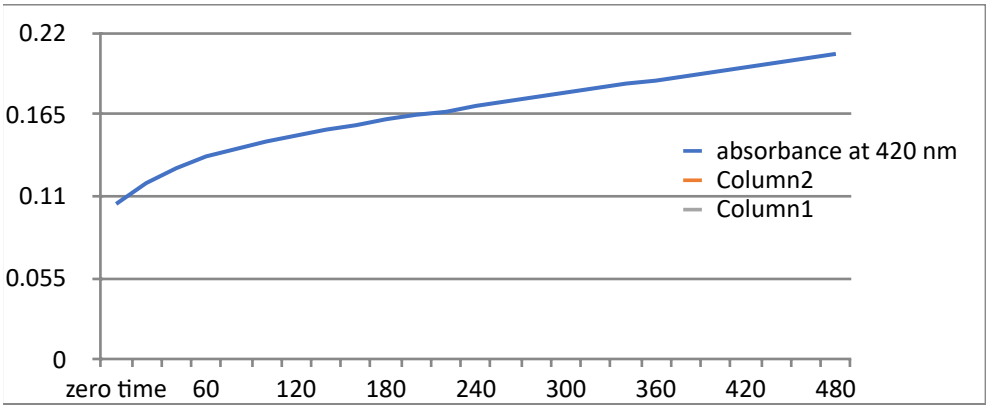
Fractions	280nm	403nm
1	0.020	----
2	0.047	0.009
3	0.817	0.144
4	1.619	0.277
5	0.810	0.212
6	0.521	0.204
7	0.422	0.156
8	0.340	0.090
9	0.315	0.066
10	0.256	0.060
11	0.232	0.036
12	0.213	0.009
13	0.189	0.006

Table 7. After Gel filtration chromatography the O.D of different fractions are collected at 280nm and 403nm with 0.25M acetate buffer.

Fractions	403nm	280nm
1	0.009	0.032
2	0.015	0.117
3	0.095	1.009
4	0.008	1.065
5	0.052	0.696
6	0.032	0.499
7	0.024	0.379

8	0.019	0.311
9	0.016	0.289
10	0.017	0.276
11	0.014	0.282
12	0.011	0.252
13	0.006	0.192

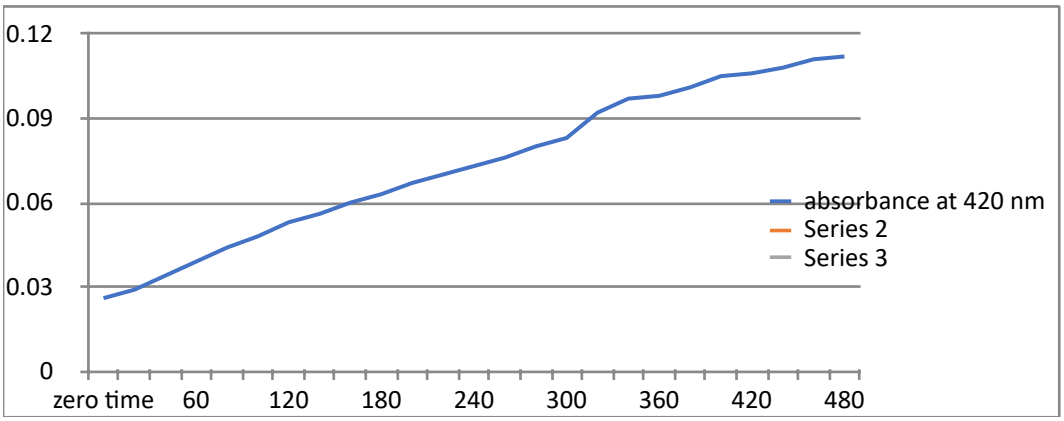
The graph for enzyme activity of eluted fraction "A" obtained for 0.25 M acetate buffer.



Calculations:

A420/20 sec= (0.026+0.029+0.034+0.039+0.044+0.048+0.063+0.056)/8
= 0.329
=0.329×3
=0.987U/100µl
=9.87U/ml

The graph for enzyme activity of eluted fraction "B" obtained for 0.25M acetate buffer.



