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ISOLATION AND AFFINITY PURIFICATION OF PEROXIDASE FROM DAIKON

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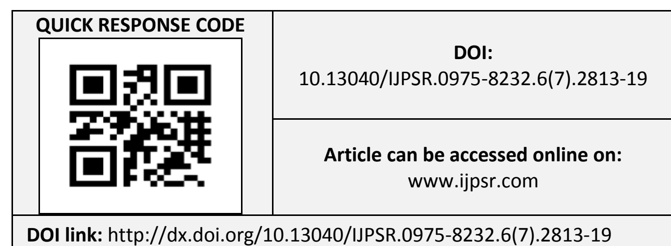
ABSTRACT: Peroxidase enzyme plays an important role in pharmaceutical, Industrial and various research laboratories. This enzyme can be purified by various different method. Our research trend was to purify the enzyme in a convenient and easy way. For this purpose a new affinity column was prepare by immobilizing PABA to N-HydroxySuccinamaideagarose gel. The presence of peroxidase was investigated on various Bangladeshi vegetables, such as, daikon, cabbage, cauliflower, tomato and sweet potato. Based on a previous screening experiment, daikon contains the highest amount of peroxidase. The juice of daikon was first extracted by tincture press and subjected to ammonium sulphate precipitation. The precipitate was dissolved in 25 mM phosphate buffer, pH7.4 containing 500 mMNaCl, 1 mM $MnCl_2$ and 1 mM $CaCl_2$. The crude extract thus obtained was subjected to an inhibitor affinity chromatography (IAC) method. Here, we have taken advantage of the affinity of peroxidase toward *p*-aminobenzoic acid hydrazide (PABAH) and subsequent recovery of the peroxidase by a known destabilizing agent ascorbic acid. The partially purified sample was applied to the column containing *p*-aminobenzoic acid hydrazide immobilized on agarose equilibrated with the same buffer. After washing the column with this buffer, daikon peroxidase was eluted with 5 mM ascorbic acid in the initial buffer. The eluted fraction showed 60 folds purification as judged by peroxidase activity measurement

INTRODUCTION: Peroxidase has a wide range of application in health science, food industry and in diagnostic purposes. It has been used in delignification of paper pulp. It is use in laundry detergents for bleaching of dyes in solution and thus preventing surplus dye from one garment to deposit on and decolorize other garments.



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Other potential applications are polymerization of chlorinated aromatic compounds in the treatment of waste-water, enhancement of flavors in food and polymerization of lignin in the production of various types of composite materials, where the use of artificial resins thus may be avoided. Various medical diagnostic kits contain appreciable amount of peroxidase in their compositions.

In spite of having the reported demand for peroxidase, no such efforts to produce peroxidase from Bangladeshi vegetables been reported in the literature to our knowledge. We have found that some indigenous vegetables contain considerable

amount of peroxidase and dikon contains height amount of peroxidise [1, 2] that can be purify in a very low cost. The objective of this study is to extend our previous work towards the development of a new technique to isolate and purify peroxidase from daikon in a convenient way. Then the purified enzyme [3, 4] can be used to develop low cost glucose diagnostic kit and also others diagnosis and industrial purposes.

Daikon (from Japanese daikon, literally "large root") is a mild-flavored East Asian giant white radish. Though most widely known as daikon, the radish is also known under other names, including daikon radish, Japanese or Chinese radish, winter radish, mooli (Hindi, Punjabi, Korean) or moo (Korean), moorro (Gujarati), moolah (Bengali). Daikon or Bengali moolahapproximately 20 to 35 cm (8 to 14 inches) long and 5 to 10 cm (2 to 4 inches) in diameter.

FIG 1: DAIKON

MATERIALS AND METHOD:

Collections and processing of daikon:

Daikon is a vegetable that available in our local market. The vegetable was purchased from local vegetables market and washed thoroughly with distilled water. After thorough cleansing, the vegetable was cut into small pieces as small as possible. The small pieces were blended with an electric blender. The blending was done for cell rapture and easy excretion of juice. The blended materials were collected in a large beaker.

