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Inactivation of Lactate Dehydrogenase from Pig Heart by *o*-Phthalaldehyde

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Abstract: Treatment of lactate dehydrogenase (LDH) with *o*-phthalaldehyde resulted in a time-dependent loss of enzyme activity. The inactivation followed pseudo first-order kinetics over a wide range of the inhibitor. The second-order rate constant for the inactivation of LDH was estimated to be 1.52 $(mol/L)^{-1} \cdot s^{-1}$. The modified enzyme showed a characteristic fluorescence emission spectrum with a maximum at 405 nm upon excitation at 337 nm, consistent with the formation of isoindole derivatives by the cross-linking of proximal cysteine and lysine residues. The loss of enzyme activity was concomitant with the increase in absorbance at 337 nm. Stoichiometric study of the reaction showed that complete loss of activity was accompanied by formation of approximately four moles of isoindole derivatives per mole of LDH subunits. One of the substrates, NADH, partially prevented the enzyme from reacting with *o*-phthalaldehyde, whereas the other substrate, pyruvate, did not provide any protection. Protection experiments suggest that one of the cysteine-lysine pairs modified by *o*-phthalaldehyde is near the NADH binding site of LDH.

Key words: lactate dehydrogenase (LDH); *o*-phthalaldehyde; inactivation

Introduction

Lactate dehydrogenase (EC 1.1.1.27, LDH) plays a vital role in the energy flow of higher organisms. It is a tetrameric enzyme that catalyzes the reversible dehydrogenation of lactate, converting it to pyruvate. The enzyme exists in five isozymic forms which are not usually found all together in one tissue or organ. At least three different types of LDH subunits (M, H and X) are known in vertebrates, but the X subunits are usually present in only one or at most a few tissues[1,2].

Various chemical reagents have been used to probe specific amino acid residues involved in the catalysis of LDH. Previous studies have shown that chemical modification of arginine[3], histidine[4], and cysteine[5,6] residues resulted in inactivation of the enzyme. Although X-rays of LDH crystals showed that the sulfhydryl group of Cys is not involved in the combination of the substrates[7,8], its intactness is still important for the enzyme to exhibit full activity. The inactivation of LDH isozymes has been studied by many researchers through covalent modification of essential sulfhydryl groups. Modification of these groups by *N*-ethylmaleimide[9,10] or *p*-chloromercuribenzoate (*p*CMB)[5,6] leads to inactivation of LDH. By allowing *N*-(*N*′-acetyl-4-[35S] sulphamoylphenyl)-maleimide to react with LDH, Holbrook and Pfleiderer[6] were able to show that each LDH subunit contains one essential SH-group.

Our interest is to probe the involvement of cysteine and lysine residues at the catalytic center of lactate dehydrogenase. The tests used *o*-phthalaldehyde, a fluorescent bifunctional reagent that reacts with proximal cysteine and lysine

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residues at locations about 0.3 nm apart to form isoindole derivatives resulting in complete inactivation of the enzyme. The present investigation showed that LDH is inactivated in the presence of *o*-phthalaldehyde. The formation of isoindole derivatives results in a characteristic fluorescence emission spectrum. One of the substrates, NADH, partially protects LDH from inactivation by *o*-phthalaldehyde, which indicates that a lysine residue is located in close proximity to the cysteine residue in the NADH binding region of LDH.

1 Materials and Methods

1.1 Materials

Lactate dehydrogenase was prepared from pig heart according to the procedure of Pesce et al.[11] The final preparation was homogeneous on polyacrylamide gel electrophoresis in the presence and absence of sodium dodecyl sulfate. NADH, pyruvate, and *o*-phthalaldehyde were Sigma products (St. Louis, MO, USA). All other reagents were local products of analytical grade used without further purification.

1.2 Methods

Enzyme concentration was determined by measuring the absorbance at 280 nm using the absorption coefficient $A^{1\%}_{1cm}$ = 14.0. Enzyme activity was determined at 30℃ by measuring the absorbance change at 340 nm accompanying the oxidation of NADH using the molar absorption coefficient ϵ_{340} = 6.22 × 10^3 $(mol/L)^{-1} \cdot cm^{-1}$ as described previously[12]. The reaction system contained 0.25 mmol/L of NADH, 0.7 mmol/L of sodium pyruvate and 0.1 mol/L phosphate buffer, pH 7.5. Enzyme concentration and activity were determined with an analytic spectrophotometer Specord 200 UV VIS (Jena, Germany).

