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Partial purification and characterization of esterase from the seeds of *Phaseolus vulgaris*

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Abstract
Carboxylesterases are hydrolases which catalyze the hydrolysis of various types of esters. In this study, carboxylesterases enzyme was isolated from the soaked seeds of *Phaseolus vulgaris*. Partial purification of esterases from *Phaseolus vulgaris* was done by conventional protein purification techniques such as CM-Cellulose ion exchange chromatography and Sephadex G-75 gel filtration Chromatography. Kinetic parameters such as pH, temperature, Km and Vmax of the purified enzyme was studied. Optimum pH value was determined as 6.5 and was found to be stable between pH range of 6-7. Optimum temperature was examined as 37°C with a ranging from 15 ºC - 37 ºC. The Km and Vmax values were found to be 0.15mM and 0.013µmoles/min, respectively. Finally, half-maximal inhibitory concentration IC^{50} was found to be 2.8×10^{-6}.

Keywords: *Phaseolus vulgaris*, carboxylesterases, purification studies, ion exchange, gel filtration, SDS page

1. Introduction
Enzymes are biocatalysts that catalyze biological and metabolic reactions. According to Enzyme commission classification of enzyme lipases and esterase comes under the class hydrolase and catalyzes the hydrolysis of ester bonds [1].
Ester compounds containing short-chain carboxylic acids are hydrolyzed by esterase (EC 3.1.1.1) whereas acyl-glycerides with long-chain are catalyzed by lipases (EC 3.1.1.3). However, the enzyme's 3D structures share the α/β hydrolase fold [2].
Carboxylesterases catalyze the hydrolysis of a wide variety of endogenous and exogeneous substrates including esters, thioesters, carbamates and amides. These are widely distributed in nature, found in animals, plants and microorganisms. They occur in multiple molecular forms and exhibit a number of unique enzyme characteristics such as substrate specificity, region specificity and chiral specificity [2, 3].
The functions of carboxylesterase enzymes have also been implicated in carbon source utilization, pathogenicity and detoxification. Especially, the potential application of these enzymes for the synthesis of short chain esters has attracted the interest of a broad range of industrial fields like foods, pharmaceuticals and cosmetics. These enzymes are also involved in fruit ripening, abscission, cell expansion, reproduction as well as hydrolysis of ester containing xenobiotic molecules [4]. Other significant functions of the carboxylesterases include metabolism and subsequent detoxification of many agrochemicals, pharmaceuticals, metabolism of a number of therapeutics, including the cholesterol-lowering drug, lovastatin, the anti-influenza drug oseltamivir, narcotic analgesic meperidine, cocaine and heroin, and resolution of racemic mixtures by transesterification, or the enantioselective hydrolysis of esters for obtaining optically pure compounds[7]. In addition to carboxyl ester hydrolase activity, reports have shown that some carboxylesterase enzymes possess amidase, dehydratase and phosphatase activity as they can utilize acetanilide, hydroxy isoflavones and organophosphates respectively [3, 4].
In the present work, purification, characterization of carboxylesterase isolated from the 72hrs soaked seeds of *Phaseolus vulgaris* was studied. Carboxylesterases from *Phaseolus Vulgaris* seed was partially purified by salting out method using ammonium sulfate fractionation followed by chromatographic techniques. The activity of the enzyme and its molecular weight determination was carried out using Native PAGE and SDS PAGE methods respectively [5]. The purified sample was characterized by determining their kinetic parameters such as pH, temperature, substrate concentration and maximum velocity.

Carboxylic esterase is identified by studying the inhibition response to DFP (diisopropylphosphofluoridate), serine and PCMB (p-chloromercuribenzoate). The present study aimed to observe and quantify the presence of esterase in germinated *Phaseolus vulgaris* seeds, assess their total and specific activity since no recent study has been carried out to characterize the properties of the carboxylesterase enzyme present in *Phaseolus Vulgaris*.

2. Materials and Methods

2.1 Plant materials

VL Rajma 125 (Chitra) of *Phaseolus vulgaris* were collected from Poorna Organic Pvt. Ltd. No 898, AECS Layout, Kundalahalli, Bangalore Bangalore KA 560037 IN.

