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RESEARCH ARTICLE

Purification and properties of alkaline phosphatase of silkworm *Bombyx mori*

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Abstract Alkaline phosphatase (AKP), from the succus entericus of silkworm, was purified using 10%–50% ammonium sulfate fractions, ion exchange chromatography of DEAE-Sepharose, and size exclusion chromatography of Sephacryl S-200. The purification fold was 464 times and specified activity was 3 936 U/mg. Optimum pH value of the phosphatase was 10.5, and was stable between pH 7.5 and 11. The optimum temperature of the phosphatase was 40°C and it was unstable over 50°C. K_m value of the phosphatase was 1.25 mmol/L. In a given condition, the phosphatase was selectively modified by PCMB, NBS, PMSF, TNBS, SUAN, DTT, BrAc, and IAc, the results indicate that PMSF, SUA, BrAc, IAc, and TNBS could obviously inhibit the activity of the phosphatase, and the degree of inhibition depended on the concentration of these reagents. There was little effect on the activity of phosphatase after treatment by PMSF, DTT, and NBT. We primarily conclude that mercapto and imidazole are essential for AKP from silkworm. Also, Lys residue and disulfide bands are necessary to protect the catalysis of the AKP.

Keywords silkworm, alkaline phosphatase, purification, properties

1 Introduction

Alkaline phosphatase (AKP) spreads extensively because animals, plants, and even microbes can produce it. The purified AKP is used in the study of nucleic acid, toxicology, and medicine. It is also used for gene sequencing, gene recombination, and isolation. Frequently, it acts as an important tool in heredity engineering and enzyme-linked immunosorbent assay (Sambrook and Russell, 2002). AKP added in cosmetics is beneficial for the regeneration and metabolism of cells. Diseases can alter AKP and this is clearly obvious in serum, this makes AKP an evidence for some diseases. At present, there a lot of studies about AKP from bacteria and animals, but there are a few studies on AKP from lepidopters. The AKP with the highest activity was screened as material from different kinds of *Bombyx mori*. In order to promote the benefit of silkworm, AKP was effectively purified, and its properties were investigated.

The structure, function, and mechanism of catalysis of AKP are very important for this research.. Chemical modification is the usual method in investigating the functional groups of the active site. There have been a number of reports about the modification of AKP (Zhang, 1997; Tait and Vallee, 1966; Engatron, 1964; Yu and Yan, 1986; Chen and Yan, 1986). However, there are a few investigations on the functional group of AKP from silkworm. Therefore, AKP from silkworm was modified by chemical reagents, and the functional groups were primarily determined. The results provide, not only a reference for physiological and biochemical research on silkworm, but also evidences on the development and application of AKP.

2 Materials and methods

2.1 Materials

Before the materials were determined, the activity assay and analysis on isozyme eletrophoresis of AKPs from over 100 kinds of silkworm were conducted from the silkworm gene bank. The kind of PMY000 was used as material. All of these silkworms were four days old and at the fifth stage (provided by Gene Bank in Key Laboratory of Sericultural Science of Agriculture Ministry, Southwest China University, China).

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2.2 Activity assay

AKP was measured using the previously described methods (Institute of medical analysis in Shanghai, 1979). We used disodium phenyl phosphate as the substrate. One milliliter of carbonate buffer (pH=10.0, 0.1 mol/L) mixed with 1 mL disodium phenyl phosphate (0.01 mol/L) was preheated for 5 min at 370°C, and then 0.1 mL enzyme solution was added. We then blended the solution evenly and incubated it at 370°C. Fifteen minutes later, we added 3 mL potassium ferriccyanide into the mixture to end the reaction. The absorbance at 510 nm was measured spectrophotometricaly. One unit of AKP was defined as the amount of AKP produced per 1 μmol benzene per minute under standard conditions.

2.3 Protein estimation

Protein concentration was determined by spectrophotometry (Layne, 1957) and the Bradford (Bradford, 1976) method using BSA as the standard.

2.4 Enzyme purification

2.4.1 Preparation of crude enzyme solution

The succus entericus of the silkworm were used as initial sample. The pH value of succus entericus was regulated by frozen Tris-HCl buffer (0.05 mol/L, pH=8.6), and the succus entericus was subjected to 10% ammonium sulphate precipitate. The supernatant obtained after 10% precipitate was further subjected to 50% ammonium sulphate precipitate. The precipitate retaining the activity was resolved by Tris-HCl buffer (0.05 mol/L, pH=8.6) and dialysis against the same buffer. After dialysis, the crude enzyme solution was obtained.

