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# Alkaline phosphatase isozymes in the midgut of silkworm: purification of high pH-stable microvillus and labile cytosolic enzymes

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**Summary.** Genetically defined alkaline phosphatase (ALP) isozymes from the larval midgut tissues of *Bombyx mori* were purified and characterized. The membrane-bound form (m-ALP) was solubilized with 1% Triton X-100, then purified by DEAE-Sephacel, Con A-Sepharose 4B and Ultrogel AcA 34 column chromatography. The soluble form (s-ALP) was purified by DEAE-Sephacel, epoxy Toyopearl coupled with phosphonic acid and Ultrogel AcA 34 column chromatography. About 840- and 650-fold purification were achieved for m-ALP and s-ALP, respectively, and both ALPs were homogeneous as judged by polyacrylamide gel electrophoresis. Both forms were found to be similar (MW = 68000 in gel permeation chromatography, and a single subunit as a monomer in denaturing SDS-polyacrylamide gels with MW = 58000 for m-ALP and MW = 61000 for s-ALP). The pH optima of ALPs were shown to lie at 10.9 (m-ALP) and 9.8 (s-ALP), the former being extremely stable even in pH 10–12 which accords with the physiological milieu in *Bombyx* midgut lumen. The $K_m$ values of the m-ALP and s-ALP for *p*-nitrophenyl phosphate were 1.99 and 1.49 m*M*, respectively. Both ALPs had a similar substrate specificity. L-Cysteine strongly inhibited both ALPs, but inhibitory effects of L-phenylalanine, L-homoarginine and L-leucine were undetectable for s-ALP and very weak for m-ALP. A comparison of enzymatic properties on two ALPs suggested that each isozyme plays different roles.

**Key words:** Alkaline phosphatases – *Bombyx mori* – Insect midgut – High gut pH – Isozyme purification

*Abbreviations: m-ALP* membrane-bound alkaline phosphatase; *s-ALP* soluble alkaline phosphatase

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## Introduction

Alkaline phosphatases (ALP: EC 3.1.3.1) are widely distributed in various organisms. These enzymes are considered to be involved in fundamental biochemical processes but their precise physiological function is not yet clear (McComb et al. 1979). Relatively little is known about ALPs in insects, although extensive studies have been performed on mammalian enzymes. The alimentary canal, which is mostly midgut in lepidopteran larvae, contains ALPs as in mammals. Nakamura (1940) has shown ALP activity in the silkworm, *Bombyx mori*, and the properties of this enzyme have been studied by several investigators (Sugai 1957; Horie 1958; Sridhara and Bhat 1963). Electrophoretic analysis of ALP showed that silkworm midgut has at least two ALP isozymes. One is a slow-migrating band and the other is fast-migrating (Yoshitake et al. 1966). In our laboratory these two isozymes have been separated by differential centrifugation, gel permeation chromatography and electrophoresis, and their properties investigated. The slow-migrating isozyme was found to be membrane-bound, and the fast-migrating isozyme to be soluble (Eguchi et al. 1972a, b; Eguchi 1975).

A number of reports have been published concerning purified preparations of ALP from mammalian tissues (Cathala et al. 1975; Holmgren and Stigbrand 1976; Komoda et al. 1981; Hua et al. 1986). In insects, however, because of the difficulty in obtaining sufficient material, most of the published results are based on experiments in crude enzyme preparations (Wolfersberger 1984). To elucidate the possible functions of midgut ALPs and the relationship between the two isozymes, the present study deals with the purification and characterization of ALPs from the midgut tissues of

silkworms and compared their properties. In addition, characteristics of the silkworm ALPs are discussed from the viewpoint of lepidopteran midgut physiology and comparisons with mammalian enzymes.

## Materials and methods

Silkworms (*Bombyx mori*) were reared on mulberry leaves at 24±1 °C. The 5th instar larvae of a normal strain, Aojuku, were used at 5 days after the 4th larval ecdysis.

*Tissue preparations.* All subsequent procedures were carried out at 4 °C or on ice. The midgut tissues were dissected and washed in a 0.75% NaCl solution, blotted on filter paper and homogenized in a Teflon-glass homogenizer. A 10% (w/v) homogenate in 10 m*M* Tris-HCl buffer (pH 8.0) containing 0.25 *M* sucrose was centrifuged at 105000 × *g* for 1 h, and the resulting pellet and supernatant were collected. This pellet was resuspended in 10 m*M* Tris-HCl buffer (pH 8.0) and made up to a half volume of the original homogenate, to which was added the same volume of 2% (w/v) Triton X-100. After standing at 30 °C for 1 h, the mixture was centrifuged at 105000 × *g* for 1 h and the supernatant used for purification of m-ALP. The supernatant after the first 105000 × *g* centrifugation was used for purification of s-ALP.

