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NATIVE AND CHIMERIC METAL-BINDING LACTATE DEHYDROGENASE AS DETECTION AND PROTECTION TOOLS FOR OXIDATIVE STRESS INDUCED BY FENTON'S REACTION

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ABSTRACT

In the present study, a simple and reliable antioxidant screening technique based on lactate dehydrogenase (LDH) oxidation by Cu^{2+}-mediated Fenton's reaction has successfully been developed. Oxidation of LDH by hydroxyl radical consequently leads to enzymatic inactivation, while addition of antioxidants can protect and regain enzyme activity. This method demonstrated a high feasibility on detecting of antioxidative activity of lipophilic (e. g. α-Tocopherol and β-Carotene) and hydrophilic compounds (e. g. glutathione, mannitol and thiourea) in a single assay. Results from linear correlation curves revealed that the IC_{50} were in the order of β-carotene (3.45 μg/ml) > α-Tocopherol (52.31 μg/ml) > Mn(II)-bacitracin (109.37 μg/ml) > glutathione (122.63 μg/ml). Detailed investigations revealed that oxidation of LDH resulted in enzyme degradation, which was metal- and time-dependent mechanism. Therefore, further experiments were conducted to determine whether extension of the N-terminus of LDH with metal-binding regions possesses protective effect against the inactivation process. Genetic construction of chimeric LDH carrying two and four repetitive sequences of cadmium binding peptide (CdBP), designated as $CdBP_2$LDH and $CdBP_4$LDH, has been carried out. From our findings, the $CdBP_2$LDH and the $CdBP_4$LDH exhibited protective action and enzyme activity regained 20-30 % and 70 % higher than that of the native LDH, respectively. Two possible mechanisms have been proposed to play important role in protection against metal-mediated Fenton's reaction: i) changing in redox potential of Cu^{2+} in metal-peptide complex, and ii) taking away of Cu^{2+} ion from the crucial amino acids by metal saturation at the cadmium-binding peptides.

Keywords: antioxidants, lactate dehydrogenase, Fenton's reaction, metal binding peptides

INTRODUCTION

Free radicals and reactive oxygen species (ROS) are molecules that play imperative roles of cellular processes in aerobic organisms. Imbalance of radical generation causes secondary oxidation and subsequently leads to cellular dysfunction and cell death (Sorg, 2004). Among this, hydroxyl radical ($OH^{\bullet}$) generated via the Fenton's reaction executes strong oxidative stress in numerous diseases such as atherosclerosis, neurodegenerative diseases, cancer, and autoimmune diseases (Valko et al.,

2007). Restoration of oxidative balance by antioxidant supplementation has extensively been proposed (Elias et al., 2008; Prachayasittikul et al., 2008; Valtuena et al., 2008).

To search for antioxidants from the vast of synthetic and natural compounds, various methods and/or reporter molecules have been applied to achieve easier, faster and more cost-effective than electron spin resonance and electron paramagnetic resonance (for recent review please see Magalhaes et al., 2008). These include β-phycoerythrin (Cao et al., 1993), crocin (Bors et al., 1984), green fluorescent protein (Izawa and Inoue, 2004), DNA (Srinivasan et al., 2002), alkaline phosphatase (Mordente et al., 1987) or the combined measurement between substrate utilization and conjugate diene peroxide generation (Nishida et al., 1996). In some circumstances, special tests to detect specific radicals scavenging activity have also been extended, e. g. singlet oxygen scavenging capacity assay and peroxynitrite scavenging capacity assay etc. (Huang et al., 2005). However, limitations particularly on the stability of compounds, durability of usage, and their applicability for screening of potential antioxidant compounds in different phases of assay reactions become an obstacle for rapid selection and large scale expansion.