2.4.2 DEAE–sepharose chromatography

DEAE-sepharose treatment was done according to instructions. After the column was packed (26 mm×140 mm), the column was equilibrated with five bed volumes of Tris-HCl buffer. Crude enzyme solution (10 mL each time) was loaded in the columns and eluted by a linear gradient of NaCl solution (0–0.4 mol/L) containing Tris-HCl buffer. The flow rate was 30 mL/h and 5 mL fractions per tube were collected; fractions were monitored at 280 nm.

2.4.3 Sephacryl S-200 chromatography

After the column was packed (16 mm×835 mm) according to instructions, the column was equilibrated for 12 h. The dialysed and concentrated sample after DEAE-sepharose chromatography was resolved and applied on the column (5 mL each time). The sample was eluted by 0.1 mol/L NaCl containing Tris-HCl buffer. The flow rate was 18 mL/h and 3 mL fractions per tube were collected; fractions were also monitored at 280 nm.

2.5 Molecular weight determination

Molecular weight was determined as previously described (Zhang, 1979). Oligomeric molecular weight was determined by SDS-PAGE method (Wang and Fan, 2002) and the concentration of the gel was 7.5%.

2.6 Isoelectrofocusing electrophoresis

The gel was prepared as described (Yang, 2001). The concentration of the gel was 7.5% and the pH gradient was 3.5–10.0. The protein was stained by 0.05% comassie bright blue R-250.

2.7 Kinetic analysis on AKP

Optimal pH and temperature as well as stability of AKP under different pH and temperature were determined as previously described (Tang, 1997), respectively. V_{max} and K_m values were also determined by the Hanes method (Chen and Zhou, 2001), and the range of concentration of the substrate was 0.3–4.76 mmol/L.

2.8 Investigation of functional groups of AKP

Under a given condition, several amino acid residues were selectively modified by phenylmethylsulfonylfluoride (PMSF), 2,4,6-trinitrobenzene-sulfonicacid (TNBS), *p*-chloromercuribenzoate (PCMB), dithiothreitol (DTT), *N*-bromosuccinimide (NBS), succinic anhydride (SUAN), bromoacetic acid (BrAc), and iodoacetic acid (IAc). Meanwhile, enzyme activity changes were measured.

3 Results

3.1 Purification

The AKP from succus entericus of silkworm was purified after ammonium sulphate precipitation, DEAE-sepharose chromatography (Fig. 2), and sepharose S-200 chromatography (Fig. 3). The purified AKP showed a single band in SDS-PAGE (Fig. 3) and isoelectrofocusing eletrophoresis (Fig. 9), which suggested that the AKP had been purified to homogeneity. The specified activity of purified AKP was 464.15-fold higher than that of the crude enzyme solution and 18.44% of activity was recovered (Table 1).

