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PURIFICATION AND CHARACTERIZATION OF SUCCINYL THIOKINASE FROM PIG HEART

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Key words: hard clam, succinyl thiokinase, hypoxia, isolation.

ABSTRACT

In our earlier studies, the concentration of succinate in the body fluid of hard clams was used as an anoxia-stress indicator. The concentration of succinate was monitored by a color kit the key component of which is succinyl thiokinases (STK). Therefore, finding a source of STK is very important for developing the color kit. STK from pig heart was purified over 850-fold to apparent homogeneity. It has a dimeric structure with a relative molecular mass of 69,200, and there are two types of subunits, α and β, with respective apparent molecular weights of 38,000 and 47,000. Among the isolation steps, the purification factor (14.6) of a CHT ceramic hydroxyapatite column was the highest. STK is heat-labile, and the addition of 2.4 M ammonium sulfate made it more heat-stable than that in 20% glycerol at 60°C. No STK activity was found in the absence of $MgCl_2$. Fifty percent of the activity of STK was inhibited by 8 mM $CaCl_2$ in the presence of 20 mM $MgCl_2$. The stability of STK stored in glycerol was greater than that stored in ammonium sulfate at 4°C. Based on exponential fit data, respective half-lives of STK stored in control, 10% glycerol, 20% glycerol, and 30% glycerol were 1.6, 3.7, 7.5, and 8.2 months.

I. INTRODUCTION

Hard clams are a very important component of the cultured malacofauna in Taiwan. Massive die-offs of clams unexpectedly occur in seasons with high temperature. This greatly impacts the incomes of farmers. However, the exact causes for the massive deaths of clams are still unknown. High stocking densities ($1\sim2 \times 10^6$ seeds/ha) and supplementation with powdered fish meal and fermented organic matter in ponds can result in a reducing layer in the sediments and possibly in low dissolved oxygen (DO) concentrations in bottom waters of ponds [15]. Indeed, a low DO concentration range of 1.5~3.5 mg O_2/L was observed in the bottom water of a clam pond at 24°C in the early morning [21]. Although hard clams have the ability to tolerate some anoxia, anoxia is a type of physiological stress. After anoxic exposure, succinate greatly accumulates in the body fluid of hard clams and can be used as an indicator of anoxic stress [20, 21]. Therefore, monitoring the concentration of succinate in clam body fluid is very important in the management of clam culture. The concentration of succinate can be determined by high-performance liquid chromatography (HPLC) and by a coupled-enzyme reaction [20, 28]. The former can only be carried out in the laboratory, while the latter can be done in the laboratory and in the field. The coupled-enzyme reaction contains three kinds of enzymes: succinyl thiokinase (STK), pyruvate kinase, and lactate dehydrogenase [8]. NADH is oxidized in the presence of succinate in the coupled-enzyme reaction. The concentration of residual NADH is indicated by the formation of formazon due to the reduction of tetrazolium salts by NADH [27, 28, 37]. Therefore, the first step of developing a color kit for determining the succinate concentration is to prepare STK from pig heart, since STK is commercially unavailable.

STK, also termed succinyl-CoA synthetase, is a tricarboxylic acid (TCA) cycle enzyme that catalyzes the substrate-level phosphorylation of GDP by inorganic phosphate, utilizing the splitting of succinyl-CoA as an energy source. The reaction catalyzed by STK is reversible. Succinyl-CoA can be cleaved into succinate and CoA which can also be recombined into succinyl-CoA, depending on whether there is a positive or negative free-energy change. In terms of the specificity of nucleotide substrates, there are two forms of STK: the A-form STK from bacteria uses ATP as a substrate [5, 34, 35], while the G-form STK from mammals uses GTP as a substrate [6]. The enzyme from *Escherichia coli* and other gram-negative bacteria was reported to have a molecular weight (MW) of approximately 142,000 and to be an $\alpha_2\beta_2$ tetramer [7, 36], while the enzyme from porcine heart was found to be an αβ dimer consisting of an α monomer with an MW of 30 kDa and a β monomer with an MW of 41 kDa [7].

Since STK was first found by Kaufman *et al*. [17], the enzyme has been extensively isolated from *E. coli* [14, 31], rat liver [4], pigeon [16], hog kidney [25], and pig heart [8, 10,

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12]. Complementary (c)DNA of the β-subunit of pig-heart STK was also cloned by Bailey *et al*. [3]. STK was provided by Sigma-Aldrich before 2004, but it is commercially unavailable now.

In this paper, we report the isolation of STK from pig heart. There are only six steps in the isolation. A 850-fold purification over the crude extract was achieved with about a 25% recovery of activity. Homogenous STK was found using sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE). The heat stability of STK and the effect of storage on its activity are provided. In order to determine the effect of seawater on the color kit, the effects of ions (Na^+, K^+, Ca^{2+}, and Mg^{2+}) on the activity of STK were also evaluated.

II. MATERIALS AND METHODS

1. Chemicals

Substrates, the cofactor, and reagents were purchased from Sigma Chemical (St. Louis, MO, USA) and Merck (Darmstadt, Germany). Ceramic hydroxyapatite type I was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Sephacryl S-200, Blue-Sepharose 6 Fast Flow, Octyl-Sepharose, and Sephadex G-25 columns were purchased from GE Healthcare (Central Plaza, Singapore).