*Column chromatography.* DEAE-Sephacel (Pharmacia, Uppsala, Sweden) equilibrated with 10 m*M* Tris-HCl buffer (pH 8.0) containing 0.1 *M* NaCl was poured into a column (2.9 × 25 cm). Each enzyme source of ALP was applied and the column was washed with the same buffer, then eluted with a linear gradient from 0.1 to 0.5 *M* NaCl in 10 m*M* Tris-HCl buffer (pH 8.0).

With regard to m-ALP, the sample was applied to a concanavalin A (Con A)-Sepharose 4B (Pharmacia) column (1.45 × 12 cm) equilibrated with 10 m*M* Tris-HCl buffer (pH 8.0) containing 0.15 *M* NaCl, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$ and 1 m*M* $MnCl_2$ (buffer A). The m-ALP was eluted with a linear gradient (0–25 m*M*) of α-methyl-D-mannoside in 10 m*M* Tris-HCl buffer (pH 8.0) containing above metal ions.

For the affinity chromatography of s-ALP, 4-aminobenzylphosphonic acid epoxy Toyopearl was adopted. The 4-aminobenzylphosphonic acid (Sigma, St. Louis, USA) was coupled to epoxy Toyopearl 650M (TOSOH Co., Tokyo, Japan) according to the method of Landt et al. (1978). The sample was loaded onto a column (1.0 × 11.5 cm) equilibrated with 10 m*M* Tris-HCl buffer (pH 8.0) containing 10 m*M* $MgCl_2$ and 10 m*M* $CaCl_2$ (buffer B). After washing with the same buffer, the column was eluted with 10 m*M* Tris-HCl buffer (pH 8.0) without $MgCl_2$ and $CaCl_2$.

Ultrogel AcA 34 (LKB, Bromma, Sweden) was equilibrated with 10 m*M* Tris-HCl buffer (pH 8.0) containing 0.15 *M* NaCl. The sample was applied to a column (2.2 × 54 cm) and eluted with the same buffer at the flow rate of 25 $ml \cdot h^{-1}$.

*Enzyme assay.* ALP activity was measured by the rate of hydrolysis of *p*-nitrophenyl phosphate (p-NPP) as previously described (Eguchi et al. 1972a, b; Eguchi 1975). The 0.1 *M* sodium borate buffer (pH 11.0) was employed for the determination of m-ALP activity and the same buffer (pH 10.0) containing 10 m*M* $MgCl_2$ for the s-ALP assay. In general, ALP is considered to be a metaloenzyme which requires a certain concentration of cations such as $Zn^{2+}$ or $Mg^{2+}$ (McComb et al. 1979). However, the presence of 10 m*M* $MgCl_2$ in the reaction mixture had no significant effect on m-ALP activity when p-NPP was used as a substrate. One unit of enzyme activity was defined as the formation of 1 μmol *p*-nitrophenol per min at 30 °C under the assay conditions.

To test the substrate specificity, α-naphthyl phosphate disodium salt (α-NP), glucose-1-phosphate disodium salt (G1P), glucose-6-phosphate disodium salt (G6P), sodium-α-glycerophosphate (α-GP), sodium-β-glycerophosphate (β-GP), adenosine-5′-monophosphate (AMP), adenosine-5′-triphosphate (ATP) and p-NPP were used as substrates. In all cases, the inorganic phosphate released from the substrate after 30 min incubation at 30 °C was estimated by the method of Takahashi (1955). Protein concentration was estimated by the method of Lowry et al. (1951) using bovine serum albumin as a standard. In the presence of interfering materials the Lowry method as modified by Bensadoun and Weinstein (1976) was adopted.

*Polyacrylamide gel electrophoresis (PAGE).* Non-denatured electrophoresis of purified m-ALP and s-ALP was carried out on a 7% polyacrylamide disc gel at pH 8.9 (Davis 1964). After electrophoresis, gels were stained for ALP activity with a solution of 0.1% α-NP and 0.1% Fast blue B salt in 0.1 *M* sodium borate buffer (pH 11.0 for m-ALP; pH 10.0 for s-ALP). Gels were stained for proteins with Coomassie brilliant blue R-250. SDS-PAGE was performed on 10% gel by the method of Laemmli (1970).