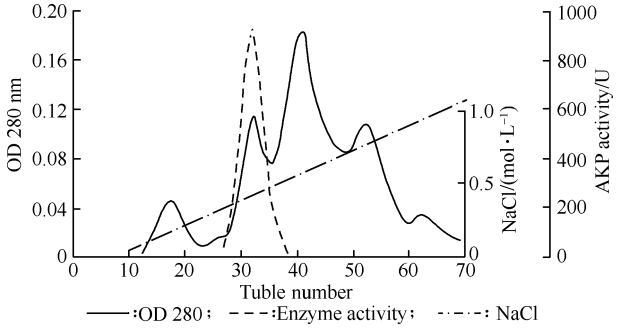


Fig. 1 DEAE-Sepharose ion-exchange chromatography of AKP from silkworm

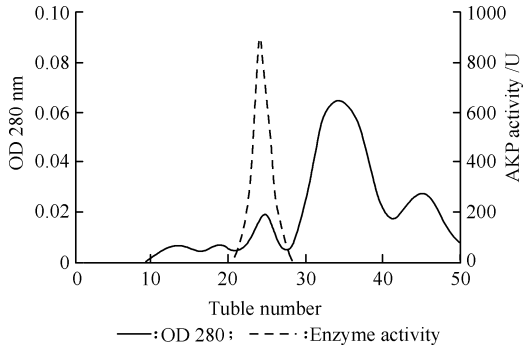


Fig. 2 Sepharcryl S-200 chromatography of AKP from silkworm

Table 1 Purification of alkaline phosphatase from silkworm

Steps	Total protein /mg	Total activity /U	Specific activity /(U·mg^{-1})	Recovery /%	Purified fold
Sample solution	7 044.90	59 764.55	8.48	100.00	1.00
Crude enzyme solution	310.71	55 650.68	179.11	93.11	21.12
DEAE—Sepharose chromatography	29.33	20 739.30	707.16	34.70	89.16
Sephacryl S-200 chromatography	2.80	11 020.85	3 936.01	18.44	464.15

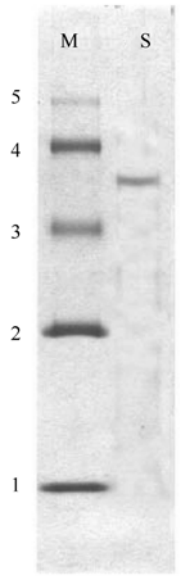
3.2 Molecular weight and oligomeric status

The molecular weight of purified AKP was 110 kDa, which was calculated from gel filtration by Sephacryl S-200 using standard molecular weight markers. It was a dimer of 2–55 kDa subunits as evidenced by SDS–PAGE (Fig. 3).

3.3 Enzyme properties

3.3.1 Optimal pH and stability of pH

The AKP activity was carried out in different pH values between 7.5 and 12. The activity reached the maximum at pH 10.5, and the optimal pH range was from 10.2 to 10.7 (Fig. 4). The AKP was stable at pH 7.5–11.0, while its activity is lost for a few minutes if beyond the range (Fig. 5).



M: Marker; 1: Lysozyme 14.4 kDa; 2: Carbonic Anhydrase, Bovine Erythrocytes 31 kDa; 3: Ovalbumin 43 kDa; 4: Bovine serum albumin 67 kDa; 5: Phosphorylase b; S: AKP submit 55 kDa

Fig. 3 SDS-PAGE of AKP

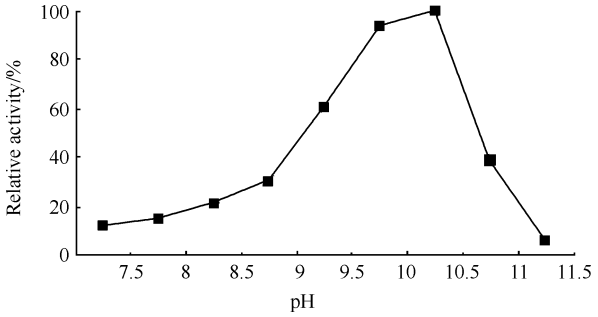


Fig. 4 Effect of pH value on AKP activity

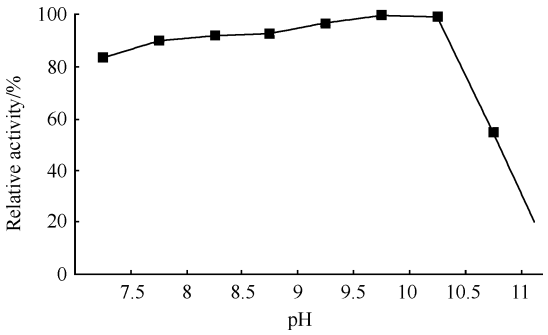


Fig. 5 pH stability of AKP in silkworm

3.3.2 Optimal temperature and temperature stability

The activity reached the maximum at 40°C (Fig. 6), and the AKP was stable between 25 and 50°C within 5 h. Beyond 55°C, especially 60°C, the activity decreased significantly (Fig. 7).

