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ISOLATION AND PURIFICATION OF ALKALINE SERINE PROTEASE FROM THE MID GUT MICROBES OF BOMBYX MORI LARVA

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ABSTRACT

Commercial protease enzymes are usually produced from bacteria due to their high rate of production and simple downstream processing. Alkaline proteases have the ability to work efficiently in extreme industrial conditions and environmental friendly. In the recent years as an alternative to chemicals, researchers are trying to find suitable protease enzymes. Hence the aim of this study was to isolation and purification the alkaline serine protease from the midgut microbes of Bombyx mori larva. Results revealed the presence of Gram positive short tubular bacteria with endospores in the midgut of Bombyx mori larva, which can be an excellent source of alkaline protease. Purification of alkaline protease was done using precipitation of ammonium sulphate, dialysis, column chromatography with DEAE-cellulose column and gel filtration. From the SDS-PAGE study the molecular weight of the purified protease was found to be around 62 kDa

Keywords: Isolation, Purification, Microbes, Protease, Bombyx mori, larva

1. INTRODUCTION

Proteases result in more eco-friendly operations when compared to chemical based materials, as they generate less byproducts and wastes. Alkaline proteases are widely used in industries due to their great hydrolyzing ability and tolerance to alkaline conditions (Zhang, et al., 2023). These enzymes have great commercial applications in various industrial sectors and are expected to increase in the modern world (Cheng, et al., 2010).

Serine alkaline proteases are produced by several bacteria, fungi, yeast, plants and animals. The prime significance of these bacterial proteases is their ability to survive and optimal functioning even in extreme environmental conditions (Farooq, et al., 2021). Proteases isolated from microbes are known for their thermal stability (Ullah, et al., 2022), resistant to organic solvents, are also work efficiently in extreme pH, less time consumption, high yield, less space requirement, cost-effective, etc (Ali et al, 2016; Nisha and Divakaran, 2014). Capacity to produce huge quantity of proteases by the non pathogenic bacteria, directly into the media greatly reduces the downstream processing (Maurer, 2004). Due to these properties, proteases produced from microbes are playing major role in food, leather tanning, pharmaceutical, fiber, detergent industry etc (Mathew and Gunathilaka, 2015; Al-Dhuayan, et al., 2021). In the global market bacteria contribute about 60 % of the total serine protease production (Farooq, et al., 2021).

Considering the wide range of applications of these proteases, in the recent years researchers are aiming to explore new and high yielding serine proteases for producing commercial enzymes with enhanced properties. Hence an effort was made in the present study to isolate and purify the serine protease from the midgut microbes of *Bombyx mori* larva.

2. MATERIALS AND METHODS

2.1. Sample collection and screening

Silk moth *Bombyx mori*, larva collected for the study from Kolar district in Karnataka, India was used for isolation of gut microbe. Using casein agar media, pour plate method was followed to isolate the microbes from the fourth instar larva of silk moth which belongs to the family Bombycidae. Based on the Gram staining technique and morphological features various types of bacteria were identified. Gram positive and well developed short tubular bacteria with endospores were selected among the available colonies for further study.

2.2. Purification

150 ml of autoclaved cooled LB-broth was used to produce serine protease enzyme. After inoculated with the culture, it was incubated at 35^{o}C for 24 hours. Then the LB-broth culture was centrifuged for 15 minutes at 10,000 rpm and the supernatant was collected for further study.

2.2.1. Ammonium salt precipitation

The above collected supernatant was subjected to salt precipitation using ammonium sulphate. In an Eppendorf tube, supernatant was collected and labelled as crude. After measuring the leftover supernatant in a measuring cylinder, it was poured into a glass beaker and subjected to magnetic stirring at 850 rpm. The procedure was completed by adding ammonium sulphate salt pinch by pinch over the course of 3-4 hours and the supernatant was chilled overnight. The next day, whole solution was centrifuged at 8500 rpm for 15 minutes. The supernatant was discarded and to the pellet 10 mM of Tris HCl was added. The salt precipitated enzyme solution was further subjected to dialysis.

