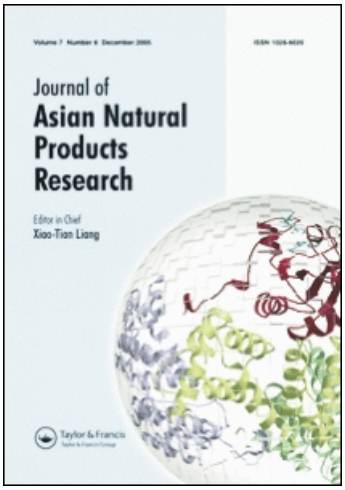

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EFFECT OF LEONURINE ON THE ACTIVITY OF CREATINE KINASE

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The effects of leonurine (**1**) on the activity of creatine kinase (CK) have been studied. The results show that leonurine inhibits enzyme activity in concentration- and time-dependent manners (at 0.75 and 1.51 mmol from 12 to 72 h). There are two mechanisms for the inhibition process. Compound **1** first acts as a non-competitive inhibitor and then as an irreversible inhibitor. Changes of CK were not found in 10% SDS-PAGE, but the amount of dimeric CK decreased in 10% non-SDS gel. The results suggest that **1** can inhibit CK activity by degrading its dimeric structure.

Keywords: Leonurine; Creatine kinase; Guanidyl group; Inactivation

INTRODUCTION

Leonurine (**1**) has long been known to be present as an alkaloid in Chinese motherwort (*Leonurus artemisia* and *Leonurus heterophyllus*) [1]. Motherwort has been widely used in China for centuries to treat dysmenorrhea, menoxenia and other female gynecological disorders [2]. The reported pharmacological effects of **1** include uterotonic action [1], anti-platelet aggregation [3] and inhibition of vascular contractile responses [4].

Creatine kinase (ATP: creatine kinase N-phospho-transferase, EC 2.7.3.2) plays an important role in energy metabolism in vertebrates and has been widely investigated. Creatine kinase (CK) catalyzes the reversible transfer of a phosphoryl group from MgATP to creatine, leading to phosphocreatine and MgADP. Guanidine hydrochloride (GuHCl) is a chemical denaturant often used in the investigation of protein folding [5]. There have been many studies of the changes of conformation and activity of CK denatured by GuHCl [6–8]. Since there is a guanidyl group in leonurine, we hypothesize that **1** can also affect CK function. Our findings suggest that **1** is an effective inhibitor of CK, probably acting by degrading the dimer to subunits of CK.

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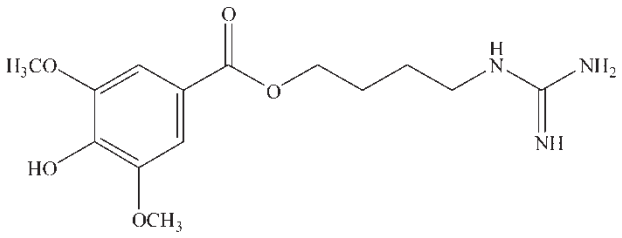

MATERIAL AND METHODS

Materials

The dried leaves of *Leonurus heterophyllus* were extracted with EtOH and concentrated *in vacuo* to leave a brownish fluid that was partitioned with n-BuOH–H_2O (1:1). The n-BuOH-soluble fraction was chromatographed over silica gel (3.5 × 50 cm column) eluted with $CHCl_3$–MeOH (5:1). Compound **1** (purity > 98%) was obtained from the fraction with R_f 0.5 by recrystallization with MeOH. Its structure was determined from its mp, IR, ^{1}H NMR and ESI-MS (*m/z*: 312 $[M + H]^+$) spectral data [9]. Rabbit muscle CK was purified as previously described [10]. The enzyme concentration resolved in Tris-HCl buffer (pH 8.0) was determined by measuring the absorbance at 280 nm with $A^{1\%}_{1cm} = 8.8$ [11]. ATP was purchased from Sigma. All other reagents were local products of analytical grade and were used without further purification.