Herein, a novel screening method for antioxidant compounds has been developed using lactate dehydrogenase from the thermophilic bacterium, *Bacillus stearothermophilus*; BSLDH, as an indicator and Cu^{2+}-mediated Fenton's reaction as radical generator. The BSLDH has been selected because of the following reasons: i) it is very stable protein (thermostability up to 70-80 °C and high stability against 6-8 M urea) which is easy to handle, ii) protein production and purification can potentially be managed, iii) its catalytic reaction can be controlled in both aqueous and organic phases, and iv) monitoring of NADH (supplied as cofactor) can simply be performed at 340 nm. To this system, the hydroxyl radicals are generated via Fenton's reaction resulting in oxidative inactivation of BSLDH. Antioxidative potency of the antioxidants can proportionally be inferred from the observed remaining LDH activity. Furthermore, protective activity derived from synthetic metal-binding peptide against oxidative inactivation has been investigated using the chimeric LDH carrying repetitive sequences of Cadmium-binding peptide (CdBP) (Mejare et al., 1998a; Prachayasittikul et al., 2001). Plausible mechanisms on the alteration of oxidation-reduction potential and capability to strip metal away from the vital site have been proposed.

MATERIALS AND METHODS

Materials

Oligonucleotides encoding Cd-binding peptide (CdBP) (Mejare et al., 1998a; Prachayasittikul et al., 2001) were synthesized by the Biomolecular unit, Lund University, Sweden. Chelating Sepharose Fast Flow was obtained from Amersham Biosciences. Mn(II)-bacitracin was synthesized as previously reported (Piacham et al., 2006). All other chemicals were of analytical grade and commercially available.

Chimeric gene construction

Construction of two and four repetitive sequences of Cd-binding peptide fused to lactate dehydrogenase was performed by cleaving *lct* gene (coding for *Bacillus stearothermophilus* lactate dehydrogenase; Clarke et al., 1985) from H3HpUC19 plasmid and ligating into the multicloning sites of pTrc99A (Amersham Biosciences, USA) at *Bam*HI and *Pst*I sites, generating pTrcLDH. Oligonucleotide I (5′-AATTCC AATTCCCAGAAAGTATTCCATTCCCA GAAAGTATTCGCTCTAGAGTCCGGG TTCCGCCG-3′ and 5′-GATCCGGCGGA ACCGGACTCTAGAGCGAATACTTTCT GGGAATGGAAATACTTTCTGGGAAT GG-3′) coding for the double repeats of Cd-binding peptide with *Xba*I restriction site was annealed and inserted into *Eco*RI and *Bam*HI sites of pTrcLDH, resulting in

pTrcCdBP$_2$LDH. For construction of the four repeats of Cd-binding lactate dehydrogenase, oligonucleotide II (5′-CTAGAGCA TTCCCAGAAAGTATTCCATTCCCAGA AAGTATTCCTGCAGG-3′ and 5′-GATC CCTGCAGGAATACTTTCTGGAATACT TTCTGGGAATGCT-3′) carrying two additional Cd-binding peptide with *Pst*I restriction site was inserted into pTrcCdBP$_2$LDH via *Xba*I and *Bam*HI sites, yielding pTrcCdBP$_4$LDH.

Enzyme expression and purification

LDH was purified according to Mejare et al. (1998b) with some modification. Briefly, the plasmid pUL1841 carring native LDH gene (Carlsson et al., 1993) was transformed into *E. coli* TG1. Isolated colony was inoculated into LB broth supplemented with 100 mg/l ampicillin and grown at 37°C with shaking (120 rpm). Gene expression was induced by addition of 1 mM of Isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were harvested at late log phase of the culture and resuspended in 50 mM sodium phosphate buffer, pH 7.4 containing 0.3 M NaCl. The cell suspensions were sonicated and supernatant was collected by centrifugation (14,000 rpm, 15 minutes). The protein solution was heated to and further incubated in 70 °C for 10 minutes. Insoluble precipitate was removed to derive the crude extract by centrifugation at 14,000 rpm for 15 minutes. The crude extract was loaded on a metal chelating Sepharose 6B column (0.7×8 cm) charged with 20 mM of desired metal ion. Cu^{2+} was used for purification of native LDH while Zn^{2+} was used for the chimeric CdBP$_2$LDH and the CdBP$_4$LDH purification. After washing with at least 10 column volumes of 50 mM phosphate buffer (pH 7.4), bound protein was eluted by applying a linear pH gradient of phosphate buffer pH 7.4-3.5. Fractions possessing enzymatic activity were pooled and dialyzed in phosphate buffer pH 7.4 at 4 °C for overnight before stored at -20 °C until used.

