ISOLATION AND ESTIMATION OF SERRATIOPEPTIDASE AND PROPHENOLOXIDASE ENZYME ACTIVITY IN DIFFERENT RACES OF SILKWORM BOMBYX MORI L.

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ABSTRACT
Extraction and estimation of serratiopeptidase and prophenoloxidase enzymes from five different races of silkworm Bombyx mori L. [Bivoltine (PM, NK2), Multivoltine (CSR2, MH1) and Multi-hybrid (RP9×NK2)] has been carried out. Serratiopeptidase activity was analysed by casein hydrolysis method since it is a protease enzyme, while prophenoloxidase by DNS method and total protein content by Lowry's method. It is found that serratiopeptidase and prophenoloxidase production was more in multi-hybrid (RP9×NK2) compared with those of bivoltine and multivoltine breeds.

KEYWORDS: Bivoltine, multivoltine, multi-hybrid, serratiopeptidase, prophenoloxidase.

INTRODUCTION
In the late 1960s an Enterobacterium bacteria named Serratia marcescens E15 was found in the intestine of silkworm Bombyx mori L. It was used by the silkworm to dissolve the pupa and emerge as a moth, and it was also found to excrete a potent proteolytic enzyme which was later named as serratiopeptidase from the reason of its origin.[1] Serratiopeptidase has been used for the treatment of multiple medical conditions like fibrocystic breast disease, fibromyalgia, migraines, throat infections, carpal tunnel syndrome, ovarian cysts, nose, ear, asthma, inflammatory bowel diseases (IBD) including, Crohn's disease, colitis and cystitis, enlarged prostate, pain and postoperative inflammation.[2] Some researchers believe that this enzyme can play an important role in prevention and removal of arterial plaque.[3] Serratiopeptidase has been reported to exhibit fibrinolycic and proteolytic activities through dissolving the complement protein and increasing the plasmin activity by inhibiting the
plasmin in-activators. The addition of metal ions like zinc, manganese and cobalt to serratiopeptidase stabilizes and ensures its efficacy. This enzyme is heat labile and gets inactivated completely on exposure to 55°C for 15 min. Serratiopeptidase has been called the “miracle enzyme” or “super enzyme” due to its wide range of activities in human body as well as it shows major antimicrobial activity against various microorganisms including bacteria like, *E.coli, Salmonella typhi, Staphylococcus aureus, Bacillus spp., Klebsiella pneumonia* and fungal species like *Mucor, Penicillium notatum* and *Aspergillus niger.*[5]

Prophenoloxidase was first reported in 1953 as a natural activator for the conversion of the phenol oxidase by the precursor prophenoloxidase activator enzyme for the synthesis of melanin in *Drosophila melanogaster.*[6,7] Melanin is involved in sealing of wounds and encapsulation of non-self materials in insects as part of their humoral immune response. This mechanism was first studied in silkworm.[8,9] This enzyme is used widely in waste water treatments too.[10]

The present study is focused on isolation and estimation of serratiopeptidase and prophenoloxidase enzymes from different races of silkworm *Bombyx mori* L.

**MATERIALS AND METHODS**

**Extraction of integuments**

Hundred different stages (4th instar, 5th instar and pupa) of 5 races of silkworms bivoltine (PM, NK2), multivoltine (CSR2, MH1) and multi-hybrid (RP9×NK2) were dissected in ice-cold 0.1M sodium phosphate buffer (pH 7.5). The integuments were meticulously separated with a sterile scalp into a sterile petriplate to remove all tissues free of cuticles. Then it was subsequently rinsed thrice in fresh Ringer’s solution and then blotted with tissue paper to remove excess liquid. Finally the integuments were weighed and separated into two halves for extraction and estimation of serratiopeptidase and prophenoloxidase.

The washed integuments were grounded finely in 50ml of distilled water on broken pieces of ice. The grounded sample was centrifuged at 5000 rpm for 15 min. The supernatant was used as the serratiopeptidase crude enzyme sample for further analyses.

For the prophenoloxidase enzyme sample, the integuments were grounded in acetate buffer of pH 5.2 on broken ice pieces using mortar and pestle. 15mM of CaCl₂ salt was added to the cuticular extract to make final concentration of 10mM, followed by overnight incubation at -
4°C. The incubated content was dialysed in 10mM Tris buffer at 4°C and the dialysate was used as crude prophenoloxidase enzyme.\textsuperscript{[11]}

**Determination of serratiopeptidase activity**

One Unit (U) of serratiopeptidase was measured to one milligram of tyrosine that is produced by one milligram of serratiopeptidase from casein substrate in one minute. The protease assay was carried out to determine the protease activity in serratiopeptidase\textsuperscript{[12]} with slight modifications. Briefly, a stock of ten mg/ml of substrate in distilled water was prepared and incubated at 30°C for 30 min independently with respective controls. The reaction was quenched by adding 3 ml of 10% TCA followed by 30 min incubation at room temperature and centrifuged at 5000 rpm for 10 min. The absorbance of supernatant was measured at 280 nm. Bovine serum albumin was used as a standard to obtain a calibration curve.