2. Pig heart

Pig hearts (200~300 g) were freshly purchased from a slaughterhouse. Fatty tissues and connective tissues were removed from the heart which was then rinsed with homogenization buffer (10 mM K_2HPO_4 (pH 6.8) and 0.5% glycerol) and cut into small pieces on ice. Pieces of the heart were homogenized in a ratio of 1:3 (w/v) with cooled homogenization buffer on ice. Homogenates were centrifuged at 9000 ×*g* for 30 min, and the supernatant was filtered through a nylon net filter with a 10-μm mesh and collected as the crude extract.

3. Enzyme Isolation

The temperature was maintained at 4°C during all manipulations unless stated otherwise. The crude extract was applied to a ceramic hydroxyapatite column (2.6 × 40 cm) pre-equilibrated with CHT buffer containing 50 mM K_2HPO_4 (pH 6.8) and 0.5% glycerol. The column was eluted with a linear gradient of 50~500 mM K_2HPO_4 (pH 6.8) in CHT buffer. The flow rate was 1 ml/min. The volume of each fraction was 6 ml. Active STK fractions were combined and slowly precipitated by the addition of ammonium sulfate powder to a final concentration of 70% saturation. The precipitate was stirred on ice overnight and then centrifuged at 12,000 ×*g* for 10 min. The supernatant was removed. The pellet was suspended in an adequate volume of Sephacryl S-200 buffer with 0.25 M Tris-acetate (pH 7.4), 100 mM NaCl, and 0.5% glycerol. The suspension was passed through a Sephacryl S-200 column (2.6 × 100 cm) pre-equilibrated with Sephacryl S-200 buffer. The flow rate was 1 ml/min. The volume of each fraction was 5 ml. The active STK fractions were collected from this column, concentrated in an Amicon cell (PM 10 membrane), and passed through a Sephadex G-25 column pre-equilibrated with Blue-Sepharose buffer containing 30 mM $NaHCO_3$ (pH 6.4) and 0.5% glycerol to remove the salts. The desalted sample was then applied to a Blue-Sepharose 6 Fast Flow column (1.6 × 20 cm) pre-equilibrated with Blue-Sepharose buffer. The column was eluted with a linear gradient of 0~1 M NaCl in Blue-Sepharose buffer. The flow rate was 1 ml/min. The volume of each fraction was 3 ml. The active STK fractions were combined and concentrated in an Amicon cell. Eighty percent saturated ammonium sulfate was slowly added to the active STK concentrate to a final concentration of 20% saturation. This preparation was then applied to an Octyl-Sepharose column (1.6 × 20 cm) pre-equilibrated with Octyl-Sepharose buffer containing 0.25 M Tris-acetate (pH 7.4), 20% saturated ammonium sulfate, and 0.5% of glycerol. The column was eluted with a linear gradient of 20%~0% saturated ammonium sulfate in Octyl-Sepharose buffer. The flow rate was 2 ml/min. The volume of each fraction was 4 ml. The active STK fractions were combined and concentrated in an Amicon cell. The concentrated sample was supplemented with 10% glycerol and stored in a -60°C freezer until use.

4. Assays of STK Activity

The reaction catalyzed by STK is as follows:

$$\text{succinate} + \text{CoA} + \text{GTP} \xrightarrow{\text{succinyl thiokinase}} \text{succinyl-CoA} + \text{GDP} + \text{Pi}$$

The reaction in the forward direction was assayed by measuring the increase in absorbance at 235 nm, which is due to the formation of a thioester bond of succinyl-CoA, using a Hitachi U3100 spectrophotometer equipped with an online data processing computer system (Tokyo, Japan). One activity unit was defined as the amount of enzyme forming 1 μmole of succinyl-CoA per min at 25°C. The conversion factor of the absorbance-change to concentration was 4.0 absorbance units per millimole [8]. The assay conditions were: 50 mM Tris-succinate (pH 7.4), 10 mM $MgCl_2$, 0.1 mM GTP, and 0.1 mM CoA. The reaction was initiated by adding the enzyme at 25°C. The reaction was monitored at 235 nm for 1 min, and the reaction velocity was calculated using a time-scanning computer program.

5. Protein Determination

Samples were assayed with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Bovine serum albumin (BSA) was used as the standard.

6. Identification of Enzymes

The molecular weight of native STK was determined using a Sephacryl S-200 column. The standard marker consisted of the following proteins (with the relative molecular mass in parentheses): aldolase (158,000), conalbumin (75,000),

ovalbumin (43,000), and cytochrome c (12,384). SDS-PAGE (with a separating gel consisting of 10% acrylamide at pH 8.8 and stacking gel consisting of 5% acrylamide at pH 6.8) was performed according to the method of Laemmli [19]. The marker consisted of the following proteins (with the relative molecular mass in parentheses): α-lactalbumin (14,400), trypsin inhibitor (20,100), carbonic anhydrase (30,000), ovalbumin (45,000), albumin (66,000), and phosphorylase b (97,000). Protein bands in SDS-PAGE were stained with silver nitrate [26].