2.2 Chemicals

Acetone, methanol, acetic acid, acrylamide, N, N methylene bis acrylamide, CM cellulose, G-75 Sephadex, ammonium per sulphate, 1-naphthyl acetate, fast blue RR salt, Folin Ciocalteau reagent, BSA, coomassie brilliant blue R250, β-mercaptoethanol, diazo blue, sodium lauryl sulpahte, dicholrovos, sodium hydroxide, sodium potassium tartarate, copper sulpahte. All chemicals were of analytical grade.

2.3 Methods

2.3.1. Preparation of crude enzyme extract: Enzyme used for the study was extracted from 72h germinated *Phaseolus vulgaris*. A 10% extract was prepared by taking 10g of germinated, dehulled seeds and homogenizing it with 90ml of 30mM chilled phosphate buffer of pH-7. The homogenate was centrifuged at 6000rpm for 10 minutes at 4 °C and filtrate obtained was filtered using a muslin cloth and appropriate dilution of the supernatant was used [8].

2.3.2 Standard curve for α-naphthol

α-naphthol is an organic compound having a hydroxyl group is attached to the carbon atom that is adjacent to the neighboring ring structure.

Here the compound is used as substrate for esterase activity and it develop a colored solution. Different aliquots of standard α-naphthol (0.1µmoles/ml) from 0.0 to 1.0ml is pipetted into different test tubes and volume is made up to 5ml with phosphate buffer (pH 7) incubate with 1ml of DBLS reagent for 15 minutes. Absorbance was read at 600nm.A graph of concentration of α-naphthol (n moles) against absorbance was drawn to obtain standard graph.

2.3.3 Standard curve for protein

A standard curve of a protein is used to accurately determine the concentration of the sample using the standard absorbance value. Protein estimation was carried according to Lowry *et al.*, (1951), using BSA as standard. Here, different aliquots of standard BSA from 0.2 to1.0ml is pipetted into different test tubes, volume is made up to 1ml using distilled water, incubate with 5ml of copper reagent after standing for 10 minutes, incubate with 0.6ml of FC reagent color developed after standing for 30 minutes was read at 660nm and amount of protein was calculated using calibration curve.

2.3.4 Determination of rate of esterase activity

The rate of activity was determined by incubating 0.25mM α-naphthyl acetate with 0.2ml to 1.6ml volume of enzyme in a 6.0ml reaction mixture for different time intervals. The reaction was arrested by adding 1.0 ml of DBLS reagent and incubated for 15 minutes. Absorbance was read at 600 nm. A graph of concentration of α-naphthol (µ moles) as a function of time against absorbance was drawn and rate was measured from the slope of the graph.

Protein concentration was determined according to the method of Lowry *et al*., using bovine serum albumin (BSA) as standard.

2.4 Partial purification methods

2.4.1 Ammonium sulphate precipitation: 50ml of crude enzyme extract was subjected to 0-30% saturation by slowly adding 8.8g of finely powdered ammonium sulphate by stirring on a magnetic stirrer in cold conditions. Then contents were allowed to stand for 30 minutes at 4 °C and centrifuged at 6,000rpm for 10 minutes at 4 °C. The obtained pellet was considered as pellet 1 and was mobilized in 3ml of 30mM phosphate buffer of pH 7 and kept for dialysis overnight. The supernatant was measured and whole 49ml of the supernatant was subjected to 30-80% saturation by slowly adding 17.19g of finely powdered ammonium sulphate by stirring on a magnetic stirrer, in a cold condition. Allow the content to stand for 30 minutes, at 4 °C. The obtained pellet was named as pellet 2 and was mobilized in 4 ml of 30mM phosphate buffer of pH 7 and was kept for dialysis overnight. Both the pellets from dialysis bag was taken and centrifuged at 6000 rpm for 10 minutes at 4 °C to remove the debris. Supernatant obtained from 30-80% precipitation and pellet 1 and 2 were collected and assayed for activity and total protein [9].

2.4.2 Ion exchange chromatography

1ml of ammonium sulphate fractionate, that is pellet 2 which showed higher activity was loaded in 10ml of CM-Cellulose column equilibrated with 50mM acetate buffer of pH
5.5 at room temperature. Fractions of 1.0ml each were collected at flow rate of 30ml/h. After sample loading, two bed volume of equilibrating buffer was run down. After the break through fraction, elution was carried out by step-wise elution method using 0.2M to 0.5M NaCl in 50 mM acetate buffer pH 5.5. The 1 ml fractions collected were assayed for esterase activity and estimated for protein. The elution profile was plotted and the fractions corresponding to major peak were pooled separately and further subjected to purification [11].