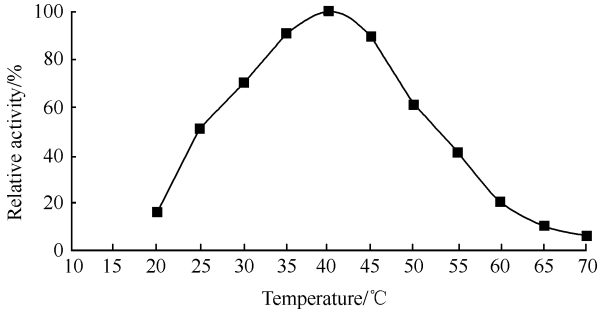


Fig. 6 Temperature-activity of AKP from silkworm

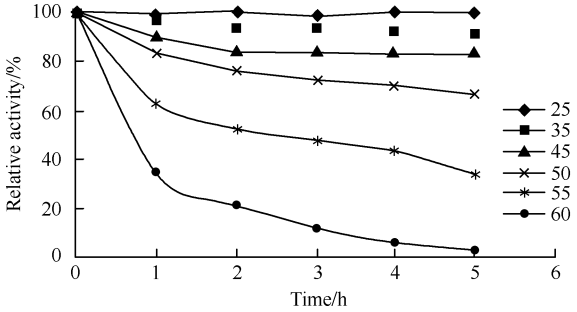


Fig. 7 Stability of temperature of AKP from silkworm

3.3.3 K_m and V_{max}

Using disodium phenyl phosphate as the substrate, both the K_m and V_{max} of AKP were obtained as 1.25 mmol/L, respectively (Fig.8).

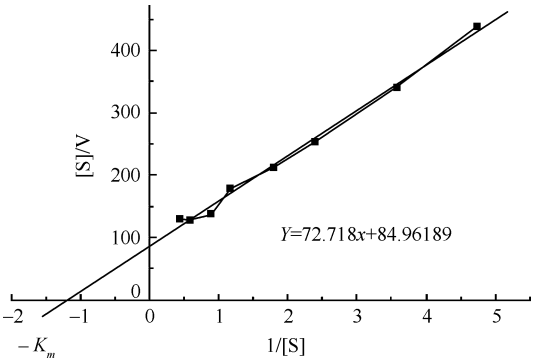


Fig. 8 K_m determination of AKP from silkworm

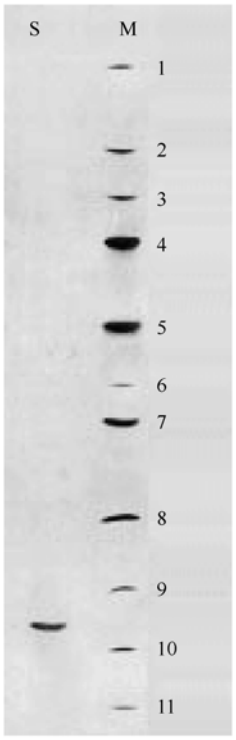
3.4 Isoeletric point determination

The PI of AKP determined by isoelectrofocusing eletrophoresis was pH 4.7 (Fig. 9).

3.5 Investigation on functional groups of AKP

3.5.1 Modification of mercapto of AKP by PCMB

PCMB is a kind of effective reagent that can react with mercapto specifically under partial acid condition (Chen and Yan, 1990). To investigate the correlation between mercapto and enzyme activity, the AKP was modified by PCMB in this paper. The results showed the activity decreased after modification, and the decreasing activity depended on the concentration of PCMB (Fig. 10). When the concentration of PCMB reached 1 mmol/L, the activity of AKP decreased to 5%. With higher concentration of PCMB and longer reaction time, the activity kept slowly decreasing. It was likely that mercapto was essential for enzyme activity, and AKP was a kind of mercapto enzyme.



M: 1. Trypsinogen; 2. Lentil lectin-basic band; 3. Lentil lectin-middle band; 4. Lentil lectin- acidic band; 5. Myoglobin-basic band; 6. Myoglobin-acidic band; 7. Human carbonic anhydrase B; 8. Bovine carbonic anhydrase B; 9. Beta-lactoglobulin A; 10. Soybean trypsin inhibitor; 11. Amyloglucosidase; S. AKP of silkworm

Fig. 9 Isoelectric point determination of AKP from silkworm by IEF-PAGE

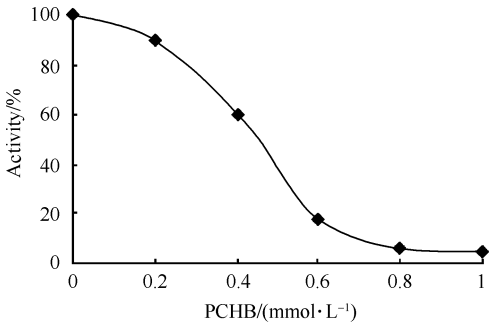


Fig. 10 Effect of PCMB on AKP activity

3.5.2 Modification of Trp residue of AKP by NBS

Since NBS could specifically modify Trp residue of protein

molecular and make enzyme denatured (Freishim and Huennekens, 1969; Viswanatha, 1960), in this paper, in order to investigate the relationship between Trp residue and enzyme activity, the AKP was modified by NBS at different concentrations. The result indicated that with the increasing of NBS concentration, the activity did not decrease significantly (Fig. 11). Even when the concentration of NBS was up to 1 mmol/L, only 19% enzyme activity was inhibited, which suggested modification by NBS had little effect on AKP activity.

