Activity stain method for sepiapterin deaminase in the silkworm

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A new activity stain method specific for sepiapterin deaminase was developed. By use of this method, distribution of sepiapterin deaminase in larval integument of the silkworm was studied in many strains and a few mutants including Kiuki mutant. All the silkworm larvae so far examined showed the enzyme activity. The present work reveals that the enzyme consists of isozymes, and although the enzyme protein shows the same molecular weight of 70,000, they have different isoelectric points. No enzyme protein specific for Kiuki mutant was found.

Sepiapterin deaminase was discovered in the lemon mutant of the silkworm, Bombyx mori (Tsusue, 1967). The enzyme was purified from the integument of lemon larvae and its properties were described (Tsusue, 1971). The yellow larval colour of the mutant lemon was found to be due to the pteridines, sepiapterin and sepialumazine (Tsusue and Akino, 1965). These pigments are substrate and product of the enzyme. A lack of sepiapterin reductase causes accumulation of sepiapterin in the lemon mutant. Sepiapterin deaminase activity was also found in the integument of the normal silkworm larvae (Tsusue and Mazda, 1977; Mazda et al., 1980). In many silkworm mutants and strains, approximately normal amounts of sepialumazine were accumulated in the integument except in the Watako mutant in which no sepialumazine was found. Sepialumazine together with xanthommatin determines the ground colour of silkworm larvae of all strains (Ohashi et al., 1983).

Another silkworm mutant, Kiuki, has a light yellow colour due to the accumulation of large amount of sepialumazine in its integument (Mazda et al., 1980). The yellow larval colour of the mutant is controlled by an incomplete dominant gene symbolized by Sel (Mazda et al., 1981). The linkage analysis showed an independent relationship between the Sel gene and chromosome-1 through 23 (Mazda et al., 1981). Chromosome-24 was designated for the locus of the Sel gene and another mutant tyw was suggested to be linked with Sel (Eguchi et al., 1986).

Although sepiapterin deaminase activity in the Kiuki silkworm depends on its genetic background, the Sel gene seems to enhance enzyme activity (Mazda et al., 1981). To examine whether or not the Sel gene is a structural gene for the enzyme, the isozyme distribution pattern in several strains of the silkworm was surveyed. For this purpose an activity stain method specific for the enzyme was developed. The method enabled a single larva to be analyzed for the isozymes.

Materials and Methods

Experimental animals

Various strains of Bombyx mori were reared in the Sericultural Experiment Station at Kobuchizawa, Japan. Larvae were reared routinely on fresh mulberry leaves at 25°C. On the last day
of the fourth instar, larvae were dissected and
the integument separated from the other tissues
and stored at −80°C until use.

Chemicals

Crystalline sepiapterin was prepared from the
mutant sepia of Drosophila melanogaster by the
previously described method (Tsusue and Akino,
1965). Ampholine was purchased from LKB,
Sweden. Toyo Pearl HW 50 S was from Toyo
Soda, Japan. Bovine serum albumin was from
ICN pharmaceuticals, Inc., USA. Human gamma
globulin, ovalbumin, horse heart myoglobin and
cytochrome c were from Sigma, USA. Acryl-
amide, N, N'-methylene-bis acrylamide, N, N,
N', N'-tetramethylethylenediamine and ribofla-
vin were of electrophoresis grade from Wako
Pure Chemical, Japan.

Preparation of sepiapterin deaminase

All subsequent procedures were carried out at
4°C. Frozen integument (ca. 3 g) was thawed,
cut into small pieces with scissors and homoge-
nized in a glass homogenizer with 4 volumes of
0.05 M potassium phosphate buffer, pH 7.0, for
3 min. The homogenate was centrifuged at 17,000
g for 20 min. The resulting supernatant was
collected after filtration with a sintered glass
filter to remove fat. The precipitate after cen-
trifugation was rehomogenized and centrifuged
in the same way. The supernatant was collected
and combined with the first extract. Solid am-
monium sulfate was added to the supernatant
solution to give 40% saturation, the mixture was
stirred for 30 min. The precipitate was removed
by centrifugation at 17,000 g for 20 min. Addi-
tional solid ammonium sulfate was added to the
resulting supernatant solution to give 80% satu-
rating. The precipitated proteins were collected
by centrifugation as above and dissolved with
0.5 ml of 0.02 M potassium phosphate buffer,
pH 7.0, and dialyzed overnight against 500 ml
of the same buffer. The dialyzed solution was
centrifuged at 13,000 g for 30 min and the super-
natant solution was used as the enzyme source.

