

Estimation and Purification of 88 kDa Chitinase Isozymes from Silkworm *Bombyx Mori* L.

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ABSTRACT: Chitinase is a common proteolytic enzyme produced by insects during ecdysis and exists in different isozyme form. In the present study 88 kDa chitinase was extracted and estimated from 4th and 5th instar larvae and pupae of 5 races namely Bivoltine (NK2, CSR2), Multivoltine (PM, MH1) and Multi-hybrid (RP9×NK2) of silkworm, *Bombyx mori* L. The crude extract was analysed for comparison of total activity by DNS method using chitin as substrate followed by protein content and specific activity. Notable variation was recorded in the quantity and activity of the enzyme among different stages of same race and also among different races of the silkworm. Further, purification of the 88 kDa chitinase from the 5th instar larvae of each race was carried out using DEAE-sephadex Anionic column chromatography. This was followed by gel filtration chromatography for extraction of 88 kDa chitinase isozymes and for comparison of their specific activity. Purified chitinase enzyme of the multi-hybrid race RP9×NK2 showed the highest enzyme and specific activity.

KEYWORDS: Chitinase isozymes, specific activity, NK2, CSR2, PM, MH1, RP9×NK2.

I. INTRODUCTION

Chitin is the second largest bio-polymer next to cellulose, which is widely found in the integuments of arthropods, nematodes, molluscs and even in cell walls of algae, certain fungi and in most of the exoskeleton and shells of crustaceans [1]. Chitinases are been synthesized in various organisms with perceptible functionalities, including cell differentiation, digestion, defence ability against harmful pathogens, etc [2]. In plants, chitinases acts as a biocontrol agent against plant pathogens and thereby activating self defence ability against various fungal and bacterial pathogens. The plant chitinase [3] was said to be in lesser molecular weight compared to the insect chitinase, since of its endo forms. Likewise, there are wide ranges of microorganisms [4] producing chitinases to degrade chitin and used as source of nutrient for their growth and proliferation. The enzyme chitinase (EC 3.2.1.14) produced by insects hydrolyses chitin and is used for degradation of the exoskeleton during molting. Several studies have been carried out to understand and clarify the mechanism of molting mainly using the silkworm, *Bombyx mori* and the tobacco hornworm *Manduca sexta* [5].

This enzyme is believed to be glycosyl hydrolases of family 18 and 19, which slightly vary in their amino acid sequence and catalytic mechanism [6]. They exhibit wide range of variation and diversity in their enzymatic properties, domain structure and in their amino acid sequence [7]. Furthermore, [8] had reported that *B. mori* 88 kDa chitinase degraded to 54 and 65 kDa, and the 65 kDa protein may be degraded to a 54 kDa protein during storage. Since the isozymes are expressed from a single gene, it was reported that during the post translation period, the isozymes of chitinase enzyme were purified from 5th instar larvae of *B. mori* [9].

Current study has been made for comparison of the 88 kDa chitinase enzyme activity at 4th and 5th instar larvae and pupae of the different races of silkworms and purification of the enzyme.

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II. MATERIALS AND METHODS

Fourth, fifth instar larvae and pupae (100 Nos. each) of different races of silkworm *B.mori* L. namely Bivoltine (NK2, CSR2), Multivoltine (PM, MH1) and Multi-hybrid (RP9×NK2) were collected from Bidadi silkworm rearing unit, Bangalore, during the months of April and May. Chitinase enzyme was initially estimated in above mentioned developmental stages of silkworm for comparison. Later, sample of the chitinase enzyme of molecular weight 88 kDa was purified from 5th instar larvae.

