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Utilization of Size Exclusion Chromatography for the Recovery of Microbial Pectinases

Kalim Ullah[1*], Omer Khiam[1], Muhammad Bilal[1], Azhar Uddin[2], Talha Salar[2], Kaleem Ullah[2], Simeen Monib[2], Monibullah[3], Husna Bibi[2] and Misbahullah[2]

[1]Institute of Biotechnology and Genetic Engineering the University of Agriculture, Peshawar, Pakistan
[2]Department of Microbiology, Abdul Wali Khan University, Timergara, Pakistan
[3]Department of Microbiology, University of Agriculture, Faisalabad, Pakistan

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***Corresponding Author:**
Kalim Ullah
Institute of Biotechnology and Genetic Engineering, University of Agriculture, Peshawar, Pakistan
kalimrahi88@gmail.com

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ABSTRACT

Size exclusion chromatography (SEC) is an effective analytical technique employed for the purification of biomolecules. In size exclusion chromatography (SEC), biomolecules are sorted according to their size. **Objectives:** To investigate the purification of pectinases from a microbiological source by size exclusion chromatography (SEC). To evaluate the amount of pectinase and total proteins in the collected fractions through measurement and qualitative analysis. **Methods:** Utilizing Sephadex G-25 as the stationary phase and a 0.05 M sodium phosphate buffer with increasing concentrations of NaCl as the mobile phase. Using a 3,5-dinitro-salicylic acid (DNS) assay and a pectin-containing agar plate assay, the existence of pectinases in the fractions that were taken was verified quantitatively and subjectively. **Results:** The increasing order of salt concentration was 0.15, 0.5, 0.8, 1 and 1.6 M NaCl concentration. At 0.15 and 0.5 M salt concentrations, desired proteins were strongly combined to the stationary phase of the Sequential Injection Chromatography (SIC) column and eluted at the last fraction while at 0.8, 1 and 1.6 M sodium chloride concentration pectinases were eluted in the early fractions as compared to the buffers containing a lower concentration of sodium chloride. **Conclusions:** It was concluded that the suitable NaCl concentration for the purification of pectinase enzyme through SEC was 0.8 M because at these concentrations pectinases can be separated very short time and at a low cost.

INTRODUCTION

Pectinases are the enzymes which can break down pectin. Pectin is a compound in the plant cell wall. It can lower the viscosity of the juice. The first commercial application of pectinases was to prepare wines and fruit juice in 1930 [1]. Pectinases are derived from different kinds of microorganisms [2, 3]. Pectinases are also reported to be produced in combination with other industrially significant enzymes by the same microbial isolate [4, 5]. Pectin enzymes can be classified into three groups mention the name of groups. This enzyme's activity on protein results in the formation of extremely polymerized soluble pectin [6, 7]. Approximately 70 million dollars were spent on pectinases worldwide [8], which accounted for only 5% of all enzyme sales worldwide. Aspergillus niger is typically used to make industrial pectinase enzymes (polygalacturonase, pectin esterase, and pectinlyase strains). Unexpected reactions may arise from the proteolytic or hydrolytic adverse effects of crude enzymes [9-11]. Commercial pectinases are used to extract and clarify sparkling clear juices (apple, pear, and grape juices), cloudy juices (citrus, prune, tomato, and nectar juices), and unicellular products by selectively hydrolyzing the middle lamella polysaccharides to preserve plant cell integrity [12]. But they do not have to be commercialized. Pectinases are among the most significant industrial enzymes [13].

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This study aims to inoculate microorganisms to produce the enzyme pectinase. To use gel filtration chromatography at various salt concentrations to separate the fermentation broth's supernatant from the microbial culture. To evaluate the amount of pectinase and total proteins in the collected fractions through measurement and qualitative.