Determination of molecular weight

The molecular weight of sepiapterin deaminase
was estimated as described by Andrews (1964),
using Toyo Pearl HW 50 S as the chromatograph-
ic medium. A column (1.5 × 86 cm) was equi-
librated with 0.02 M potassium phosphate buffer,
pH 7.0, containing 0.2 M KCl. One ml of en-
zyme solution after ammonium sulfatefractiona-
tion step was applied to the column. The flow
rate was maintained at 8 ml/hr and 1-ml frac-
tions were collected. Activity of sepiapterin de-
aminase in fractions from the column was photo-
metrically assayed by the method previously de-
scribed (Tsusue, 1971). The following proteins
were used as markers: human gamma globulin
(mol. wt 150,000), bovine serum albumin (67,
000), ovalbumin (43,500), horse heart myoglo-
bin (18,800) and horse heart cytochrome c (12,
384). Elution of the marker proteins was moni-
tored by determining absorbance at 280 nm.

Isoelectric focusing

Gel isoelectric focusing was performed using
glass tubes (5 × 120 mm). One volume of Am-
pholine in a range of pH 3.5-10 was mixed with
4 volumes of Ampholine in a range of pH 5-7
and the mixture was added to the acrylamide
gel components. The components of the gel were
as follows: 5% acrylamide, 0.25 % N, N'-meth-
ylene-bis acrylamide, 2% Ampholine, 0.06% N,
N', N'-tetramethylethylenediamine and 0.0005
% riboflavin. The above mixture was poured
into glass tubes and polymerized by photochemi-
cal reaction under a fluorescent lamp. The solu-
tion of 60% glycerol and 4% Ampholine was
mixed with the same volume of dialyzed enzyme
solution and 150 µl was applied to the top of the
polymerized gel. Above the layer of the sample
solution, a protective solution was overlaid to a
height of 3 mm, it consisted of 15% glycerol, 2
% Ampholine and 0.8% sodium glutamate. The
anode was at the upper end of the glass tube
and the cathode was at the bottom of the glass
tube. As the electrode solution 0.01 M phosphor-
ic acid was used for the anode and 0.1 M sodium hydroxide for the cathode. Electrophoresis was conducted at 4°C. A constant voltage was applied overnight to the gel at 200V, followed by increasing the voltage to 400V for 1 hr.

After electrophoretic focusing, one of gel was taken from the glass tube and cut transversely into 5-mm slices. The slices were placed in small test tubes and 1 ml of distilled water was added. Ampholine was then extracted from the sliced gel by shaking with water at room temperature for 2 hr. The pH value of the solution was measured by pH meter at 4°C. Another gel was analyzed for sepiapterin deaminase activity by paper electrophoresis.

**Results**

*Activity staining of sepiapterin deaminase*

After isoelectric focusing, the activity of sepiapterin deaminase on the gel was analyzed by paper electrophoresis as follows: Gel was cut longitudinally into two pieces by a Canalco longitudinal gel slicer II (Miles Laboratories, Inc., USA). All subsequent procedures were carried out at 4°C. The gel was immersed in 1 M potassium phosphate buffer, pH 8.0, for 30 min to change the pH value in the gel to the optimum pH of sepiapterin deaminase (Tsusue, 1971). The buffer solution was changed three times. After 30 min the buffer solution was removed and the gel was immersed in 10⁻³ M sepiapterin solution for 20 min with occasional stirring. The gel was rinsed three times with 0.02 M potassium phosphate buffer, pH 7.0, and the flat surface placed on filter paper (Toyo No. 51 A, 16 × 40 cm). Electrophoresis of the gel-paper combination was carried out at 500 V for 2.5 hr in the dark, using 0.02 M potassium phosphate buffer, pH 7.0. After electrophoresis the areas of sepiapterin and sepialumazine on the filter paper were detected by their fluorescence when irradiated with ultraviolet light of wavelength 365 nm. Under the above conditions, only sepialumazine migrated to the anode on the filter paper. Therefore, the detection of sepialumazine, the product of the enzymatic reaction by sepiapterin deaminase, served to locate the enzyme in the gel. The isoelectric points of sepiapterin deaminase were determined by measuring the pH value of the corresponding portion of the gel in which the enzyme was localized.

