Distribution of Aldolase Isozymes and Some Related Enzymes in Eggs and Post-Embryonic Organs of Bombyx Mori

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GENERAL INTRODUCTION

In a study which aims at understanding the mode of developmental gene expression, it is often required to focus on an enzyme as a molecular target. The present author was interested in fructose 1,6-bisphosphate aldolase [E.C. 4.1.2.13] (it will be called aldolase in what follows) and analyzed its changes in activity and distribution in *Bombyx mori* with special reference to isozyme forms.

Many proteins as phenotypes of respective single or plural genes have been studied in *B. mori*. The biosynthesis of yolk proteins and their programmed degradation are especially well investigated (Izumi *et al.*, 1994). Eggs of *B. mori* contain large amounts of the major yolk proteins vitellin, 30 kDa proteins and egg-specific protein (ESP) (Zhu *et al.*, 1986). Vitellogenin is synthesized by fat body cells of sexually matured females, released in the hemolymph, taken up by the developing oocytes and becomes vitellin. A group of structurally related proteins with molecular weights of about 30,000 have also been detected in the hemolymph; these are partly accumulated in the oocyte as another kind of yolk proteins. The follicular epithelial cells secrete ESP into developing oocytes at the middle pupal stage. Each protein exhibited a unique profile of degradation during embryogenesis. Recently several proteases for yolk protein utilization have been detected (Izumi *et al.*, 1994). For example, a trypsin-like serine protease undergoes a limited hydrolysis of ESP (Indrasith *et al.*, 1988). The structure and biosynthesis of yolk proteins and the regulatory mechanism of yolk degradation
are important objects, attracting attention of many investigators who studied gene expression in a sex-, stage- or organ-dependent manner.

There exist several different forms of enzymes catalyzing the same reaction. Isozymes differ from one another in terms of amino acid sequence that arise from genetically determined differences. The gene expression for isozymes of multi-locus type is mostly regulated in an organ-specific manner. In addition, the expression of many isozymes are developmentally controlled. Isozymes mirror a refined cell differentiation, and an analysis of their changing patterns during development may offer important insights into the enzymatic regulation of cellular metabolism. For example, the lactate dehydrogenase (LDH) provides a good example of the occurrence of isozymes and have been well characterized in vertebrates (Markert et al., 1975). The enzyme is a tetramer of 140,000 in molecular weight. In most tissues LDH takes five forms separable by electrophoresis. These arise from the five possible ways of assembling a tetramer from the two types of subunits H and M (H4, H3M, H2M2, HM3 and M4). H4 predominates in the heart muscle and is often called LDH-1, whereas M4 predominates in the skeletal muscle and is often called LDH-5 (Markert and Moller, 1959). The pattern of isozyme distribution in embryonic tissues differs from the adult pattern. Most embryonic tissues initially contain LDH-5 as a principle component; as development proceeds LDH activity is gradually transposed toward the LDH-1 (Markert and Ursprung, 1962). It should be noted here that LDH has not served as a good marker for the silkworm embryogenic development, since the Bombyx eggs was characterized by the lack of LDH (Chino, 1960).
The present author studied aldolase which also provides a marked isozyme system. Moreover, aldolase is one of the glycolytic enzymes and it has a crucial role in the regulation of carbohydrate metabolism. The remaining part of GENERAL INTRODUCTION will be devoted to a review about previous knowledge of aldolase and its isozymes.

Aldolase catalyzes conversion of the six-carbon unit D-fructose 1,6-bisphosphate (FBP) to the two three-carbon units D-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate during glycolysis. There is a clear distinction between aldolases from higher organisms (Class I) and those from fungi and bacteria (Class II) (Rutter, 1964). *Escherichia coli* contains both class I and II aldolases (Stirling and Perham, 1973) and that *Euglena gracilis* includes plastidic class I and cytosolic class II aldolases (Mo et al., 1973).

Class II aldolases are dimeric proteins and the subunit molecular weight is 38,000. In this class, a metal ion is required for activity. There is no significant homology between classes I and II.

Class I aldolases can attack not only FBP but also D-fructose 1-phosphate (F1P), which is cleaved to glyceraldehyde and dihydroxyacetone phosphate. The native form of a mammalian class I aldolase has a molecular weight of about 160,000, a tetramer of subunits each with a molecular weight of about 40,000 (Penhoet et al., 1967). Animal tissues have three distinct isozymes A, B and C. In tissues where aldolases A and C (or A and B) are co-expressed, the five possible tetrameric forms are present (Lebherz and Rutter, 1969, 1973; Penhoet et al., 1966). The identification of these isozymes has been made from the substrate specificity, electrophoreic
mobility, tissue distribution and when necessary specific immuno-
logical property. These isozymes have been studied extensively
from the viewpoint of differential gene expression (Lebherz and
Rutter, 1969; Hori et al., 1987). Aldolase A was found in most tis-
sues studied and predominantly distributed in the skeletal muscle.
Aldolase B was restricted to the liver and kidney. The tissue distri-
bution of aldolase C varied somewhat more widely and generally
present in brain. In the human liver, aldolase A was predominantly
observed in early embryonic stages with a small amount of al-
dolases B and C. Before birth, aldolase A activity is abruptly de-
creased and aldolase B begins to rise in amount and becomes a
predominated form in the adult liver (Rutter et al., 1963;
Matsushima et al., 1968). Aldolase A reappeared but aldolase B,
which is abundant in the normal adult liver, disappeared in growing
hepatoma (Gracy et al., 1970; Horecker et al., 1972; Schapira, 1981).
The recurrent of the fetal tissue type of isozyme occurs in cancer
(Schapira, 1981).

Purified aldolase isozymes were found to have different cata-
lytic properties. In particular, the relative activity toward the two
substrates FBP and F1P (the FBP/F1P activity ratio) changed from
isozyme to isozyme. The ratio is believed to reflect the sugar-phos-
phate metabolism of the organs to which the respective isozymes
belong and has been utilized as an important index in studies of al-
dolase isozymes (Lebherz and Rutter, 1969; Horecker et al., 1972).
Aldolase A has a much higher activity toward FBP than F1P giving
the ratio of 50. In contrast, aldolase B cleaves FBP and F1P almost
equally with the ratio of about 1. Aldolase C has an intermediate
ratio of about 10. The ability of aldolase B to use F1P as a substrate
can be correlated with the role of the liver in the metabolism of fructose, since absence or inactivation of this enzyme due to mis-sense, deletion, frameshift, premature stop-codon or splice-site mutation leads to a hereditary fructose intolerance (Cross et al., 1988; Dazzo and Tolan, 1990; Cross and Cox, 1990; Kajihara et al., 1990).

The complete amino-acid sequences of Class I aldolases are known. The amino acid sequences of three isozymes composed three different sequence units: C.C.S. (common conserved sequence), I.G.S. (isozyme group specific sequence) and divergent sequences (Kitajima et al., 1990; Takasaki and Hori, 1990; Kusakabe 1994). The C.C.S. units are conserved through the three isozymic groups and constituted a barrel structure in which the active site of the enzyme exists (see below). The I.G.S. units are highly conserved within a singe isozymic group and, in particular, amino acid residues 34-108 (I.G.S.-4) have an important role in determining the characteristics of aldolase A as revealed in studies of enzymes with chimerical construction and those after the site-directed mutagenesis (Kitajima et al., 1990; Takasaki and Hori, 1990). X-ray structural analysis showed that each subunit is composed of eight strands of parallel β-sheet forming a barrel (Sygusch et al., 1987). The active site residue Lys-229 is located in the interior of this barrel. Analysis of the isotope-labeled enzyme uncovered that a Schiff base is formed between dihydroxyacetone phosphate and the ε-amino group of Lys-229 (Lai and Horecker, 1972). The C-terminal portion of a chain of aldolase (A and B) is thought to be folded over the surface of the subunit, thus it extends close to the active site in the β-barrel and affects the activity (Rutter et al., 1961; Takahashi et al., 1989).
Molecular cloning and structural analysis of vertebrate aldolase gene have been elucidated in human (Mukai et al., 1987, 1991), mouse (Paolella et al., 1986), rat (Tsutsumi et al., 1985; Joh et al., 1986; Mukai et al., 1991), Xenopus leavis (Atsuchi et al., 1993) and sheep mesonephros (Gianquinto et al., 1994). Vertebrate aldolase isozymes are encoded by three distinct genes that are located on different chromosomes; the genes for the human aldolases A, B and C are located on chromosomes #16 (Kukita et al., 1987), #9 (Henry et al., 1985) and #17 (Tolan et al., 1985), respectively. These genes have essentially the same structures with nine protein-coding exons. It is possible that these three isozymes have arisen from a common ancestral gene during evolution (Tolan et al., 1985).

The molecular anatomy of aldolase promoters have been done. The expression of the human aldolase A gene is controlled by three alternative promoters. The two ubiquitously active promoters are both active in fetal tissues and in adult tissues. The third promoter is highly specific to the skeletal muscle. The expression of the aldolase A gene is turned on at birth and the enzymatic activity becomes strong, reaching a maximal level in the adult muscles (Salminen et al., 1994). Like aldolase A gene, aldolase B gene is controlled by two promoter regions, one for basal factors and another for liver-specific factors (Ito et al., 1990; Tsutsumi et al., 1993; Yabuki et al., 1993).

In contrast to vertebrate aldolase, our knowledge on the enzymes in invertebrates has been rudimentary. An aldolase was purified from pupae of Drosophila melanogaster and has been well characterized. This enzyme belongs to class I and contains four
identical subunits with a molecular weight of about 40,000 (the molecular weight for the native form is 158,000). The N-terminus (tyrosine) is blocked by acetylation. Optimum pH is at a broad range between 6.8 and 8.0. The $K_m$ values for both of FBP and F1P ($2.7 \times 10^{-5}$ M and $1.85 \times 10^{-2}$ M, respectively) closely resembled those of the rabbit A-type aldolase, but the FBP/F1P activity ratio was apparently similar to the mammalian C-type aldolase, which is a speculative ancestor of modern aldolase isozymes (Brenner-Holzach and Leuthardt, 1972; Brenner-Holzach, 1979). The complete amino acid sequence was already elucidated and showed 71% identity toward rabbit muscle aldolase (Malek et al., 1985).