2.4.3 Gel filtration chromatography

0.5ml blue dextran was run to determine the void volume of gel filtration column. Ion exchange pooled sample was concentrated by ammonium sulphate precipitation and 1ml of the sample was loaded onto 50ml Sephadex G-75 column. Fraction of 1ml each were collected at a flow rate of 10ml/h. Elution was carried out by 0.1M NaCl in 50mM phosphate buffer, pH 7. 1 ml fractions were collected and assayed for esterase activity and proteins. The elution profile was drawn and fractions corresponding to major peak were pooled separately and run on gel.

2.4.4 Native Poly Acrylamide Gel Electrophoresis (Native-Page)

Analytical Native PAGE was carried at different stages of purification according to method of Davis (1957). Different amounts of proteins were electrophoresed on discontinuous-dis-gel system of 7.5% resolving gel and 5.0% stacking gel. The gels were stained for protein using coomassie brilliant blue R-250, and de-stained overnight using 7% acetic acid and 10% methanol. Esterase activity on polyacrylamide gels was detected by the methods of Hunter & Marker (1957). In-

gel, esterase activity was detected by immersing the gel in the solution of α-naphthyl acetate, 0.2% of fast blue RR, until brownish activity band was developed. Then the reaction was stopped and gel bands were fixed using 7% acetic acid.

2.4.5 Denaturing poly acrylamide gel electrophoresis (SDS-Page)
SDS-Page was carried according to Laemmli, U.K. (1970) known amount of esterase precipitation from Rajma Crude enzyme extract, ammonium sulphate fractionate, CM-Cellulose purified fractionate and gel filtration fractionate were electrophoresed on a 10% Resolving Gel (Solution A: 2.5ml, Solution C: 3.33ml, distilled water: 4.02ml, 10% SDS: 0.1ml, 10% APS 0.1ml and TEMED: 5µl) and 5% Stacking Gel (Solution B: 1.25ml, Solution C: 0.833ml, distilled water: 2.82ml, 10% SDS: 0.05ml, 10% APS: 80µl and TEMED: 5µl). After electrophoresis the gels were stained for protein using Coomassie brilliant blue R-250 and de-stained overnight in a solution containing 7% acetic acid and 10% methanol [12].

2.5 Kinetics parameter of partially purified esterase
Kinetics were carried out using purified sample.

2.5.1 Determination of Km and V max
A known amount of partially purified enzyme was incubated with different substrate concentrations in a 6.0 ml reaction mixture for 15 minutes at room temperature.
The reaction was arrested by the addition of 1.0 ml of DBLS and color developed on standing for 20 minutes was read at 600 nm. A plot of 1 /V against 1 /S was drawn to graphically determine Km and Vmax.

2.5.2 Determination of optimum temperature
A known amount of enzyme was preincubated at different temperature (10 °C-70 °C) for 30 minutes. At optimum temperature the reaction was started by adding 0.3 mM substrate, and after 15 minutes the reaction was arrested by the addition of 1.0 ml of DBLS reagent and color developed on standing for 20 minutes was read at 600nm. A plot of temperature (°C) against absorbance was drawn to graphically determine stability of the enzyme.

2.5.3 Determination of optimum pH
A known amount of enzyme was preincubated at different pH. The reaction was started by adding 0.3 mM substrate for 15 minutes at room temperature. The reaction was arrested by the addition of 1.0 ml of DBLS reagent and color developed on standing for 20 minutes was read at 600nm. A plot of pH against absorbance was drawn to graphically determine optimum pH.

2.5.4 Determination of pH stability
known amount of enzyme was preincubated at different pH for 30 minutes. The reaction was started by adding 0.3 mM substrate in 30 mM phosphate buffer of pH 7.0, and after 15 minutes the reaction was arrested by the addition of 1.0 ml of DBLS reagent and color developed on standing for 20 minutes was read at 600nm. A plot of pH against absorbance was drawn to graphically determine stability of the enzyme activity.