Enzyme activity assay

LDH activity assay was performed in 0.1 M 2-(N-morpholino) ethanesulphonic acid (MES) buffer, pH 6.5, containing 0.2 mM NADH and 30 mM pyruvate (Olin et al., 1995). Declining of absorbance at 340 nm was measured and simultaneously recorded via UV-visible spectrophotometer (Shimadzu UV-1601). One unit of enzyme was defined as the amount of enzyme that reduced 1 µM of pyruvate per minute at 25 °C.

Enzyme inactivation by reactive oxygen species (ROS)

Effect of ROS generated from Fenton's reaction (Feldberg et al., 1985) on LDH was evaluated by incubating 20 U/ml LDH for 3 minutes prior to determining enzymatic activity with various concentrations of Cu^{2+} (1 µM to 1 mM) and H_2O_2 (0.2625 % to 3.15 %). Optimized concentrations of copper (100 µM) and hydrogen peroxide (0.525 %) were selected for further investigations. In certain circumstances, the effect of ROS on protein degradation was investigated on SDS-PAGE (Laemmli, 1970). Briefly, appropriate amount of enzyme of protein was incubated for 3 minutes at ambient temperature with the mixture of 0.525 % H_2O_2 and Cu^{2+} ranging from 50-200 µM. The reaction was stopped by mixing with loading buffer and further subjected to denaturing SDS-PAGE. Preincubation of protein solution with 10 mM EDTA prior to applying metal-mediated Fenton's reaction was carried out for control.

Protective activity of antioxidants

To test for the efficiency of antioxidants in protecting the ROS-treated LDH enzymes, various antioxidants were individually incubated with enzymes for 10 minutes and the Fenton's reaction was subsequently applied. This system was performed in either aqueous or aqueous solvent mixture. Phosphate buffered saline (PBS) was used to investigate the efficiency of water-soluble antioxidants while 10 % DMSO or

10 % methanol was used for testing of lipophilic antioxidants.

RESULTS

Inactivation of LDH by metal-mediated Fenton's reaction

In this study, inactivation of LDH activity by metal-mediated Fenton's reaction was investigated. Generation of ROS (in particular, hydroxyl radical) as a consequence of redox reaction between Cu (II) and hydrogen peroxide was proved to exert marvelous suppressing effects on the LDH activity. As represented in Table 1, a rapid declining (within 3 minutes) of LDH activity up to 96 % was observed in the presence of 100 µM Cu^{2+} together with 0.525 % H_2O_2 (reaction no. 10). Higher concentrations of either copper ions or hydrogen peroxide alone provided non-significance change on the catalytic activity of the enzyme.

Due to the fact that assay of LDH activity was relied on monitoring the oxidation of NADH to NAD^+, while Fenton's reaction derived radicals might also demonstrate capability to oxidize NADH. Therefore, it is of great importance to clarify that radicals specifically attacked enzyme, and there was no radical left to interfere with the assay reaction. Our results clearly showed that only tiny effect (0.21 %) on the NADH oxidation was detected in the absence of LDH (reaction no. 4, Table 2). This non-specific oxidation was approximately 500 times lesser than LDH control system (100 %), and 15 times lower than Fenton's inactivated LDH control (3.99 %). Such effect might be negligible since no significant difference from the buffer control (reaction no. 3) was observed.

Screening of antioxidative activity by simultaneous monitoring of LDH activity

The feasibility of using this simplified technique as a potential tool for screening of hydrophilic and lipophilic antioxidants was evaluated. Table 3 demonstrated the antioxidative activities of tested compounds in aqueous buffer system. It was demonstrated that glutathione gave the highest protective effect (97.79 %) with 2, 2.5 and 16 times greater than the other radical quenchers: mannitol (48.28 %); thiourea (28.02 %) and sodium azide (6.07 %), respectively. Superoxide dismutase, first line of scavenging enzyme against ROS, demonstrated 17.36 % remaining LDH activity, indicating that superoxide radicals did not play crucial role in the inactivation. By contrarily, the protective activities of EDTA (98.91 %), histidine (97.47 %) and catalase (95.72 %) were more pronounced, possibly by disruption of Fenton's reaction upon Cu^{2+} chelation and degradation of H_2O_2.