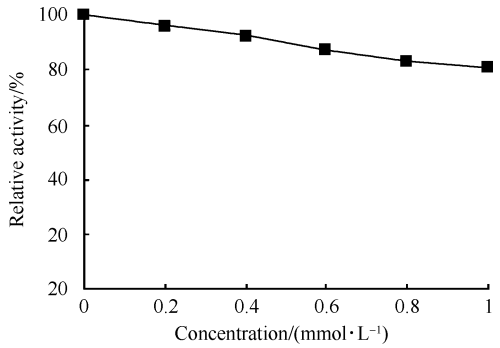


Fig. 11 Effect of NBS on AKP activity

3.5.3 *Modification of amido of AKP by TNBS and SUAN*

The AKP activity decreased when modified by TNBS and SUAN and the decreasing of AKP activity depended on the concentration (Fig. 12). Results showed that 1 mmol/L TNBS and SUAN resolved in carbonate buffer (0.05 mol/L, pH=10.0) could inhibit 51% and 86% of AKP activity, respectively. With higher concentrations of PCMB and longer reaction time, the activity kept slowly decreasing.

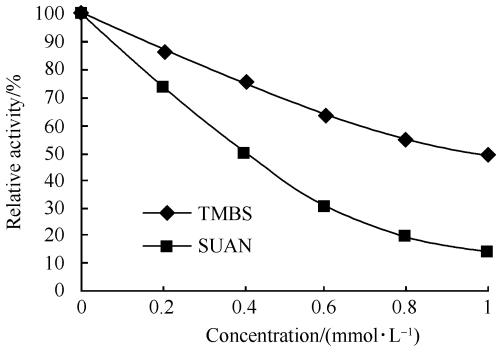


Fig. 12 Effect of TNBS and SUAN on AKP activity

3.5.4 *Modification of disulfide bonds of AKP by DTT*

DTT could react with disulfide Bonds. At 37°C, AKP incubated with DTT for 20 minutes under alkaline condition (pH=10.0, 0.05 mol/L carbonate buffer). With the increase in DTT concentration, AKP activity decreased significantly. When the concentrations were 2.5 mmol/L and 12.5 mmol/L, AKP activity was inhibited 39% and 83% after 20 min, respectively. When the concentration was higher than 12.5 mmol/L, the activity did not change (Fig. 13).

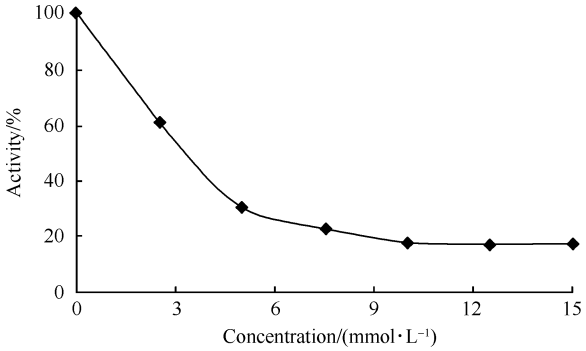


Fig. 13 Effect of DTT on AKP activity

3.5.5 *Modification of His residue by BrAc and IAc*

Under partial acid condition, BrAc and IAc reacted with His residue specifically. In this paper, AKP was modified by 0.1 mmol/L BrAc and IAc respectively in the acetate buffer (pH=5.0, 0.1 mol/L) at 37°C. Twenty minutes later, enzyme activity decreased. When the concentration of BrAc and IAc reached 0.5 mmol/L, the activity was inhibited 68% and 64%, respectively (Fig. 14).