METHODS

An experimental design was held from August 2024 to January 2025. Balance, Falcon tubes, PH meter, pipettes, tips, Eppendorf tubes, centrifuge, burette, vortex, beaker, magnetic stirrer, test tubes, water bath, racks, spectrophotometer, and cuvette. Sigma-Aldrich provided the isopropanol, ethanol, methanol, Tris, or sodium dodecyl sulphate. Merck was the supplier of ammonium sulphate. We bought sodium hydroxide from Riedel-de Haen in Seezle. Sodium chloride and yeast extract were bought. The supplier of glycine was Phyto Technology Laboratory Services, located in Shawnee, Kansas, in the United States. Hydrochloric acid was purchased from Scharlau , and Tris-HCl. The presence of pectinases was confirmed on nutrient agar plates. The agar plates were prepared using the composition NaNO3 (0.2%) KH2PO4 (0.1%) KCL (0.05%) MgSO4 (0.05%) Trypton (0.5%) pectin (0.5%) After the screening, 50 ml liquid broth medium having composition peptone(1%)yeast extract(0.5%)Nacl(1%)were inoculated by a loop full of the colony from the agar plate. The media were autoclaved at 121C °C for 20 minutes. The composition of the medium for pectinase production includes NaNO3 (0.2%), KH2PO4 (0.1%), KCL (0.05%), MgSO4 (0.05%), tryptone (0.5%) citrus peel powder (2.5%). After preparation of the media, it was autoclaved at 121°C for 20 minutes. The freshly prepared inoculum quantity of 0.3 mL/100 mL broth was added to the medium. The media was then incubated for at least 48 hours and then checked for the presence of pectinases. The cultured broth was then centrifuged at 7500 rpm for 10 minutes until a clear supernatant was obtained. Pectinase isolation was accomplished in several steps. The supernatant of a crude culture was applied to a Sephadex G-25 disposable column that was preequilibrated with 0.05 M sodium phosphate buffer. The protein elution and binding were performed with the same buffer. To check the effect of sodium chloride concentration on pectinase elution, sodium chloride concentration was increased stepwise. The salt concentration was further increased to 0.15, 0.5, 0.8, 1 and 1.6 M of NaCl. Twenty different fractions in each run were collected and further checked for pectinase activity and total protein concentrations. The unbound enzymes were eluted first. The combinations were incubated in separate test tubes (a and b) at 70°C in a shaking water bath for 10 minutes. After incubation, the test tubes were cooled under tap water, and only the control combination received 0.3 ml of crude enzyme. After adding the DNS solution to test tubes a and b, they were incubated in boiling water for 10 minutes. After incubation, the test tubes were cooled under tap water and transferred to 1.5 ml Eppendorf tubes for 5 minutes of centrifugation. Calculate pectinase units by comparing the difference in optical density (OD) at 575 nm between test and control combinations using the formula: Pectinase unit = optical density × 22/3. Additionally, total protein was computed as (optical density- 0.0247) / 0.0487. In the chromatographic experiment, gel filtration in group separation mode removed tiny molecules from bigger ones, such as salts or labels. Gel filtration chromatography beads were placed into a commercial 1.0 ml column and equilibrated with 10 column volumes of sodium phosphate buffer. Packing Column is a very perilous stage in gel filtration chromatography. A well-packed column is vital for high-resolution fractionation in gel filtration chromatography. For column packing Sephadex G-25 as supplied, fill up to one column volume and equilibrate with the help of 10 column volumes of (0.15 M sodium phosphate) buffer. In the chromatographic experiment, a mobile phase consisting of 0.05 M sodium phosphate buffer and varying concentrations of NaCl (0.15, 0.5, 0.8, 1, and 1.6 M) was utilized as both a binding and elution buffer. Key considerations included the buffer composition, pH, ionic strength, and the presence of denaturing agents or detergents, which could induce conformational changes in the target molecules. The protein sample was purified and free from particulate matter through centrifugation. After pre-equilibration, 300 ml of the protein sample was loaded onto the column using a pipette, and non-bound materials were eluted with 10 column volumes of buffer, while compounds of interest were collected in fractions using a simple step elution procedure. Following protein loading, different fractions of up to 1 ml were collected via a stepwise increase of sodium phosphate buffer for subsequent analysis of protease activity. To regenerate the gel filtration column, it was washed with 0.2 M sodium hydroxide (NaOH) and non-ionic detergents, then stored at 4°C in the dark with an antimicrobial agent (0.02-0.05% sodium hydroxide or 20% ethanol). The protein amount was determined using the Bradford assay, which measures the capability of proteins to bind to Coomassie Brilliant Blue, forming a complex with a significantly higher extinction coefficient than that of free dye. At 595 nm, absorbency was determined with a UV-visible spectrophotometer. 100 mg of Coomassie Brilliant Blue G250 was dissolved in 50 milliliters of 94% ethanol and 100 milliliters of 84% phosphoric acid to create the Bradford reagent. Then diluted to a final volume of 1 liter. To assess pectinase activity in the collected fractions, a DNS assay was performed according to standard procedures