**Determination of prophenoloxidase activity**

Prophenoloxidase activity was determined by colorimetric method. The reaction mixture consists of 0.1 ml sample, 0.9 ml distilled water and 1 ml of 10% 3, 5-dintrosalicylic acid solution. Absorbance was measured at 490nm after incubation at 35°C for 10 min. One unit of the enzyme was defined as the amount causing increase in absorbance of 0.01 under the above condition. The activated homogeneous cuticular prophenoloxidase gives a specific activity of A520 per 5min for milligrams of protein.\textsuperscript{[11]}

**Protein quantification**

The total protein content of serratiopeptidase present in the integuments was estimated by Lowry’s method.\textsuperscript{[13]} Bovine serum albumin was used as a standard to obtain a calibration curve.\textsuperscript{[4]}

**RESULTS AND DISCUSSION**

Results of the *in vitro* assay for activity of serratiopeptidase of the cuticular extract of the 3 different developmental stages (4\textsuperscript{th} & 5\textsuperscript{th} instar and pupa) of the silkworm races are presented in Table 1. All the 5 races of silkworms showed maximum enzyme activity of the enzyme at 5\textsuperscript{th} instar followed by 4\textsuperscript{th} instar and minimum at the pupal stage. Among the different races of silkworms tested, the multi-hybrid RP9×NK2 was found to be producing high amount of serratiopeptidase, followed by CSR2 and NK2. Lowest production of the enzyme was recorded in PM. Probably, the individuals utilize the enzyme for eventual breaking of the
pupal case or pupa\(^1\) and hence the sample recorded lower levels of serratiopeptidase activity in comparison with 5\(^{th}\) instar larvae.

Table 1: Evaluation of serratiopeptidase activity and protein content among silkworm races.

<table>
<thead>
<tr>
<th>Silkworm races</th>
<th>Enzyme activity (units)</th>
<th>Protein content (in ‘g’)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4(^{th}) instar</td>
<td>5(^{th}) instar</td>
</tr>
<tr>
<td>PM</td>
<td>5400</td>
<td>10,000</td>
</tr>
<tr>
<td>NK2</td>
<td>13200</td>
<td>23700</td>
</tr>
<tr>
<td>CSR 2</td>
<td>14600</td>
<td>24600</td>
</tr>
<tr>
<td>MH 1</td>
<td>11288</td>
<td>21288</td>
</tr>
<tr>
<td>RP9×NK2</td>
<td>16600</td>
<td>26600</td>
</tr>
</tbody>
</table>

The quantity of serratiopeptidase protein in different races of silkworms was subjected for investigation (Table 1). The total quantity of this protein also followed same trend as that of the serratiopeptidase enzyme activity, with RP9×NK2, recording highest quantity of the protein, followed by CSR2 and NK2. PM race recorded lowest quantity of the enzyme. This result is in agreement with the earlier report of Kaur and Singh\(^4\), confirming the linear relationship between enzyme activity and enzyme concentration.

Activity levels of prophenoloxidase among the selected 5 races of silkworms are presented in Table 2. Prophenoloxidase activity also showed similar trends as serratiopeptidase, with RP9×NK2 recording highest activity for this enzyme, followed by CSR2 and NK2. PM race recorded least level of production of the enzyme. Unlike serratiopeptidase, prophenoloxidase activity was least at 4\(^{th}\) instar, highest at 5\(^{th}\) instar and moderate at pupal stages of PM, NK2 and CSR2. However, MH1 and RP9×NK2 showed highest activity of the enzyme at 5\(^{th}\) instar, lowest activity in pupal stage and moderate activity at 4\(^{th}\) instar stage.

Table 2: Evaluation of prophenoloxidase activity and protein content among silkworm races.

<table>
<thead>
<tr>
<th>Silkworm races</th>
<th>Enzyme Activity (units)</th>
<th>Protein content (in ‘g’)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4(^{th}) instar</td>
<td>5(^{th}) instar</td>
</tr>
<tr>
<td>PM</td>
<td>1040 U</td>
<td>11400 U</td>
</tr>
<tr>
<td>NK2</td>
<td>2600 U</td>
<td>22600 U</td>
</tr>
<tr>
<td>CSR 2</td>
<td>2800 U</td>
<td>27800 U</td>
</tr>
<tr>
<td>MH 1</td>
<td>2176 U</td>
<td>20176 U</td>
</tr>
<tr>
<td>RP9×NK2</td>
<td>3200 U</td>
<td>29200 U</td>
</tr>
</tbody>
</table>

Results of the estimation of prophenoloxidase enzyme among different stages of the selected silkworm races are presented in Table 2. Quantity of this protein was found to be highest at
pupa stage followed by 5th instar and 4th instar larvae. The concentration level of prophenoloxidase has followed different trend than that of serratiopeptidase.

CONCLUSION
In the present study a comparative evaluation of the quantity and activity of serratiopeptidase and prophenoloxidase enzymes in five different races of silkworms have been analysed. It is confirmed that higher levels of serratiopeptidase and prophenoloxidase in multi-hybrid race (RP9×NK2) compared with those of bivoltine and multivoltine races was observed.

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