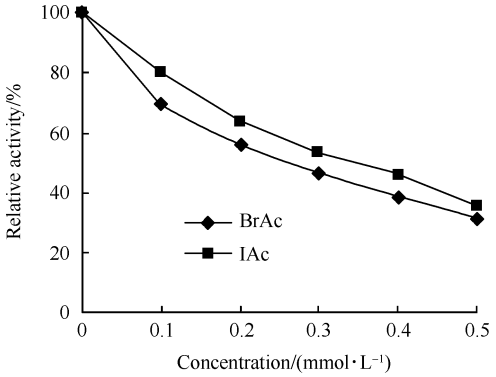


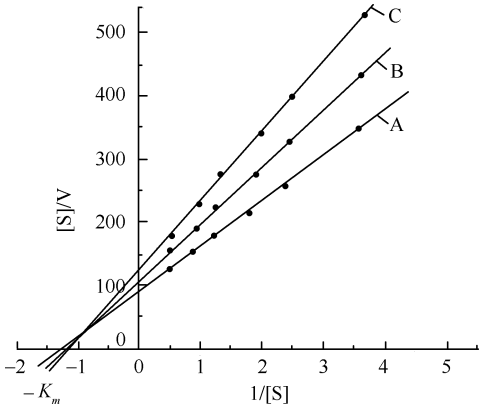
Fig. 14 Effect of BrAc and IAc on AKP activity

3.5.6 *Inhibition kinetics of PMSF and DTT to AKP*

In the carbonate buffer (0.05 mol/L, pH=10.0), AKP reacted with PMSF (0.1 mmol/L) and DTT (1 mmol/L). The activity was measured at different substrate concentrations. The results suggested inhibition of PMSF and DTT to AKP was non-competing (Fig. 15), and DTT was a stronger inhibitor.

3.5.7 *The effect of organic solvent on AKP activity*

The effect of four effectors (methanol, ethanol, glycol, and isopropyl alcohol) on the activity of AKP was determined.



A: [I]=0; B: [PMSF]=0.1 mmol/L; C: [DTT]=1.0 mmol/L

Fig. 15 Inhibition kinetics curve of PMSF and DTT to AKP

The results showed these effectors inhibited the AKP activity significantly (Fig. 16). With increase in organic solvent concentration, the enzyme activity decreased linearly. When the concentration of methanol, ethanol, glycol, and isopropyl alcohol reached 50%, the enzyme activity was inhibited more significantly. At the moment, 85%, 91%, 78%, and 94% of the activity were inhibited, respectively. The strongest inhibitor was isopropyl alcohol, followed by ethanol, methanol, and glycol.

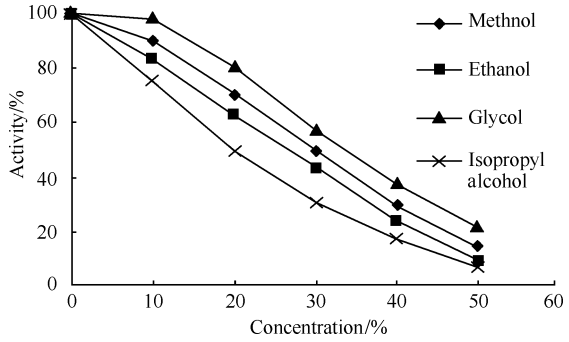


Fig. 16 Effect of methanol, ethanol, glycol, and isopropyl alcohol on AKP activity

4 Discussion

At present, there are a number of reports on purification and properties of AKP. The optimal pH value and temperature for AKP from succus entericus of silkworm were consistent with that of loach (Tang, 1997), *Anodonta woodlana* (Zhang and Liu, 1996), *Silurus meridionalis* (Chen, 1994), and Lateen (Li, 1989), while different from chavannes (Zhao, 2001) and Greencrab (Chen, 1998). This indicated properties of enzyme depend on the kinds of animals. The K_m value was 1.25 mmol/L, which suggested that the affinity of silkworm AKP to substrate was higher than that of lateen, *Silurus meridionalis,* and loach.

The physiological function of AKP had been researched for many years by native or foreign scientists. The physiological function of AKP varied in different tissues. AKP is a kind of membrane-binding protein and a kind of non-specific enzyme that can hydrolyze many kinds of phospholipids. AKP plays an important role in calcium absorption, formation of calcium phosphate, and secretion and formation of chitin, so it is necessary for the growth and survival of organisms. At present, it is usually considered that AKP is involved in the transportation of substance and utilization of phosphorus. AKP also plays an important role in the formation of bones, metabolism of carbohydrate, absorption, and synthesization of carbohydrate (Zhang and Zhang, 2003). The properties of AKP are dependent on heredity background and environment, so the study on AKP from different organisms, not only enrich enzymology, but also supply guidance for yield.