Recently, the aldolase gene of $D. melanogaster$ were isolated and characterized (Shaw-Lee et al., 1992; Kim et al., 1992; Kai et al., 1992). The protein-coding region is composed of the five exons 2, 3, 4α, 4β and 4γ. The insect exon 2 was formed by the fusion of exons 2 to 7 of vertebrate aldolase gene. Sequence comparisons of the alternative fourth exons showed that the duplication leading to the multiple exons must have occurred in quite old times (Shaw-Lee et al., 1992). The gene is capable of generating mRNAs for the three isozymes α, β and γ by alternative usage of the final three C-terminal exons. The purified pupal enzyme was shown to be isozyme γ but isozymes α and β have not yet been found. The usual type mRNAs for the three isozymic forms and two novel RNA species, the $\alpha\beta$-type mRNA and $\beta\gamma$-type mRNA, were detected in vivo by Northern and reverse transcriptase-PCR analyses. The $\alpha\beta$-type mRNA is an a-type mRNA in which exon 4β remains unspliced, while the $\beta\gamma$-type mRNA is a $\beta$-type mRNA with the exon 4γ remains unspliced. The recombinant enzyme from the $\alpha\beta$-type
mRNA species in *E. coli* is enzymatically active (Sugimoto, submitted for publication). The mRNA species were expressed at different developmental stages of the fly, and also occurred showing the tissue-specific expression in adult flies (Kai *et al.*, 1992). Detailed analyses of organ and development-specific expression of *D. melanogaster* aldolase isozymes, may be a tedious task because of the small body volume and short life span of the fruit fly. In the silkworm system, however, it is expected that the organ distribution of the isozymes can be assessed easily and data can be discussed in the light of cell type-specific sugar metabolism. The present study was undertaken on the basis of this criterion.

The first chapter includes a modification of the assay method for aldolase activity. Using this method, the author analyzed activity changes of aldolase during embryonic development of *B. mori*. The results confirmed that two aldolase isozymes were interchanged during embryogenesis. In Chapter II, the author noticed the activity changes during embryogenic development of hexokinase and some other enzymes related to aldolase, and compared the results with that of aldolase. Chapter III deals with the occurrence and distribution of aldolase isozymes in organs of larvae, pupae and adults. The silkworm aldolase was shown to exist as two distinct molecular varieties and to form five sorts of hybrids in some organs. In Chapter IV, the author compared organ distribution of some enzymes related to aldolase. Chapter V describes the partial purification of the two aldolase isozymes and their enzymatic properties. By the Southern blotting analysis with a highly conserved human aldolase sequence (cDNA) as a probe, the silkworm aldolase gene was preliminary estimated. In GENERAL
DISCUSSION the author debated the significance of activity changes and organ distribution of aldolase isozymes in connection with carbohydrate utilization.
Chapter I

Interchange in the type of aldolase isozymes during embryogenesis

INTRODUCTION

Changes of the zymogram band of silkworm aldolase during embryogenesis were investigated using crude extracts of eggs and newly hatched larvae (subsequently the latter will be termed neonates). The results obtained in the present chapter confirmed the presence of two types of aldolase isozymes; one was replaced by the other at a late stage of embryogenesis. Then the level of aldolase activities during embryogenesis was assessed again using crude extracts.

Previously, the activity levels of aldolase have generally been measured by a spectrophotometric method reported by Rajkumar et al. (1966). This method is simple but the present study indicated that it was inapplicable to crude extracts. In the first part of the present chapter, the author tried to rearrange the assay method. This modified method was thoroughly utilized in the experiments.

MATERIALS AND METHODS

Chemicals

FBP, NADH, glycerol-3-phosphate dehydrogenase, triosephosphate isomerase and glyceraldehyde-3-phosphate dehydrogen-
ase were purchased from Boehringer (Mannheim, Germany). F1P was obtained from Sigma Chemical Co. (St. Louis, U.S.A.). Cellulose acetate membranes (Sephraphore III, 5.7 × 12.7 cm) were obtained from Gelman Science (Ann Arbor, U.S.A.). Polyvinylidene difluoride (PVDF) membranes (Immobilon Transfer, 0.45 μm pore size) were obtained from Nihon Millipore (Tokyo, Japan). Peroxidase-F(ab') 2 of goat anti-rabbit IgG (H+L) were purchased from Zymett (Code. 62-1820, Lot. 10708106, San Francisco, U.S.A.). Other chemicals and enzymes were procured from Wako Pure Chemicals (Osaka, Japan).

**Animals**

Eggs of a hybrid race, Gunpo × Shugyoku, purchased in a newly deposited state from Aizusanshu Co. (Fukushima, Japan) were kept at 25°C to start diapause. On day 30, the eggs were chilled to 5°C and, on day 120 of chilling, these were transferred to 25°C to allow development. These eggs will be called “post-diapause eggs.” About 95% of the eggs hatched on day 9 of development. Some of the hatched larvae of Gunpo × Shugyoku were reared on an artificial diet (Nippon Nosan Kohgyo, Yokohama, Japan) at 25°C and used for the sources of ovarian specimens.

Also used were eggs deposited by the moths of a hybrid between a Japanese race (N137) and a Chinese race (C137). The pupae, supplied by Mr. T. Kawabata of the Kyushu Institute of Agricultural Experiment Station, were kept at 25°C, and after emergence the adults were randomly copulated for 1 hr. The mother moths inseminated were chilled at 5°C for 16 hr to synchronize the start of oviposition. The moths allowed to oviposit at 25°C for 30
min. The chilling did not affect the following embryonic development. A batch of eggs were kept at 25°C for 20 hr. To initiate the embryogenesis, the eggs were treated with hot HCl (spec. grav. 1.075, at 46°C for 5 min) and transferred to 25°C. These eggs will be termed the "artificial non-diapause eggs." More than 98% of the eggs hatched on day 10 after the hot acid treatment.

The embryonic stages were allocated according to Takami and Kitazawa (1960) as listed in Table 1. Eggs at each stage and neonates (as well as ovaries) were kept at −80°C until used for extraction. It should be mentioned here that the presently used eggs (and neonates) were of a hybrid race or, in the case of N137 × C137, the second filial generation. Thus the population would be genetically heterogeneous. Care was taken to mix eggs given by several moths as randomly as possible in expectation of getting averaged results.

Dissection

Some of the eggs were each dissected by the method according to Miya et al. (1972). The chorion was removed with a razor's edge in 10 μl ice-cold 0.75% saline, then the serosal membrane was incised. The embryo and serosal membrane was taken out with forceps and collected as an "embryo rich fraction" and a "serosal membrane rich fraction," respectively. Yolk materials were collected from the saline through a micro-pipette and save as a "yolk rich fraction." The three fractions were stored at −80°C until use.
<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1</td>
<td>Fertilization</td>
</tr>
<tr>
<td>Stage 2</td>
<td>Cleavage</td>
</tr>
<tr>
<td>Stage 3</td>
<td>Cellular blastoderm formation</td>
</tr>
<tr>
<td>Stage 4</td>
<td>Yolk cell formation</td>
</tr>
<tr>
<td>Stage 5</td>
<td>Pigmentation of serosal cells</td>
</tr>
<tr>
<td>Stage 6</td>
<td>Formation of ectoderm and mesoderm</td>
</tr>
<tr>
<td>Stage 7</td>
<td>Formation of telson</td>
</tr>
<tr>
<td>Stage 17</td>
<td>Appendage development; differentiation of thorax and abdominal legs</td>
</tr>
<tr>
<td>Stage 20</td>
<td>Head and thorax become distinguished</td>
</tr>
<tr>
<td>Stage 21</td>
<td>Blastokinesis; embryo undergoes reversal</td>
</tr>
<tr>
<td>Stage 25</td>
<td>Pigmentation of mandible; formation of taendia inside tracheae and tracheoles</td>
</tr>
<tr>
<td>Stage 26</td>
<td>Head pigmentation I; head capsule darkening</td>
</tr>
<tr>
<td>Stage 27</td>
<td>Head pigmentation II; ingestion of serosa and residual yolk material</td>
</tr>
<tr>
<td>Stage 28</td>
<td>Body pigmentation; darkening of thorax and abdomen</td>
</tr>
<tr>
<td>Stage 30</td>
<td>Hatching</td>
</tr>
</tbody>
</table>

According to Takami and Kitazawa (1960).
Preparation of crude extracts

For the assay of enzyme activity, eggs or neonates (usually 100 individuals) were homogenized with a Potter-Elvehjem type microhomogenizer in 10 volumes of 100 mM Tris-HCl buffer, pH 8.0, containing 10 mM ethylenediamine tetraacetate (EDTA) at 4°C. The supernatant solutions obtained by centrifugation at 12,000g for 20 min at 4°C were used as crude extracts for experiments. As a positive control, tissues of the chicken were processed similarly.

Spectrophotometric determination of aldolase activity

The level of aldolase activities in crude extracts were measured by the spectrophotometric method of Rajkumar et al. (1966) after a modification, the details of which will be described in RESULTS. One unit (U) of enzyme was defined as the amount that caused the change of 1 µmole substrate per min.

Assay of aldolase activity by staining method (zymogram)

Aldolase activity was analyzed by an activity staining method as described previously (Takasaki and Hori, 1990). Aldolase isozymes were separated by electrophoresis on cellulose acetate membrane strips. The strips were “activated” prior to use by sinking the dry strips into the electrophoresis buffer (60 mM sodium barbital buffer, pH 8.6, containing 10 mM 2-mercaptoethanol). After about 5 min, the strip was placed on a piece of blotting paper, and excess buffer was allowed to drain. The crude enzyme solution (about 30 to 50 mU aldolase) was run on the strip at 250 V for 45 min at 4°C in the electrophoresis buffer. Aldolase activity was detected by placing the strip on the surface of an agar plate containing the assay
reagents, which were prepared as follows. Agar (10 mg) was dissolved in hot 10 ml 4 mM Tris-HCl buffer, pH 8.0, containing 1.6 mM sodium arsenate. The solution was rapidly cooled and mixed with 10 ml 16 mM FBP, 150 µg glyceraldehyde-3-phosphate dehydrogenase, 3.6 mM NAD, 5.0 mg nitroblue tetrazolium chloride and 300 mg phenazine methosulfate. The plates were then incubated at 37°C for 5 to 20 min in the dark, since the phenazine methosulfate is light sensitive. The aldolase on the strip cleaves its substrate FBP to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Glyceraldehyde 3-phosphate is then oxidized by glyceraldehyde-3-phosphate dehydrogenase with the concomitant reduction of NAD to NADH. The NADH produced then reduces oxidized phenazine methosulfate; this in turn reduces oxidized nitroblue tetrazolium to the reduced form, that is highly colored (blue) state. Thus, wherever aldolase is present on the strip, a blue band appears in the adjacent agar.