Extraction of crude Peroxidase:

The blended materials were poured into Tincture Press Machine (HP-5, Karlkolb, Germany). The juice was collected by compression using Tincture Press at a maximum pressure of 400 bars. The juice was then filtered to remove any dirt material suspended in the juice. The clear supernatant was referred as crude extract.

Measurement of activity of the crude extract:

In this study, it is very important to confirm that the isolate contain active forms of enzyme. Therefore, we first measured the peroxidase activity of the crude extract isolated from cabbage. When we added hydrogen peroxidase to the enzyme solution in the liquid cell of the spectrophotometer, a sharp increase in the absorption at 436 nm was observed indicating the formation of tetraguaiacol from guaiacol, whereas absorption remained almost unchanged in the absence of crude extract. This indicates that the crude extract contain appreciable amount of peroxidase.

The volume of different reagents during assay of peroxidase (for 1 ml test) is given below:

TABLE 1: AMOUNT OF DIFFERENT REAGENTS FOR PEROXIDASE ASSAY

Sno.	Reagent	Volume
1	25 mM phosphate buffer, pH7.4 containing 500 mM NaCl, 1 mM	916 µl
2	4-amino phenazone (Dye), (37.4mM)	6.7 µl
3	Phenol solution (0.75 mM)	7.3 µl
4	Substrate (4.9 mM H_2O_2)	20 µl
5	Crude extract	50 µl
	Total reaction volume	1.00 ml

Partial purification of crude extract:

Purification of enzyme is vital in the study of their function and expression. Separation involves removing any contaminants that are present in the mixture; these may be other enzyme or completely different molecules. Enzymes are all made of amino acids of various lengths. The amino acids can be positioned in any order in the sequence giving an almost infinite number of enzymes [5, 6] that can be produced.

The sequence of amino acids alters the properties of the enzyme molecule in the form of charge, hydrophobicity, geometry, and solubility. The different enzymes can have similar shapes or isoelectric points. For this reason no one technique can be used to fully purify a mixture of enzymes [7,

[8, 9, 10,] therefore a sequence of physical and chemical procedure need to be carried out.

Outline of ammonium sulphate precipitation:
Precipitation of enzymes is a useful method for initial purification of enzymes from small organic molecules. Ammonium sulphate is one of the best known and used chemical for precipitating and concentrating enzymes. It is commonly used since it is highly water-soluble, relatively cheap and available at high purity. Furthermore, it has no adverse effects upon enzyme activity. Increasing the salt concentration to a very high level will cause enzymes to precipitate from solution without denaturation if done in a gentle manner. An enzyme in a buffer solution is very highly hydrated, in other words, the ionic groups on the surface of the enzyme attract and bind many water molecules very tightly:

When a lot of salt (ammonium sulfate), is added to the protein solution, the salt ions attract the water molecules away from the enzyme. This is partly since the salt ions have a much greater charge density than the proteins. So as the salt is added and these small ions bind water molecules, the protein molecules are forced to interact with themselves and begin to aggregate.

So when enough salt has been added, the enzymes will be begun to precipitate. If this is carried out at a cold temperature like in ice, the enzymes will precipitate. Thus, the enzyme can be collected by centrifugation and then dissolved in solution using a buffer with low salt content.

Purification of peroxidase by affinity chromatography:
Required chemicals:

I. 4-amino ethyl benzoate (Benzocaine)
II. Sodium bi-carbonate
III. Sodium chloride
IV. Ethanol amine
V. Hydrazine hydrate
VI. Acetic acid
VII. Ascorbic acid

Preparation of Affinity Column:
Hi-Trap NHS-activated HP 1ml column is made of polypropylene, which is biocompatible and non-interactive with biomolecules. The top and bottom frits are manufactured from porous polyethylene. It was delivered with a stopper on the inlet and a snap-off end on the outlet. The separation can be easily achieved using a syringe with the supplied luer adaptor, a peristaltic pump, or in a chromatography system.

Packing material of the column:
Hitrap NHS-activated column was packed with NHS-activated sepharose.