2.5.5 Determination of IC^{50}
The enzyme was incubated with equal volume of different concentration of Inhibitor (Dichlorovos) for 10 minutes. The reaction was started by adding 0.3 mM substrate, and after 15 minutes the reaction was arrested by addition of 1ml of DBLS reagent and color developed on standing for 20 minutes was read at 600nm. A plot of PI against % inhibition was drawn to graphically determine IC^{50}.

Result
The purification table 2 showed decrease in % yield and increase in fold purification after each step, also indicating enzyme was purified

Ion-exchange chromatography
Ammonium Sulphate fraction of crude esterase leads to improved specific activity of 0.052 IU/mg of protein and yield of 13.08%. CM-Cellulose Matrix-Ion-exchange chromate-graphy (Cation exchange chromatography) yielded a peak of esterase activity. The specific activity of the peak was 0.655 IU/mg of protein.

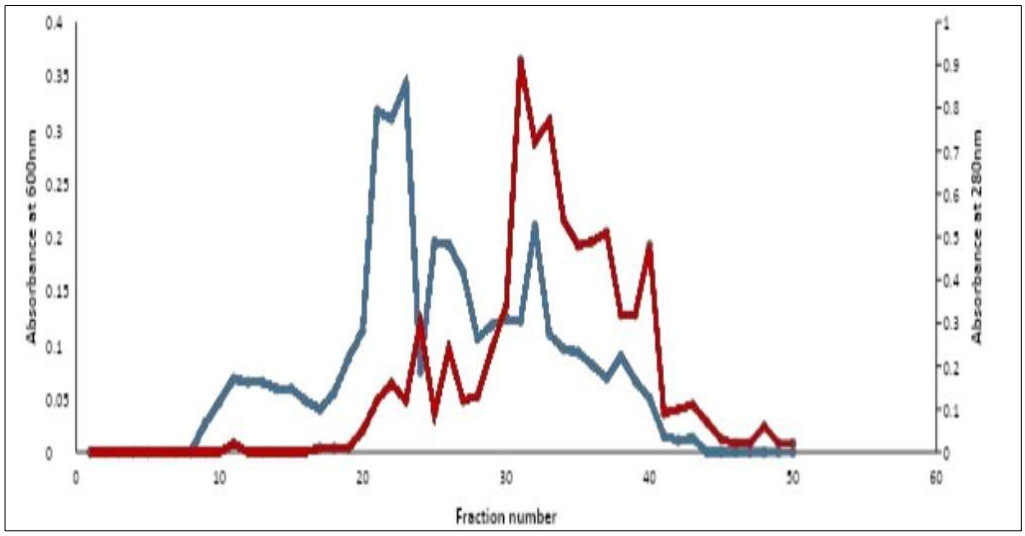


Fig 1: Calibration curve generated for Ion-exchange Chromatography (Cation exchange chromatography) of Ammonium sulphate fractionate of *Phaseolus Vulgaris* esterase. Blue line Total protein and red line total activity.

Table 2: Purification Table of *Phaseolus Vulgaris* esterase (Per gram tissue)

Sample	Total Volume (ml)	Total Activity (IU)	Total Protein (mg/g)	Specific Activity (IU/mg of protein)	Percentage Yield	Fold Purification
Crude	64	2.66	62.112	0.042	100	1
Ammonium sulphate precipitation (30-80%)	7.8	0.384	6.73	0.052	13.08	1.230
Ion Exchange Chromatography	2.8	0.27	0.412	0.655	10.15	15.59
Gel Filtration Chromatography	2.7	0.213	0.22	0.968	8	22.85

Gel filtration

Gel filtration chromatography (Sephadex G-75) yielded a peak of esterase activity. The specific activity of the peak was 0.96 IU/mg of protein.