1.2.1 Reaction of *o*-phthalaldehyde with LDH

o-Phthalaldehyde solution was prepared fresh daily by dissolving *o*-phthalaldehyde in 1% methanol (mass fraction) as described previously[13]. The modification was carried out at 25℃ by incubating LDH with *o*-phthalaldehyde of different concentrations. At the indicated time intervals, an aliquot of the solution was added to an equal volume of stop solution containing 20 mmol/L cysteine and 10 mmol/L β-mercaptoethanol. Then an aliquot was withdrawn to determine the residual enzyme activity. A control without *o*-phthalaldehyde was run concurrently.

Protection experiments were performed in a similar manner except that the enzyme was pre-incubated with given concentrations of NADH or pyruvate for 15 min before the modification was initiated by the addition of *o*-phthalaldehyde. The residual activity was determined as described above.

1.2.2 Stoichiometry of *o*-phthalaldehyde reaction with LDH

The stoichiometric investigation was carried out by incubating LDH with *o*-phthalaldehyde for 50 min at 25℃. The absorbance at 337 nm was recorded continuously and the loss of activity was measured in a parallel experiment. The number of formed isoindole derivatives was calculated using ϵ_{337} = 7.66 × 10^3 $(mol/L)^{-1} \cdot cm^{-1}$. The relative molecular mass of LDH was taken as 140 × 10^3[14].

1.2.3 Fluorescence spectral measurements

The corrected fluorescence spectra were measured using a Hitachi 850 spectrofluorimeter. Fluorescence emission and excitation spectra were recorded as described elsewhere[15]. LDH was modified with *o*-phthalaldehyde as described above. The fluorescence spectra were recorded in 1-cm light path cuvette at 25℃.

2 Results

2.1 Inactivation course of LDH by *o*-phthalaldehyde

The LDH was eventually inactivated by *o*-phthalaldehyde. The loss of enzyme activity followed pseudo first-order kinetics (Fig. 1). The ultimate residual activity in Fig. 1 was about 2%, less than that of other enzymes inactivated by *o*-phthalaldehyde[15,16]. This result means that *o*-phthalaldehyde is an efficient inhibitor to LDH. Increasing *o*-phthalaldehyde concentrations led to

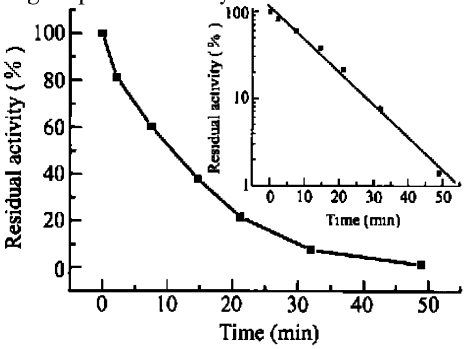

Fig. 1 Inactivation of LDH by 400 μmol/L *o*-phthalaldehyde. Residual activity was determined as described in Methods. The final enzyme concentration was 0.91 μmol/L. The inset is a semilogarithmic plot of the results.

accelerated loss of enzyme activity. The pseudo first-order rate constants, k_{obs}, obtained at each *o*-phthalaldehyde concentration were replotted as a function of *o*-phthalaldehyde concentration in Fig. 2. The second-order rate constant for the inactivation of the enzyme by *o*-phthalaldehyde was 1.52 $(mol/L)^{-1} \cdot s^{-1}$ as determined from the slope of this plot.

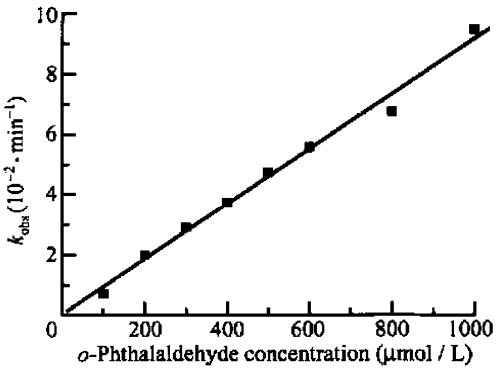

Fig. 2 Variation of the pseudo first-order rate constant (k_{obs}) for the inactivation of LDH with *o*-phthalaldehyde concentration.