*Amino acid analysis.* Aliquots of the purified m-ALP and s-ALP were hydrolyzed in 6 *M* HCl for 24, 48, 72 h at 110 °C and chromatographed on a Hitachi 835 amino acid analyzer.

## Results

### *Purification of membrane-bound and soluble alkaline phosphatases*

The silkworm strain (Aojuku) used in this study possessed both types of alkaline phosphatase (ALP) with similar levels of activity. The membrane-bound type (m-ALP) was almost solubilized with 1% (w/v) Triton X-100 without any loss of activity (Table 1). The solubilized and soluble (cytosolic) fractions of midgut homogenate were fractionated with 30–60% ammonium sulfate saturation, dialyzed and separated by DEAE-Sephacel column chromatography, respectively (Fig. 1). Each ALP was eluted from the column as a single symmetrical peak in different positions. The m-ALP was eluted with about 0.3 *M* NaCl and s-ALP with about 0.4 *M* NaCl. Each peak fraction was collected and precipitated with 65% saturated ammonium sulfate and dissolved in buffer A for m-ALP purification or buffer B for s-ALP purification.

Subsequently, affinity chromatography was used for the second stage in the purification of both ALPs. The m-ALP fraction was applied to a Con A-Sepharose 4B column. The adsorbed enzyme was eluted with a linear gradient of α-methyl-D-mannoside at about 10 m*M* (Fig. 2). The s-ALP

**Table 1.** Purification of membrane-bound and soluble alkaline phosphatases from the silkworm midgut

| Purification step | Total activity (unit)[a] | Total protein (mg) | Specific activity (unit·mg$^{-1}$) | Yield (%) | Purification factor |
|---|---|---|---|---|---|
| Membrane-bound ALP | | | | | |
| 1. 105000 *g* precipitate | 570 | 1190 | 0.479 | 100 | 1 |
| 2. Solubilized supernatant | 594 | 697 | 0.857 | 104 | 1.79 |
| 3. $(NH_4)_2SO_4$ 30–60% fraction | 763 | 214 | 3.57 | 134 | 7.45 |
| 4. DEAE-Sephacel column | 742 | 35.1 | 21.1 | 130 | 44.1 |
| 5. Con A-Sepharose 4B column | 729 | 2.32 | 314 | 128 | 656 |
| 6. Ultrogel AcA 34 column | 582 | 1.44 | 404 | 102 | 843 |
| Soluble ALP | | | | | |
| 1. 105000 *g* supernatant | 142 | 798 | 0.178 | 100 | 1 |
| 2. $(NH_4)_2SO_4$ 30–60% fraction | 207 | 575 | 0.36 | 146 | 2.02 |
| 3. DEAE-Sephacel column | 108 | 43.5 | 2.48 | 76 | 13.9 |
| 4. Affinity column[b] | 62.9 | 0.551 | 114 | 44 | 640 |
| 5. Ultrogel AcA 34 column | 18.4 | 0.158 | 116 | 13 | 652 |

[a] 1U = 1 μmol p-nitrophenol·min$^{-1}$
[b] 4-aminobenzyl phosphonic acid epoxy Toyopearl column

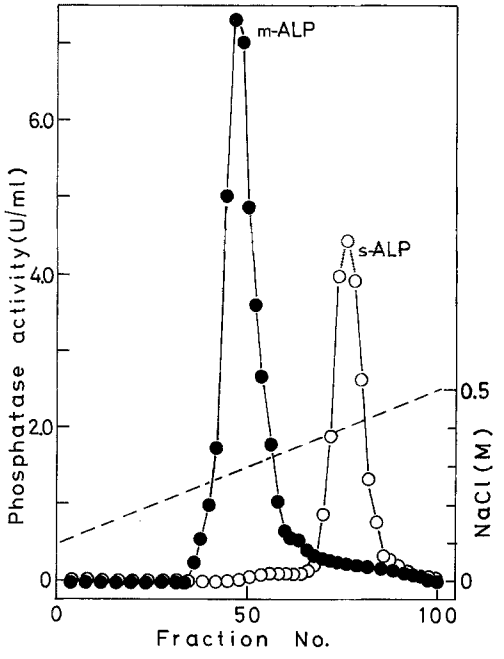


**Fig. 1.** DEAE-Sephacel column chromatography of membrane-bound (m-ALP) and soluble alkaline phosphatase (s-ALP) of the silkworm, *Bombyx mori*. The m-ALP and s-ALP fractions were separately applied to a column (2.9(i.d.) × 25 cm) equilibrated with 10 m*M* Tris-HCl buffer (pH 8.0) containing 0.1 *M* NaCl. Each ALP was eluted from the column with a linear gradient (dashed line) of 0.1–0.5 *M* NaCl. The flow rate was 35 ml·h$^{-1}$ and a 7-ml fraction each was collected

fraction was applied to an affinity column of 4-aminobenzyl phosphonic acid epoxy Toyopearl. The s-ALP was specifically adsorbed on the column and eluted with the buffer B without divalent cations (Fig. 3). Each peak of ALP was collected, concentrated with a membrane filter and then applied to a Ultrogel AcA 34 column. Both ALPs were recovered from the column with a single sharp peak (data not shown).