CK Activity Assay

The CK activity was determined at 25°C by following proton generation due to the reaction of ATP and creatine with the indicator Thymol blue at 597 nm. The reaction mixture contained 48 mmol creatine, 4 mmol ATP, 5 mmol Mg acetate, 0.01% Thymol blue and 5 mmol glycine–NaOH (pH 9.0) [6]. CK at different concentrations incubated with **1** or water (control) for various times at 25°C was added to 1 mL of reaction mixture. Absorption spectra were measured with a Bio-Rad SmartSpec 3000 spectrophotometer. The relative activity of CK was calculated from the ratio of sample treated with **1** *versus* control at same concentration and time.

Polyacrylamide Gel Electrophoresis Analysis

Polyacrylamide gel electrophoresis was used with an electrophoresis unit (EPS 301, Amersham Pharmacia Biotech). For both the non-SDS and SDS gels, 2% stacking gels and 10% resolving gels were used. Samples were run at a maximum current of 30 mA and a maximum voltage of 200 V at room temperature for 4 h for the non-SDS gels and for approximately 80 min for the SDS gels.

RESULTS

Inactivation of CK by Leonurine

The leonurine concentration was 1.51 mmol; **1** was dissolved in water or Tris-HCl buffer (pH 8.0) which had no effect on the reaction mixture. There were no obvious changes in CK

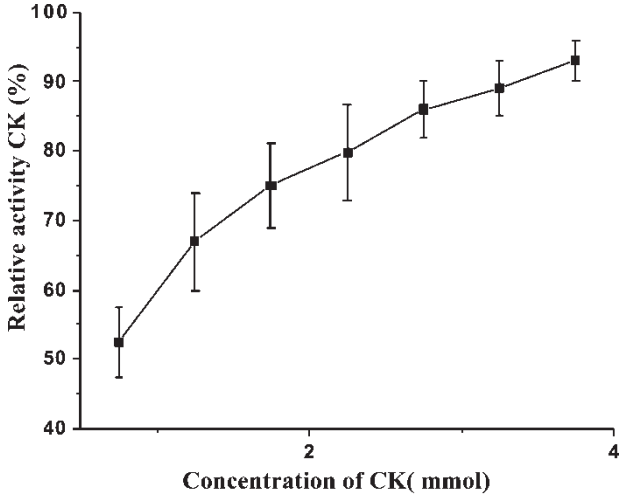

FIGURE 1 Activity of CK incubated with leonurine. The CK solution was incubated with 0.75 mmol leonurine for 12 h before being added to 1 ml of the reaction mixture. Relative activity is the ratio of reaction rate of CK incubated with leonurine to CK incubated with water.

activity after treatment with **1** for 6 h. The inhibitory effects of **1** treatment could be observed for up to 12 h of incubation with CK. The inhibitive effects of **1** decreased with increasing concentrations of CK (Fig. 1). The inhibitory effects increased directly with leonurine concentrations of 0.75 mmol and 1.51 mmol (Fig. 2). The inhibitory effect on prolonged exposure (from 12 to 24 h) to 1.51 mmol leonurine was gradually enhanced (Fig. 3a). CK lost its function after treatment with leonurine for 96 h. However, CK retained 90% activity after 96 h without 1.51 mmol **1** (Fig. 3b).

Two Phases of CK Inactivation by Leonurine

The semi-logarithmic plot obtained from Fig. 3(b) showed that CK inactivation by **1** had both slow and fast phases (Fig. 4a). In the slow phase that occurred within the first 48 h the inactivation rate constant was $5.14 \times 10^{-3}\ s^{-1}$. Afterwards, the inactivation rate constant was $13.25 \times 10^{-3}\ s^{-1}$. In the slow phase, **1** showed a non-competitive inhibitory effect (Fig. 4b). In the fast phase, **1** showed an irreversible inhibitory effect (Fig. 4c).