2.2 Fluorescence spectral analysis of *o*-phthalaldehyde-modified LDH

o-Phthalaldehyde-modified LDH showed a typical fluorescence emission spectrum (λ_{ex} = 337 nm) with a maximum at 405 nm and a typical fluorescence excitation spectrum (λ_{em} = 405 nm) with a maximum at 337 nm (Fig. 3). These results were consistent with the formation of the isoindole derivative that involves the participation of the

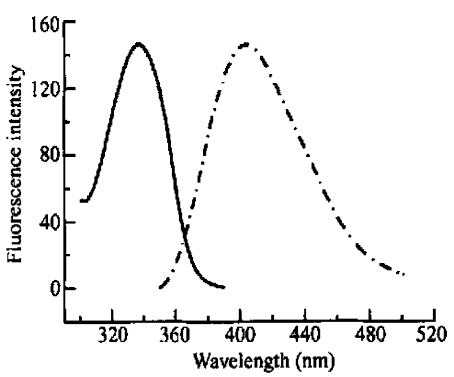

Fig. 3 Fluorescence excitation and emission spectra of *o*-phthalaldehyde-modified LDH. The final LDH concentration was 0.91 μmol/L. The enzyme was incubated with 1000 μmol/L *o*-phthalaldehyde at 25℃ for 60 min before the fluorescence spectra were recorded. The excitation spectrum (solid line) was recorded with an emission wavelength of 405nm and the emission spectrum (dash dot line) was recorded with an excitation wavelength of 337 nm.

proximal —SH group and the ε-NH_2 group of cysteine and lysine[17,20]. However, *o*-phthalaldehyde reacts with only the primary amino group when not in the presence of thiol compounds resulting in non-fluorescent product formation[21]. The molar transition energy was calculated as[17]

$$\frac{E_T}{kJ \cdot mol^{-1}} = 2.985 \frac{\lambda_{em}}{nm} - 1087.28.$$

E_T for this system was found to be 121.6 kJ/mol. This value was close to that of synthetic isoindole (121 kJ/mol) in the hydrophobic environment of hexane, indicating that the microenvironment around the residues involved in the isoindole formation was hydrophobic in nature.

2.3 Stoichiometry of *o*-phthalaldehyde reaction with LDH

The UV-visible spectrum for *o*-phthalaldehyde-modified LDH is characterized by an absorption at 337 nm which is not present in the spectrum of the unmodified enzyme. The stoichiometry study monitored the increase in absorbance at 337 nm during the inactivation reaction (Fig. 4a). The

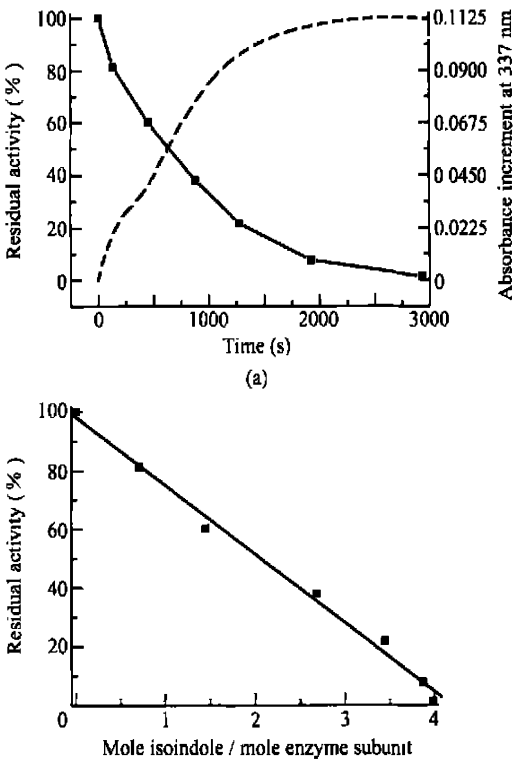

Fig. 4 Stoichiometry of *o*-phthalaldehyde with LDH. (a) Time course of inactivation of lactate dehydrogenase (■) and the increase in absorbance at 337 nm (dash line) following treatment with 400 μmol/L *o*-phthalaldehyde (see Methods). The final enzyme concentration was 0.91 μmol/L. (b) Stoichiometry of *o*-phthalaldehyde inactivation. Data was taken from (a).

correlation between isoindole formation and enzymatic activity is shown in Fig. 4b. The inactivation process showed a linear relationship between isoindole formation and the loss of enzyme activity with stoichiometry of 4 mol isoindole per mol enzyme subunit. In addition, the formation processes of these four isoindole derivatives were indistinguishable with respect to the reaction rate during the inactivation course.

