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AQUEOUS TWO PHASE EXTRACTION OF PROTEASE FROM NEEM LEAVES [*Azadirachta indica*]

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ABSTRACT:

Neem (*Azadirachta indica*) leaves contains 7.1% of protein, hence proteolytic activity was studied in neem leaves using casein as substrate. It was found neem leaves is an experimental material for the study of biological activities of proteins, this paper reports the presence of proteolytic activity in neem leaves. Aqueous two phase partitioning of protease from neem leaves was studied on systems containing polyethylene glycol PEG 6000-K_2HPO_4 and PEG 8000-K_2HPO_4 to obtain comparative results. The results revealed that specific enzyme activity and amount of protein was found to be high in the top phase of the PEG 8000/ Phosphate system and it was about 124.4 U/mg and 900 µg respectively. Also, effect of kinetic parameters on protease were studied and optimum temperature, pH, Half life period were found as 40°C, 6.5, and 9 minutes respectively.

Keywords: Protease, Azadirachta indica, aqueous two phase system, casein, polyethylene glycol and kinetic parameters.

Abbreviations: Polyethylene glycol (PEG), Trichloroacetic acid (TCA), Aqueous two phase system (ATPS) and molecular weight (MW).

1.INTRODUCTION:

Proteases are the enzymes that breakdowns proteins. Protein is a macromolecule made up of amino acids linked by peptide bonds and it is responsible for the translation of information encoded in DNA into physical form [1,2]. Proteases are involved in plant life cycle regulation and aging process, recycling damaged plant proteins, modifies proteins to perform specific functions, defense [3,4] with

wide applications in several industries such as leather industry, food industry, pharmaceutical industry and preparation of laundry detergents because it is stable and active even in the presence of surfactants, bleaching agents and fabric softeners [5,6].Plant proteases are stable over wide range of pH, temperature, substrate specificities because of these properties it is used for human digestive problems which does not requires enterical coating and works by proactive approach. Plant enzymes can be efficiently extracted in larger amount at lower cost [7].

Azadirachta indica (neem) is an evergreen tree of dry areas of Asia and East Africa [8] and known for its medicinal properties such as anti-malarial, anti-fungal, anti-tumour and anti-bacterial activity [9]. Neem finds wide application in the production of medicines, cosmetics, pesticides, manure and animal feed [10].The composition of fresh neem leaves is protein-7.1%, fat-1%, fibre-6.2%, carbohydrates-22.9%, minerals-3.4% and moisture-59.4% [11].

Aqueous two phase systems (ATPS) are formed when two immiscible polymers or a polymer when mixed with chaotropic or kosmotropic salt in water [12]. ATPS provides very mild environment for biological materials because of the presence of 85-95% of water and non-volatile components [13,14]. Formation of phase is affected by pH, temperature and ionic strength. Partitioning of compounds occurs rapidly because it depends on the surface properties like size, charge and hydrophobicity [15]. ATPS has several advantages such as it stabilizes target molecule, lower interfacial stress, high yield, high purity, low energy requirements and low labour cost [16].

In this work, the extraction of protease from neem leaves in an aqueous two phase system containing polyethylene glycol and dipotassium hydrogen phosphate was studied using PEG of various molecular weight like PEG 6000 and PEG 8000 to obtain comparative results. PEG-phosphate system has several advantages over PEG-Dextran system such as lower cost and lower viscosity so the process becomes amenable for scale up. Denaturation of proteins by salt can be prevented by using salt at lower concentration [17,18].

2. MATERIALS AND METHODS:

2.1. Chemicals: Polyethylene glycol (MW 6,000 and 8,000), Dipotassium hydrogen phosphate (k_2HPO_4), Potassium dihydrogen phosphate(KH_2PO_4), Casein, 10% Trichloro acetic acid(TCA), Sodium hydroxide(NaOH), Sodium carbonate(Na_2CO_3), Sodium potassium tartrate, copper sulphate($CuSO_4$) and Folin phenol reagent. All the chemicals were obtained from Himedia laboratories, Mumbai, India are of analytical grade.

2.2. Plant material: Neem (*Azadirachta indica*) leaves were obtained from the garden of sastra university,Thanjavur. Leaves were washed with sterile distilled water and ground to paste using mortar. To this paste, double the amount of extraction buffer (20 mM tris HCl) was added and centrifuged at 6,000 rpm for 10 minutes at 4°C. The supernatant of the sample was precipitated using ice cold acetone, the precipitated sample was subjected to centrifugation at 6,000 rpm for 10 minutes at 4°C and the pellet was washed with ice cold acetone to remove the pigments and lipids. Finally, the total protein concentration was determined using Bradford method (Bradford1976).