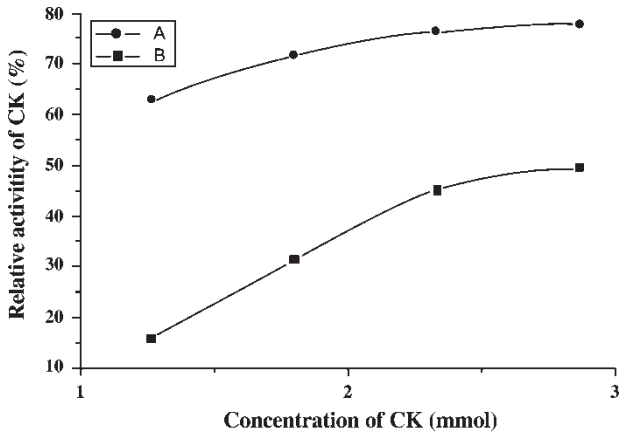

FIGURE 2 Inactivation due to leonurine. CK solution incubated with leonurine (A, 0.75 mmol; B, 1.51 mmol) for 24 h.

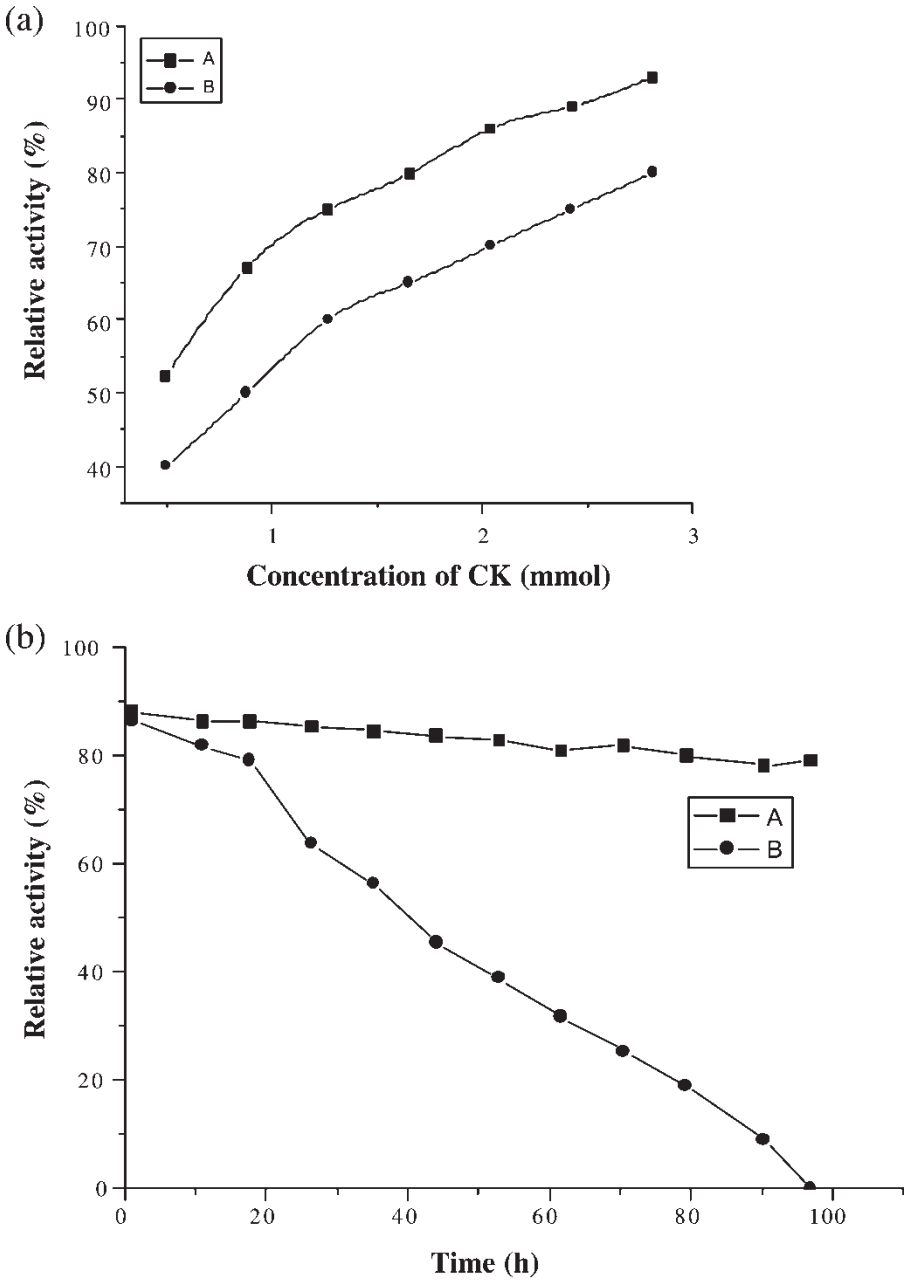

FIGURE 3 Variation of CK inhibition by leonurine with incubation time. (a) Enzyme solution incubated with 1.51 mmol leonurine for 12 (A) or 24 h (B) before being added to 1 ml of the reaction mixture. (b) Variation of relative activity of CK incubated without (A) or with (B) 1.51 mmol leonurine.

Changes of CK on PAGE

The CK structure was not obviously changed after treatment with **1** as shown by 10% SDS PAGE, showing that **1** had no effect on the CK subunits (Fig. 5). However, the result on 10% non-SDS PAGE showed that the amount of dimeric CK incubated with **1** decreased (Fig. 6).