NHS-activated sepharose is designed for the covalent coupling of ligands containing primary amino groups. The medium is based on highly cross-linked agarose beads with 6 atoms spacer arms attached to the matrix by epichlorohydrine and activated by N-hydroxysuccinimide. The substitution level is ≈10 μmol NHS-groups/ml medium. Nonspecific adsorption of proteins to HiTrap columns is negligible due to the hydrophilic properties of the base matrix. The activated medium is supplied in 100% isopropanol to preserve the stability of the activated medium prior to coupling.

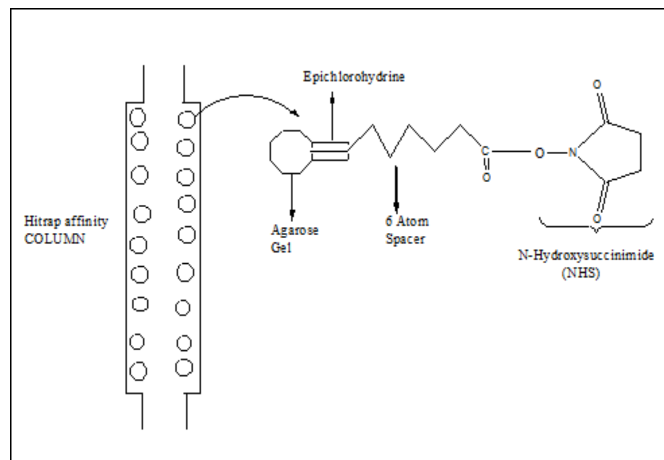

FIG. 2: Hi-TRAP AFFINITY COLUMN

Preparation of reagents:
i) Standard coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3):
1.68 gm. $NaHCO_3$ and 2.951gm.NaCl were dissolved in 100 ml distilled water and pH was adjusted to 8.3 by adding 1M HCl drop wisely with properstirring.

ii) mMHCl: 8.837μl 35% HCl was mixed with 100ml distilled water.

iii) Buffer A (0.5 M ethanolamine, 0.5M NaCl, pH 8.3):
0.05 ml ethanolamine and 2.951gm.NaCl were dissolved in 100ml distilled water and pH was adjusted to 8.3 by adding 5M Hcl drop wisely with proper stirring.

iv) Buffer B (0.1M acetate, 0.5M NaCl, pH 4): 573.62µl acetic acid and 2.951gm. NaCl were dissolved in 100 ml distilled water and pH was adjusted to 4 by adding 1 M HCl drop wisely with proper stirring.

v) Buffer (0.5 M Hydrazine hydrate, 0.5M NaCl):1.22 ml hydrazine hydrate and 1.48gm. NaCl were dissolved in 50 ml distilled water.

vi) 0.25 M Ascorbic acid solution: 4.403 gm. ascorbic acid (M.W: 176.13) was dissolved in 100 ml 25 mM phosphate buffer, pH7.4 containing 500 mMNaCl, 1mM $MnCl_2$ and 1mM $CaCl_2$

Ligand coupling:
The column can be operated with a syringe, peristaltic pump or chromatography system. Isopropanol (100%) was used to prevent deactivation of the NHS-groups.

i) The ligand (Benzocaine) was dissolved in the coupling buffer to an accurateconcentration (0.5–10 mg/ml for proteins). The concentration depends on the ligand being used. Optimal volume is 1 for HiTrap 1 ml column.

ii) The top-cap was removed and a drop of ice cold 1 mMHCl was applied to the top of the column to avoid air bubbles.

iii) The Hi Trapluer adaptor (or tubing from a system) was connected to the top of the column.

iv) The snap-off end was removed at the column outlet.

v) The isopropanol was washed out with 2 ml of 1mM ice-cold HCl. The operation was repeated for three times. The flow rate was maintained at 1 ml/min (1/2 drop/sec), otherwise the medium can be irreversible compressed.

vi) Immediately 1 ml of the ligand solution obtained in step 1 was injected onto the column.

Vii) Then the column was sealed and was allowed to stand for 15-30 minutes at +25 °C. In order to perform maximum coupling, ligand solution was recirculated by connecting a second syringe to the outlet of the column and gently pumping the solution back and forth for 15–30 minutes.