2.2.2. Dialysis

Dialysis membrane was soaked in 100 ml of hot water in a conical flask for some time. Carefully retrieve the bag with forceps and as it cools, one end of this was tied and check for leakage using water. Then the bag was filled with the pellet (salt precipitated enzyme) solution and zipped in the other end to keep it intact. The bag was placed in a beaker with full of water and allowed overnight to de-osmosis. The next day every half an hour, the water in the beaker was changed and subjected for magnetic stirring. Dialysis was performed by changing the distilled water every half an hour for 2 hours. After dialysis, the dialysis bag was pricked and the solution was collected in an Eppendorf tubes for further examination. The remaining solution was subjected to ion exchange chromatography.

2.2.3. Ion exchange chromatography

Initially the activity column was treated with methanol. Following that, the column was filled with water and sonicated for 15 minutes. The process was carried out repeatedly until the free flow of water from the column. The column was completely depleted of water and then filled with DEAE-cellulose gel. It was fully given the freedom to calm down on its own. By using Tris-HCl, NaCl, and distilled water, 7 different concentrations of elutions buffer were prepared (table 1). Elution 1 was added to the column to bring it into equilibrium. After the gel settles down, it was completely eluted. With the help of marker, the upper layer of the sample was marked on the column after the dialysis was added. Then the remaining elutions were added and collected in separate tubes until the elution reached the top layer of the sample. 1ml of each of the elutions was placed in Eppendorf tubes to be refrigerated.

Table 1: Elution buffer concentration for ion exchange chromatography

Sl. no	1M Tris HCl	1M NaCl	Distilled water
1	250 µl	250 µl	4.50 ml
2	250 µl	250 µl	4.50 ml
3	250 µl	250 µl	4.25 ml
4	250 µl	250 µl	4.00 ml
5	250 µl	250 µl	3.75 ml
6	250 µl	250 µl	3.50 ml

7	250 µl	250 µl	3.25 ml

For crude, salt precipitation, and dialysis samples, enzyme assay was performed to determine the best elution value. Protein estimation for the above obtained elutes were performed by Lowry's method.

Protein estimation by Lowry's method

BSA stock was prepared in the standard concentration of 1mg/ml. From the standard 1ml was taken and diluted to 4ml with distilled water to get working standard of concentration 200 µg/ml.

Alkaline copper reagent preparation

Reagent A was prepared by adding 2% Na_2CO_3 in 1N NaOH. Reagent B was prepared by adding 0.5% $CuSO_4$ in 1% potassium sodium tartrate. Both of these solutions were mixed in the ratio of 50:1. This makes reagent C, i.e., alkaline copper reagent. The solution C should be prepared freshly just before the start of experiment.

For standard, different aliquotes of BSA ranging from 0.2 ml to 1ml was pipetted out into different test tubes. The volume was made up to 1ml using distilled water. Similarly, 0.1 ml of sample crud, salt precipitation, dialysis, Ion Exchange 2 (IE-2)-Ion Exchange 7 (IE-7) were pipetted into the test tubes. To the test tubes, 5 ml of alkaline copper reagent was added and incubated at room temperature for 10 minutes. Following this 0.5 ml of Folin Ciocalteu reagent was added to the test tubes and incubated in dark room for 30 minutes. The absorbance was read spectrophotometrically at 660 nm using suitable blank and protein concentration was calculated.

Following estimation of protein, enzyme assay for the sample's crude, salt precipitation, dialysis, IE 2 to IE 7 was performed and spectrophotometric readings for enzyme activity were recorded at 660 nm.