Calculations:

A420/20 sec=
(0.067+0.070+0.073+0.076+0.080+0.083+0.092)/7

= 0.541

=0.541×3

=1.623U/100µl

=16.23U/ml

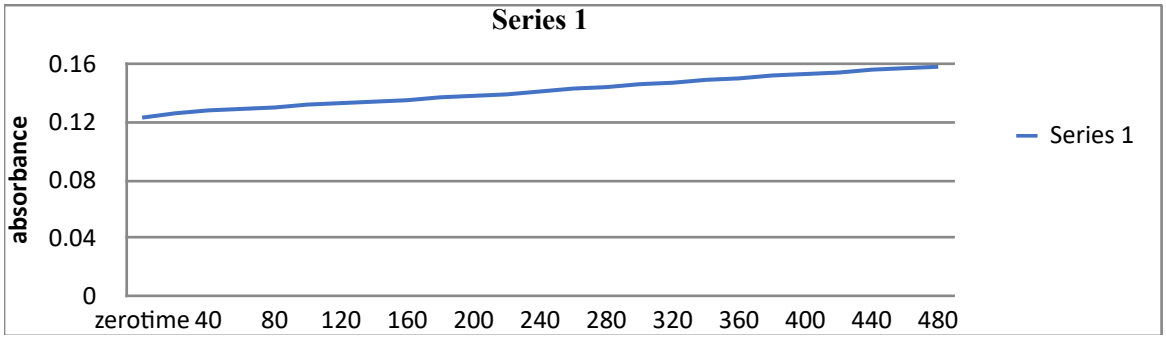
The O.D of different fractions eluted with different concentration gradient of 0.005MNaCl, 0.10M NaCl and 0.25M NaCl in acetate buffer in the column packed with CMC at 403 and 280 nm are shown in Table 8.

After ion exchange chromatography with CMC the O.D of different fractions are collected at 280nm and 403nm with different concentration gradient of 0.005MNaCl, 0.10M NaCl and 0.25M NaCl in acetate buffer. The fractions with maximum O.D were pooled together in fractions and named as C, D, E. The degree of purification of desalted enzyme was increased by applying it to DEAE-cellulose column for ion exchange chromatography. Peroxidase exists in many isozymic forms and majority of them are anionic (Evans, 1968). The most often used cellulosic anion exchanger is DEAE-cellulose (Voet, 1990). A number of other ion exchange resins i.e. DEAE ephadex, carboxy methyl cellulose etc. are also available. But ion exchange cellulose is preferred because the finer particle size and higher density of the microgranular cellulose result in more compact adsorbent bed which provides higher resolution (Jakoby, 1971). But in our research experiment DEAE-Carboxy methyl cellulose gives best result with respect to enzyme activity. The enzyme activity was measured used pyrogallol assay of fraction 'C', 'D' and 'E' is shown in figure 1, 2 and 3.

Table 8. The O.D of different fractions eluted with different concentration gradient of 0.005MNaCl, 0.10M NaCl and 0.25M NaCl in acetate buffer in the column packed with CMC at 403 and 280 nm

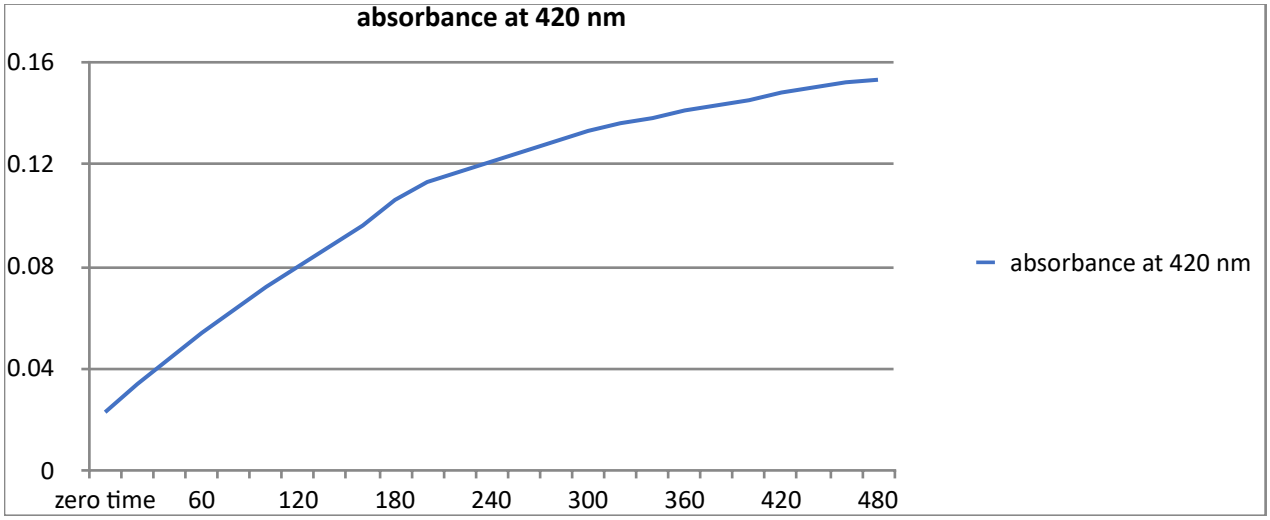
Fractions	0.005M NaCl		0.10 M NaCl		0.25M NaCl	
	403nm	280nm	403nm	280nm	403nm	280nm
1	0.103	0.424	0.008	0.166	0.008	0.101
2	0.187	0.751	0.006	0.258	0.007	0.133
3	0.039	0.505	0.019	0.171	0.028	0.150
4	0.005	0.258	0.023	0.151	0.040	0.276
5	------	0.096	0.011	0.063	0.016	0.065