RESULTS

The presence of pectinase enzyme in cultured broth was

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confirmed on nutrient agar plates using the well diffusion method. The clear zones around the wells indicated the presence of pectinases in the broth. Results found that by increasing the supernatant, the diameter of the zone elevated significantly, as shown in figure 1.

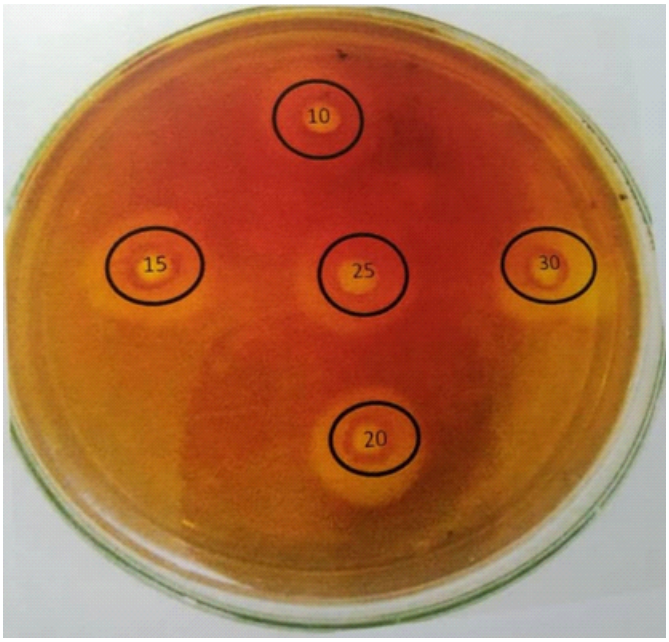

Figure 1: Plate assay for Pectinases: Zone Diameter After 48 Hours of Incubation

Results show that the fractions collected at the beginning have lower pectinase activity as well as lower concentrations of total protein. However, in the latter fractions, the pectinase enzyme activity was higher, as well as total protein. It was observed that a gradual increase occurred, and the highest activity (0.75) of pectinase enzyme was found in fraction no.19. The Pectinase enzyme unit and total proteins in the collected fractions using a gel G-25 column are presented in table 1.

Table 1: Standard Values of Libido

Fractions	Test	Control	OD	Pectinase Unit	Total Protein
1	0.06	0.04	0.02	0.14	0.00
2	0.08	0.08	0.01	0.04	0.00
3	0.06	0.02	0.04	0.27	0.25
4	0.01	0.09	0.00	0.00	0.00
5	0.04	0.06	0.00	0.30	0.00
6	0.05	0.00	0.05	0.38	0.56
7	0.06	0.05	0.01	0.08	0.00
8	0.06	0.02	0.04	0.29	0.29
9	0.01	0.02	0.00	0.00	0.00
10	0.04	0.07	0.00	0.00	0.00
11	0.08	0.02	0.06	0.42	0.66
12	0.07	0.02	0.05	0.40	0.60
13	0.05	0.02	0.03	0.25	0.19
14	0.03	0.02	0.01	0.06	0.00
15	0.09	0.02	0.07	0.48	0.83
16	0.07	0.02	0.05	0.37	0.54
17	0.03	0.02	0.01	0.10	0.00
18	0.08	0.02	0.06	0.41	0.64
19	0.13	0.02	0.10	0.75	1.59
20	0.10	0.02	0.08	0.56	1.05

Results illustrate the pectinase activity of the collected fraction through gel filtration sephadex G-25 column using 0.05M phosphate buffer along with 0.15 M NaCl, as binding buffer, as well as elution buffer elution pattern from G-25 column with size exclusion chromatography utilizing 0.05 M phosphate buffer along with 0.15 M NaCl. Fractions collected after elution were then checked for pectinase activity as well as for total protein. Black bars represent pectinases activity while gray bars represent total proteins, as shown in table 2.