7. The Effect of Temperature on the Activity of STK

The effect of temperature on STK's enzyme activity was performed by preincubating purified STK, STK with 20% glycerol, and STK with 2.4 M ammonium sulfate at temperatures ranging 30~70°C for 5 min. The enzymatic activity was determined in buffer of 50 mM Tris-succinate (pH 7.4), 10 mM $MgCl_2$, and 0.1 mM CoA. The reaction was initiated by the addition of 0.1 mM GTP at 25°C. The reaction was monitored at 235 nm for 1 min. The activity of STK without heat-pretreatment was set to 100%.

The heat stability of STK was determined by respectively preincubating purified STK with 20% glycerol at 55°C and purified STK with 2.4 M ammonium sulfate at 60°C for time periods ranging 0~60 min. The other assay conditions were the same as those described above.

8. Effects of Ions on the Activity of STK

The effect of Mg^{2+} ions on STK's activity was determined by the enzymatic activity in the assay buffer of 50 mM Tris-succinate (pH 7.4) and 0.1 mM CoA as well as with various concentrations of $MgCl_2$. The other assay conditions were the same as those described above. In the presence of various concentrations of $MgCl_2$, the maximum activity of STK was set to 100%.

Effects of $CaCl_2$, NaCl, and KCl on STK's activity were evaluated by determining the enzymatic activity in assay buffer of 50 mM Tris-succinate (pH 7.4), 20 mM $MgCl_2$, and 0.1 mM CoA as well as various concentrations of $CaCl_2$, NaCl, and KCl. The other assay conditions were the same as those described above. The STK activity in the presence of 20 mM $MgCl_2$ and in the absence of $CaCl_2$, NaCl, or KCl was set to 100%.

The activity of STK incubated with 10 mM $CaCl_2$ was recovered by the addition of $MgCl_2$. The recovery assay was performed by determining the enzymatic activity in the assay buffer of 50 mM Tris-succinate (pH 7.4), 10 mM $CaCl_2$, and 0.1 mM CoA as well as various concentrations of $MgCl_2$. The other assay conditions were the same as those described above. STK's activity in the presence of 10 mM $MgCl_2$ and in the absence of $CaCl_2$ was set to 100%.

9. The Effect of Storage on the Activity of STK

The enzyme was stored in buffer containing 250 mM Tris-acetate (pH 7.4) and 0.1% NaN_3 with three concentrations of $(NH_4)_2SO_4$ (of 0.8, 1.6, and 2.18 M) or glycerol (10%, 20%, and 30%) at 4°C for 1 year. The enzymatic activity was assayed twice a month. The assay conditions of the activity were the same as those described above. The activity of the enzyme on day 0 was set to 100%.

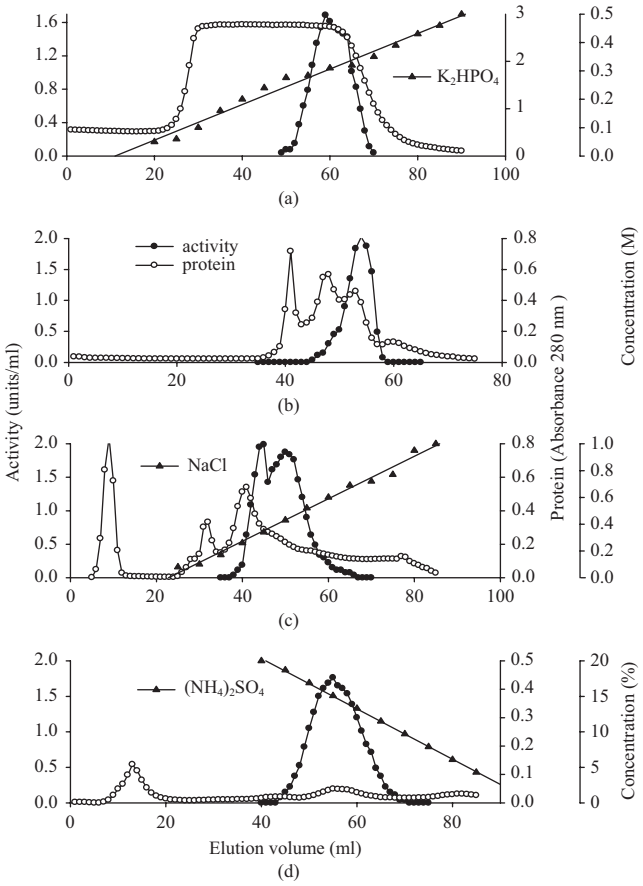

Fig. 1. Chromatography of succinyl thiokinase on a ceramic hydroxyapatite column (a), Sephacryl S-200 column (b), Blue-Sepharose column (c), and Octyl-Sepharose column (d). Details are given in "Material and Methods".