The summary of purification of both ALPs is shown in Table 1. Approximately 840-fold and 650-fold purification were achieved for m-ALP and s-ALP, respectively. Both ALPs increased their apparent yields following ammonium sulfate fractionation (Table 1). Silkworm midgut tissue itself contains 20–40 m*M* $Mg^{2+}$ and $Ca^{2+}$ (Giordana and Sacchi 1978). These cation levels may affect ALP activity but the high yields of enzyme recovery were reproducible. Purified enzymes were subjected to PAGE analysis (Fig. 4). Each ALP showed a single protein band coincident with the activity band.

### *Enzymatic properties of purified ALP*

Several properties of the purified enzymes were investigated and the following results were obtained.

*Molecular weight estimation.* The molecular weight of each ALP was determined by gel permeation chromatography on a Ultrogel AcA 34 column. The value of Ve/Vo was plotted against the known molecular weight of standards in a graph from which the molecular weights of both ALPs were estimated to be about 68000 (data not shown). This was confirmed by high performance liquid chromatography using TSK gel-G 3000 SW (a molecular sieving column, 7.5 mm (i.d.) × 60 cm, TOSOH Co.). Furthermore, under reducing condition, SDS-PAGE analyis demonstrated a single

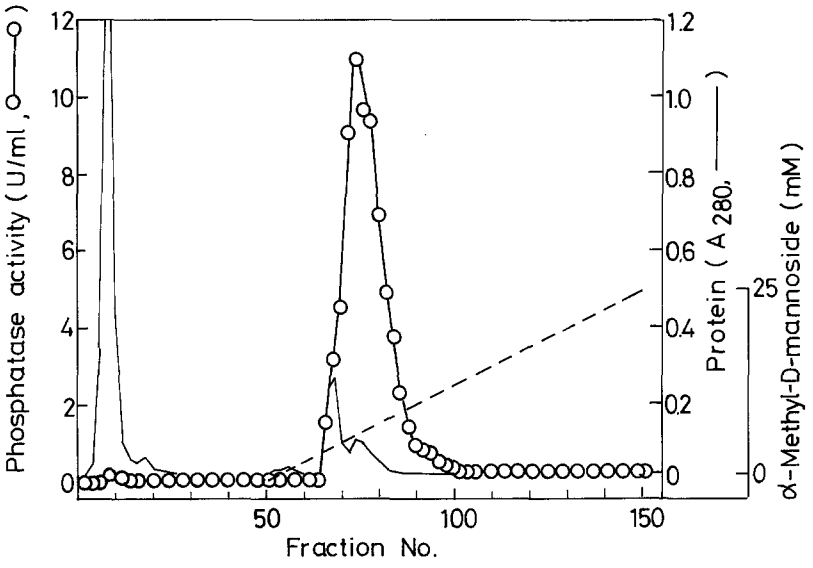


**Fig. 2.** Concanavalin A-Sepharose 4B column chromatography of membrane-bound alkaline phosphatase (m-ALP). The peak fraction of m-ALP after DEAE-Sephacel column chromatography was applied to a column (1.45(i.d.) × 12 cm) equilibrated with 10 m*M* Tris-HCl buffer (pH 8.0) containing 0.15 *M* NaCl, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 1 m*M* $MnCl_2$. The m-ALP (open symbols) was eluted with a linear gradient (dashed line) of 0–25 m*M* α-methyl-D-mannoside. The flow rate was 20 ml·h$^{-1}$ and 3-ml fractions were collected. A solid line shows the absorbance at 280 nm