2.2.4. Gel filtration

Following a methanol rinse, the water-filled gel filtration column was subjected to 10-minute sonication process. Till water came out, the sonication was repeated several times. The column was topped off with sefedex G-50 gel and given time to settle. The column's top section was noted using a marker pen and then elution sample was added to it. Glycine buffer was prepared, set to pH 7 and added to the gel filtration column considerably above that level. Collect the elution into 1 ml of 40 Eppendorf tubes till the solution level in the column comes down to the gel marked level. Take 0.1 ml of solution from each Eppendorf tube and make up to 3 ml using distilled water. Now OD of each tube was noted at 280 nm. Protein estimation and enzyme assays were carried out on particular samples acquired after chromatography to estimate the ideal protein content and enzyme activity, depending on the OD value.

2.3. Determination of molecular weight

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) technique was used for the determination of molecular weight of the purified protein. Based on the OD value gel filtration sample 8 and sample 32 were selected as protein samples for the study.

3. RESULTS

3.1. Isolation

Following the application of screening techniques, based on morphology and Gram staining method, many strains of bacterial colonies were observed. Among the available colonies, well developed Gram positive, short tubular with endospore bacterial colony was selected for further study.

3.2. Purification

3.2.1. Standard protein estimation by Lowry's method

Details of protein and enzyme assay for crude, salt, dialysis and ion exchange chromatography samples (IE 2 - IE 7) are summarized in table 2-4. In both protein and enzyme assay IE 3 is showing highest OD value 0.998 and 0.415 respectively.

Table 2: Protein estimation by Lowry's method

Test tube	Working BSA in (µl)	Sample (µl)	DW (µl)	Reagent C (µl)	Incubate for 10 min at RT	FC reagent (µl)	Incubate for 30 min in dark room	OD at 660 nm
Blank	-	-	1000	5000		500		0.040
1	200	-	800	5000		500		0.091
2	400	-	600	5000		500		0.165
3	600	-	400	5000		500		0.243
4	800	-	200	5000		500		0.252
5	1000	-	-	5000		500		0.326

Table 3: Protein estimation for samples by Lowry's method

Sample	Working BSA in (µl)	Sample (µl)	DW (µl)	Reagent C (µl)	Incubate for 10 min at RT	FC reagent (µl)	Incubate for 30 min in dark room	OD at 660 nm
Blank	-	100	900	5000		500		0.040
Crude	-	100	900	5000		500		1.099
Salt	-	100	900	5000		500		1.485
Dialysis	-	100	900	5000		500		1.143
IE 2	-	100	900	5000		500		0.775
IE 3	-	100	900	5000		500		0.998
IE 4	-	100	900	5000		500		0.985
IE 5	-	100	900	5000		500		0.632
IE 6	-	100	900	5000		500		0.320
IE 7	-	100	900	5000		500		0.229

Table 4: Enzyme assay for crude, salt, dialysis and ion exchange chromatography samples

Treatment	Substrate (ml)	Incubate for 5 min at 37°C	Enzyme (ml)	Incubate for 10 min at 37°C	TCA (ml)	DW	Incubate for 30 min at 37°C	Filter	Filtrate (ml)	Na_2Co_3 (ml)	Incubate for 10 min at RT	FC Reagent (ml)	Incubate for 30 min in dark room	Centrifuge at 6000 rpm for 10 min	O.D at 660 nm
B	5		-		0.5	0.5			2	5		0.5			0
Crude	5		0.5		0.5	-			2	5		0.5			0.401
Salt	5		0.5		0.5	-			2	5		0.5			1.140
Dialysis	5		0.5		0.5	-			2	5		0.5			0.469
IE 2	5		0.5		0.5	-			2	5		0.5			0.125
IE 3	5		0.5		0.5	-			2	5		0.5			0.415
IE 4	5		0.5		0.5	-			2	5		0.5			0.345
IE 5	5		0.5		0.5	-			2	5		0.5			0.329
IE 6	5		0.5		0.5	-			2	5		0.5			0.148
IE 7	5		0.5		0.5	-			2	5		0.5			0.156