**Analyses of enzyme properties**

Values for the Michaelis-Menten constant $K_m$ were determined by the Lineweaver-Burk plots of the reaction rates determined with varying concentrations of FBP as a substrate. Optimal pH for the FBP reaction was determined by the spectrophotometric assay in 50 mM glycylglycine buffer at pH 5.0 to 8.0 and 50 mM glycine-NaOH buffer at pH 8.0 to 10.0. The temperature during reaction was fixed at 30°C.
Preparation of antiserum

Aldolase had previously purified from eggs at early embryo­genesis by salting out, affinity chromatography with a phospho­cellulose column and gel filtration with a TSK G3000SW column (Toso, Tokyo, Japan) and subjected to the production of rabbit anti­serum (Nishimura, 1992). This anti-aldolase serum recognized al­dolase preparations purified from eggs and neonates of B. mori (later these will be called S and F isozymes, respectively) as well as those denatured by boiling or by sodium dodecyl sulfate (SDS) (Nishimura, 1992). The identity of the antiserum and its reactivity were confirmed in the present study (details will be described in Chapter V).

Immunological procedures

Eggs and neonates were homogenized in the modified Laem­mli's sample buffer (Laemmli, 1970) composed of 500 mM Tris-HCl buffer, pH 6.8, containing 2% 2-mercaptoethanol, 2% SDS, 20% glycerol and 0.001% bromophenol blue. The mixture was cen­trifuged at 12,000g for 5 min at 10°C, and the supernatant was heated at 100°C for 3 min.

The solution was subjected to polyacrylamide gel elec­trophoresis in the presence of SDS (SDS-PAGE), according to Laemmli (1970). The stacking gel was composed of 5% acrylamide and 2.5% N,N'-methylene bisacrylamide in 125 mM Tris-HCl buffer, pH 6.8, plus 0.1% SDS, and the separation gel was of 12% acrylamide and 2.5% N,N'-methylene bisacrylamide in 375 mM Tris-HCl buffer, pH 8.8, plus 0.1% SDS. Electrophoresis was car­ried out at a constant voltage of 120 V with the electrode buffer
composed of 25 mM Tris, 192 mM glycine and 0.1% SDS at pH 8.3. Commercially available purified rabbit muscle aldolase A (molecular weight of 40,000, No. 102644) as a molecular marker was run in parallel.

For Western blot analysis proteins separated by SDS-PAGE were transferred to a PVDF membrane using Towbin's transfer buffer (Towbin et al., 1979) at 120 mA for 1 hr at room temperature. The membrane was immersed in phosphate buffered saline (PBS) containing 5% non-fat dry milk (Milk-PBS) at room temperature for 2 hr. The filter was then kept overnight at 4°C in solution containing the anti-aldolase S serum as the first antibody which had been diluted at 1 : 1,000 with Milk-PBS. After the reaction was completed, the membrane was washed twice with PBS containing 0.05% Tween-20 (T-20-PBS) and once with PBS. Then it was treated with the goat anti-rabbit IgG conjugated with horseradish peroxidase as the second antibody which had been diluted at 1 : 1,000 with Milk-PBS. The membrane was washed twice with T-20-PBS and once with PBS, and incubated at room temperature in a developer (Taketa et al., 1986), consisting of 50 mM sodium phosphate buffer, pH 7.0, plus 0.5 mg/ml NADH, 0.5 mg/ml phenol, 0.25 mg/ml nitroblue tetrazolium and 10% H₂O₂. Within 30 min, purple color appeared as a band indicating the immunoreactive protein. The color was produced by the reduction of tetrazolium salt, instead of the ordinary color reaction of peroxidase involving the oxidation of substrate to form colored products.
RESULTS

I-1. Establishment of assay method of aldolase activity

First, aldolase activity was determined by the method of Rajkumar et al. (1966) using the artificial non-diapause eggs and neonates (N137 × C137) as materials. To a 400 μl mixture containing 50 mM glycylglycine buffer, pH 7.5, 0.1 mM NADH, 10 mM EDTA, 0.45 mg glycerol 3-phosphate dehydrogenase, 4.54 mg triosephosphate isomerase and 2 mM FBP (or 10 mM F1P), crude enzyme solution (25 μl) was added and the rate of absorbancy decrease at 340 nm was recorded with Hitachi U-3000 spectrophotometer at 30°C. The rate was constant for at least five min. The slope was thought to be dependent upon the aldolase activity and the ratio of activities toward the two substrates FBP and F1P was calculated. The value for the crude egg extract on day 1 of embryogenesis was 3 and that for the crude neonate extract was 1. However, repeated measurements indicated that this spectrophotometric assay was inadequate, since the initial decrease in absorbancy at 340 nm was found to involve a nonspecific decrease due to the crude extracts. Thus the assay procedure was modified, where preincubation was done before the addition of substrate. A crude extract (25 μl) plus a mixture (400 μl), consisting (at final concentrations) of 50 mM glycylglycine buffer, pH 7.5, 0.1 mM NADH, 10 mM EDTA, 0.45 mg glyceraldehyde-3-phosphate dehydrogenase and 4.54 mg triosephosphate isomerase, was subjected to the measurement of absorbancy at 340 nm at 30°C until the decrease became constant (60 sec or longer). Then 50 μl of 20 mM
FBP or 100 mM F1P was added and the monitoring of the absorbancy was continued. The difference of slope obtained before and after the addition of the substrate was taken to represent the net reaction rate. By this modified method, the activity values toward the substrates (in particular F1P) were decreased, making the FBP/F1P activity ratio larger, 6 for eggs and 30 for neonates instead of the smaller values obtained by the original Rajkumar method. The results by both methods are summarized in Table 2. These differences between the original and modified methods were reproducible in repeated experiments, some of which were conducted by using the post-diapause eggs (and neonates) of Gunpo × Shugyoku.

As a control, the activity ratios were measured using crude extracts of chicken tissues, where a non-specific decrease in absorbancy at 340 nm before the addition of the substrate was also found. The FBP/F1P activity ratio for the crude extract of skeletal muscle, liver and heart by using original Rajkumar method were 40.2, 0.5 and 26.2, respectively. In contrast, the values were 61.0, 1.4 and 50.9, respectively, obtained by the modified method (Table 2).

I-2. Interchange of aldolase types during embryogenesis

Changes in banding pattern of aldolase isozymes during embryogenesis and oogenesis

The eggs (Gunpo × Shogyoku) were allowed to enter diapause without hot-acid treatment and activated by a long term chilling to start the post-diapause development. The time when
TABLE 2. Comparison of “reaction rates” vs. FBP and F1P, and of the activity ratios (FBP/F1P), as measured by the original and modified methods.

<table>
<thead>
<tr>
<th>Extract</th>
<th>Aldolase type</th>
<th>Original method</th>
<th>Modified method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FBP*</td>
<td>F1P*</td>
</tr>
<tr>
<td>Silkworm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day-1 egg</td>
<td>S</td>
<td>6.5</td>
<td>2.1</td>
</tr>
<tr>
<td>Neonate</td>
<td>F</td>
<td>18.5</td>
<td>18.5</td>
</tr>
<tr>
<td>Chicken</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle</td>
<td>A</td>
<td>18,900</td>
<td>470</td>
</tr>
<tr>
<td>Liver</td>
<td>B</td>
<td>96</td>
<td>191</td>
</tr>
<tr>
<td>Heart</td>
<td>A-C†</td>
<td>3,400</td>
<td>130</td>
</tr>
</tbody>
</table>

*The apparent or net activities towards the two substrates FBP and F1P were expressed in terms of the decrease in absorbancy at 340 nm × 10³ /min/mg protein. Protein was estimated by the absorption at 280 nm (1 O.D. was regarded as 1 mg protein/ml). †The chicken heart contains the isozymes A and C. For the silkworm, N137 × C137 was used. See I-2 for the aldolase types S and F.
transferred from 5°C to 25°C will be termed day 0. The eggs and neonates were homogenized and the supernatants were analyzed for the zymogram pattern of aldolase (Fig. 1). Each lane contained one egg equivalent. Two types of aldolase bands with different electrophoreic mobility were detected. A slowly-migrating band, named the S type was consistently seen on days 1 to 5. No difference was observed before day 5 on the intensity of the S-type band. On the other hand, a fast-migrating band appeared on days 7 to 8 and after hatching. This band was named the F type. Thus, aldolases S and F were interchanged on days 6 to 7 (Stages 25 to 27, the latter is the head pigmentation stage). On day 6, there was no marked aldolase band. The author could confirm that the interchange also occurred in the artificial non-diapausing eggs (N137 × C137) treated with hot HCl at 20 hr after oviposition (patterns not shown). Also in this case the switchover was seen at Stage 26 (on day 8 of development or 2 days before hatching). The S-type band was detected in eggs until day 7 of development after the hot acid treatment and the F-type band observed on day 9 and neonates; no intensive bands were detected on day 8.