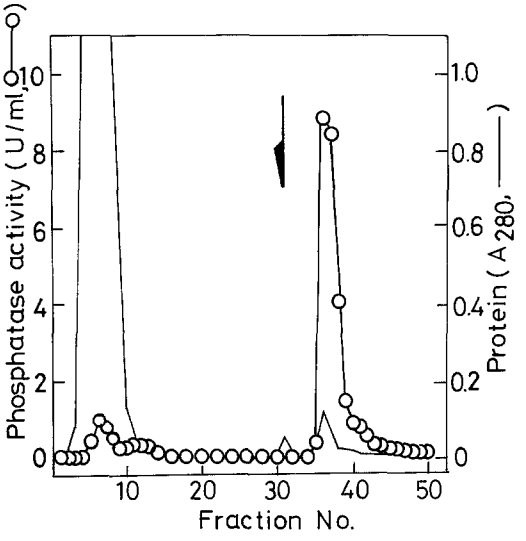


**Fig. 3.** The affinity chromatography of soluble alkaline phosphatase (s-ALP) on a column of 4-aminobenzyl phosphonic acid epoxy Toyopearl (1.0(i.d.) × 11.5 cm). The column was equilibrated with 10 m*M* Tris-HCl buffer (pH 8.0) containing 10 m*M* $MgCl_2$ and 10 m*M* $CaCl_2$. The s-ALP fraction after DEAE-Sephacel column chromatography was applied and the column washed with the same buffer. The s-ALP (open symbols) was specifically eluted from the column with 10 m*M* Tris-HCl buffer (pH 8.0) without divalent cations (arrow). The flow rate was 10 ml·h$^{-1}$ and 3-ml fractions were collected. A solid line shows the absorbance at 280 nm

polypeptide with MW = 58000 for m-ALP and MW = 61000 for s-ALP (Fig. 4).

*Optimum pH and pH stability.* The relationship between pH and enzyme activity is depicted in Fig. 5. The pH optima of these ALPs were shown to lie at 10.9 for m-ALP and 9.8 for s-ALP. These values are fairly comparable to those obtained from crude enzymes (Eguchi et al. 1972a). As shown in Fig. 5, s-ALP displayed a broad curve from the neutral region toward optimum pH, while m-ALP had a sharp peak in a more alkaline region.

Concerning pH stability (Fig. 6), m-ALP was extremely stable in alkaline regions (pH 7–12). On the other hand, s-ALP was gradually inactivated toward more alkaline conditions. Under acidic conditions, below pH 6, both ALPs were found to be unstable.

*Thermal stability.* The enzyme activity of m-ALP decreased when temperature was higher than 50 °C (15 min incubation) and was lost completely at temperatures over 70 °C. The s-ALP was remarkably labile; its activity was almost lost at 40 °C for 15 min.

*Relation between substrate concentration and enzyme activity.* The enzyme activities of both ALPs were determined at various concentrations of p-NPP and followed saturation kinetics. The $K_m$ values were determined to be 1.99 ± 0.11 m*M* at pH 11.0 for m-ALP, and 1.49 ± 0.04 m*M* at pH 10.0 for s-ALP, respectively. The values of $V_{max}$ were 579.0 ± 11.3 units·mg protein$^{-1}$ for m-ALP, and 117.0 ± 2.7 units·mg protein$^{-1}$ for s-ALP, respectively.

*Substrate specificity.* Table 2 represents the rates of hydrolysis of various substrates of both ALPs. They have similar specificities. α-NP is mostly hydrolyzed, particularly by s-ALP. With m-ALP, the addition of 10 m*M* $MgCl_2$ did not cause any increment of enzyme activity, but rather inhibited activity. The hydrolysis of ATP in both ALPs decreased markedly with 10 m*M* $MgCl_2$ and the hydrolysis