A buffer [0.5 M hydrazine hydrate, 0.5 M Sodium chloride, pH 8.3] solution was passed through the column (to produce inhibitor) to react the hydrazine hydrate with the benzocaine.

Washing and deactivation:
Any excess active groups that have not coupled to the ligand was deactivated and washed out the non-specifically bound ligands, by the following procedure:

Buffer A: 0.5 M ethanolamine, 0.5 M NaCl, pH 8.3

Buffer B: 0.1 M acetate, 0.5 M NaCl, pH 4

Washing with buffer A and B:
For washing of the column buffer A and B were injected alternatively as follows:
i) Injection of 3 × 2 ml of Buffer A.

ii) Injection of 3 × 2 ml of Buffer B.

iii) Injection of 3 × 2 ml of Buffer A.

iv) Then the column was allowed to stand for 15–30 min at room temp. Then again buffer A and B were injected alternatively.

v) Injection of 3 × 2 ml of Buffer B.

vi) Injection of 3 × 2 ml of Buffer A.

vii) Injection 3 × 2 ml of Buffer B.

viii) Finally, 2 ml of a buffer with neutral pH was injected to adjust the pH (phosphate buffer).

[The column was then ready for use. The column can be stored by using 0.1% NaN3, pH 7.]

Attachment of the spacer arm and inhibitor molecule into the column:
Specific enzyme can be highly purified by biospecific adsorption and subsequent desorption (affinity chromatography). The best results are obtained when the inhibitor, which has to be bound to solid support (agarose), adequately fits the receptor, or in case of an enzyme, the active site. However the best inhibitor is useless if bound too

close to the solid support. Cuatrecasas first had the idea of separating the inhibitor from the solid support by a 'spacer' of considerable length. In case of acetylcholinesterase, a spacer of about 45-58Å has been proved to be adequate. The conventional spacer- synthesis, however, is relatively time-consuming, as it requires several steps. To overcome this difficulty, we used commercially available cross-linked agarose beads with 6 atoms spacer attached to the matrix by epichlorohydrine, which has a considerable length.

Operation of the column:

5 ml of the crude sample was injected to column equilibrated with 25mM phosphate buffer, pH 7.4, containing 500 mMNaCl, 1 mM $MnCl_2$ and 1 mM $CaCl_2$ at a flow of 0.2 ml/min. The adsorption of peroxidase to the column was evaluated by taking a sample of the eluate every 5 min and analysing the content for peroxidase activity. Washing of the column in order to remove not bound material was carried out by pumping 25 mM phosphate buffer, pH 7.4, containing 500 mMNaCl, 1 mM $MnCl_2$ and 1 mM $CaCl_2$ at a flow rate of 1 ml/min until the absorbance in the eluate at 280 nm returned to base-line.

After binding, the gel-bound peroxidase is needed to remove from the column. The enzyme can be removed from the column by using a strong enzyme- inhibitor. For this purpose we choose ascorbic acid, but ascorbic acid precipitates the enzyme at higher concentration, therefore an investigation was done to find out the appropriate concentration of ascorbic acid at which it will inhibit the peroxidase activity without precipitating the enzyme.

Elution of gel-bound peroxidase from the column:

Gel-bound peroxidase was removed by elution with a strong enzyme inhibitor, ascorbic acid. Therefore it is necessary to determine the concentration of ascorbic acid that could able to release the gel-bound peroxidase by destabilizing it and at the same time the peroxidase could not be precipitated. An appropriate concentration and volume of ascorbic acid was found out by adding different concentration and volume with the enzyme solution.

RESULTS AND DISCUSSION:

Measurement of activity of the partially purified peroxidise:

In order to quantify the enzymatic activity, a specified amount of isolated peroxidase was added to the reaction mixture in the liquid cell and the absorbance was monitored at 546 nm with time. When the enzyme consumes all the added substrate the curve remained almost constant after 10 min as shown in **Fig.3.** The minor deviation in the constant part of the curve is due to decomposition of quinoneimine by contaminant enzymes.