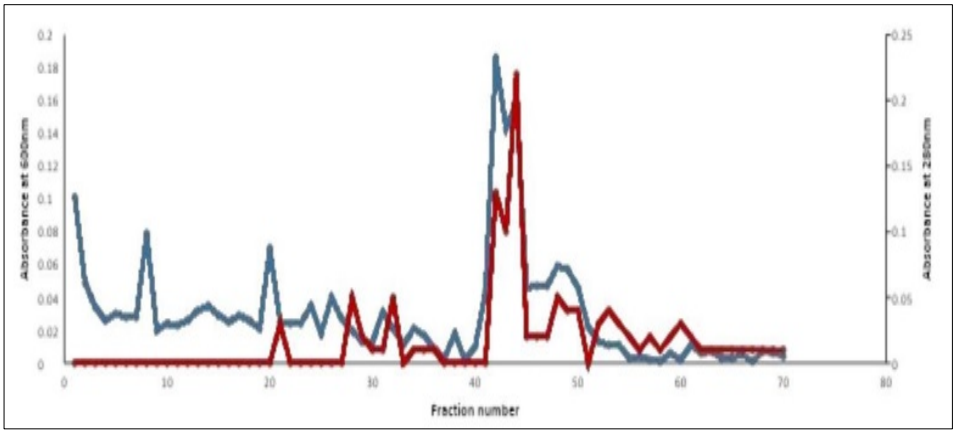


Fig 2: Gel filtration chromatography of ion exchange pooled sample. (Blue line-total protein and red line-total activity).

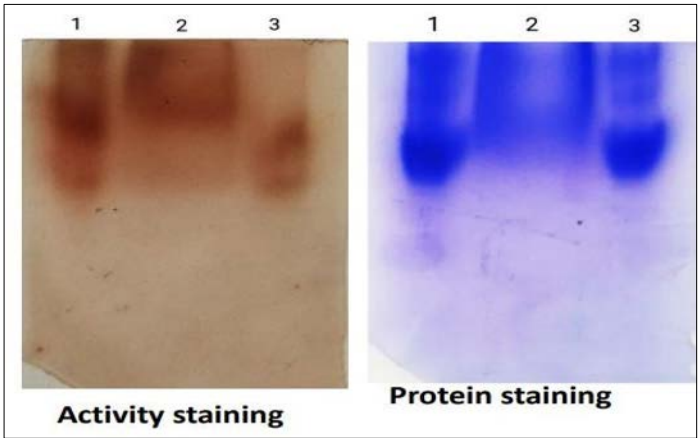


Fig 3: Native PAGE: Lane 1-*Phaseolus Vulgaris*, Ammonium sulphate fractionate, Lane 2-CM-Cellulose purified fractionate and Lane 3-Gel filtration fractionate. Electrophoresed gel was stained for esterase and protein using Coomassie Brilliant Blue-R250

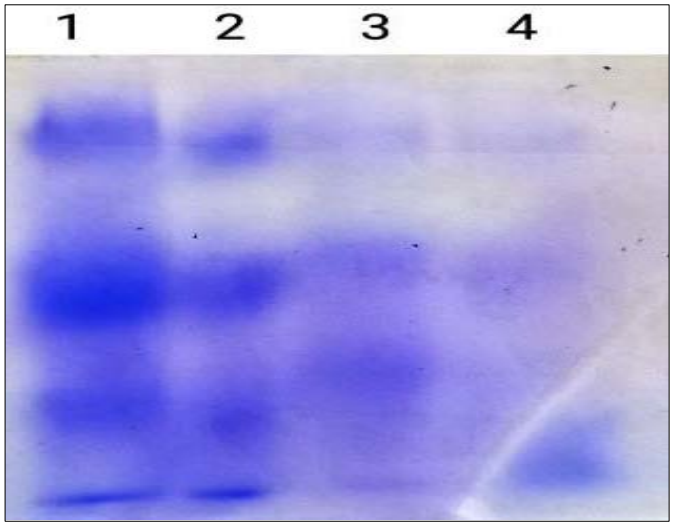
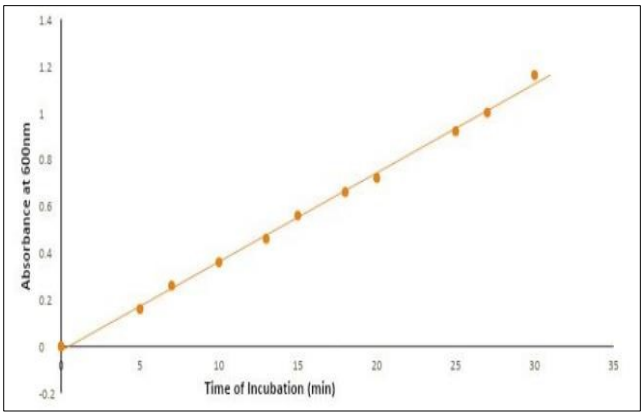
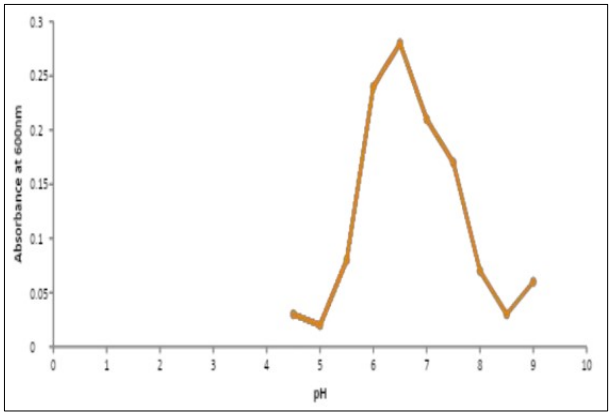