To understand the localization of aldolases S and F in an egg, zymogram analysis was repeated after the post-diapause eggs were separated into the three fractions rich in yolk, embryo or serosal membranes. Fig. 2 shows the patterns at late embryogenic stages. The type-S band was present in the yolk and embryo-rich fractions at Stage 24 (before the onset of head pigmentation), but it was not detected in the yolk-rich fraction at Stage 27, when the embryo begins to use yolk. In contrast, the type-F aldolase was detected in the embryo-rich fraction at Stage 27. Solely the type-S band was
FIGURE 1. Zymogram patterns of aldolases during embryogenesis. Lanes 1 to 8 contained extracts of eggs during the post-diapause development (numbers denote the stages and the respective days of embryogenesis). Lane 9, extract of neonates. S and F along the right margin indicate the positions of aldolases S and F, respectively. Post-diapause eggs of Gunpo × Shugyoku were used.
FIGURE 2. Distribution of aldolase activity among three fractions of eggs at late stages of embryonic development. Y, E and S indicate the fractions rich in yolk, embryo and serosal membranes, respectively. ST. 24, Stage 24; ST. 27, Stage 27. Fractions collected from fifty eggs were used for each analysis. Post-diapause eggs of Gunpo × Shugyoku were used.
detected in the yolk-rich fractions of eggs at early embryonic stages (patterns not shown). The whole extracts of the ovaries from day-4 and day-9 larvae at the fifth instar (the latter were shortly before the larval-pupal ecdysis), as well as from day-3 pupae, exhibited no type-F band but distinctly the type-S aldolase band (Fig. 3). The whole ovaries from day-6 pupae, including vitellogenic and chorio­genic follicles, also consistently exhibited the type-S band alone.

*Developmental changes in activity level during embryogenesis*

The changing levels of aldolase activity during embryogenesis were determined (Fig. 4). The activities were expressed on a whole egg (and neonate) basis. In the post diapause eggs, aldolase activity was found at a higher level on day 1 (about 0.35 mU/individual) and then gradually decreased. On day 6, the activity was at a minimum (but not zero, about 0.1 mU/individual). It increased again and reached its highest level on the neonatal stage (0.6 mU/individual). In the artificial non-diapause eggs, the fluctuation patterns of the total activity per egg through the embryogenic period were basically similar to those in the post-diapause eggs, but the activity remained at a relatively constant level for 7 days. A minimum value was recorded on day 8. Until larval hatching, the activity increased rapidly.

The low activity level on day 6 or 8 (also the apparent lack of active band on the zymogram, Figs. 1 and 2) may not be an artifact associated with the presence of endogenous proteinases and/or inhibitory factors. This inference was confirmed by the finding in mixing experiments, where the aldolase activities were additive when the homogenate from day-8 eggs was mixed with each of
FIGURE 3. Zymogram patterns of aldolase activity during oogenesis. Lanes 1, 2 and 3 are extracts of ovaries from day-4 larvae at the 5th instar, day-2 larvae after the onset of spinning and day-3 pupae, respectively. Lanes 4 to 10 are extracts of follicles. Every ten follicles from terminal were taken out of the ovarioles of day-6 female pupae. The terminal follicles (Lane 10) had finished in vitellogenesis and engaged in choriogenesis. Lane 11 denotes unfertilized eggs taken out of newly eclosed female imagoes. Specimens were prepared from Gunpo × Shugyoku reared by an artificial diet.
other homogenates tested or different eggs were co-homogenized (Table 3).

The localization of aldolase activity in the three fractions of eggs was examined also by the spectrophotometric method. Most of aldolase activities were found in the yolk-rich fractions from diapausing and day-3 eggs (Fig. 5). The total aldolase activities in the three fractions were not agreement with the whole egg activities in the diapausing eggs, probably because of the difficulty in collecting complete materials in particular yolk.

Comparison of properties of both types of aldolases

Some enzymatic properties were investigated using two kinds of crude extracts, from the eggs on day 1 after the long-term chilling and neonates; these were taken to represent the S and F type of enzymes, respectively. The results are summarized in Table 4. Both enzymes belong to the metal-independent aldolase, so-called class I, since the addition of 5 mM EDTA to the reaction mixture did not affect the zymogram pattern and the spectrophotometrically determined activity level. Optimum pH was in a broad range between 7.5 and 9.0. The FBP/F1P activity ratio was 6.0 for aldolase S, whereas 27.5 for aldolase F. The $K_m$ values for FBP of aldolases S and F were $8.2 \times 10^{-6} M$ and $2.0 \times 10^{-5} M$, respectively. Those for F1P were not determined.

Changes in value of FBP/F1P activity ratio during embryogenesis

Fig. 6 shows the changes in value of the FBP/F1P ratio of the artificial non-diapausing eggs. From day 0 to 7 after the hot-HCl
TABLE 3. Effects of mixing of different preparations to day-8 extract.

<table>
<thead>
<tr>
<th></th>
<th>Reaction rate vs. FBP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>1.05</td>
</tr>
<tr>
<td>Day 8</td>
<td>0.55</td>
</tr>
<tr>
<td>Day 10</td>
<td>1.66</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
</tr>
<tr>
<td>Day 1 + Day 8</td>
<td>1.55</td>
</tr>
<tr>
<td>Day 8 + Day 10</td>
<td>2.22</td>
</tr>
<tr>
<td><strong>Experiment 3</strong></td>
<td></td>
</tr>
<tr>
<td>Homogenized (Day 1 + Day 8)</td>
<td>1.53</td>
</tr>
<tr>
<td>Homogenized (Day 8 + Day 10)</td>
<td>2.28</td>
</tr>
</tbody>
</table>

Experiment 1: Aldolase activity was measured as described in text, with 25 μl crude extract. Experiment 2: Different preparation was added to day-8 extract, both 25 μl. Experiment 3: Co-homogenation of day-8 plus day-10 eggs. After centrifugation, the supernatant (50 μl) was used for experiment. Experiments 2 and 3 gave additive results. Artificial non-diapause eggs of N137 x C137 were used.
FIGURE 5. The localization of aldolase activity in eggs. The samples are W; whole egg, S; serosal membrane, E; embryo and Y; yolk. Each data represents mean of two separate samples. Post-diapause eggs of Gunpo x Shugyoku were used.
### TABLE 4. Properties of silkworm aldolases S and F.

<table>
<thead>
<tr>
<th></th>
<th>Aldolase S</th>
<th>Aldolase F</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ for FBP (µM)</td>
<td>8.2</td>
<td>20</td>
</tr>
<tr>
<td>FBP/F1P activity ratio</td>
<td>6.0</td>
<td>27.5</td>
</tr>
<tr>
<td>EDTA effects</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>7.5 - 9.0</td>
<td>7.5 - 9.0</td>
</tr>
</tbody>
</table>

Animals used were Gunpo × Shugyoku. N137 × C137 gave substantially similar results. Aldolase S was from day-1 eggs and aldolase F from neonates.
FIGURE 6. Changes in value of FBP/F1P activity ratio during embryogenesis in artificial non-diapausing eggs. Day 0 denotes unfertilized eggs taken out of newly eclosed female imagoes and day 10 indicates neonates. Bars stand for S.E. \((N=3 \text{ to } 5)\). N137 \(x\) C137 was used.
treatment, the value of the ratio which was consistently at about 6 exceeded 30 on day 9 and at the neonate stage. On day 8, the reaction rates of both FBP and F1P were very low and the ratio varied from experiment to experiment.

**Immunoblotting analysis of aldolase during embryogenesis**

The aldolase-antiserum used was that previously prepared from purified aldolase S, but it reacts also to aldolase F as described in MATERIALS AND METHODS (see also Chapter V). Aldolases S and F had a molecular weight of 40,000 (Chapter V). In all immunoblotting analyses of crude extracts prepared during post-diapause embryogenesis, the anti-aldolase serum strongly reacted with the protein band with a molecular weight of 40,000 (Fig. 7). The intensity in this immunoreactive band was constant on days 1 to 5. On days 6 and 7, the intensity became somewhat weaker (with concomitant appearance of smaller bands) and again increased up to day 9, the time when larvae hatched. It is highly likely that the band with the molecular weight of 40,000 represents aldolase protein. Its changes in intensity seemed to be in agreement with the changes in aldolase activity (cf. Figs. 1 and 4A). The author infers that the fluctuation of activity mirrors essentially that of the amount of aldolase molecule.
FIGURE 7. Immunological detection of aldolase protein. Western blot analysis was done with crude extracts after SDS-PAGE. Lanes 1 to 8 contained extracts of eggs during the post-diapause development (numbers denote the stages and the respective days of embryogenesis). Lane 9, extract of neonates. Plausible aldolase protein (the molecular weight of 40,000) is marked with an arrow. Rabbit muscle aldolase (40,000 in mol. weight) co-migrated with this band (not shown). The positive bands with smaller sizes might be artifacts or degradation products of aldolase. Gunpo × Shugyoku was used.
DISCUSSION

1. Assay method for the silkworm aldolase

The presently applied assay method for aldolase activity is based on the principle that limiting amounts of aldolase are incubated with excess glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase and NADH. Dihydroxyacetone phosphate is formed from FBP or F1P by aldolase and quantitatively converted to α-glycerol phosphate with the concomitant oxidation of a stoichiometric amount of NADH to NAD. The coupled reaction proceeds at a measurable rate that is followed spectrophotometrically by loss of absorption at 340 nm only in the presence of aldolase. The quantity of NADH oxidized is equated with dihydroxyacetonephosphate formed, and in turn related to the moles of substrate cleaved during the reaction period (2 moles of NADH are oxidized for every mole of FBP cleaved, and 1 mole of NADH is oxidized for each F1P cleaved). The conventional measuring procedure for aldolase activity (Rajkumar et al., 1966), which depends on the above principle, was reassessed. The crude extracts from silkworm eggs and neonates had some NADH reductive activities that was not depending on aldolase activity. A similar phenomenon was also observed using crude extracts from chicken tissues. The dialyzed 30 to 80% saturated ammonium sulfate fractions (or the void fractions of gel filtration) of the crude extracts showed similar characteristics. When the commercially purified rabbit aldolase preparations were added to the reaction mixture without substrate, no decrease of absorbancy at 340 nm was observed (details not shown). The author suggests that the
nonspecific decrease may be due not to endogenous substrate but to some high-molecular-weight material(s). These may be included in the crude extract from various animal tissues.