2.3. Aqueous two phase extraction: PEG (MW 6,000 and 8,000) of 50% concentration and 40% K_2HPO_4 was prepared using pH 7 buffer. In a sterile test tube, 5.0 ml of PEG (MW 6,000 and 8,000), 5.0 ml of K_2HPO_4 and 2.0 ml of leaf extract was taken and left

undisturbed for 10 minutes at room temperature. This mixture was centrifuged at 4,000 rpm for 10 minutes. Top phase and bottom phase were collected and subjected to casein assay and Bradford method (Bradford 1976)

2.4. Protein assay: Protein concentration was estimated using Bradford method (Bradford 1976). Bradford reagent was prepared by dissolving 100 mg of Coomassie Brilliant Blue G 250 in 50 ml of 95% ethanol and 100 ml of 85% phosphoric acid. It is made upto 1.0 liter by adding sterile distilled water. Protein content was measured by adding 100µl of leaf extract to 2.0 ml of Bradford reagent and 900µl of pH 7 buffer. Absorbance was measured at 595 nm using UV visible spectrophotometer (Spectronic UV-160A, Shimadzu, Japan). Protein content was measured using standard graph of BSA (10-100 µg/ml) [19].

2.5. Casein assay: In a sterile test tube, 5.0ml of 1% casein was taken and 250µl of leaf extract (protease) was added. The reaction mixture was incubated for 10 minutes at room temperature. After incubation, 5.0ml of 10% TCA was added and absorbance was measured at 280 nm.

2.6. Kinetic studies:

2.6.1 Effect of temperature on enzyme activity: Casein assay was carried out at various temperatures such as 40˚C, 50˚C, 60˚C, 70˚C and room temperature to determine the optimum temperature.

2.6.2 Effect of pH on enzyme activity: Casein assay was carried out at various pH ranging from 4, 4.5, 5, 5.5, 6, 6.5 and 7 to determine the optimum pH. pH was adjusted using phosphate buffer.

2.6.3 Effect of heat on enzyme activity: Casein assay was carried out for the sample maintained at 70˚C at regular intervals (5, 10, 15, 20, 25 and 30 minutes) to determine the heat stability and half life of the enzyme.

2.7.Formulas:

2.7.1. Determination of enzyme activity:

Enzyme activity = A280 nm / min * 0.001 @ specific conditions.

2.7.2. Determination of partition coefficient:

Partition coefficient is defined as the ratio of concentration of protein in top phase and concentration of protein in bottom phase.

3. Results and discussions:

3.1. Effect of ATPS on the yield and activity of protease: Protease from neem leaves was extracted using PEG 6000 and PEG 8000 50% (w/w) and 40% (w/w) phosphate ATPS. The maximum yield of 84% and the maximum specific enzyme activity of 124.4 Units/mg with 900 µg protein in PEG 8000/Phosphate top phase was obtained as shown in Table 1 and 2.This values are higher when compared with PEG 6000 and it clearly indicates that PEG 8000/Phosphate system enriches specific activity, amount and yield of protein at PEG rich top phase.

TableA.1. Activity, purification factor and yield of protease in the top phase of PEG/K_2HPO_4 system

Sample	Conc. (µg/µl)	Total activity (U/mg)	Total protein (µg)	Specific activity (U/mg)	Yield (%)	Purification factor (µg/µl)
Crude extract	0.55	60	550	109.09	100	--
PEG 6000	0.8	90	800	112.5	52.47	1.03
PEG 8000	0.81	112	900	124.4	84.65	1.14

TableA.2. Activity, purification factor and yield of protease in the bottom phase of PEG/K_2HPO_4

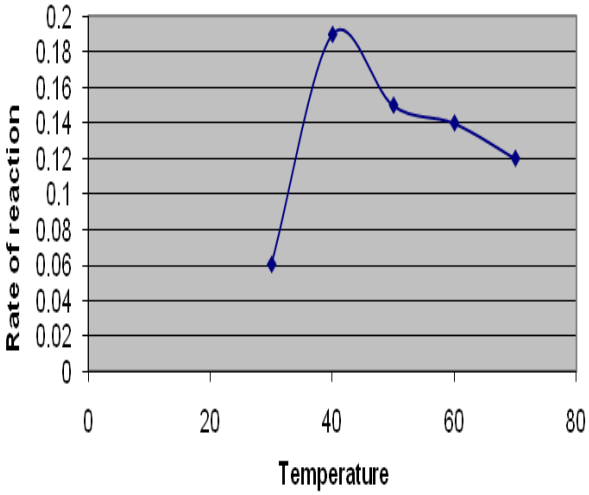
Sample	Conc. µg/µl	Total activity (U/mg)	Total protein (µg)	Specific activity (U/mg)	Yield (%)	Purification factor (µg/µl)
Crude extract	0.55	60	550	109.09	100	--
PEG 6000	0.55	50	550	90.9	15.34	0.83
PEG 8000	0.81	80	815	98.15	47.52	0.89

3.2. Kinetic studies:

3.2.1. Effect of temperature on enzyme activity:

Figure 1 shows the protease activity measured at different temperatures (40- 60°C) for 10 minutes using casein as the subsrate to determine the optimum temperature for protease activity and the maximum activity was found to be at 40°C.