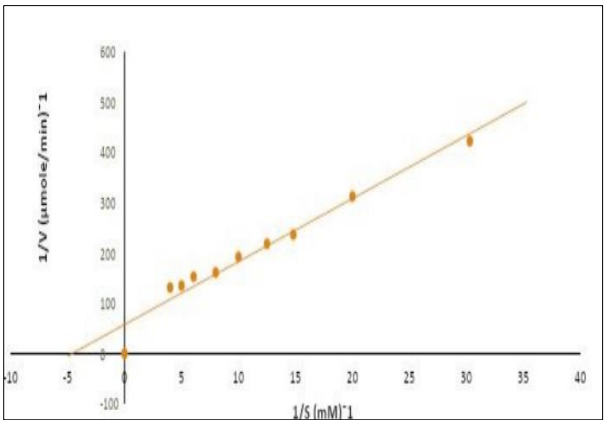
Fig 4: SDS-PAGE: Lane 1-*Phaseolus Vulgaris*, Crude Enzyme extract, Lane 2-Ammonium sulphate fractionate, Lane 3-CM-Cellulose purified fractionate and Lane 4-Gel filtration fractionate. Electrophoresed gel was stained for protein using Coomassie Brilliant Blue-R250



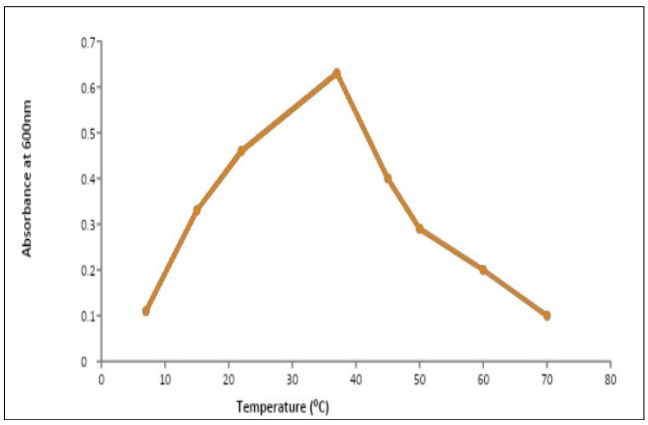
a. Determination of Time curve



b. Determination of Optimum pH



c. Determination of Km AND Vmax



d. Determination of Optimum Temperature

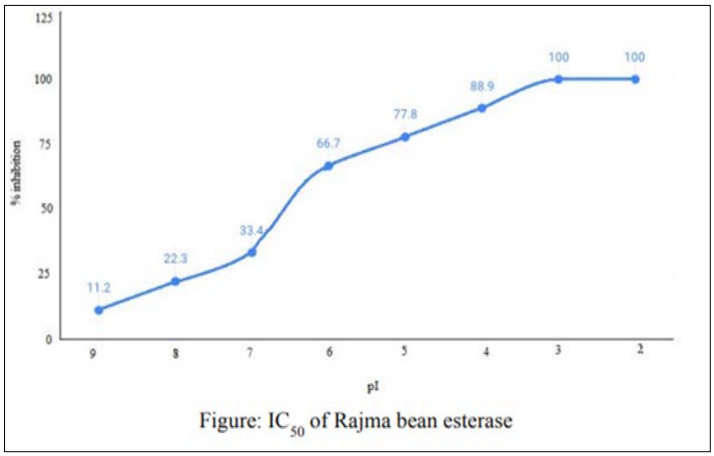


Figure: IC_{50} of Rajma bean esterase

e. Determination of IC_{50}

Fig 5: a) Effect of time on *Phaseolus Vulgaris* esterase, b) Effect of Optimum pH of partially purified *Phaseolus Vulgaris* esterase, c) LB-plot of Ammonium sulphate fractionate of *Phaseolus Vulgaris* esterase depicting Km and Vmax, d) Effect of temperature stability of partially purified *Phaseolus Vulgaris* Esterase, e) IC_{50} of *Phaseolus Vulgaris* esterase was found to be 2.8×10^{-6}