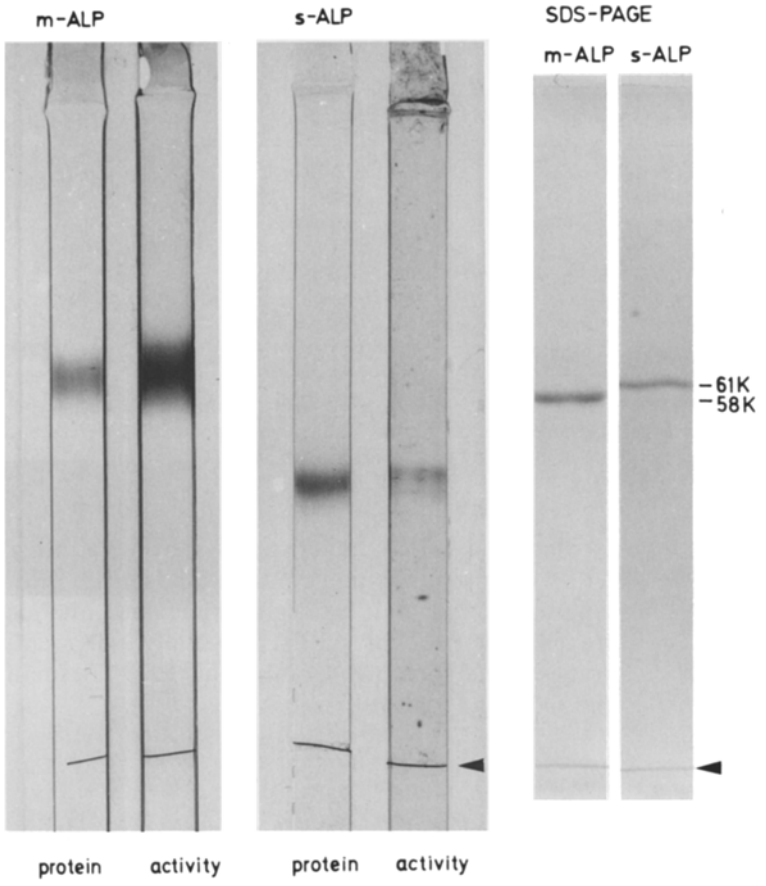


**Fig. 4.** Polyacrylamide gel electrophoresis of purified alkaline phosphatases from the silkworm midgut. The membrane-bound and soluble alkaline phosphatases were applied to respective 7% polyacrylamide gels. After electrophoresis, the gels were stained for protein and enzyme activity. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 10% gels with 0.1% SDS. Dye fronts are marked by arrowheads

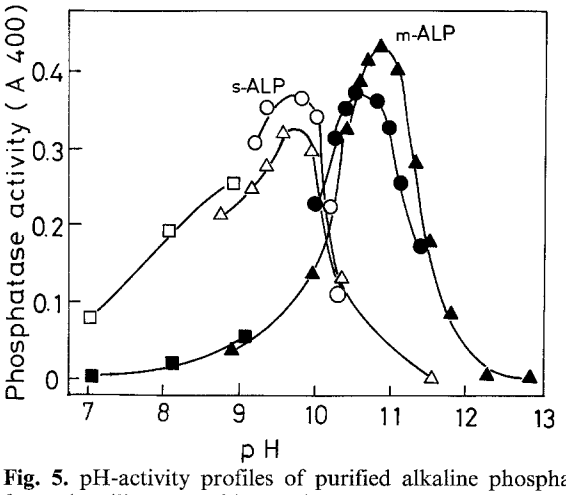


**Fig. 5.** pH-activity profiles of purified alkaline phosphatases from the silkworm midgut. The m-ALP (filled symbols) and s-ALP (open symbols) were assayed with the following buffers; 50 m*M* borate-HCl (■, □) 50 m*M* borate-NaOH (●, ○), 50 m*M* glycine, NaCl–NaOH (▲, △)

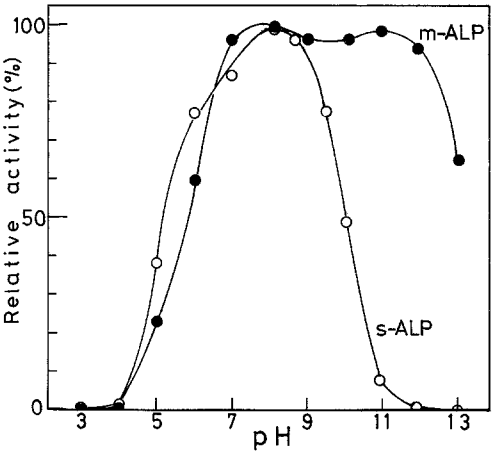


**Fig. 6.** pH-stability profiles of purified alkaline phosphatases from the silkworm midgut. The m-ALP (filled circles) and s-ALP (open circles) were incubated at various pHs for 30 min at 30 °C, and then aliquots were added to the borate buffer at pH 11.0 for m-ALP, or pH 10.0 for s-ALP, and assayed

of AMP was decreased in m-ALP but enhanced in s-ALP.

*Effect of inhibitors.* The m-ALP and s-ALP activities were assayed with various amino acids (5 m*M*), $K_2HPO_4$ (10 m*M*) and urea (2 *M*). The influence of amino acids was similar in both ALPs. Membrane-bound ALP was partially inhibited by L-phenylalanine, L-homoarginine and L-leucine, whereas there was little or no effect on s-ALP.