- 1. Extraction of crude enzyme:** The integuments of the 4th and 5th instar larvae were obtained by dissecting and removing the midgut region. Immediately after dissection the integuments were washed in Ringer's solution, whereas the pupae was directly homogenized using extraction buffer (0.02M citric acid in 0.02M sodium phosphate pH 8.3) before centrifugation [10]. The homogenate was centrifuged at 5000rpm for 20 min at -4°C in order to obtain the crude enzyme. The supernatant i.e. the crude enzyme was stored at -4°C until used for further analysis.
- 2. Chitinase enzyme activity assay:** The chitinase enzyme activity was assessed by Miller's DNS method [11] using colloidal chitin as the substrate for the chitinase. 1ml of crude enzyme was added to 1ml 0.5% colloidal chitin in 0.1M citrate buffer (pH 7) in duplicates and samples were incubated at 37°C for 45 min. After the incubation, the reaction was terminated by adding 2ml of 5% DNS solution. The optical density of the samples at 575nm was measured after incubating the tubes at 50°C in water bath for 15 min. The activity was determined comparing the standard N-acetyl-D-glucosamine calibration curve, where one unit of enzyme activity was determined by the amount of enzyme that releases one micro mole of glucose per minute under the above mentioned conditions [12].
- 3. Total protein quantification:** Total protein concentration was measured by Lowry's method [13] with BSA as standard protein solution.
- 4. Enzyme purification:** In the initial step of purification the integument of the larvae were homogenized in the extraction buffer. The crude enzyme extract was concentrated by 50-80% ammonium sulphate precipitation. After centrifugation at 10000rpm for 10 min at -4°C, the precipitate was dialysed against the extraction buffer. The dialysate was mixed in the DEAE-sephadex column material and equilibrated with 0.1M sodium phosphate buffer (pH 7.5). The total mixture was gently stirred overnight, before poured into the glass column (3* 50 cm). The sample column was washed with 2-3 bed volumes the same buffer in order to remove the unbound proteins. The enzyme was eluted by linear gradient of 0.01M sodium phosphate buffer containing 0.1- 0.5M NaCl at the flow rate of 40ml/hour. The active fractions were dialysed against 0.05M sodium phosphate buffer (pH 7). Then the dialysate was centrifuged at 10000 rpm for 10 min and the corresponding supernatant was subjected for further purification by gel filtration chromatography.
- 5. Gel filtration chromatography:** The gel filtration column was equilibrated by deionised water till it reaches pH 7. The isozymes of chitinase were eluted with linear gradient of 0.1- 0.5M NaCl. The sample was eluted in 5ml fractions and protein content was measured spectrometrically at 280nm. The fractions showing peaks were pooled together and dialysed in distilled water at 4°C overnight. The dialysate was centrifuged at 10000 rpm for 10 min and the corresponding sample was used as purified enzyme sample and analysed further.
- 6. Gel electrophoresis:** SDS-PAGE was carried out according to the protocol described by [12, 14]. Stacking and resolving gels were made up of 5% and 12.5% (W/V) polyacrylamide and 0.1% (W/V) SDS. Samples were boiled for 3 min with gel loading buffer containing 25% (V/V) glycerol, 1% SDS in 0.6M Tris- HCl (pH 6.8) with 5% (V/V) 2-beta mercaptoethanol. Then the samples were loaded for normal protein staining and another for chitinase activity. Coomassie Brilliant Blue R 250 was used for protein staining. Standard protein markers were used for molecular weight determination.

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III. RESULTS AND DISCUSSION

Table 1. Comparison of the quantity and activity of chitinase in different races of *B.mori* L.

Races of Silkworm		PM	NK2	CSR2	MH1	RP9×NK2
4 th instar larvae	Total activity [Units]	390	990	1040	800	1190
	Protein content [grams]	5.9	9.4	11.7	6.6	12.7
	Specific activity [Units]	0.066	0.105	0.089	0.121	0.094
5 th instar larvae	Total activity [Units]	790	1260	1400	870	1790
	Protein content [gms]	9	12	11	8.2	15.1
	Specific activity [Units]	0.088	0.105	0.127	0.106	0.119
Pupae	Total activity [Units]	1200	1600	1850	1218	2020
	Protein content [gms]	10.1	13.1	12.1	10.2	17.1
	Specific activity [Units]	0.119	0.122	0.153	0.119	0.118

From the Table 1 it can be observed that the protein content as well as activity level varies among different developmental stages of the same race of silkworm and also among different races.

Protein content, total activity and specific activity showed an ascending trend from 4th instar to 5th instar and pupal stages in *B. mori* race PM. The race NK2 also recorded similar trend except specific activity, which remained same in both the larval stages studied. However, the pupal stage showed an increase in all the three parameters investigated. CSR2 recorded a slight reduction in the quantity of chitinase from instar 4th to 5th. The rest of the parameters showed an upward trend from 4th instar to pupal stage. Specific activity of chitinase showed a wavering trend in the race MH1 with decrease in the specific activity from 4th to 5th instar larval stages followed by a slight increase in pupal stage. The race RP9×NK2 showed an increase in quantity and unit activity of the enzyme from 4th to 5th instar and to pupae. But the specific activity showed a decline from 5th instar larva to the pupal stage. Wide variations have been observed in the quantities and activities of chitinase among different races of *B. mori* L.

The difference in the amount and activity of chitinase among different developmental stages may be correlated with the changing requirement of the enzyme for ecdysis from one developmental stage to the next. Some of the fluctuating trends of the enzyme quantity and activity in few races observed during the current study warrants further validation studies before coming to a conclusive remark. The difference in the extent of activity as well as quality of the enzyme between races can be attributed to their genetic configuration.

Table 2. Purification of 88 kDa chitinase from different races of *B. mori* L.