Discussion

The purified *Phaseolus vulgaris* seed esterase separated by PAGE were stained with 1-naphthyl acetate in conjunction with OPs, carbamates and sulphydryl inhibitors [1]. The esterase is inhibited only by OPs and hence it is classified as carboxylesterase. Similar observations were noticed in case of insect carboxylesterases and plant carboxylesterases [6].

Carboxylesterases were isolated and purified from various sources including plants, animals, and microorganisms. Multiple forms of esterase catalyzing the hydrolysis of carboxylic esters of short chain fatty acids have been demonstrated in several plant tissues including leaves and seeds of *Phaseolus* species.

The purification steps employing classical/conventional protein chemistry methods resulted in partial purification of esterase. The purpose of purification is to isolate specific enzymes from a crude extract of cells containing many other unwanted components in order to obtain the maximum specific activity with the best possible recovery of the initial activity. Esterase exists in multiple forms and exhibit variable substrate specificity. Before purification of the enzyme, initial kinetics is to be formed to standardize the working condition. Here, the enzyme of study common bean esterase was purified partially by ammonium sulphate precipitation (30-80%).

At the temperature 37 °C Phaseolus Vulgaris esterase activity shows highest value and at the temperature above at 40 °C enzyme activity significantly decreases. Below the optimum temperature the molecules have less kinetic energy and consequently collide less frequently, resulting in less

reactions. A rise in temperature increases the reaction speed while the enzyme conserves its native structure. However, most enzymes are denatured at temperatures above 40 °C - 60 °C and lose their catalytic power.

Primary purification was carried out by applying ammonium sulphate precipitation, Acetone precipitation and pH precipitation. Out of these, ammonium sulphate precipitation yielded 13.08%. Ammonium sulphate fractionation helped to get rid of most unwanted proteins, and gave better yield and fold purification. It is acidic in nature and has low hygroscopicity and chemical stability. Then the ammonium sulphate fractionate was subjected to CM-Cellulose Ion exchange chromatography which yielded better specific activity and improvement in fold purification. Most of the carboxylesterases studied so far, have low pI values contain large proportions of acidic amino acids. The isoelectric pH value and binding affinity to cation exchanger probably indicate the presence of large proportions of basic amino acids in this enzyme [8].

Later the ion exchange pooled sample was further purified using G-75 Sephadex Gel filtration chromatography. The kinetic characterization was carried out using ammonium sulphate fractionate [9]. The homogeneity of the preparation was checked by Native-PAGE and SDS-PAGE. Native gel electrophoresis was carried out using 7.5% resolving gel. Bands corresponding to activity and protein were obtained indicating their purification by the reduction of bands. Then the samples were subjected to SDS PAGE using 10% resolving gel.

The purification table showed decrease in % yield and increase in fold purification after each step, also indicating enzyme was purified. There are so many other chromatographic techniques are there based on their criteria we have to choose that. In that one of the most commonly used technique is Affinity Chromatography and it is also used in the purification of a biomolecules with respect to specific binding of that biomolecule due to chemical structure

In the first step of purification will found divers types of bands of various molecular weights, on gel. After the various purification steps, we should see the disappearance of certain bands concomitant with the increasing concentration of a certain band representing our protein. If we have successfully purified our protein we should arrive at a constant specific activity and a single band on a gel. Actually, these types of techniques are used in food industries. Because it determines the economic value of the food product and it can impact the economic feasibility of new industries for alternative protein product.

Conclusion

Protein purification involves extraction and purification. In extraction process, there are different procedures to disrupt cells or tissues as well as different extraction solvents, depending on the nature of the cells or tissues. From the present academic project carried out, we have standardized conventional purification steps for esterase, which is involved in isolation and partial purification and characterization of carboxyl esterase from Rajma bean.

Conflict of Interest: No conflict of interest

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