**Table 2.** Comparison of hydrolytic activity of purified alkaline phosphatases for various substrates with or without $MgCl_2$

| Substrates | Membrane-bound | | Soluble | |
|---|---|---|---|---|
| | No $MgCl_2$ | 10 m*M* $MgCl_2$ | No $MgCl_2$ | 10 m*M* $MgCl_2$ |
| *p*-NPP | 100 (12.8) | 100 (10.3) | 100 (9.17) | 100 (10.7) |
| α-NP | 116 | 118 | 178 | 164 |
| G1P | 45.7 | 30.8 | 50.7 | 53.3 |
| G6P | 80.5 | 58.4 | 68.6 | 73.0 |
| α-GP | 11.4 | 6.7 | 19.3 | 17.9 |
| β-GP | 40.9 | 26.4 | 64.0 | 51.2 |
| AMP | 41.1 | 17.1 | 44.1 | 66.8 |
| ATP | 57.5 | 2.6 | 32.8 | 3.2 |

The membrane-bound (m-ALP) and soluble alkaline phosphatase (s-ALP) activity were assayed with 50 m*M* borate buffer (pH 11.0 for m-ALP, pH 10.0 for s-ALP) containing indicated organic phosphates at 2 m*M*. Values are expressed as percentage for the hydrolysis of *p*-nitrophenyl phosphate (p-NPP). Abbreviations of other substrates are shown in Materials and methods. The value in parenthesis is the enzyme activity (μmol Pi·min$^{-1}$)

**Table 3.** Amino acid compositions of membrane-bound and soluble alkaline phosphatases purified from the silkworm midgut

| Amino acid | No. residues per mol protein | |
|---|---|---|
| | Membrane-bound | Soluble |
| Asp/Asn | 53 (10.1) | 44 ( 8.3) |
| Thr | 40 ( 7.6) | 34 ( 6.0) |
| Ser | 30 ( 5.8) | 46 ( 9.3) |
| Glu/Gln | 56 (11.0) | 58 (12.1) |
| Pro | 19 ( 3.6) | 22 ( 3.9) |
| Gly | 48 ( 9.4) | 57 (11.3) |
| Ala | 52 (10.0) | 63 (11.3) |
| 1/2 Cys | 7 ( 1.3) | 6 ( 1.0) |
| Val | 42 ( 8.0) | 30 ( 5.2) |
| Met | 10 ( 2.0) | 8 ( 1.4) |
| Ile | 13 ( 2.5) | 17 ( 3.0) |
| Leu | 40 ( 7.6) | 48 ( 8.1) |
| Tyr | 11 ( 2.1) | 21 ( 3.6) |
| Phe | 18 ( 3.5) | 13 ( 2.4) |
| Lys | 17 ( 3.3) | 11 ( 2.0) |
| His | 22 ( 4.2) | 29 ( 4.9) |
| Arg | 35 ( 6.6) | 33 ( 5.5) |
| Trp | 7 ( 1.4) | 4 ( 0.7) |
| Total | 520 | 544 |

The values in parentheses are mol%, calculated by assuming the MW = 58000 for m-ALP, MW = 61000 for s-ALP

The effect of L-cysteine was most prominent; most of the activity of both ALPs were lost. The presence of $K_2HPO_4$ inhibited m-ALP activity by about 90%, and s-ALP activity by about 75%. Addition of urea depressed m-ALP activity by 40%, but 90% of s-ALP activity was depressed.

*Amino acid analysis.* Table 3 shows the amino acid composition of two forms of ALP. Both isozymes showed 60% of polar and 40% of non-polar amino acids and had similar compositions. A high content of alanine is a characteristic shared with other ALPs. Comparable high contents of aspartic acid/asparagine, glutamic acid/glutamine and alanine are characteristic of ALPs, especially the membrane-bound form (McComb et al. 1979; Besman and Coleman 1985).

## Discussion

Intestinal ALPs have been reported to be mainly associated with the microvillus plasma membrane as well as enzymes from other organs (Ey and Ferber 1977; Hanna et al. 1979; Besman and Coleman 1985), but little is known about soluble forms of the enzyme (Yedlin et al. 1981) except for intralumenal ALP (Besman and Coleman 1985; Ehle et al. 1985). In insects, the midgut ALP is also presumed to be located at the microvillus plasma membrane (Wolfersberger 1984), and few investigators consider that ALP exists in the soluble compartments (Eguchi et al. 1972a, b). Silkworm midgut contained m-ALP and s-ALP to various degrees which are genetically defined (Yoshitake et al. 1966; Eguchi and Yamashita 1977). To understand the biochemical relationship between two isozymes and their roles in midgut cells, we have purified and examined the properties of ALPs.