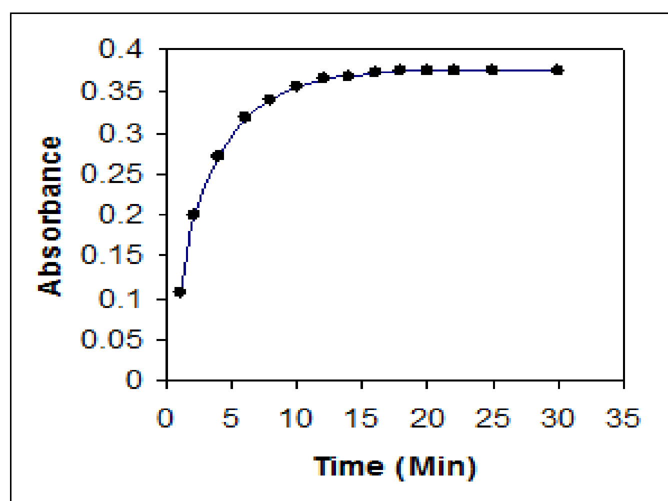

FIG. 3: CHANGES OF ABSORBANCE WITH TIME DURING KINETIC MEASUREMENT OF PARTIALLY PURIFIED PEROXIDASE FROM DAIKON.

Determination of ascorbic acid concentration for elution of enzyme from the column:

Ascorbic acid is a strong inhibitor of peroxidase. It is now well accepted that ascorbic acid inhibit the peroxidase activity by destabilizing its tertiary structure. In addition, ascorbic acid in high concentration, completely denature the enzyme as a result enzyme begins to precipitate. The results are presented in **Table 2**.

TABLE 2: DETERMINATION OF ASCORBIC ACID CONCENTRATION FOR ELUTION OF ENZYME

Sl No.	250 µl reaction mixture prepared by adding…..		Concentration of ascorbic acid (mM)	Appearance of the solution
	Volume of Peroxidase µl	Volume of ascorbic acid µl		
1	245	5	5	Clean
2	240	10	10	Cloudy

4	230	20	20	Cloudy
5	225	25	25	Cloudy
6	220	30	30	Cloudy
7	200	50	50	Cloudy
8	150	100	100	Cloudy

It was clear from the above table that 5 mM concentration (compared to other concentration) of ascorbic acid does not precipitate the enzyme. For this reason 5mM ascorbic acid was used to elude the enzyme molecule from the column.

Comparison of activity of peroxidase after different stage of purification:

To compare the activity of enzyme at different stage of purification, a test was done. The peroxidase solution was added to the reaction mixture in the liquid cell with some specific reagent as mentioned in the **Table 1.** The test was done separately for crude extract, partially purified and purified enzymeand the absorbance was monitored at 546nm with time. From **Fig.4** it is found that, the absorbance has been increased gradually for all cases and when the enzyme consumes all the added substrate the curve remained almost constant after 10 minutes. From the curve, the crude extract shows the minimum absorbance and the purified enzyme shows the maximum absorbance that is about 50 to 60 fold than that of crude extract.

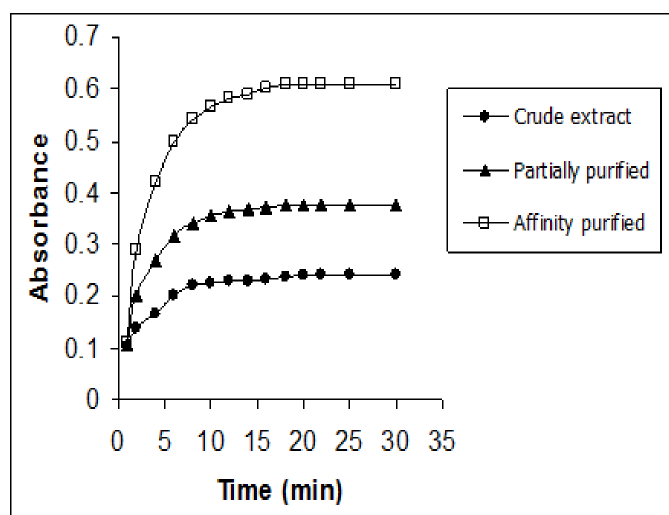

FIG. 4: COMPARISON OF THE RATE OF ENZYMATIC ACTIVITY OF PEROXIDASE AFTER DIFFERENT STAGE OF PURIFICATION

UV-Vis spectrum of peroxidase at different stage of purification:

Uv-Vis spectra of peroxidase at different stage of purification are presented in **Fig.4.** The spectrum was taken in 25mM phosphate buffer, pH 7.4 containing 500 mM NaCl, 1 mM $MnCl_2$ and 1mM $CaCl_2$. Usually peroxidase showed a small absorption band in the blue region of the optical absorption spectrum, called Soret band arise from the heme molecule attached to theprotein. Spectral analysis of all the enzymes revealed the presence of Soret band with a λ_{max} at 415 nm, indicating that all the isolated peroxidase were heme-containing enzyme.

TABLE 3: ESTIMATION OF THE YIELD OF PURIFICATION AT DIFFERENT STAGE

Sample	Amount of enzyme added to 1 ml reaction mixture	Slope of the initial part of the reaction	Purification fold
Crude extract	50 µl	0.016	1
Purification by ammonium sulphate precipitation	50 µl	0.032	2
Affinity purified	50 µl	0.096	60

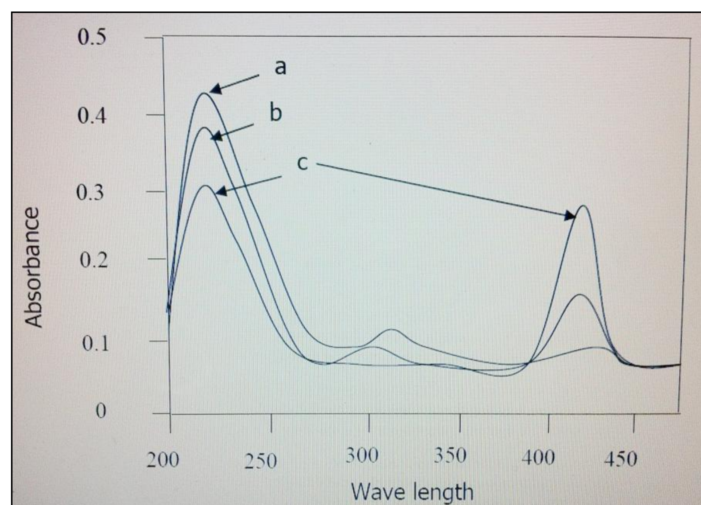

FIG 5: UV-VIS SPECTRUM OF PEROXIDASE, (a) CRUDE EXTRACT (b) PARTIALLY PURIFIED ENZYME (c) AFFINITY PURIFIED ENZYME

The location of the Soret absorption band of iron heme can provide the information about the denaturation of heme enzyme. When enzyme is denatured, the Soret band will shift or disappear. The presence of small Soret band also indicates that all the enzymes partially purified from different

vegetables were in a native condition. In addition peroxidase consists of several amide (peptide) backbone with various side chains on the α-carbons between each amide. The dominant chromophore of the amide group has a weak nπ* transition at about 220 nm and an intense ππ* transition at about 195 nm. Thus, absorption maximum at around 210 nm mainly arises from the peptide bonds present in the enzyme. The enzyme peroxidase also contains phenylalanine, tyrosin and tryptophan in its molecular structure. These aromatic amino acids show the electronic transitions at around 270 nm, which is also present in all the absorption spectrum.

CONCLUSION: Peroxidase enzyme has a wide range of application and it plays an important role in pharmaceutical and medical diagnosis, industrial purpose and also various research laboratories. But according to our demand, this enzyme is not available along with high cost. So we have tried to isolate and purify the enzyme by a simple and convenient way, so that, we can apply it in various sector at a very cheap rate. We can make glucose diagnostic kit, we can treat the industrial waste water and so on.

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