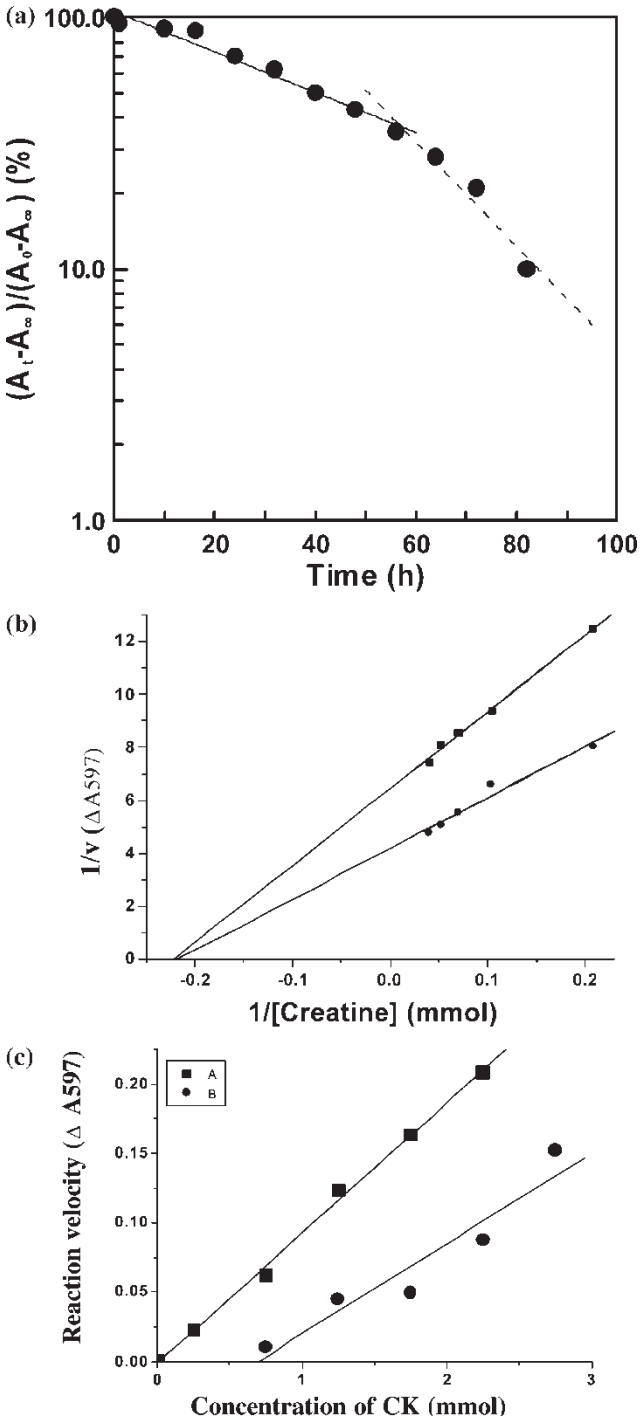

FIGURE 4 Two phases of CK inactivation by leonurine. (a) Semi-logarithmic plot of data in Fig. 3(b). (b) Lineweaver–Burk plots for inhibition of CK due to leonurine within 48 h. Reaction velocity of CK added to 1.51 mmol leonurine (A) or water (B); (A) and (B) were incubated for 24 h before being added to 1 ml of the reaction mixture. (c) Irreversible inhibition due to leonurine after incubation for 48 h. Reaction velocity of CK added to water (A) or 1.51 mmol leonurine (B); (A) and (B) were incubated for 72 h before being added to 1 ml of reaction mixture.