2.4 Protective effect of substrate on LDH inactivation

Protection studies explored the binding site of *o*-phthalaldehyde in LDH. The effects of substrates on the reaction of *o*-phthalaldehyde with LDH are shown in Fig. 5. The corresponding pseudo first-order rate constants for inactivation in these protection experiments are summarized in Table 1. The enzyme sample pre-incubated with 200 μmol/L NADH retained approximately 35% of the original enzymatic activity with 3 mol isoindoles formed per mol enzyme subunit. The results showed that NADH partially protected the enzyme against inactivation, while pyruvate did not provide any protection. Furthermore, the fluorescence emission intensity due to the isoindole derivative formation was also greatly diminished in the presence of NADH (Fig. 6). The protection experiment results indicated that the inactivation of LDH by *o*-phthalaldehyde was due to modification of proximal cysteine and lysine residues situated near the NADH binding site.

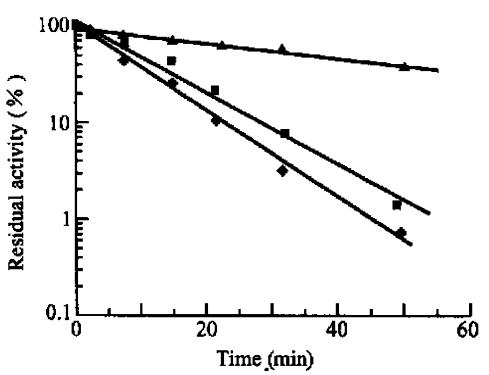

Fig. 5 Semilogarithmic plot of kinetic course of inactivation of the enzyme by 400 μmol/L *o*-phthalaldehyde in the absence and presence of substrates. The enzyme was pre-incubated with neither NADH nor pyruvate (■), with 200 μmol/L pyruvate (◆) and with 200 μmol/L NADH (▲) before the addition of *o*-phthalaldehyde. The final enzyme concentration in the incubation system was 0.91 μmol/L.

Table 1 Inactivation rate constants of LDH by 400 μmol/L *o*-phthalaldehyde in the absence and presence of substrates

Pre-incubation with substrates	k_{obs} (10^{-2} · min^{-1})
None	3.75
NADH (200 μmol/L)	0.73
Pyruvate (200 μmol/L)	4.48

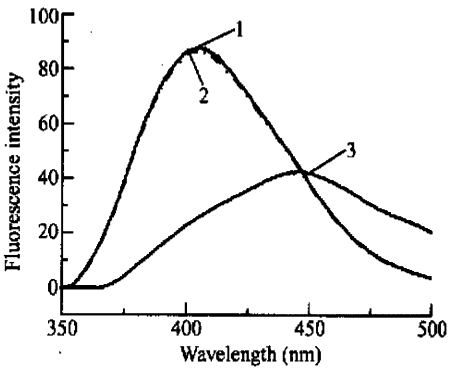

Fig. 6 Fluorescence emission spectra (λ_{ex} = 337 nm) of *o*-phthalaldehyde-modified LDH in the absence and presence of substrates. The final enzyme concentration was 0.57 μmol/L. The enzyme was incubated with 250 μmol/L *o*-phthalaldehyde for 60 min at 25℃. Curves in this figure represent the fluorescence emission spectra of *o*-phthalaldehyde-modified LDH in the absence of both NADH and pyruvate (1), the presence of only pyruvate (2) and the presence of only NADH (3).

3 Discussion

o-Phthalaldehyde has been extensively used as a bifunctional reagent to probe the role of cysteine and lysine residues involved in the active center of many enzymes[13, 15, 16, 18, 22-24]. It has also been used as a fluorescent probe to follow conformational changes at the active center[25]. The present study indicated that porcine heart lactate dehydrogenase was inactivated by *o*-phthalaldehyde in a pseudo first-order process. Replots of the kinetic data yielded a second-order rate constant of 1.52 $(mol/L)^{-1} \cdot s^{-1}$. The data in Fig. 2 formed a line passing through the origin, which strongly suggests that this modification reaction is a second-order reaction rather than an affinity labeling reaction. The spectroscopic properties of the inactivated enzyme were consistent with the formation of fluorescent isoindole derivatives via *o*-phthalaldehyde binding to proximal —SH group and —NH_2 group of cysteine and lysine in the enzyme. There was a direct correlation (linear relationship) between isoindole formation and the loss of enzymatic activity. The stoichiometry

result proved that four moles isoindole derivatives per mole enzyme subunit were formed during the course of modification. According to the methods of Tsou[26], only one of these four Cys-Lys pairs per subunit is essential for the enzyme to maintain full activity. Native and completely modified LDH showed identical mobilities when examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. No higher relative molecular mass species appeared from cross-linking of enzyme monomers (data not shown). Thus, modification of the enzyme by *o*-phthalaldehyde results in the formation of cross-links between cysteine and lysine residues within the same polypeptide chain.