Effect of temperature on enzyme activity

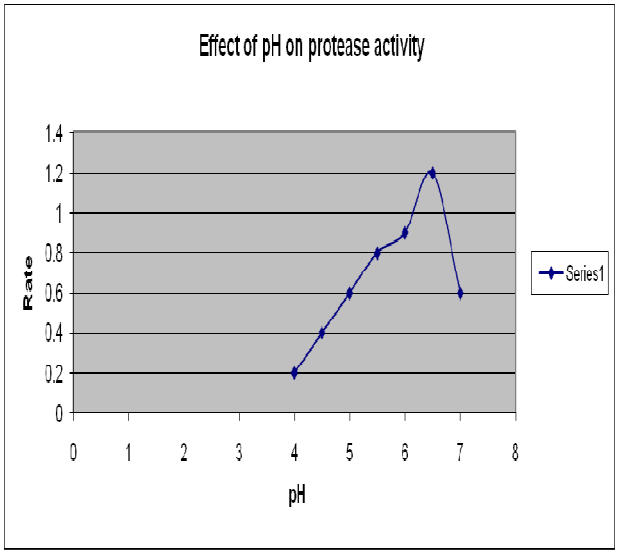


Rate of reaction and temperature is represented in g/ lit.min and ˚Celsius respectively.

Figure1: Effect of temperature on enzyme activity.

3.2.2. Effect of pH on enzyme activity:

Figure 2 shows the protease activity measured at different pH (4-7) using casein as the substrate to estimate the optimum pH for the protease activity and maximum proteolytic activity was found to be at pH 6.5.

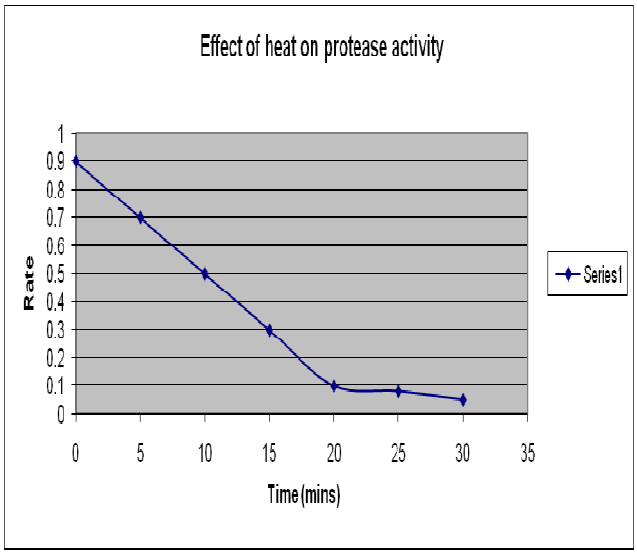


Rate of reaction is represented in g/lit.min.

Figure 2: Effect of pH on protease activity.

3.2.3. Effect of heat on enzyme activity:

Stability of enzyme at 70°C was studied at various time intervals and Figure 3 shows as time increases, there is a gradual decrease in the proteolytic activity of the enzyme.



Rate of reaction is represented in g/lit.min.

Figure 3: Effect of heat on protease activity at 70˚C.

. 3.2.4. Estimation of half life period of protease:

Half life is the time period taken by the enzyme to reduce half of its activity. It was estimated as 9 minutes by plot between ln (Vm/Vmo) vs Time (mins) as shown in figure5.

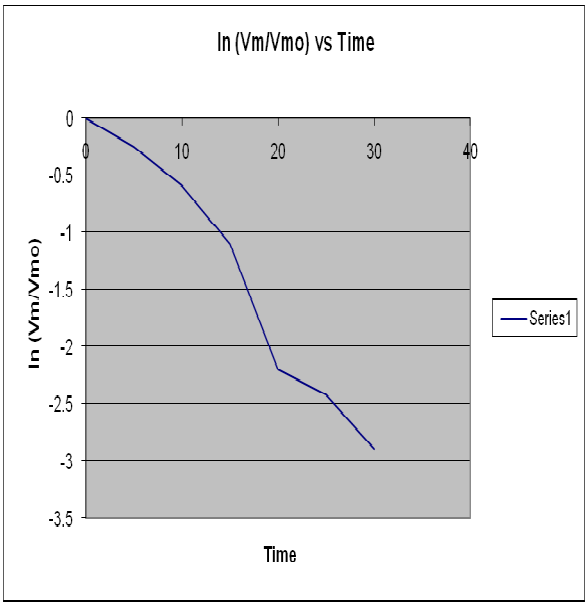


Figure 4: ln (Vm/Vmo) vs Time (mins).

4. CONCLUSION:

Aqueous two phase system containing polyethylene glycol and potassium dihydrogen phosphate system was used to extract protease from neem leaves. From the tested systems: PEG6000- K_2HPO_4 and PEG8000- K_2HPO_4, the latter was found to be the most effective in comparison with total protein content, yield and specific enzyme activity partitioned to top phase. Also kinetic studies which is the fundamental aspect to choose an enzyme in the industrial view was also performed.

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