So far as we know, this is the first report on purification of insect ALP, although partial purification was done in *Drosophila melanogaster* (Harper and Armstrong 1972, 1973). Two ALP isozymes from silkworm midgut were isolated in a pure form, judging from native-PAGE and SDS-PAGE (Fig. 4). By introducing affinity chromatography (Figs. 2 and 3) for subsequent purification of each ALP, the specific activity was greatly increased without significant loss of the yield (Table 1). The m-ALP is quite stable throughout the

purification procedure as indicated by total activity and the % yield (Table 1). This seems to be reflected in the pH stability (Fig. 6) and heat stability of this isozyme. The lower stability of s-ALP with respect to pH and temperature was undoubtedly the reason for the low recovery of s-ALP (Table 1).

Most ALPs so far studied have been shown to exist in a dimeric form comprising two very similar or identical subunits (Cathala et al. 1975; Holmgren and Stigbrand 1976; Komoda et al. 1981; Hua et al. 1986). Silkworm ALPs are conceived to be a single polypeptide as a monomer with a molecular weight of about 60000 (Fig. 4). This result differs from those for mammalian and bacterial enzymes (McComb et al. 1979). A significant difference between the molecular weight estimation using gel permeation chromatography and SDS-PAGE was observed, which may be due to the glycoprotein nature of both ALPs. In fact, m-ALP was clearly adsorbed to the Con A-Sepharose column (Fig. 2) and the purified m-ALP showed a single positive band by Con A-peroxidase bridge staining (s-ALP also had the same properties, unpublished data). The entity of native ALP molecules in silkworms could be revealed more precisely using other techniques.

In mammals, organ-specific ALP and its inhibition by certain amino acids was reported (McComb et al. 1979). Silkworm midgut ALPs, however, were not inhibited by L-phenylalanine, L-homoarginine and L-leucine, which are utilized as tissue-specific inhibitors in mammalian enzymes. Our experiment revealed that L-cysteine strongly inhibits both ALPs in silkworms. Cysteine-sensitive ALPs were also reported in *Drosophila melanogaster* (Harper and Armstrong 1973) but the inhibition mechanism is not clear.

Considering the location of m-ALP at the brush border surface, it must be continuously suffused with a highly alkaline solution of digestive fluid (pH 11–12) (Eguchi et al. 1986). As described above, m-ALP was extremely stable at high alkalinity and showed higher optimum pH than s-ALP (Figs. 5 and 6). In addition, m-ALP was resistant to digestion with digestive fluid proteinase but s-ALP was labile (Eguchi et al. 1972b; Eguchi 1975). This implies an adaptability of m-ALP to the physiological environment of the midgut lumen in silkworms, as in the tobacco hornworm, *Manduca sexta* (Dow 1984). The question of inconsistency of the high pH optima of various ALPs and lower pH values in mammalian tissues in situ are still unresolved. In the silkworm midgut system, however, there would seem to be a need for elucidating the physiological role(s) of ALP in alkaline milieu. With regard to this point, we have considered the possibility of other enzyme action(s) in our purified ALPs, such as a certain cation-stimulated ATPase (Wieczorek et al. 1986).

The exact localization of s-ALP is uncertain, although it has been shown to be distributed in the cytosolic fraction (Eguchi et al. 1972a). The idea that the s-ALP is a precursor of m-ALP, or that s-ALP is a proteolytic product of m-ALP is unlikely to be the case for the following reasons: (1) the purified m-ALP and s-ALP had different Rf values on polyacrylamide gel (Fig. 4), which was identical with that found in the original homogenate; (2) the interconversion of each ALP during purification was not observed; (3) the ALP in the digestive fluid originated from m-ALP in the midgut tissue, not from s-ALP (Eguchi et al. 1972b; Eguchi 1975); (4) a certain silkworm mutant possessed mainly m-ALP and was deficient in s-ALP, and vice versa (Yoshitake et al. 1966; Eguchi 1975); (5) ALP isozymes are controlled by two distinct genes on the same chromosome (Eguchi and Yamashita 1977). Consequently, it is reasonable to assume that m-ALP and s-ALP are distinct isozymes. In mammals, s-ALP is specifically present at an early developmental stage (Yedlin et al. 1981; Besman and Coleman 1985). As described above, ALPs of the silkworm midgut were found to show another feature comparable with mammalian enzymes. Further work is in progress to elucidate the immunological relationship between two forms of ALPs and the biosynthesis of these isozymes.

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