The protection experiment provided useful data for ascertaining the location of the *o*-phthalaldehyde reaction in LDH. The results indicated that one of the substrates, NADH, provided partial protection against *o*-phthalaldehyde modification, while the other substrate, pyruvate, did not provide any protection. In fact, pyruvate even promoted inactivation of the enzyme by *o*-phthalaldehyde. The enhancement mechanism for enzyme inactivation by pyruvate remains unclear. Conformational changes in the enzyme molecule might be a contributing factor. The results of the substrate protection experiments provided evidence that one pair of proximal cysteine and lysine residues modified by *o*-phthalaldehyde was located at the nucleotide domain of LDH.

The involvement of the cysteine residue in the active center has been previously proven[6], but the presence of lysine in the active center in close proximity to the essential cysteine has never been identified before. Previous work showed that there are 24 lysine residues per subunit of porcine heart LDH, one of which (Lys 58) is situated in the active center near the NADH binding domain[27]. Thus, this residue is most likely the one involved in the isoindole derivative formation at the active center. The reactive thiol present in the active center was found to be an essential cysteine (Cys 165) that was related to the enzyme activity[28]. A previous report proved that this cysteine did not participate in the binding to the substrates, but chemical modification resulted in steric hindrance and the subsequent loss of enzyme activity[7]. Thus this cysteine was suggested to be one of the essential amino acid residues involved in the active site. In this investigation, the formation of the isoindole derivative from proximal cysteine and lysine residues also resulted in steric hindrance in the active site and subsequent loss of the enzyme activity, which reconfirmed the importance of the intactness of this residue for the enzyme to maintain its full activity.

In summary, *o*-phthalaldehyde inactivates pig heart lactate dehydrogenase by cross-linking proximal cysteine and lysine residues to form fluorescent isoindole derivatives. One of the cysteine-lysine pairs modified by *o*-phthalaldehyde is near the NADH binding site of LDH.

References

[1] Goldberg E. Lactic and malic dehydrogenases in human spermatozoa. *Science*, 1963, **139**: 602-603.

[2] Shaklee J B, Kepes K L, Whitt G S. Specialized lactate dehydrogenase isozymes: The molecular and genetic basis for the unique eye and liver LDHs of teleost fishes. *J Exp. Zool.*, 1973, **185**: 217-240.

[3] Yang P C, Schwart G W. Inactivation of lactate dehydrogenase by butanedione. *Biochemistry*, 1972, **11** (12): 2218-2224.

[4] Chilson O P, Kitto G B, Kaplan N O. Factors affecting the reversible dissociation of dehydrogenases. *Proc. Natl. Acad. Sci. USA*, 1965, **53**: 1006-1014.

[5] Pfleiderer G, Jeckel D. Coupling reaction of lactate dehydrogenase after reversible masking of the SH-groups: Reaction with *p*-diazobenzenesulfonic acid for marking essential histidine or tyrosine residues. *European J. Biochem.*, 1967, **2**: 171-175.

[6] Holbrook J J, Pfleiderer G. Significance of SH groups for enzymatic activity (3): A method for the radioactive marking of the essential cysteine residue in native lactate dehydrogenase (isozyme I) in pig heart. *Biochem. Z.*, 1965, **342**: 111-114.

[7] Vinals G, Bolle X D, Depiereux E, Feytmans E. Knowledge-based modeling of the *D*-lactate dehydrogenase three-dimensional structure. *Protein Struct. Funct. Genet.*, 1995, **21**: 307-318.

[8] Abad-Zapatero C, Griffith J P, Sussman J L, Rossmann M G. Refined crystal structure of dogfish M_4 apo-lactate dehydrogenase. *J. Mol. Biol.*, 1987, **198** (3): 445-467.

[9] Holbrook J J. The importance of SH-groups for enzymic activity (V): The coenzyme-binding capacity of pig heart lactate dehydrogenase, isozyme I, after inhibition by various maleinimides. *Biochem. Z.*, 1966, **344**: 141-152.

[10] Allison W S. Structure and evolution of triose phosphate and lactate dehydrogenases. *Ann. N. Y. Acad. Sci.*, 1968, **151**: 180-189.