The FBP/F1P activity ratios, obtained by the modified method, were larger than the corresponding ones measured when the original method was applied. This was also the case for the crude extracts from chicken skeletal muscle, liver and heart where aldolase isozymes A and B exist, respectively. The higher values seemed to be more plausible since the ratios of the purified aldolase isozymes A and B (from the muscle and liver, respectively) are approximately 50 and 1, respectively (Lebherz and Rutter, 1969). The effects of crude extracts were more marked for F1P than for FBP, since larger amounts of crude extracts were needed for the assay with F1P.

The modified spectrophotometric method was found to be suitable for the measurements of optimal catalytic activities of aldolase using crude extracts from vertebrate or invertebrate tissues. The author applied this method to the following series of study.

2. Embryonic development and aldolase isozymes

In this chapter, the author tried to search for aldolase isozymes during embryogenic development in *B. mori*. Two types of aldolases S and F were detected during embryogenesis by zymogram experiment. Zymogram analysis of whole eggs indicated an exchange of aldolase bands from the type-S to the type-F. The FBP/F1P activity ratio changed from 6 to 30 at the head pigmentation stage. The type of aldolase interchanged at this stage. The eggs before Stage 25 had only the type-S aldolase activity,
which was detected mainly in yolk. At younger stages, aldolase S in yolk may play a part as a glycolysis enzyme in supplying nutrient materials for embryos from the glycogen granules, which are localized in the yolk cells (Miya et al., 1972; Kunkel and Nordin, 1985). The type-S activity was also detected during oogenesis. At early embryonic stages, the presence of aldolase S may depend upon the maternal substances.

The poly(A)+ RNAs were isolated from the silkworm oocyte and these were translatable in a cell-free protein synthesis system containing radioactive amino acids (Fujii and Kawaguchi, 1982; Yara et al., 1994). Results after the administration of actinomycin D suggested that protein synthesis at early developmental stages depended on maternal mRNAs (Kawaguchi and Fujii, 1984). Northern blot hybridization analysis with a recently obtained cDNA clone for B. mori aldolase (see GENERAL DISCUSSION) as a probe suggested the presence of maternal aldolase mRNA in unfertilized and early embryogenic eggs (Yara, 1994). Also B. mori lectine gene transcripts were detected during oogenesis and early embryogenesis by Northern blot analysis (Amanai et al., 1994). However, maternal substances are not limited to mRNA. For example, unfertilized eggs of the silkworm contain chymotrypsin inhibitors (Aratake et al., 1990). The maternal inhibitors are transported from the hemolymph into the developing ovaries and become dominant components in the eggs, where these remain stable during the early embryogenic development and the de novo synthesis of chymotrypsin inhibitors occurred on the latter half of embryogenic development. It is obscure whether maternal aldolase protein(s) is present or not in eggs of B. mori.
The type-S enzyme became almost undetectable while the embryo uses yolk. The activity level of aldolase determined on the whole egg basis decreased to a minimum (not zero) at Stage 26. No intense bands on zymogram and minimum aldolase activity were detected at the head pigmented stage. The aldolase activity paralleled the intensity of "aldolase" band in Western blot analysis. The changes in aldolase activity thus seemed to depend upon the biosynthesis (and/or degradation) of aldolase proteins. Aldolase is not completely absent at the period of switchover. Thus the metabolism could continue in the presence of low aldolase activity. The activity of aldolase after Stage 26 rose again until hatching. The rise is thought to be due to an increase in amount of type-F aldolase, since the F band is the major component at this stage (see Fig. 1). However, when the embryo-rich fraction was analyzed, also the type-S band was seen one day before hatching (see Fig. 2). The extract of whole eggs at Stage 27 often gave the weak type-S band (not shown). The author infers that aldolase S detected here is also a member of embryonic enzymes and in time becomes a larval component like aldolase F, since both bands are present after hatching.

Some enzymatic properties of S and F enzymes were compared by using the crude extracts from the silkworm eggs and neonates. They differed in the FBP/F1P activity ratio and thermostability. As for the activity ratio, the value for aldolase S of the silkworm (6) was found to be intermediate between that of aldolase B of the vertebrate liver (1) and of aldolase C of the brain (12) (Lebherz and Rutter, 1969). On the other hand, the value for aldolase F was as high as 30. Although similar in optimum pH,
aldolases S and F are considered to be isozymes. The interchange of aldolase types thus may involve the differential gene expression (including post-transcriptional processing) to produce distinct isozymes. In mammalian species, the three aldolase isozymes A, B and C, specific to the muscle, liver and brain, respectively (Lebherz and Rutter, 1969), have also different affinities for FBP and F1P, and change their distribution patterns during embryogenic development. In early embryonic stages, aldolase A appears predominantly in the liver, while the amount of aldolase B rises and becomes a major form in the adult liver (Rutter et al., 1963; Matsushima et al., 1968) (see General Introduction for details).

The author was interested in the problem as to whether other enzymes exhibit the changing pattern in activity level like aldolase during embryogenesis of the silkworm. Alkaline phosphatase [E.C. 3.1.3.1], detectable in B. mori eggs (Sugai, 1957; Chino, 1961; Mihara et al., 1988), has been shown to express a minimum level prior to hatching (Koga et al., 1988), like that shown in the present results. The common features of different enzymes prompted the author to the further analyses of B. mori enzyme systems. The results will be described in the next chapter.

**SUMMARY**

Aldolase occurred as two distinct molecular varieties in B. mori in zymogram analysis: a slowly migrating (S) band observed in the ovary and in yolk of the eggs during embryonic development until the stage of head pigmentation and a fast migrating (F) band
appearing after this stage in the embryo. The transition pattern was reproducible in repeated experiments, which were done with the “artificial non-diapause eggs” and “post-diapause eggs.” Enzymatic studies using crude extracts of eggs in the early embryogenic eggs and of neonates indicated that these isozymes differed in the ratio of activities towards the two substrates, namely FBP/F1P activity ratio (6 for S and 30 for F).
Chapter II

Changes in activity of hexokinase and some other enzymes related to aldolase during embryogenesis

INTRODUCTION

The present chapter deals with the occurrence and changes of some enzyme activities related to aldolase, all probably functioning in the carbohydrate utilization during embryogenesis of B. mori. This approach was chosen in the belief that maximal activity measurements of enzymes reflect intrinsic flux capacity for metabolic pathways (Sugden and Newsholme, 1973, 1975). This principle will be again utilized in Chapter IV.

Alkaline phosphatase discussed in the previous chapter was omitted since its specificity is too vast. The selected enzymes other than aldolase includes hexokinase [ATP:D-hexose 6-phophotransferase, E.C. 2.7.1.1] as a key enzyme for the glucose metabolism (Grossbard and Schimke, 1966), phosphofructokinase (PFK) [E.C. 2.7.1.11] as a key enzyme for the FBP metabolism (Storey, 1982), NADP-sorbitol dehydrogenase (NADP-SDH, another name aldose reductase) [E.C. 1.1.1.21] and NAD-sorbitol dehydrogenase (NAD-SDH) [E.C. 1.1.1.14] as enzymes for the polyol pathway generating F1P (NAD-SDH is a key enzyme for this pathway; cf. Yaginuma and Yamashita, 1979) and glucose 6-phosphate dehydrogenase (G6PDH) [E.C. 1.1.1.49] as a rate-limiting enzyme for the pentose monophosphate shunt (Levy, 1979). Also the activities of NAD-
isocitrate dehydrogenase (NAD-iCDH) [E.C. 1.1.1.41] and NADP-isocitrate dehydrogenase (NADP-iCDH) [E.C. 1.1.1.42] were measured as representatives for the TCA-cycle and related pathways.

MATERIALS AND METHODS

Chemicals

NADP and enzyme preparations used for the coupling reactions were purchased from Boehringer (Mannheim, Germany). ATP and isocitrate were from Sigma (St. Louis, U.S.A). Sephadex G-25 Superfine was obtained from Pharmacia LKB Biotechnology (Uppsala, Sweden). Other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan).

Animals

For study of hexokinase, Shunrei × Shogetsu, a hybrid silk-worm (Aizusanshu Co., Fukushima, Japan) were used. On day 2 after oviposition, the eggs were transferred to 5°C and kept for 4 months. To brake the diapause completely the eggs were subjected to the hot-HCl treatment (spec. grav. 1.10 at 48°C for 5 min) and transferred to 25°C to start the post-diapause development. About 98% of the eggs hatched on day 9 of development. For study of other enzymes, commercial hybrid race, Gunpo × Shugyoku (see Chapter I), was used. Newly deposited eggs were kept at 25°C for 30 days to let them enter diapause, then chilled at 5°C for 120 days to terminate diapause and transferred to 25°C. Thereafter, about
95% of the eggs hatched on day 9. Eggs were kept at -80°C until use.

**Preparation of crude enzyme solution for hexokinase**

Eggs or neonates (usually 100 individuals) were homogenized in 500 μl of ice cold PKM (10 mM sodium phosphate buffer, pH 7.0, containing 100 mM KCl and 1 mM 2-mercaptoethanol). The supernatant solution, obtained by centrifugation at 12,000g for 15 min at 4°C, was used for the determination of the level of enzyme activity. Then the supernatant was applied to a column of Sephadex G-25 Superfine, equilibrated with PKM. The column was eluted with PKM and the void fraction was used to characterize the hexokinase activity.

**Activity staining method for hexokinase**

Hexokinase activity was analyzed by the modified method of Grossbard and Schimke (1966). An appropriate volume of crude extract was brought to 10% glycerol using concentrated stock solution and subjected to polyacrylamide gel electrophoresis without addition of SDS (Native-PAGE) according to Davis et al. (1964). The slab was composed of a separation gel with 7.5% (w/v) acrylamide and 0.27% (W/V) N,N'-methylene bisacrylamide in 375 mM Tris-HCl buffer, pH 8.8, connected with a stacking gel with 2.48% polyacrylamide and 0.62% N,N'-methylene bisacrylamide in 125 mM Tris-HCl buffer, pH 6.8. Electrophoresis was carried out at a constant voltage of 50 V for 16 hr at 4°C with the electrode buffer composed of 25 mM Tris and 192 mM glycine, pH 8.3. Gels were stained for hexokinase activity at 37°C in the dark for 30 min by
immersion in 100 mM Tris-HCl buffer, pH 7.4, containing 5 mM MgCl₂, 10 mM glucose, 3.7 mM ATP, 0.13 mM NADP, 0.55 U/ml glucose-6-phosphate dehydrogenase, 0.03 mg/ml phenazine methosulfate and 0.5 mg/ml nitroblue tetrazolium chloride. The development of purple color as a band indicates the hexokinase activity.