III. RESULTS

STK was separated from a large amount of contaminating proteins through ceramic hydroxyapatite columns (Fig. 1(a)) and a Sephacryl S-200 column (Fig. 1(b)). STK was eluted with a concentration of 0.3~0.5 M NaCl in a Blue-Sepharose column (Fig. 1(c)) and by 18%~10% saturated ammonium sulfate in an Octyl-Sepharose column (Fig. 1(d)). The enzyme was purified by 850-fold to a final specific activity of 212 U (mg protein)$^{-1}$ and was judged to be homogenous by SDS-PAGE (Fig. 2). Determination of the molecular weight of native STK was carried out by Sephacryl S-200 gel filtration. One species, equivalent to 69.2 kDa, was found (Fig. 3(a)). SDS-PAGE showed that highly purified STK contained two types of subunits, α and β (Fig. 2). Their respective apparent molecular weights were 38 and 47 kDa in this study (Fig. 3(b)).

Table 1. Purification of succinyl thiokinase from pig heart.

Steps	Total volumes (ml)	Activities (units/ml)	Total activities (units)	Proteins (mg/ml)	Total proteins (mg)	Specific activities (units/mg)	Yields (%)	Purification factors
Crude homogenate	932 ± 7.6	1.88 ± 0.11	1750 ± 15	7.55 ± 0.75	7030 ± 650	0.25 ± 0.02	100	1
Hydroxyapatite chromatography	174 ± 11	8.95 ± 0.68	1560 ± 19	2.47 ± 0.15	430 ± 46	3.65 ± 0.41	89	14.6
Ammonium sulfate fractionation	34 ± 0.87	42.8 ± 2.0	1460 ± 29	0.5 ± 0.05*	197 ± 17	7.43 ± 0.76	83	2.04
Sephacryl S-200 chromatography	10 ± 0.5	119 ± 5.2	1180 ± 31	5.93 ± 0.71	59.3 ± 6.7	20 ± 1.7	68	2.69
Blue-Sepharose chromatography	11.5 ± 1.0	48 ± 5.2	549 ± 16	1.12 ± 0.11	12.9 ± 1.3	42.9 ± 4.6	31	2.15
Octyl-Sepharose chromatography	5.8 ± 0.64	76.5 ± 7.8	438 ± 8.3	0.36 ± 0.01	2.09 ± 0.29	212 ± 27	25	4.94

*: Protein determination after salt removal by Sephadex G-25 column.

Data from three preparations are presented as the mean ± standard deviation.

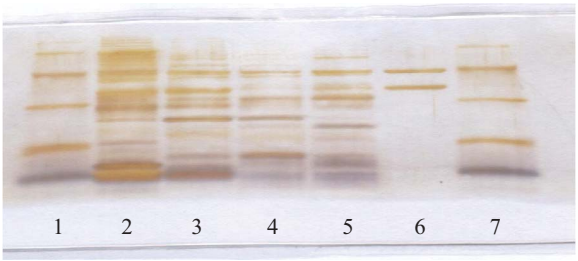

Fig. 2. SDS-gel electrophoretogram of samples after (2) crude extraction (1.55 μg), and extraction through (3) a hydroxyapatite column (1.32 μg), (4) a Sephacryl S-200 column (0.64 μg), (5) a Blue Sepharose column (0.54 μg), and (6) an Octyl Sepharose column (0.24 μg). The protein standards (α-lactalbumin, trypsin inhibitor, carbonic anhydrase, ovalbumin, albumin, and phosphorylase b) are shown in lanes 1 (0.49 μg) and 7 (0.49 μg). The gel was stained for proteins by the silver staining method.

Specific activities, yields, and purification factors of pig-heart STK in each step during isolation are presented in Table 1. Among these isolation steps, the purification factor (14.6) of a CHT ceramic hydroxyapatite column was the highest. The purification factors of ammonium sulfate fractionation, the Sephacryl S-200 column, and the Blue Sepharose column were between 2 and 3. The last step, an Octyl-Sepharose column, also showed a high purification factor (4.94).

The effect of temperature on STK activity was examined by preincubating STK, STK with 20% glycerol, and STK with 2.4 M ammonium sulfate at various temperatures for 5 min. More than 90% of the activity of STK and that of STK with glycerol pretreated at ≤ 50°C for 5 min remained (Fig. 4). The activity of STK and that of STK with glycerol sharply decreased to < 10% at pretreatment temperatures of 55 and 60°C for 5 min, respectively. However, ammonium sulfate seemed to make STK more and less sensitive to heat at pretreatment temperatures of ≤ 50 and > 55°C, respectively. Approximately 50% of the activity of STK with ammonium sulfate pretreatment at 60°C for 5 min remained.