[11] Pesce A, Mckay R H. The comparative enzymology of lactate dehydrogenase (I). Properties of the crystalline beef and chicken enzymes. *J. Biol. Chem.*, 1964, **239**(6): 1753-1761.

[12] Wang Xicheng, Jiang Li, Zhou Haimeng. Minimal functional unit of lactate dehydrogenase. *J. Protein Chem.*, 1997, **16**(3): 227-231.

[13] Huynh Q K. Mechanism of inactivation of *Escherichia coli* 5-enolpyruvoylshikimate-3-phosphate synthase by *o*-phthalaldehyde. *J. Biol. Chem.*, 1990, **265**(12): 6700-6704.

[14] Jaenicke R, Knof S. Molecular weight and quaternary structure of lactate dehydrogenase (3). Comparative determination by sedimentation analysis, light scattering and osmosis. *European J. Biochem.*, 1968, **4**: 157-163.

[15] Pandey A, Sheikh S, Katiyar S S. Identification of cysteine and lysine residues present at the active site of beef liver glutamate dehydrogenase by *o*-phthalaldehyde. *Biochim. Biophys. Acta.*, 1996, **1293**: 122-128.

[16] Sheikh S, Mukunda K, Katiyar S S. Evidence for proximal cysteine and lysine residues present at the nucleotide domain of rabbit muscle creatine kinase. *Biochim. Biophys. Acta.*, 1993, **1203**: 276-281.

[17] Palczewski K, Hargrave P A, Kochman M. *o*-Phthalaldehyde, a fluorescence probe of aldolase active site. *Eur. J. Biochem.*, 1983, **139**: 429-435.

[18] Chen C Y, Emig F A, Schramm V L, Ash D E. Inactivation of chicken mitochondrial phosphoenolpyruvate carboxykinase by *o*-phthalaldehyde. *J. Biol. Chem.*, 1991, **266**: 16645-16652.

[19] Puri R N, Bhatnagar D, Roskoski R Jr. Adenosine cyclic 3′, 5′-monophosphate dependent protein kinase: Fluorescent affinity labeling of the catalytic subunit from bovine skeletal muscle with *o*-phthalaldehyde. *Biochemistry*, 1985, **24**: 6499-6508.

[20] Belousova L V, Fedosov S N, Rostovtsev A P, Zaitseva N V, Myatlev V D. Non-equivalency of SH groups essential for the activity of mitochondrial creatine kinase. *Biokhimiya*, 1986, **51**: 478-493.

[21] Simons S S Jr, Johnson D F. Reaction of *o*-phthalaldehyde and thiols with primary amines: Fluorescence properties of 1-alkyl(and aryl) thio-2-alkylisoindoles. *Anal. Biochem.*, 1978, **90**(2): 705-725.

[22] Puri R N, Bhatnagar D, Roskoski R Jr. Inactivation of yeast hexokinase by *o*-phthalaldehyde: Evidence for the presence of a cysteine and a lysine at or near the active site. *Biochim. Biophys. Acta.*, 1988, **957**: 34-46.

[23] Ahn J-Y, Chio S, Cho S-W. Identification of lysine residue involved in inactivation of brain glutamate dehydrogenase isoproteins by *o*-phthalaldehyde. *Biochimie*, 1999, **81**(12): 1123-1129.

[24] George S P, Ahmad A, Rao M B. Involvement of a lysine residue in the active site of a thermostable xylanase from Thermomonospora sp. *Biochem. Biophys. Res. Commun.*, 2001, **282**: 48-54.

[25] Zhou Haimeng, Zhang Xiaohong, Yin Yong, Tsou Chenglu. Conformational changes at the active site of creatine kinase at low concentrations of guanidinium chloride. *Biochem. J.*, 1993, **291**: 103-107.

[26] Tsou Chenglu, Zhao Kangyuan. Quantitative relationship between alteration of functional groups in protein and its corresponding activity. *Acta Biophysica Sinica.*, 1989, **5**(3): 318-323. (in Chinese)

[27] William E, Michael C R, Susan S T, Hans-Joachim T, Helmut M, Walter K, Hans-Hermann K. Structural adaptations of lactate dehydrogenase isozymes. *Proc. Natl. Acad. Sci. USA*, 1977, **74**: 2677-2681.

[28] Adams M J, Ford C C, Koekoek R, Lentz P J, McPherson A Jr, Rossmann M G, Smiley I E, Schevitz R W, Wonacott A J. Structure of lactate dehydrogenase at 2-8 A resolution. *Nature*, 1970, **227**(263): 1098-1103.