Races of Silkworm (5 th instar larvae)		PM	NK2	CSR2	MH1	RP9×NK2
Initial crude extract of larvae	Total activity [Units]	790	1260	1400	870	1790
	Protein content [grams]	9	12	11	8.2	15.1
	Specific activity [Units]	0.088	0.105	0.127	0.106	0.119
After DEAE- Sephadex column chromatography	Total activity [Units]	608	970	1078	670	1370
	Protein content [grams]	0.280	0.440	0.490	0.296	0.610
	Specific activity [Units]	2.17	2.21	2.2	2.26	2.24
After Gel filtration chromatography	Total activity [Units]	158	194	215	134	274
	Protein content [µg]	4.1	4.3	5.4	3.4	5.8
	Specific activity [Units]	3.85	45.1	39.81	39.06	47.24

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88 kDa chitinase purification: Table 2 presents the purification process of the 88 kDa chitinase isolated from 5th instar larvae of different races of *B. mori* L. Of the purified chitinase, the enzyme from the Multi-hybrid race RP9 x NK2 recorded highest activity than that of the race PM. Previous studies on *B. mori* had reported the purification of all three isozymes of chitinase [8, 9]. In this paper, we had conducted a comparative study on purification of enzyme 88 kDa molecular weight chitinase in different races of *B. mori* L. namely Bivoltine (NK2, CSR2) Multivoltine (PM, MH1) and Multi-hybrid (RP9 x NK2) at 5th instar stage. As per Koga et al the observation of 54kDa chitinase appear three days later after it started spinning, whereas 65kDa appears in the later stages of ecdysis and the 88 kDa appears one day before ecdysis. So we tried to purify 88 kDa proteins from earlier stages of ecdysis.

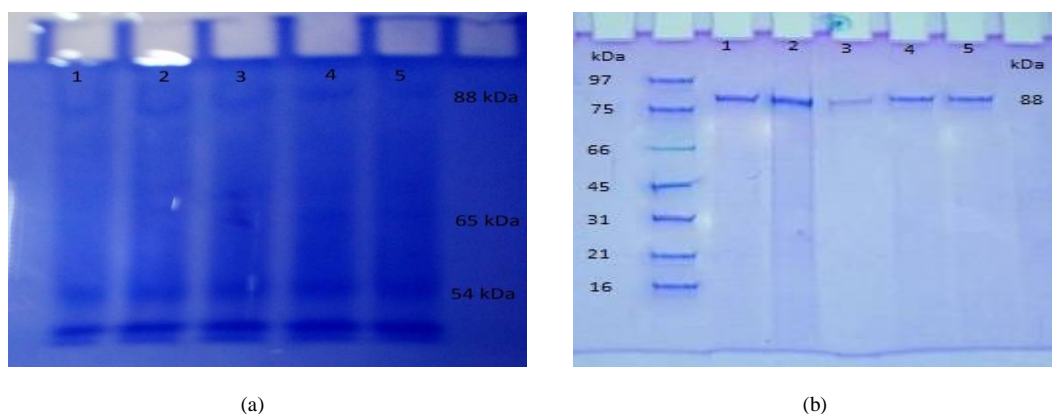


Figure 1: (a) Crude chitinase sample after initial extraction showing chitinase isozymes of 88 kDa, 65kDa and 54kDa. (b) 88 kDa chitinase isozymes purified by anion exchange chromatography and gel filtration chromatography. Samples are in order PM, NK2, CSR2, MH1, RP9xNK2

The DEAE-sephadex purified sample on SDS-PAGE showed traces of other chitinase isozymes such as 65kDa and 54kDa (Fig.1). Hence the samples were subjected for further purification by gel filtration chromatography. The principle of gel filtration is to separate the proteins according to molecular weight. The anionic column purified sample was dialysed and used as a sample in the gel filtration. The 88 kDa chitinase sample was eluted in 5 ml fraction of 0.3M NaCl gradient and eluted after 10th fraction. Since it is a higher molecular weight protein, it elutes late in the gel filtration column, while the smaller molecular weight protein elutes faster. Fig. 2 illustrated the isolation of purified 88 kDa chitinase from all the 5 races of *B. mori* L.

IV. CONCLUSION

The study has indicated variations in the quantity and extent of activity of chitinase enzyme among developmental stages of individual race of *B. mori* investigated. But uniform pattern of variation could not be traced except in the race PM. Therefore, validation of the results may be recommended. The variations in the enzyme quantity and activity may be attributed to their genetic makeup and physiological requirements. Further, the study confirmed that 88 kDa chitinase was purified from all the 5 races of *B. mori* L and enzyme activity could be compared. The 88 kDa chitinase was highly stable until 40°C and it was active between pH 6-10 towards short and long substrates. The enzyme was saturated at 60% for initial anion exchange chromatography at 0.5M NaCl gradient. Further, the other chitinase isozymes were separated by gel filtration chromatography to purify 88 kDa chitinase in 0.3 M NaCl gradient buffer. The enzyme activity was measured using chitin as substrate and found to be more active compared to highest activity reported earlier for 65 kDa chitinase for Kinshu×Showa race of silkworm [9]. The enzyme activity was more in Multi-hybrid while compared to the other Bivoltine and Multivoltine breeds.

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