3.2.2. Gel filtration

Out of the 1 ml elusion sample collected in 40 effendrof tubes, 100 µl sample from each effendrof tube was used for gel filtration and diluted using distilled water (2.9 ml of DW and 100 µl sample). OD of each tube was taken at 280 nm. Protein estimation and enzyme assay were carried out on particular samples acquired after ion exchange chromatography to estimate the ideal protein content and enzyme activity, based on the sample with greatest value. In gel filtration sample 8 and sample 32 showed highest OD value (table 5). The details of protein estimation and enzyme assay for gel filtration sample 8 and sample 32 are summarized in table 6 and table 7.

3.3. SDS PAGE

Results of the Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) for the determination of molecular weight revealed that the molecular weight of the purified protease was found to be around 62 kDa.

Table 5: Gel filtration at 280 nm

Test sample	Elusion vol (µl)	DW (ml)	OD at 280 nm
8	100	2.9	0.135
32	100	2.9	0.026

Table 6: Protein estimation for gel filtration samples

Sample	Working BSA in (µl)	Sample (µl)	DW (µl)	Reagent C (µl)	Incubate for 10 min at RT	FC reagent (µl)	Incubate for 30 min in dark room	OD at 660 nm
Blank	-	-	1000	5000		500		0.311
1	200	-	800	5000		500		0.391
2	400	-	600	5000		500		0.419
3	600	-	400	5000		500		0.453
4	800	-	200	5000		500		0.296
5	1000	-	-	5000		500		0.481
Sample 8	-	100	900	5000		500		0.735
Sample 32	-	100	900	5000		500		0.292

Table 7: Enzyme assay for gel filtration

Sample	Substrate (ml)	Incubate for 5 min at 37°C	Enzyme (ml)	Incubate for 10 min at 37°C	TCA (ml)	DW	Incubate for 30 min at 37°C	Filter	Filtrate (ml)	Na_2Co_3 (ml)	Incubate for 10 min at RT	FC Reagent (ml)	Incubate for 30 min in dark room	Centrifuge at 6000 rpm for 10 min	O.D at 660 nm
B	5		-		0.5	0.5			2	5		0.5			0
Sample 8	5		0.5		0.5	-			2	5		0.5			0.149
Sample 32	5		0.5		0.5	-			2	5		0.5			0.096

4. DISCUSSION

There is huge demand for the high yielding bacteria which can produce proteases to industrial environment. However, very little is known about the usage of midgut microbes from insects for the production of proteases. Results of the study indicated that short tubular Gram positive bacteria with endospores isolated from the midgut microbes of *Bombyx mori* larva can be a good source of protease.

Gimenez et al., (2000) and Studdert, et al., (2001) have mentioned that the molecular weight of alkaline proteases range from 20-130 kDa. Mary et al., (2017) reported the molecular weight of alkaline protease isolated from *Pseudomonas* sp. as 45 kDa and Chimbekujwo, et al., (2020) noted it as 68 kDa isolated from *Aspergillus brasiliensis*. From the results of the SDS-PAGE in the present study the molecular weight of the purified protease enzyme was found to be around 62 kDa. This result is in line with the results of the previous reports. Even though the production and purification of proteases involves many steps, the choice of technique should be economically feasible and cost effective (Manavalan, et al., 2020; Wajeeha, et al., 2021).

5. CONCLUSION

It is evidenced that Gram positive short tubular bacteria with endospores isolated from the mid gut microbes of *Bombyx mori* larva can be used as a source of protease. This protease was successfully purified using ammonium sulphate precipitation, dialysis, column chromatography and gel filtration. From the SDS-PAGE study the molecular weight of the purified protease was found to be around 62 kDa.

CONFLICT OF INTEREST

The authors declare no competing financial interest.

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None

AUTHOR CONTRIBUTIONS

Mohan Kumar H. M. Yashodha Lakshmi G. S. and Yashaswini C. conceived the study, performed the experiment and wrote the article.

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