Photometric determination of activity levels for different enzymes

All activities described below were measured by the changes in absorbancy at 340 nm with a Hitachi U-3000 spectrophotometer. The temperature at preincubation and incubation was maintained at 30°C using a cell-holder equipped with a circulating hot water bath. One unit (U) of enzyme was defined as the amount that caused the change of 1 μmole substrate per min.

Hexokinase activities were assayed by the method of Grossbard and Schimke (1966). The routine microcuvette contained, at a final concentration, 100 mM Tris-HCl buffer, pH 7.5, 0.75 mM NADP, 5 mM MgCl₂, 4 mM ATP, 25 mM glucose and 0.3 U/ml glucose-6-dehydrogenase (grade I) at a final volume of 450 μl. The mixture was preincubated for 1 min in a cuvette at 30°C and the reaction was started by adding a 50 μl aliquot of the above described crude enzyme solution.

For aldolase, eggs and organs were extracted and the activities were assayed under the conditions as specified in Chapter I.

PFK was assayed essentially according to the method of Storey (1982) using 200 eggs, which were homogenized in 500 μl PKM in a Potter-Elvehjem type microhomogenizer. The homogenate was centrifuged at 12,000 g for 30 min at 4°C and the supernatant was saved. The mixture (500 μl), which contained 50 μl
supernatant, 50 mM glycine-NaOH buffer, pH 8.6, 30 mM 2-mercaptoethanol, 50 mM MgCl₂, 50 mM KCl, 0.2 mM ATP, 0.1 mM NADH, 5 mg aldolase (rabbit muscle, No. 102644), 0.9 mg glycerol 3-phosphate dehydrogenase and 0.1 mg triosephosphate isomerase, was preincubated for 1 min, and the reaction was started by the addition of 40 mM fructose 6-phosphate.

For NAD-SDH and NADP-SDH, enzyme sources were prepared and activities were measured by the methods according to Yaginuma and Yamashita (1979). In brief, 200 eggs were homogenized in 500 µl 100 mM Tris-HCl buffer, pH 8.8. After centrifugation at 12,000g for 20 min at 4°C twice, the supernatant was passed through a small column (1 ml disposable micropipette chip) of Sephadex G-25 equilibrated with 50 mM Tris-HCl buffer, pH 8.8, to obtain the macromolecule fraction. Incubation medium for NADP-SDH consisted of 450 µl 85 mM glycine-NaOH buffer, pH 9.6, 0.75 mM NADP, 2 mM MgSO₄ and 1 M sorbitol. The reaction was started by adding of 50 µl enzyme solution. The mixture for NAD-SDH assay was 75 mM Tris-HCl buffer, pH 8.8, 1.5 mM NAD, 2 mM MgSO₄ and 400 mM sorbitol in a final volume of 450 µl. The reaction was initiated by the addition of 50 µl of enzyme solution.

G6PDH was assayed by the method of Chino (1960) after modification. Samples (100 eggs) were homogenized with a Potter-Elvehjem type microhomogenizer in 500 µl 100 mM Tris-HCl buffer, pH 8.0, and the homogenate was centrifuged at 12,000g for 30 min at 4°C and the supernatant was used as an enzyme preparation. The mixture (450 µl) containing 100 mM Tris-HCl buffer, pH 8.6, 10 mM MgCl₂, 0.33 mM NADP and 1 mM glucose 6-phosphate was preincubated for 1 min and the reaction was initiated by
the addition of 50 μl supernatant (after diluted to give an absorbancy increase of 0.01 to 0.02 for 1 min).

For NAD-iCDH and NADP-iCDH, 200 eggs were homogenized in 500 μl PKM, and the homogenate was centrifuged at 600g for 20 min at 4°C. Aliquots of the supernatant was subjected to the assay of NAD-iCDH activity. The rest was centrifuged at 12,000g for 30 min at 4°C. The supernatant was used as an NADP-iCDH preparation. The assay methods were essentially according to Miake et al. (1976). The mixture for NADP-iCDH (500 μl) was composed of 100 mM Tris-HCl buffer, pH 8.0, 1 mM DL-isocitrate, 0.1 mM MgCl₂ and 0.1 mM NADP. The mixture (500 μl) for NAD-iCDH contained 50 mM sodium phosphate buffer, pH 6.6, 10 mM DL-isocitrate, 3 mM MgCl₂ and 0.5 mM NAD. These were each preincubated for 1 min and the reaction was initiated by the addition of 50 μl enzyme preparation diluted to give an increase in absorbancy of 0.01 to 0.02 for 1 min.

RESULTS

Developmental changes in zymogram pattern of hexokinase

Crude extracts from eggs during the post-diapause development and neonates (Shunrei × Shogetsu) were assayed for zymogram pattern of hexokinase (Fig. 8). Until day 4, no bands were observed. On day 5 to 9 of the post-diapausal development, the clear cut difference between the mobility of the two enzymes on Native-PAGE was shown. The hexokinase types are designated A and B in order of increasing motility on Native-PAGE. The slowly-
FIGURE 8. Zymogram patterns of hexokinase during embryogenesis. Lanes 1 to 8 contained extracts of eggs during the post-diapause development (numbers denote the stages and the respective days of embryogenesis). Lane 9, extract of neonates. A and B along the right margin indicate the band positions of types A and B, respectively. Day 7 is the head pigmentation stage. Other positive bands might be artifacts. Shunrei × Shogetsu was used.
migrating band B was consistently seen on days 5 to 6 and at the neonatal stage. In some cases, the B-type band was observed on days 3 and 4 (not shown). On the other hand, the faster-running band A appeared on days 7 and 8. These results suggested that hexokinase of the embryogenic eggs exist in the two forms, which are interchanged at two different stages of late embryogenesis.

Developmental changes in activity level of hexokinase

Hexokinase activity in the whole egg during the post-diapause development was examined. The patterns were expressed in terms of total activities per egg (Fig. 9). There was a gradual increase with age. On day 7, the activity temporary decrease and then made a rapid increase until hatching.

Enzyme properties of two types of hexokinase

Some enzymatic properties were investigated with using the three kinds of crude extracts: from day-6 eggs and neonates to represent the type-B enzyme and from day-7 eggs to represent the type-A enzyme (Table 5). They did not differ largely with respect to apparent $K_m$ values for glucose and ATP, and optimum pH.

Properties of PFK, G6PDH, NAD-iCDH and NADP-iCDH

As preliminary experiments to establish the optimal conditions for the assay of silkworm PFK, G6PDH, NAD-iCDH and NADP-iCDH, some of their properties were determined. The major features of the present findings are summarized in Table 6. The pH dependency was surveyed in 50 mM sodium phosphate buffer for pH 6.0 to 7.5, 50 mM Tris-HCl buffer for pH 7.0 to 8.5 and 50 mM
FIGURE 9. Changes in level of hexokinase activity per egg. Values are means (±S.E.) of five determinations. Day 9 indicates neonates. Shunrei x Shogetsu was used.
TABLE 5. Properties of silkworm and rat hexokinases.

<table>
<thead>
<tr>
<th></th>
<th>Bombyx mori</th>
<th>Rat*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Type A</td>
<td>Type B</td>
</tr>
<tr>
<td>Occurrence</td>
<td>Eggs (day 7)</td>
<td>Eggs (day 5)</td>
</tr>
<tr>
<td>$K_m$ for glucose (μM)</td>
<td>100</td>
<td>75</td>
</tr>
<tr>
<td>ATP (μM)</td>
<td>410</td>
<td>740</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>7.5 - 8.6</td>
<td>7.5 - 8.6</td>
</tr>
</tbody>
</table>

* Data from Grossbard and Schimke (1966). For the silkworm, Shunrei × Shogetsu was used.
TABLE 6. Some kinetic properties of PFK, G6PDH, NAD-iCDH and NADP-iCDH.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Material</th>
<th>Optimum pH</th>
<th>$K_m$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFK</td>
<td>Neonates</td>
<td>8.6</td>
<td>ATP 17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>F6P 300</td>
</tr>
<tr>
<td>G6PDH</td>
<td>Day-1 eggs</td>
<td>8.6-9.0</td>
<td>G6P 72</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NADP 31</td>
</tr>
<tr>
<td></td>
<td>Neonates</td>
<td>8.6-9.6</td>
<td>G6P 73</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NADP 15</td>
</tr>
<tr>
<td>NAD-iCDH</td>
<td>Neonates</td>
<td>6.6</td>
<td>Isocitrate 2,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NAD 40</td>
</tr>
<tr>
<td>NADP-iCDH</td>
<td>Day-1 eggs</td>
<td>8.0</td>
<td>Isocitrate 140</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NADP 150</td>
</tr>
<tr>
<td></td>
<td>Neonates</td>
<td>8.0</td>
<td>Isocitrate 90</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NADP 150</td>
</tr>
</tbody>
</table>

Enzyme activities were measured under the conditions given in text. Results are means of determinations with three separate samples (ranges for $K_m$ were within 5% of the mean values). NAD-iCDH and NADP-iCDH were detected in the cytosol and mitochondrial fractions, respectively. Gunpo × Shugyoku was used.
glycine-NaOH buffer for pH 8.0 to 10.0. The apparent $K_m$ values were determined from Lineweaver-Burk plots. The NAD-iCDH and NADP-iCDH activities were assayed after the cytosol and mitochondria fractions were separated by centrifugation at 12,000g for 30 min. The mitochondrial fraction reduced NAD after the addition of isocitrate. In contrast, the cytosol fraction reduced NADP after the addition of isocitrate. These results indicated that NAD-iCDH is localized in mitochondria and NADP-iCDH is localized in the cytosol. PFK and NAD-iCDH in day-1 eggs were not determined.