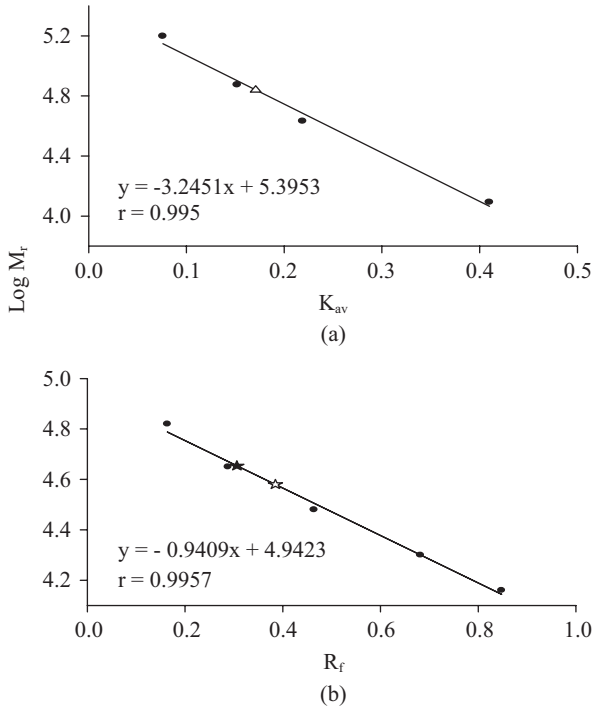

Fig. 3. Plots of K_{av} against the log of M_r corresponding to the determination of the molecular weight of purified succinyl thiokinase (Δ) by a Sephacryl S-200 column equilibrated with 0.25 M Tris- acetate (pH 7.4), 100 mM NaCl, and 0.5% glycerol (a). The protein standards were (1) cytochrome c, (2) ovalbumin, (3) conalbumin, and (4) aldolase. The voided volume was determined by the elution volume of blue dextran 2000. K_{av} = (Ve - Vo)/(Vt - Vo) where Ve is the elution volume for the protein, Vo is the column void volume, and Vt is the total bed volume. Plots of R_f against the log of M_r corresponding to the determination of molecular weight of purified succinyl thiokinase by SDS-PAGE (b). The corresponding R_f values were calculated by dividing the migration distance of the respective proteins by that of the dye marker. The protein standards were α-lactalbumin (14.4 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (45 kDa), and albumin (66 kDa). ☆: α-subunit of succinyl thiokinase; ★: β-subunit of succinyl thiokinase.

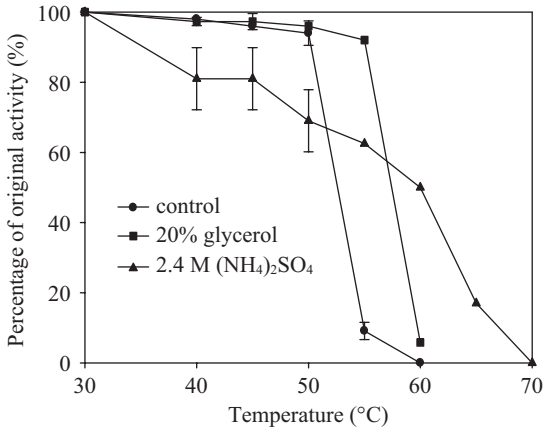

Fig. 4. Effects of 20% glycerol and 2.4 M ammonium sulfate on the activity of succinyl thiokinase pretreated at various temperatures for 5 min.

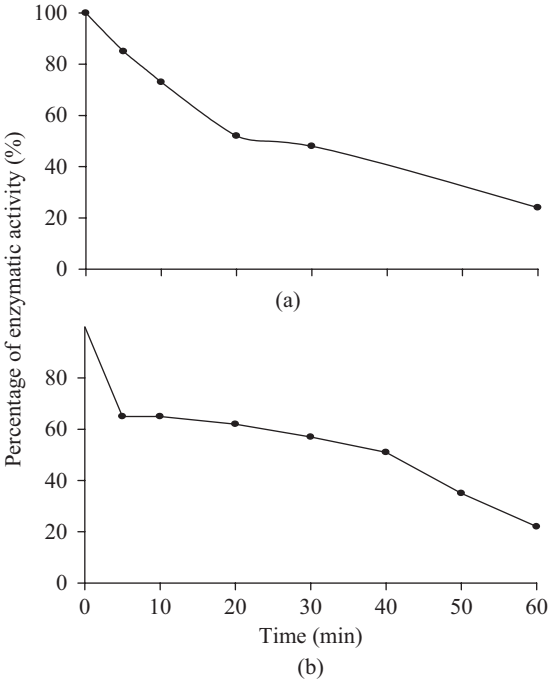

Fig. 5. Effect of pre-incubation time on the activity of succinyl thiokinase with 20% glycerol pretreatment at 55°C (a) and with 2.4 M ammonium sulfate pretreatment at 60°C (b).

In order to determine how heat-stable STK is, STK with 20% glycerol and that with 2.4 M ammonium sulfate were respectively pretreated at 55 and 60°C for 1 h (Fig. 5). Two phases of the activity of STK with 20% glycerol pretreated at 55°C were seen in the time course (Fig. 5(a)). The activity of STK sharply decreased at a rate of 2.4% of activity/min with 20 min of pretreatment, while a low rate of 0.65% of activity/min was observed after 20 min. However, three phases of the activity of STK with 2.4 M ammonium sulfate pretreated at 60°C were seen (Fig. 5(b)). After 60°C pretreatment for 5 min, the activity of STK had dramatically