The molecular weight (148 kDa and 70.5 kDa) and PI (pH=3.85 and pH=4.34) of two isozymes of AKP from yolk (Gao, 1991) were different from those of the AKP from silkworm, so the molecular weight and PI of AKPs from different organisms were different (Yan and Chen, 1985; Chen, 1994; Chen and Tang, 1995; Qin and Wei, 1986).

PMSF was a specific modifier to Ser residue, which was a necessary group of AKP from *Ericerus pela* (Zhao, 2001), *Escherichia coli*, Calf-intestine, *Sinonovacula constricty,* and *Anodonta woodiana* Heude. Notwithstanding, different evolution status of organisms, the structure and mechanism of active site of the AKP were conservative. However, there is little effect on the AKP activity after it was modified by PMSF. The difference may result from a special active structure and regulation mechanism of the AKP from silkworm.

PCMB, which can specifically react with mercapto, is often used to modify Cys residue. With the increasing PCMB concentration, the activity of AKP decreased significantly. When the concentration of PCMB reach 1 mmol/L, the enzyme activity remained only at 5%. The result indicates PCMB is a strong inhibitor of AKP, and the mercapto is an essential group of AKP from silkworm. PCMB also strongly inhibited AKP from rat intestines (Frishman and Ghost, 1967), while it shows little effect on AKP from Amphioxus and *Ericerus pela*. We deduce that the mercapto is not always so important for AKPs from different species.

As a special reagent, NBS is often used to modify Trp residue. Zhang and Liu (1996) reported that with the increase in NBS concentration, the activity of AKP decreased suddenly, and characteristic absorption peak disappeared after modification. They concluded that the oxidation of Trp led to denaturation of the AKP. However, the activity of AKP decreases little after modification by NBS, thus it is possible that Trp is not indispensable for AKP. The result further explains the special structure of AKP from silkworm, and Trp is protected from oxidizing.

Modification of the AKP by acetic anhydride and SUAN (Meigher, 1975) resulted in the modification of Lys residue and decrease in the activity. In this paper, modification of Lys by TNBS and SUAN also leads to a decrease in AKP activity. When the concentration of TNBS and SUAN is up to 1 mmol/L, 51% and 86% of activity are inhibited. The

result indicates there is some relationship between the activity and dissociative ε-NH_2 Lys residue.

DTT is a strong reducer, by which disulfide bonds of proteins can be deoxidized. The deoxidization of disulfide bonds leads to a change in construction and functions. In this paper, DTT with different concentration is used to modify AKP in the pH 10.0 carbonate buffer. As a result, the activity decreases when the concentration of the DTT was enhanced. With DTT concentration at 2.5 mmol/L, 60% of the activity remains, and with a much higher concentration as 15.0 mmol/L, 17% of the activity still remains. The results are similar with other reports (Zhang, 1997; Zhao, 2001). We concluded that all disulfide bonds are not involved in catalysis, and disulfide bonds play an important role in maintaining the construction of the active site of the AKP. According to kinetics, DTT is a non-competing inhibitor of AKP. DTT only affects the V_{max}, while it shows no effect on K_m value.

Under partial acid condition, BrAc and IAc specifically react with the imidazole of His residue and carboxymethyl derivatives would be produced after reaction. In the present study, BrAc and IAc can strongly inhibit the activity of AKP. With a concentration of 1 mmol/L of BrAc and IAc, 90% and 88% of the activity were inhibited, respectively. Therefore, BrAc and IAc can effectively modify the imidazole of His residue and denature the AKP. It is likely that imidazole is indispensable to the catalysis of AKP.

As previously discussed, mercapto and imidazole are indispensable and are possibly involved in catalysis. Lys residue and disulfide bonds likely play an important role in maintaining the construction of AKP.

The strongest inhibitor is isopropyl alcohol, while glycol is the worst. Thus, the degree of inhibition to AKP is in proportion to polarity, and hydrophobic interaction is important for the construction of enzymes. At water medium, hydrogen band, static power, and hydrophobic interaction maintain the three dimension construction, and there is a water membrane on the surface of enzymes. When organic solvents are added, the water membrane and secondary band are broken. Thus, the construction of enzymes changes. Finally, the activity of enzymes decreases.

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