*Multiple forms of the enzyme in several strains*

By using the sepiapterin deaminase preparation after ammonium sulfate fractionation, we found...
that the enzyme exists in several forms which differ in their isoelectric point. An example using sepiapterin deaminase from the Watako strain is shown in Fig. 1. The isoelectric points of sepiapterin deaminase from various strains are summarized in Table 1. For this experiment about 3 g of integument from 5-6 larvae from each strain was used. To ascertain whether sepiapterin deaminase in individual larvae exists in multiple forms, the integument from individual larva of the Kiuki and Watako strains was homogenized respectively with 2 volumes of 0.02 M potassium phosphate buffer, pH 7.0. When the homogenate was analyzed by isoelectric focusing, sepiapterin deaminase was found in multiple forms for individual. That is, each individual larva in the same strain showed the same pattern of the isozyme, and the pattern is intrinsically due to strain itself.

![Fig. 2. Determination of the molecular weight of sepiapterin deaminase by gel filtration through Toyo Pearl HW 50 S.](image)

When sepiapterin deaminase from the Kiuki and Watako strains was studied by gel filtration, the column eluates showed multiple forms for the enzyme all with the same molecular weight of 70,000 daltons as shown in Fig. 2.
When hybrid larvae, between Murasakiko and Watako strains, were used as the enzyme source, the zymogram exhibited an intermediate pattern between the two strains. We could not detect isozymes which had isoelectric points at pH 6.2, 6.3 and 6.5 in the F1 larvae, but the zymogram of F1 had more isozymes than either parent.

Discussion

The fact that the Kiuki gene increased sepiapterin deaminase activity beyond the level normally found in each strain (Mazda et al., 1981) caused us to study the silkworm enzyme by gel isoelectric focusing using the activity stain method. By this method multiple forms of sepiapterin deaminase were revealed, but we found no specific enzyme for Kiuki larva. Considering the result that large changes in the enzyme activity were found during developmental stages and rearing seasons (Tsusue et al., 1983), the relationship between the Sel gene and sepiapterin deaminase activity remains unknown.

Based on the analysis of gel filtration and on isoelectric points, the different enzyme proteins have the same molecular weight of 70,000 daltons and therefore multiforms for the enzyme are not attributed to differences in molecular weight.

The Watako strain in which no sepialumazine was detected (Ohashi et al., 1983) also showed enzyme activity. All strains and mutants so far examined had sepiapterin deaminase. This result together with wide distribution of the enzyme in larval tissues (Tsusue, 1971) suggests that the enzyme is indispensable for the silkworm.

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References


淹川新一郎・津末玄夫・山本俊雄：家蚕幼虫のセピアピテリンデアミナーゼの活性染色法

家蚕幼虫の皮膚に含まれているセピアピテリンデアミナーゼを調べるために、電気泳動法を用いた当酵素の活性染色法を開発した。この方法で家蚕幼虫の種々の系統及び突然変異種におけるセピアピテリンデアミナーゼの分布を調べたところ、調べた限りの全ての系統にこの酵素活性が見られた。一方等電点電気泳動法によりこの酵素には6.0から6.7に至る0.1きざみの等電点を持つ8種のアイソサイムがあり、系統によって特定の組み合わせを持つ事が分かった。これらのアイソサイムは同じ分子量であり、ゲル浄過の結果分子量7万と推定された。

この酵素を持たない系統が無い事は、幼虫の組織における広い分布を考え合わせて、家蚕幼虫の生活に必須のものと考えられる。浮遊虫に特有な当酵素のアイソサイムは見出されなかった。