Table 2: Table Pectinase Activity and Total Protein Concentration

Fraction No.	Pectinase Activity (U)	Total Protein (mg/ml)
1	0.08	0.30
2	0.18	0.55
3	0.00	0.15
4	0.04	0.30
5	0.00	0.55
6	0.07	0.90
7	0.00	0.20
8	0.10	0.60
9	0.00	0.40
10	0.06	0.55
11	0.24	0.90
12	0.00	0.60
13	0.40	1.00
14	0.12	0.75
15	0.34	0.60
16	0.20	0.90
17	0.06	0.55
18	0.00	0.30
19	0.70	1.50
20	0.50	1.20

The finding illustrates the pectinase activity of the collected fraction through gel filtration Sephadex G-25 column using 0.05M phosphate buffer along with 0.5 M NaCl, as binding buffer as well as elution buffer, shows purification of pectinases through the chromatographic process of the G-25 column. Supernatant of the crude culture was applied for elution from a G-25 size exclusion chromatographic column by using 0.05 M Sodium phosphate buffer along with 0.5 M NaCl. Twenty different fractions were checked for pectinase activity as well as for total proteins. Binding and elution of the target protein were performed with the same buffer. Black bars represent pectinase enzyme activity, while gray colored bars represent total protein concentration in the collected fractions, and the results are shown in table 3.

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Table 3: Pectinase Activity and Total Protein Concentration

Fraction No.	Pectinase Activity (U)	Total Protein (mg/ml)
1	0.00	0.00
2	0.00	0.00
3	0.00	0.00
4	0.00	0.00
5	0.15	0.10
6	0.25	0.20
7	0.35	0.30
8	0.00	0.00
9	0.10	0.05
10	2.30	1.00
11	0.00	0.00
12	1.10	0.60
13	1.20	0.65
14	0.60	0.40
15	1.20	0.65
16	0.30	0.20
17	0.10	0.05
18	0.20	0.15
19	2.80	1.30
20	2.00	1.10

Results illustrate the pectinase activity of the collected fraction through gel filtration sephadex G-25 column using 0.05M phosphate buffer along with 0.8 M NaCl, as binding buffer as well as elution buffer, represents the elution pattern through sephadex G-25 column size exclusion chromatography using 0.05 M phosphate buffer along with 0.8 M NaCl. The black bars illustrate pectinases activity while gray bars reveal total protein contents and findings shown in table 4.

Table 4: Estimated Pectinase Activity and Total Protein Concentration

Fraction No.	Pectinase Activity (U)	Total Protein (mg/ml)
1	0.00	0.10
2	0.00	0.00
3	0.00	0.00
4	0.90	0.45
5	1.10	0.60
6	1.40	0.70
7	2.30	0.90
8	1.00	0.40
9	1.40	0.60
10	0.70	0.30
11	1.00	0.50
12	0.80	0.40
13	0.60	0.30
14	0.90	0.45
15	0.50	0.25
16	0.70	0.35
17	0.20	0.10
18	0.60	0.30
19	0.10	0.05
20	0.00	0.10

Study illustrated the pectinase activity of the collected fraction through gel filtration Sephadex G-25 column using 0.05M phosphate buffer along with 1.0 M NaCl as binding buffer as well as elution buffer, showing elution pattern through size exclusion chromatography with G-25 column using 0.05 M phosphate buffer along with 1.0 M NaCl at PH 8.0. The samples were loaded on a size-exclusion chromatography Sephadex G-25 column. The fractions were applied to a gel filtration chromatography column. Twenty different fractions were collected and analyzed for pectinase activity and total protein content, and the results are shown in table 5.