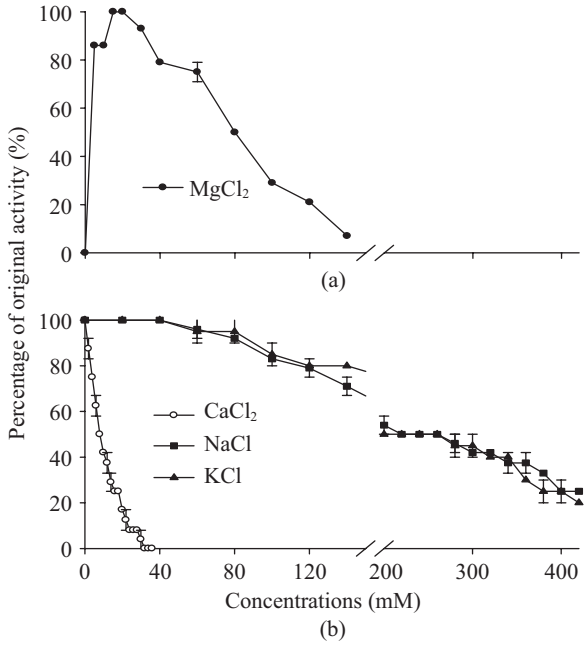

Fig. 6. Effect of $MgCl_2$ on the activity of succinyl thiokinase (a). Effects of $CaCl_2$, NaCl, and KCl on the activity of succinyl thiokinase in the presence of 20 mM $MgCl_2$ (b).

decreased to 63%. The activity of STK was relatively stable during 5~40 min of pretreatment. However, the decrease in the rate of activity of STK became greater after 40 min of pretreatment.

The color reaction catalyzed by STK is used to determine the concentration of succinate in clam body fluid the ionic compositions of which are similar to that of seawater. Na^+, K^+, Ca^{2+}, and Mg^{2+} are major cations in seawater. The effects of these ions on the activity of STK were tested. No STK activity was found in the absence of $MgCl_2$ (Fig. 6(a)). Maximum activity of STK was obtained in the presence of 15~20 mM $MgCl_2$. However, STK activity decreased when the concentration of $MgCl_2$ exceeded 25 mM. Calcium chloride had a great inhibitory effect on the activity of STK in the presence of 10 mM $MgCl_2$ (Fig. 6(b)). $CaCl_2$ at 8 mM inhibited the activity of STK by 50%. However, the inhibitory effects of NaCl and KCl on the activity of STK were minor compared to that by $CaCl_2$. NaCl or KCl at 200~260 mM inhibited the activity of STK by 50%. We also found that 63% of STK activity was recovered by the addition of 40~80 mM $MgCl_2$ in the presence of 10 mM $CaCl_2$ (Fig. 7).

STK lost half of its activity after being stored in 0 and 2.4 M ammonium sulfate for 2 months, while STK stored in 0.8 and 1.6 M ammonium sulfate for 2 months lost only 10% of its activity (Fig. 8(a)). Overall, the stabilities of STK stored in 0.8 and 1.6 M ammonium sulfate at 4°C were greater than those stored in 0 and 2.4 M ammonium sulfate. Eighty percent of the activity of STK stored in 10%~30% glycerol for 2 months remained (Fig. 8(b)). However, the activity of STK stored in 10% glycerol dramatically decreased to 40% and

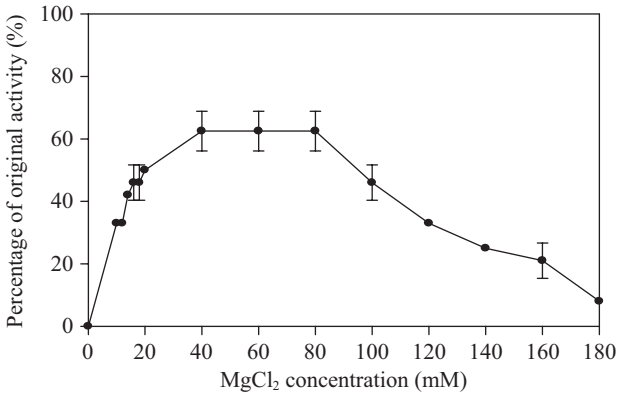

Fig. 7. Effect of $MgCl_2$ on the activity of succinyl thiokinase in the presence of 10 mM $CaCl_2$.

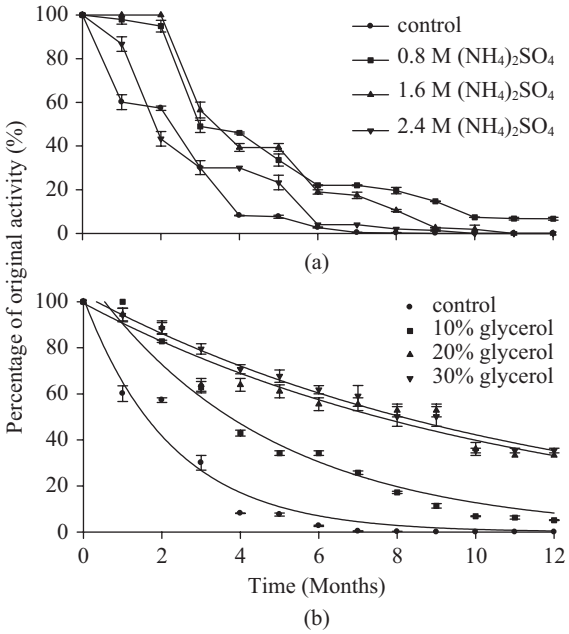

Fig. 8. Effects of storage times on the activity of succinyl thiokinase stored in ammonium sulfate (a) and glycerol (b). The exponential fits of the percentage of original activity (P) against storage time (T) were: control: $P = 101.543*e^{-0.443T}$, $R^2 = 0.97$; 10% glycerol: $P = 112.458*e^{-0.218T}$, $R^2 = 0.97$; 20% glycerol: $P = 99.359*e^{-0.0913T}$, $R^2 = 0.94$; and 30% glycerol: $P = 103.003*e^{-0.0891T}$, $R^2 = 0.98$.