Figure 1. This enzyme activity for fraction 'C' eluted from the column having a concentration of 0.005M NaCl in acetate buffer



Inference- It is continuously increasing graph and hence interprets that the fraction obtained is a mixed fraction.

Figure 2. The enzyme activity of eluted fraction "D" obtained for 0.10 M NaCl in acetate buffer



Calculations:

A_{420}/20 sec= (0.023+0.034+0.044+0.054+0.063+0.072+0.080+0.096+0.106+0.133)/11

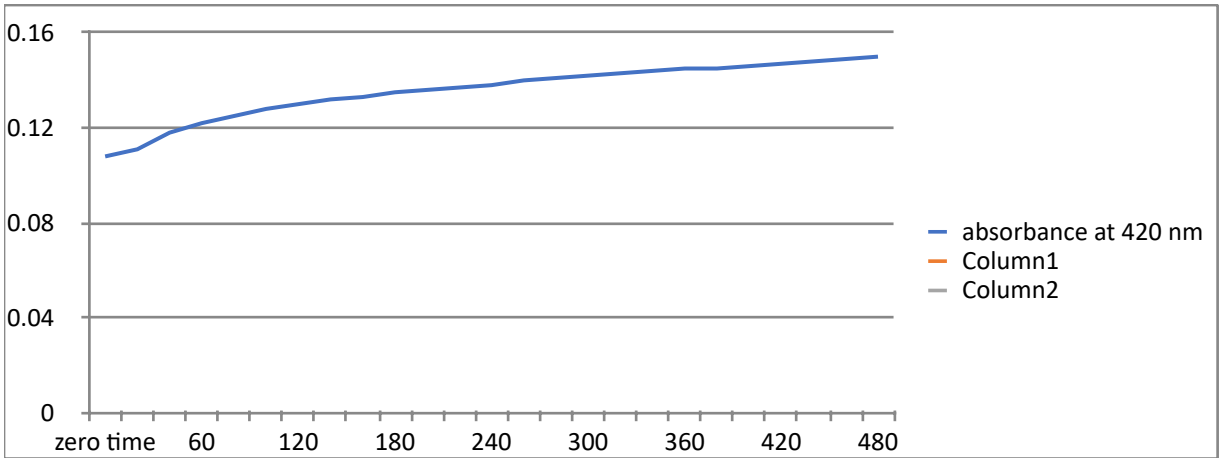
= 0.793

= 0.793×3

=2.397U/100µl

=23.97U/ml

Figure 3. The enzyme activity of eluted fraction "E" obtained for 0.25M NaCl in acetate buffer



Calculations

A_{420}/20 sec= (0.108+0.111+0.118+0.122+0.124+0.128)/6

= 0.712

=0.712×3

=2.136U/100µl

=21.36U/ml

Conclusions

Peroxidase could be extracted from radish roots with 0.1MK2HPO4. Precipitation of enzyme using ammonium sulphate followed by DEAE cellulose column, sephadex G-75 column, CMC (carboxy methyl cellulose) column for further purification of enzyme. Fractions 2 and 4 with maximum O.D from DEAE cellulose column chromatography were further purified by sephadex G-100. Gel filtration chromatography was applied by sephadex G-100 column which proved very efficient as fractions with maximum O.D were pooled to gether and named as fractions "A" and "B". The enzyme activity of eluted fractions "A" and "B" obtained for 0.25M acetate buffer is 9.87U/ml and 16.23U/ml. Fractions with maximum O.D from CMC (carboxy methyl cellulose) column were pooled to gether and named as "C", "D" and "E". Fraction "c" does not show any enzyme activity due to its mixed fraction value.The enzyme activity of eluted fraction "D" obtained for 0.10 M NaCl in acetate buffer is 23.97U/ml. The enzyme activity of eluted fraction "E" obtained for 0.25M NaCl in acetate buffer is 21.36U/ml.

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