Changes in activity level of PFK, G6PDH, NAD-iCDH and NADP-iCDH during embryogenesis

Developmental changes in activity of several enzymes were examined during post-diapause development. The patterns of total activities per egg are seen in Fig. 10. The result for aldolase was exactly the same as that shown in Chapter I (cf. Fig. 4A). PFK gave substantially the same fluctuation pattern of activity as aldolase. NAD-SDH was high in activity during the former half of embryogenesis, making a peak on day 3 and then decreased with age. Also the NADP-SDH activity was maintained at higher levels until day 6 and dropped abruptly. The level of G6PDH activity gradually decreased until day 7 and increased again. The NAD-iCDH activity shown in Fig. 11 remained at low levels for 4 days, followed by an increase towards the hatching stage. In contrast, the NADP-iCDH activity gradually increased with age (Fig. 11).
FIGURE 10. Changes in activity of some enzymes during post-diapause development. Activities were expressed in mU/egg. A, aldolase for F1P (open circles) and aldolase for FBP (closed circles); B, PFK; C, NAD-SDH (open circles) and NADP-SDH (closed circles); D, G6PDH. Each value is the mean of three or five separate samples with ± S.E. Day 0 denotes the day of transfer from 5°C to 25°C. Day 9 shows neonates. Gunpo × Shugyoku was used.
FIGURE 11. Changes in activity of NAD-iCDH (open circles) and NADP-iCDH (closed circles). Activities were expressed in mU/egg. See Fig. 20 for other comments.
DISCUSSION

Hexokinase is a rate-limiting enzyme for the glucose-utilizing pathways. Glucose enters glycolysis through phospholylation to glucose 6-phosphate and it is this reaction that hexokinase involves in. It requires ATP as a phosphate donor. Zymogram analysis indicated that two types of hexokinase are present in *B. mori* eggs. These were interchanged on days 7 and 9 (two days before and shortly before hatching, respectively, in the present sample). The former is the head pigmentation stage, when the activity level of hexokinase showed a minimum value. The pattern was reproducible in repeated experiments with artificial non-diapausing eggs of N137 × C137. These situations of hexokinase are somewhat similar to those of aldolase described in Chapter I. The expression of hexokinase during embryogenesis seemed to be controlled in relation to that of aldolase. However, the interchanging pattern for the hexokinase bands was more complex than that for aldolase, indicating that the expression of the hexokinase gene is complicated. It is reminiscent of the vertebrate hexokinase system, where there are four isozymes named I, II, III and IV. The types I to III include the so-called “low $K_m$ hexokinase (for glucose)” and type IV designated “high $K_m$ glucokinase” (Katzen *et al.*, 1965; Grossbard and Schimke, 1966). Mammalian isozymes I, II and III were found to be highly homologous with molecular weight (about 100,000) consisting of pairs of very similar peptide sequences of type IV isozymes with about a molecular weight of 50,000 (Schwab and Wilson, 1988, 1989, 1991; Thelen and Wilson, 1991). Recently, a genomic clone for type II hexokinase was isolated. The mam-
malian hexokinase gene was shown to be evolved from a glucokinase gene via duplication and fusion with conserving the basic organization (Kogure et al., 1993). Whereas glucokinase occurs only in the liver, the three low $K_m$ type of hexokinase constitute a family, uniform in property but differing in proportion from tissue to tissue (in the rat I, II and III predominate in the brain, muscle and liver, respectively). As to the $K_m$ values for glucose, the silkworm hexokinases A and B were found to be comparable to those of rat type I and type II enzymes. Thus the A and B enzymes could be classified into the low $K_m$ isozyme group. Type B was also detected in the larval muscle, but A was not detected in the muscle and fat body during the post-embryonic development (details not shown). The author's unpublished results have indicated that type B silkworm hexokinase may be a counterpart of the rat type II. A high $K_m$ type of silkworm hexokinase has not been found in the course of this experiment.

In the previous chapter the author analyzed the activity level of aldolase using eggs during embryogenesis and neonates. This was repeated in the present chapter which dealt with several enzymes related to carbohydrate metabolism. The value of the FBP/F1P activity ratio of aldolase activities until the head pigmentation stage was about 6 (Chapter I). This value was much lower than that (30) for neonates, and F1P may be catalyzed more efficiently in young eggs than in neonates. F1P could be supplied from carbohydrate stock via fructose (the polyol pathway), since in the present results the activities of two types of SDH was marked at the early half periods of embryogenesis. The latter finding is the confirmation of previous reports (Chino, 1960; Yaginuma and Yamashita,
At the same time, activity of G6PDH was detected, implying that there is also a potency for the pentose monophosphate shunt. As to PFK, some investigators have argued that its activity was scarce in the eggs at the early embryogenic stages (Kageyama and Ohnishi, 1971). However, conspicuous PFK activity could be detected here, in agreement with Suzuki and Miya (1975). We suggest that the carbohydrate metabolism may not bypass the FBP fission pathway at the early embryogenic stages. In addition, NAD-iCDH, assayed as a representative of TCA cycle members, was active, probably reflecting the energy metabolism necessary for embryogenesis.

The FBP/F1P activity ratio was as high as 30 in the eggs at the latest periods of embryogenesis, and this aldolase may be less adapted for F1P, when compared to the stages before the head pigmentation. Moreover, the total activities of NAD-SDH and NADP-SDH per egg were rather decreasing in activity. The author infers that the metabolism of F1P at these periods were gradually impaired. On the other hand, the activities of hexokinase, aldolase, PFK, G6PDH, NAD-iCDH and NADP-iCDH are all increasing until hatching, augmenting the overall capacities of carbohydrate metabolism. A stock of energy source, glycogen, is used actively and its amount became a minimum shortly before hatching (Chino, 1957). The time when glycogen was abruptly decreasing corresponds to the switchover from aldolase isozymes S to F.
SUMMARY

In the silkworm eggs, two types (low $K_m$) of hexokinase were observed. Zymogram analysis exhibited twice-occurring marked transition, at the stage of head pigmentation and the stage shortly before hatching. Besides, the level of total hexokinase activity made a temporary decrease at the stage of head pigmentation, similarly to the pattern obtained by aldolase enzyme.

The value of the FBP/F1P activity ratio until the head pigmentation stage was confirmed to be 6, lower than the value of 30 at the later stages, suggesting that F1P utilization in young eggs is rather effective. This inference was supported by the present observation that the activities of NADP-SDH and NAD-SDH, responsible for the polyol pathway generating F1P, were high in the eggs at the early half stages of embryogenesis. Also the silkworm eggs were shown to have the activities of PFK, G6PDH, NAD-iCDH and NADP-iCDH.
CHAPTER III

Distribution of aldolase isozymes in post-embryonic organs

INTRODUCTION

Aldolase occurred as two distinct molecular varieties during embryogenesis in *B. mori*, and these interchanged at a later period of development. The activity level assayed exhibited a minimum at the period of interchange. As an extension of the study along this line, the present chapter aims at (1) figuring out the distribution of aldolase variants in post-embryonic, i.e. larval, pupal and adult, organs, and at (2) enumerating more fully the multi-molecular forms of aldolase.

MATERIALS AND METHODS

Animals

Eggs deposited from female moths of a hybrid N137 × C137 (Chapter I) were kept at 25°C for 2 days, then chilled at 2.5°C for 100 days and treated with hot HCl (spec. grav. 1.10, at 48°C for 5 min). The eggs were kept at 25°C to allow the post-diapause development and the hatched larvae were reared on an artificial diet (see Chapter I) at 25°C. Larvae, pupae and adults were sacrificed and organs were dissected as described below. Sometimes larvae,
pupae and adults of Shugetsu × Gunshu (Aizusanshu Co., Fuku­shima, Japan) were used to see the reproducibility. No difference of data were found between the races used and the results for the latter were omitted.

**Dissection**

Male larvae were cut along the mid-ventral line with scissors in ice-cold silkworm Ringer's solution, containing (in mg/l) NaCl 8.62, KCl 100, CaCl2-2H2O 441, Na2HPO4-12H2O 597 and KH2PO4 227 at pH 6.5 (Narahashi, 1963). The silk glands, testes, Malpighian tubules, mid-guts, nervous tissues (nerve cords, ganglions and brain-retrocerbral complexes), fat bodies and heads were taken out, each briefly blotted on filter paper and stored at −80°C until use. The carcasses containing the muscles, cuticles and epidermal cells were saved as a “muscle” fraction. Organs from pupae and adults were collected by the methods as described above.

**Determination of enzyme activity**

The assay methods for activity by zymogram and spectrophotometry were as described in Chapter I. Protein was determined by the method of Bradford (1976) using a Bio-Rad protein assay kit with bovine serum albumin (Wako Pure Chemicals, Osaka) as a standard.
RESULTS

Distribution of aldolase isozymes on post-embryonic organs

The whole body extracts of larvae on day 1 of the 1st instar exhibited both type-S and F bands, and also weak aldolase activity bands between S and F (Fig. 12). Then, larval organs at the 5th instar were examined for zymogram patterns. Typical results are illustrated in Fig. 13. Up to five distinct bands were seen, two corresponding to S and F and three between them. The author tentatively designated the intermediate bands as II, III and IV (implying that bands I and V correspond to S and F, respectively). The type-S band was found in most of the larval organs except for the head. The mid-gut, Malpighian tubule and silk glands gave bands of II, III and/or IV-types. In contrast, the type-F band was detected in the head and muscle (plus epidermal cells and cuticle) but not in the Malpighian tubule and silk glands. The larval fat body exhibited only the type-S band. The author examined also pupal and adult organs for the banding patterns of aldolases. Overall results (including those for larvae) are summarized in Table 7. The fat bodies of pupae and adults gave only the type-S band as for larval fat body. Strong type-F band was found in the muscles (plus epidermal cells and cuticles) in pupae and adults. The composition of the five bands changed from organ to organ. The testes from larvae, pupae and adults gave all of the five bands. This is in contrast to the ovaries which had only the type-S band (see Chapter I). For other organs, there was no sex-dependent difference in aldolase activity patterns. The addition of 10 mM EDTA to the reaction plate did not affect the aldolase activity patterns, indicating
FIGURE 12. Zymogram patterns of aldolase in crude extracts of larvae on day 0 of the 1st instar to day 0 of the 4th instar. S and F on the right margin designate aldolase S and F isozymes, respectively. Abscissa indicates the instar and age in days (e.g. 1L0 means day 0 of the 1st instar). N137 × C137 was used.
FIGURE 13. Zymogram patterns of aldolase in crude extracts of larval organs. On day 4 of the 5th instar, male larvae were dissected and organs were extracted and analyzed. Lanes 1, 2, 3, 4, 5, 6, 7 and 8 denote head, muscle, mid-gut, nerve, Malpighian tubules, posterior plus middle silk glands, anterior silk gland and fat body, respectively. I(F), II, III, IV and V(S) on the right margin designate aldolase isozyme types (see text). N137 x C137 was used.
**TABLE 7. Isozyme patterns of aldolase in larval, pupal and adult organs.**