10% after a further 2 and 7 months of storage, respectively. After 8 months of storage, > 50% of the activity of STK stored in 20%~30% glycerol remained. Therefore, the stabilities of STK stored in glycerol were greater than those stored in ammonium sulfate. STK stored in 20% glycerol was as stable as that stored in 30% glycerol. Based on exponential fit data, respective half-lives of STK stored in control, 10% glycerol, 20% glycerol, and 30% glycerol were 1.6, 3.7, 7.5, and 8.2 months.

IV. DISCUSSION

1. Isolation and Molecular Weight

Cha [8] used a calcium phosphate gel prepared by the method of Tsuboi and Hudson [33] to isolate STK. The yield and purification factor were respectively 62% and 8 with this method. Cha *et al*. [10] obtained a 60% yield and a 4.3 purification factor. The major defect with the calcium phosphate gel is the low flow rate and low efficiency. This defect can be over come by using a ceramic hydroxyapatite column. CHT ceramic hydroxyapatite is a spherical, macroporous form of hydroxyapatite which is a form of calcium phosphate. The yield and purification factor were respectively 89% and 14.8 in this study. The purification factor in this step was the highest among all steps. This indicates that this column has the highest efficiency of retaining STK and removing contaminating proteins.

The steps of ammonium sulfate fractionation and use of the Sephacryl S-200 column are necessary during the preparation of STK. STK can be condensed by precipitating proteins from a large-volume preparation by ammonium sulfate fractionation. The yields and purification factors were respectively 77% and 1.7 [8] as well as 75% and 2.64 [10]. They were respectively 93% and 2.04 in this study. The Sephacryl S-200 column can remove a lot of contaminating salts from ammonium sulfate fractionation. The respective yield and purification factor of 79% and 2.76 were obtained with the Sephadex G-100 column [10]. In this study, we obtained a similar result, with the respective yield and purification factor of 81% and 2.69. Information on the application of Blue-Sepharose chromatography and Octyl-Sepharose chromatography to STK isolation is beyond our knowledge. Therefore, there is no information on comparing these two columns. According to knowledge from a review of the literature on STK isolation [4, 10-12, 16, 25, 29, 31], the number of isolation steps was the least and the recovery of activity was the highest in the present work.

The molecular weight of the native form of STK was 69.2 kDa in this study. This is consistent with the observation by Cha *et al*. [10] who also isolated STK from pig heart. However, the total molecular weight of the two subunits of STK determined by SDS-PAGE was 85 kDa, which was much higher than that of its native form. A discrepancy in the molecular weight between the native and denatured forms of STK from rat liver was also found. By the technique of gel filtration on a Sephadex G-200 column, the molecular weight of native STK from rat liver was judged to be approximately 70 kDa, while the subunit molecular weights of 46.5 kDa for the β subunit and 33.5 kDa for the α subunit were obtained by SDS-PAGE [4]. Gel filtration is sensitive to the shape and the size of the protein and also to the pH of the buffer. An effect of the buffer pH on the molecular weight of native STK was reported. Baccanari and Cha determined the molecular weight of native STK from pig heart to be 79.25 kDa at pH 8.0 and 74 kDa at pH 6.0 using a Sephadex G-100 column [2].

The molecular weight of the α-subunit of STK in the study

was 38 kDa which is larger than that (35 kDa) of STK from pigeon liver [16], that (33.5 kDa) of STK from rat liver [4], that (34 kDa) of STK from porcine heart [18], and that (32.11 kDa) of STK from pig heart tissue [32]. In this study, we found that the molecular weight of the β-subunit of STK was 47 kDa which was also larger than that (44.5 kDa) of STK from pigeon liver [16], that (46.5 kDa) of STK from rat liver [4], and that (42.5 kDa) of STK from pig heart [3].

2. Activity Assay, Heat Stability, and Storage

The activity of STK was estimated by measuring the formation of succinyl-CoA. The bond between the succinyl group and CoA has a higher absorbance at 235 nm. However, Cha [8] reported that the linearity between the formation rate of succinyl-CoA and the amount of STK did not hold when the rate exceeded 0.04 absorbance units per minute. The reason may have been due to the instability of the succinyl-CoA formed.