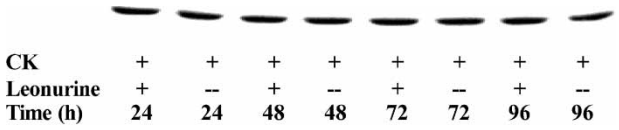

FIGURE 5 Results for CK structure with and without treatment with 0.75 mmol leonurine for various time periods on 10% SDS PAGE. CK was added to 1.51 mmol leonurine or water and then run on 10% SDS PAGE after various times.

DISCUSSION

The inhibitive effect of **1** was observed after incubation for 12 h. After incubation for 96 h, the CK entirely lost its function. Generally, CK treated with 0.5 M GuHCl would lose its function within one hour. The rate constant for inactivation by **1** was less than that by GuHCl [6]. However, after incubation for 72 h, 1 mmol GuHCl had no inhibitory effect on CK (data not shown). These results indicate that **1** could have a greater inhibitory effect than GuHCl under the same conditions.

Leonurine inhibited enzyme activity in concentration- and time-dependent manners. There were two phases in the inhibition process, as occur for CK inhibited by GuHCl [6]. The inhibition of CK by **1** was enhanced as the incubation time increased. In the first 48 h, leonurine showed a non-competitive inhibitory effect, but it showed an irreversible inhibitory effect afterwards. Since leonurine has a guanidyl group, we suggest that the important mechanism may be when the guanidyl group of leonurine combines with CK.

CK treated with **1** had no obvious structural changes on 10% SDS PAGE. The results showed that **1** did not affect the CK subunits. However, the results using 10% non-SDS resolving gel showed that the amount of dimeric CK decreased in leonurine solutions. These results suggest that **1** could degrade CK to subunits. The dimeric structure of CK is important to its function [12], so we suggest that the degradation of the dimeric CK structure by **1** inhibits the CK activity.

These results are the first to find that leonurine, an alkaloid from natural products, can inhibit CK activity by affecting the quaternary structure. The effect of **1** on CK structure will be the subject of a future study.

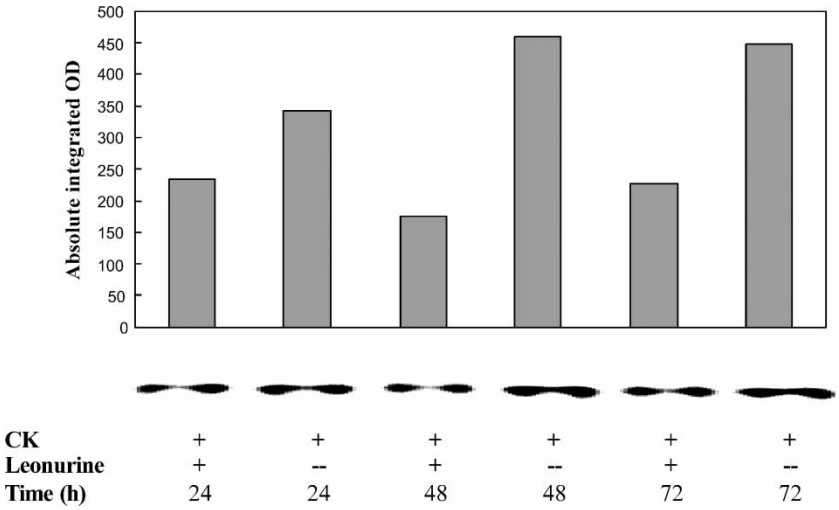

FIGURE 6 Results for CK incubated with 1.51 mmol leonurine on 10% non-SDS PAGE and its densitive scanning graph. CK was added to 1.51 mmol leonurine or water after different times and then run on 10% non-SDS PAGE.

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