<table>
<thead>
<tr>
<th>Zymogram</th>
<th>I (F)</th>
<th>II</th>
<th>III</th>
<th>IV</th>
<th>V (S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Larval (day 4 of the 5th instar)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole body</td>
<td>+++</td>
<td>±</td>
<td>±</td>
<td>±</td>
<td>++</td>
</tr>
<tr>
<td>Head</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle*</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mid-gut</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>Nervous tissues**</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>Malpighian tubule</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Silk gland</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Posterior + middle</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Anterior</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+++</td>
<td></td>
</tr>
<tr>
<td>Fat body</td>
<td></td>
<td>++</td>
<td></td>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>Testis</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Pupal (day 3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle*</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat body</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>Testis</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>Adult (newly emerged)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Head</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thorax muscle*</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abdomen muscle*</td>
<td>+++</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat body</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>+++</td>
</tr>
<tr>
<td>Testis</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

*Containing cuticles and epidermal cells.
**Containing nerve cords, ganglia and brain-retrocerebral complexes.

Use of day-8 (instead of day-4) larvae at the 5th instar (at the beginning of spinning) and day-8 (instead of day-3) pupae also gave similar results. N137×C137 was used.
again that the silkworm aldolases are of the class I. Interestingly, the fat body showed a bias to aldolase S and the muscle to aldolase F. The aldolases of these organs were further characterized and the results are described in the following two paragraphs.

**Developmental changes in aldolase activity of fat body**

Fat bodies were collected during the period from day 0 of the 5th instar to the emergence and assayed for the aldolase activity. The value reached a maximum on day 6 of the 5th instar (about 60 mU/mg protein). Before cession of feeding, activity began to decline markedly and only trace activity was found through pupal-adult development (Fig. 14). The value of FBP/F1P activity ratio fluctuated from 10 to 20. To try to understand the relationship between aldolase activity in the fat body and dietary conditions, larvae on day 3 of the 5th instar were kept without feeding during one day. Control larvae were continuously fed. By the starvation, the zymogram banding of isozyme S and the value of FBP/F1P activity ratio were not affected, but the level of activity was decreased (Table 8).

**Comparison of properties of aldolases in fat bodies and muscles**

Some enzymatic properties were investigated using the crude extracts from the fat bodies and muscles and the results are listed in Table 9. The values of the FBP/F1P activity ratio for the fat bodies were significantly lower than those for the muscles. There was no difference in the activity ratio between the same tissues at the different developmental stages. Optimum pH was broad and EDTA did not affect the activities. The apparent Michaelis constants for
FIGURE 14. Changes in activity of aldolase activity (A) and the FBP/F1P activity ratio (B) in larval, pupal and adult fat bodies. A: Enzyme activity was expressed in mU/mg protein of the enzyme solution from five independent experiments. Bars stand for S.E. B: Each value is the mean of two samples. 5L0, PP0, P0 and A0 denote day-0 larvae at the 5th instar, the onset of spining, day-0 pupae and newly emerged adults, respectively. N137 × C137 was used.

<table>
<thead>
<tr>
<th></th>
<th>5L3 (Feeding)</th>
<th>5L4 (Non-feeding)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zymogram</td>
<td>S</td>
<td>S</td>
</tr>
<tr>
<td>FBP/F1P activity ratio</td>
<td>21.1</td>
<td>20.7</td>
</tr>
<tr>
<td>Activity</td>
<td>31.7</td>
<td>64.0</td>
</tr>
<tr>
<td>(mU/mg protein)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Enzyme activity was expressed in mU/mg protein of enzyme solution. Each value represents the mean of five separate samples. S.E. were within 20% of the mean values (not shown). 5L3, 5L4 and 5L6 denote day-3, day-4 and day-6 larvae at the 5th instar. N137 × C137 was used.
<table>
<thead>
<tr>
<th></th>
<th>Fat bodies</th>
<th></th>
<th></th>
<th></th>
<th>Muscles*</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5L4</td>
<td>PP2</td>
<td>P4</td>
<td>A0</td>
<td>5L4</td>
<td>P4</td>
<td>A0</td>
</tr>
<tr>
<td>$K_m$ for FBP (µM)</td>
<td>10</td>
<td>20</td>
<td>50</td>
<td>30</td>
<td>10</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>FBP/F1P activity ratio</td>
<td>17.3</td>
<td>16.2</td>
<td>12.4</td>
<td>11.0</td>
<td>32.0</td>
<td>28.6</td>
<td>52.0</td>
</tr>
<tr>
<td>EDTA effects</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Optimum pH</td>
<td>7.0 - 8.0</td>
<td>7.0 - 8.0</td>
<td>7.0 - 8.0</td>
<td>7.0 - 8.0</td>
<td>7.5 - 9.0</td>
<td>7.5 - 9.0</td>
<td>7.5 - 9.0</td>
</tr>
</tbody>
</table>

*Containing cuticles and epidermal cells.

5L4, PP2, P4 and A0 denote day-4 larvae at the 5th instar, day-2 larvae after the onset of spinning, day-4 pupae and newly emerged adults, respectively. N137 × C137 was used.
the fat body aldolase were at substantially the same order as those for the muscle aldolase, although definite comparison of the kinetics must be done after purification.

DISCUSSION

The whole extracts of larvae, pupae and adults displayed two distinct types of aldolase, named S and F, which were found during embryonic development. From these findings, the author concluded that the S and F are two basic aldolase isozymes of the silkworm. Analysis of organ distribution indicated that aldolases S and F were found to be predominantly distributed in the fat bodies and muscles, respectively. In the vertebrate liver and skeletal muscle, aldolases B and A exist as homotetrameric forms B4 and A4, respectively. The insect fat bodies, which are often most conspicuous materials in the body capacity, are analogized to the vertebrate liver and adipose tissues (Price, 1973; Keeley, 1985). Thus aldolase S in the silkworm can be compared to the mammalian homotetrameric isozyme B. Also the silkworm aldolase F can be liken to the mammalian isozyme A.

The weak aldolase activity bands between S and F were detected in the mid-gut, Malpighian tubule and silk glands. These may be heterohybrids of S and F. This inference will be confirmed in Chapter V. Similarly, in vertebrate tissues containing two of the parental aldolases, five-membered hybrid sets were usually detected. Multiple forms of aldolase were detected also in the crab
muscle, but not in the head, thorax and abdominal regions of the honey bee (Lebherz and Rutter, 1969).

In the blowfly, *Phormia regina*, aldolase in crude extracts of the fat body and muscle were indistinguishable by Native-PAGE (Bauer *et al.*, 1978) but larval and adult fat body aldolases differ in electrophoretic mobility. The FBP/F1P activity ratio of blowfly aldolase assayed in crude extracts was about 20 for the adult (Bauer *et al.*, 1974) and those for the larval and adult fat bodies were 6.3 and 5.0, respectively (Bauer *et al.*, 1978). The latter feature was similar to that of *B. mori* described above, where the activity ratio of the fat body aldolase was significantly lower than that of muscle, and no developmental changes in ratios within the same tissues. The activity ratio for aldolase S of the silkworm (10) was intermediate between those of the blowfly fat body and of the muscle (Bauer *et al.*, 1978). On the other hand, the value for aldolase F was 60, which resembles that for the mammalian muscle type aldolase (50) (Lebherz and Rutter, 1969) and higher than that of the blowfly muscle.

The aldolase S activity in the silkworm fat body was decreased by starvation. Also in the rat liver, prolonged fasting depressed the aldolase B activity, and refeeding with carbohydrate-rich diet brought about activation (Weber *et al.*, 1980). The aldolase S activity in the silkworm fat body abruptly fell at the end of feeding period. The fat body in last instar larvae of *B. mori* accumulates a large amount of lipid, glycogen and proteins as reserves of metabolic energy for the following metamorphosis and these events are under endocrinological control (Inagaki and Yamashita, 1986, 1989). As for the alcohol dehydrogenase gene of *D. melanogaster*,
cis-acting regulatory elements are known to be required for expression. The relevant enhancers contain C/EBP and FTZ-F1 binding motifs (Falb and Maniatis, 1992; Ayer and Benyajati, 1992). C/EBP may be involved in the mammalian liver aldolase B gene expression under different dietary conditions (Gomez et al., 1994) and FTZ-F1 is a member of the nuclear steroid hormone receptor superfamily of D. melanogaster (Ueda and Hirose 1990; Lavargna et al., 1991, 1993; Ohno and Petkovich, 1992; Ayer et al., 1993; Woodard et al., 1994). What types of mechanisms for aldolase gene expression are functioning in the body of the silkworm remain to be elucidated.

**SUMMARY**

Two distinct types of aldolases, named S and F named according to the electrophoretic mobility of activity bands in Chapter I, were detected in organs during post-embryonic development. Isozyme S predominating in the fat bodies and F in the muscles. In addition, three bands were exhibited between the S and F bands on zymograms. The composition of the five bands depended upon the organ. Enzymatic properties investigated using crude extracts of the fat bodies and muscles showed that different FBP/F1P activity ratios (10-20 for the fat bodies whereas 30-50 for the muscles).