The activity profiles of STK and STK with 20% glycerol pretreated at various temperatures for 5 min were similar, but 20% glycerol seemed to make STK 5°C more heat-resistance. These profiles showed that STK pretreated at > 50°C for 5 min tended to quickly lose its compact three-dimensional structure. However, the activity profile of STK in 2.4 M ammonium sulfate greatly differed from those of STK and STK in glycerol. There was no discrete temperature to disrupt the compact three-dimensional structure of STK in the presence of ammonium sulfate. The heat-labile property of STK was also observed by Baccanari and Cha [2] who found that the activity of STK pretreated at 48°C for 5 min began to decrease, and its activity went to 0% at a pretreatment temperature of 58°C for 5 min.

STK activity is very sensitive to the process of freezing and thawing, but not to lyophilization [10]. The enzyme loses as much as 50% of its activity each time it passes through a freeze/thaw cycle. Therefore, STK should be stored in a lyophilized state or kept at 4°C. In order to prepare the color reaction kit, STK storage at 4°C is convenient for handling of the entire process. Cha *et al*. [10] found that a solution of STK in 0.25 M Tris buffer at pH 7.4~8.0 can be kept for several weeks without an appreciable loss of activity, while STK stored for 2 and 4 months at 4°C in the same buffer respectively lost 40% and 90% of its activity in this study. STK activity protected by the addition of glycerol was also reported by Baccanari and Cha [2]. They found that STK (pI 6.4 isoform) stored in 100 mM Tris-acetate buffer (pH 7.4) containing 30% glycerol retained 70% of its activity over a 2-month period. However, 88% activity of STK stored in 30% glycerol was retained during the same storage period in our study. Cha *et al*. [10] and Cha and Parks [12] pointed out that STK suspended in 80% (3.28 M) saturated ammonium sulfate at 0°C can be kept for a year, while STK stored in 2.4 M ammonium sulfate for 6 months only retained 5% of its activity at 4°C in this study. From the results of our work, we strongly suggest that STK be stored in 0.8 and 1.6 M ammonium sulfate for short-term storage (for as long as 2 months) and in 20% and 30% glycerol for long-term storage (for as long as a year) at 4°C.

3. Effects of Metal Ions on STK Activity

Magnesium ions serve as cofactors or activators for many enzymes, especially kinases, and play several physiological roles in biological systems. Substrates of hexokinases are not free forms of ATP and ADP, but are complexed forms of ATP/ADP and Mg^{2+} [30]. Most free ATP and ADP are bound by Mg^{2+} in cells [23]. In addition, magnesium ions were proposed as being needed during the phosphorylation and dephosphorylation of STK [9]. Therefore, STK activity could be greatly affected by the concentration of complexed forms of GTP/GDP and Mg^{2+}. Although information on the effect of the ionic strength on the stability constants of GTP/GDP and Mg^{2+} is scant, the stability constants of ATP/ADP and Mg^{2+} at ionic strengths of 0 and 0.1 are available [24]. The ionic strength decreases the association equilibrium constants (K) of ATP and Mg^{2+}, since the values of their log K are 5.83 at an ionic strength of 0 and 4.06 at an ionic strength of 0.1 [24]. The effects of Na^+ and K^+ on STK activity were minor. This effect could be attributed to the effect of the ionic strength. Percentages of the GTP-Mg^{2+} complex over total GTP were approximately 100% at ionic strengths of 0 and 0.1 in the presence of 0.1 mM GTP and 20 mM Mg^{2+}. STK activity roughly reached 100% in the presence of 50 mM Tris-succinate, 40 mM NaCl, and 20 mM $MgCl_2$ the ionic strength of which was 0.15.

The effect of Ca^{2+} on STK activity was much greater than those of Na^+ and K^+. One-half of the activity of STK was inhibited by 8 mM of Ca^{2+} in this study. Therefore, calcium ions may be a regulator of STK activity. A significant effect of Ca^{2+} on STK activity can be explained by the stability constants of the Ca-GTP^{2-} complex (log K = 3.58) and Mg-GTP^{2-} complex (log K = 4.02). The percentage of the Mg-GTP^{2-} complex over total GTP decreased from 100% in the presence of 20 mM Mg^{2+} to 87% in the presence of 20 mM Mg^{2+} and 8 mM Ca^{2+}. A significant decrease in the concentration of the Mg-GTP^{2-} complex may have resulted in a decrease in STK activity. Manganese ions can form a complex with GTP (log K = 4.73) as does Mg^{2+}, while Zn^{2+} does not form a complex with GTP [24]. It was reported that 6 mM Mn^{2+} was as effective as Mg^{2+} in activating STK, whereas there was no discernable activity of STK in the presence or absence of 6 mM Zn^{2+} [25].

STK is a key component of the color reaction for determining the succinate concentration in clam body fluid. Therefore, the activity of STK can be affected by the composition of seawater. Hard clams are an osmoconformer organism [13]. Ionic concentrations in their hemolymph and tissues depend more on the ambient environment than those of osmoregulator organisms. There are 55 mM Mg^{2+} and 10 mM Ca^{2+} in seawater with 36 ppt salinity [1]. Although STK activity was inhibited by Ca^{2+}, 60% of STK activity was recov-

ered by 40~80 mM Mg^{2+} in the presence of 10 mM Ca^{2+}. Fortunately, the concentration of Mg^{2+} is more than 5-fold that of Ca^{2+} ions in seawater.

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