Table 5: Pectinase Activity and Total Protein Concentration in Fractions

Fraction No.	Pectinase Activity (U)	Total Protein (mg/ml)
1	1	0.0
2	1	0.1
3	1	0.2
4	2	0.3
5	2	0.4
6	3	0.5
7	3	0.6
8	3	0.7
9	4	0.8
10	4	0.9
11	5	1.0
12	5	1.1
13	6	1.2
14	6	1.3
15	7	1.9
16	6	1.5
17	5	1.4
18	4	1.2
19	3	1.0
20	1	0.0

Illustrating the pectinase activity of the collected fraction through gel filtration sephadex G-25 column using 0.05M phosphate buffer along with 1.6 M NaCl, as binding buffer as well as elution buffer, shows purification mechanism from the sephadex G-25 column using 0.05 M phosphate buffer along with 1.6 M NaCl. Twenty different fractions were collected and then checked for pectinase activity and total proteins. Black bars represent pectinases activity while gray bars indicate total proteins, and results are shown in table 6.

Table 6: Pectinase Activity and Total Protein Concentration in Fractions

Fraction No.	Pectinase Activity (U)	Total Protein (mg/ml)
1	1	0.0
2	1	0.1

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3	1	0.2
4	2	0.3
5	2	0.4
6	3	0.5
7	3	0.6
8	3	0.7
9	4	0.8
10	4	0.9
11	7	1.2
12	5	1.1
13	6	1.3
14	5	1.4
15	4	1.5
16	5	1.6
17	7	1.7
18	4	1.4
19	3	1.2
20	1	0.9

DISCUSSION

The selected bacterial strain for pectinase production was confirmed with the help of pectin-containing agar plates [14, 15]. The pectinase function in the supernatant is shown by agar plates with clear zones surrounding the wells [16]. The pectinase enzyme in the supernatant has broken down the substrate pectin, visible by the clear zone surrounding the wells [17]. The outcome demonstrates that the area of clear zones surrounding the wells grows with the volume of supernatant, indicating a rise in the pectinase enzyme's efficiency. The hydrolysis zone produced on the pectin agar plates could be related to the results found in the literature [18]. In this investigation, gel filtration chromatography was used to purify pectinases from fermented cultured broth. The stationary phase was a gel filtration disposable column containing Sephadex G-25, while the mobile phase was 0.05 M phosphate buffer with increasing NaCl concentrations (0.15, 0.05, 0.8, 1.0, 1.6 M). Contact between the size exclusion stationary phase and proteins of interest separated them. The mobile phase pH and ionic strength were controlled to elute target proteins from chromatographic columns. Zones were not seen in total protein. Pectinase activity was measured by the DNS test and given as Soxhlet units, whereas total protein was measured by the Bradford assay [19]. Pectinase enzyme was found to be strongly bound to the stationary phase, and hence, pectinase was eluted in the last fraction, no.19. By increasing sodium chloride concentration up to 0.5 M, almost the same elution pattern of pectinase enzyme was observed. To examine how sodium chloride concentration affected pectinase enzyme elution, it was raised to 0.8 M. Pectinase activity at 0.8 M sodium chloride, with fraction 7 having the greatest activity. In our study, fraction 15 had the greatest pectinase enzyme activity and total protein content with 1.0 M NaCl. The sodium chloride concentration was raised to 1.6 M to further test its influence on pectinase enzyme elution. Greatest pectinase enzyme activity and total protein content. NaCl compounds in the buffer determine their ionic strength, which may explain this behaviour. A thick dielectric double layer and reduced protein-protein interaction (aggregation) depend on ionic strength. Sodium chloride affected protein elution volume. Salt in the mobile phase delineated protein peaks and inhibited protein-matrix interactions, improving molecular size determination [20].

CONCLUSION

It was concluded that this study can be further pursued for further purification of microbial pectinases by utilizing the same conditions for the pilot-scale purification of pectinases.

Authors Contribution

Conceptualization: KU[1], OK
Methodology: MB, TS, KU[2], HB, M[2]
Formal analysis: AU
Writing review and editing: SM, M[1]

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

All the authors declare no conflict of interest.

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