Purification of Esterase A4 from Bombyx eggs*

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The esterase A4 (Ease A4) activity of silk-worm diapause eggs was found to increase shortly before the completion of the diapause development in cold (Kai and Nishi, 1976; Kai et al., 1982, 1984a). The elevation may due to the molecular modification that have somehow been induced by the cold; Ease A4 may be clock-run. The present paper describes the purification and some properties of the Ease A4 in an attempt in the direction of the clock-run mechanism.

Materials and Methods: Shi 108 strain of the silkworm, Bombyx mori, which was scheduled to produce diapause eggs (Kai et al., 1982), was used for the Ease A4 purification.

Sephadex G-25 (fine) was equilibrated with 0.025M piperazine-HCl buffer (pH 5.25), with which the elution was carried out. For the high pressure chromatofocussing on Mono-P™ column (FPLC), the piperazine-HCl (pH 5.25) was used as the initial buffer and the Poly-buffer (pH 3.25) (Pharmacia Fine Chemicals, Uppsala, Sweden) as the elution buffer. Procedures other than heat-treatment were carried out in a cold room (5°C) or in an ice-water bath.

The esterase assay was carried out as described previously (Kai et al., 1984b). All chemicals were of analytical grade.

Results and Discussion: Table 1 presents a summary of typical purification procedure. Eggs were immersed for a few hours in cold acetone (3°C). The acetone was discarded and the eggs were crushed in a glass mortar with newly added cold acetone (more than 30 volumes). The acetone powder thus obtained was suspended in 0.025M veronal buffer (pH 7.5). The Bombyx eggs contain a number of isozymes of general esterase including Ease A4 (Kai and Hasegawa, 1972; Kai et al., 1981, 1982), therefore, the high activity found in the acetone fraction (Table 1) reflects the combined activities of the isozymes.

The acetone fraction was then heated at 85°C for 15 min, and most of the activity (almost 99%) was eliminated (Table 1). Although the

<table>
<thead>
<tr>
<th>Material</th>
<th>Total protein (mg)</th>
<th>Total activity (n mol/min)</th>
<th>Specific activity (n mol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone powder</td>
<td>1047.3</td>
<td>415.3</td>
<td>0.3965</td>
</tr>
<tr>
<td>Heat treatment</td>
<td>12.90</td>
<td>5.643</td>
<td>0.4374</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ fractionation</td>
<td>1.600</td>
<td>0.8033</td>
<td>0.5021</td>
</tr>
<tr>
<td>G-25 fraction</td>
<td>0.0735</td>
<td>0.8110</td>
<td>11.03</td>
</tr>
<tr>
<td>FPLC fraction</td>
<td>0.0031</td>
<td>0.8210</td>
<td>264.8</td>
</tr>
</tbody>
</table>

Protein was determined by the modified Lowry's method (Bensadoun and Weinstein, 1976) with bovine serum albumin as a standard.
data are not shown, the Ease A4 is very stable to the heat-treatment; more than 95% of the original activity remains after the treatment. The heat stable supernatant obtained by a centrifugation was brought to 50% saturation with ammonium sulphate adjusted to pH 3.30. After a centrifugation, solid ammonium sulphate was further added to the supernatant with stirring to give 80% saturation. The precipitate after centrifugation was washed with acetone and subsequently 80% saturated ammonium sulphate.

The washed preparation collected by centrifugation was dissolved with 0.025M piperazine-HCl buffer (pH 5.25), and then applied to Sephadex G-25 gel. In the elution with the buffer, the Ease A4 was absorbed slightly by the gel; the Ease A4 fraction appeared between the first (void volume) and the second peaks of absorbance at 280 nm. The Ease A4 fraction was further submitted to FPLC. The FPLC produced a single symmetric band. On native-poly acrylamide gel electrophoresis, the final preparation displayed a single band with Mr=0.30 to bromophenol blue in the 15% gel at pH 8.9 (Fig. 1).

It is meaningless to calculate the purification ratio and the recovery of Ease A4 throughout the purification procedure, because the original activity due to the Ease A4 is unknown.

The Ease A4 is very stable when stored in 80% saturated ammonium sulphate. But, after this step, an activation may occur on the 15th day of cold storage. So, the purification was completed within a day, or one day from acetone powder to 80% saturated ammonium sulphate fractionation step and another day from the ammonium sulphate fractionation to the final FPLC step.

The Eaes A4 was eluted at pH 3.85–3.90 on FPLC, pH 3.95 on chromatofocussing and pH 3.85 on isoelectric focussing without the action of Donnan potential (data not shown, cf. Kai et al., 1981); the isoelectric point of the Ease A4 would be pH 3.85.

Fig. 1. Polyacrylamide gel electrophoresis of purified esterase A4. The electrophoresis was performed in a slab gel apparatus using 15% gel with 1.0 mm thickness in a 0.375 M Tris-HCl buffer, pH 8.9 in the absence of sodium dodecyl sulphate. The final esterase A4 fraction of 1 µg was subjected to electrophoresis for 80 min at a constant voltage of 14.3 v/cm. After electrophoresis, the gel was soaked in 10% trichloroacetic acid for 10 min and then treated by silver staining method for detecting protein (Wray et al., 1981).

The final Ease A4 preparation has no proteolytic activities for haemoglobin, casein, α-N-benzoyl-DL-arginine-p-nitroanilide hydrochloride, L-leucine-p-nitroanilide, benzoyl-L-tyrosine-p-nitroanilide nor N-acetyl-L-alanine-p-nitroanilide at pH 4.0 (0.05 M Tris-HCl buffer), pH 7.5 (0.05 M veronal buffer) or pH 7.5 with 5 mM CaCl₂.

References