Table 1: Effects of various concentrations of Cu^{2+} and H_2O_2 on LDH activity

Testing condition	% LDH activity
1. LDH	100
2. LDH + Cu^{2+} (1 mM)	98.7
3. LDH + Cu^{2+} (100 µM)	99.8
4. LDH + Cu^{2+} (10 µM)	99.4
5. LDH + Cu^{2+} (1 µM)	99
6. LDH + H_2O_2 (3.15 %)	111.4
7. LDH + H_2O_2 (2.1 %)	109.4
8. LDH + H_2O_2 (1.05 %)	102.9
9. LDH + H_2O_2 (0.525 %)	100.46
10. LDH + Cu^{2+} (100 µM) + H_2O_2 (0.525 %)	3.99

Table 2: Effect of Hydroxyl radical on NADH oxidation

Testing condition	Oxidation of NADH to NAD^+ (340 nm)
1. LDH	100 %
2. LDH + Cu^{2+} + H_2O_2	3.99 %
3. PBS (absence of LDH)	0.27 %
4. PBS (absence of LDH) + Cu^{2+} + H_2O_2	0.21 %

Note: Oxidation of NADH to NAD^+ was monitored as relative to those of LDH activity.

Table 3: Antioxidant activity of tested compounds in aqueous buffer system

Testing condition	% LDH activity
1. LDH	100
2. LDH + Cu^{2+} + H_2O_2	3.99
Antioxidants	
3. LDH + Glutathione (10 mM) + Cu^{2+} + H_2O_2	97.79
4. LDH + Mannitol (10 mM) + Cu^{2+} + H_2O_2	48.28
5. LDH + Thiourea (10 mM) + Cu^{2+} + H_2O_2	28.02
6. LDH + Sodium azide (10 mM) + Cu^{2+} + H_2O_2	6.70
Antioxidative enzymes	
7. LDH + Catalase (100 U/ml) + Cu^{2+} + H_2O_2	95.72
8. LDH + bovine SOD (100 U/ml) + Cu^{2+} + H_2O_2	17.36
Metal chelators	
9. LDH + Histidine (10 mM) + Cu^{2+} + H_2O_2	97.47
10. LDH + EDTA (10 mM) + Cu^{2+} + H_2O_2	98.91

Further investigations were conducted to explore the applicability of such system in binary solvent (10 % DMSO or 10 % methanol in PBS). It was found that the remaining LDH activity were of 94.9 % and 101.6 % in the presence of DMSO and methanol, respectively (Table 4). Addition of Cu^{2+} and H_2O_2 into the assay condition resulted in a rapid decrease of LDH activity (reactions no. 4 and 6) to the same magnitude as buffer system (reaction 2). Supplementation of the lipophilic antioxidant compounds namely β-carotene, α-Tocopherol and Mn(II)-bacitracin at fixed concentrations gave rise to the remaining LDH activity of approximately 50 %. Further determination of the half maximal (50 %) inhibitory concentration of the compound (IC_{50}) was performed. Results from linear correlation curves revealed that the IC_{50} were in the order of β-carotene (3.45 µg/ml) > α-Tocopherol (52.31 µg/ml) > Mn(II)-bacitracin (109.37 µg/ml) > glutathione (122.63 µg/ml).

Table 4: Antioxidant activity of tested compounds in aqueous solvent mixture

Testing condition	% LDH activity
1. LDH in PBS	100
2. LDH + Cu^{2+} + H_2O_2	3.99
3. LDH in 10 % DMSO in PBS	94.9
4. LDH in 10 % DMSO in PBS + Cu^{2+} + H_2O_2	6.2
5. LDH in 10 % methanol in PBS	101.6
6. LDH in 10 % methanol in PBS + Cu^{2+} + H_2O_2	4.2
7. LDH + α-Tocopherol (43 µg/ml) + Cu^{2+} + H_2O_2	46.74
8. LDH + β-Carotene (3 µg/ml) + Cu^{2+} + H_2O_2	47.43
9. LDH + Mn(II)-bacitracin (100 µg/ml) + Cu^{2+} + H_2O_2	46.16

Note: β-Carotene was prepared in DMSO, while α-Tocopherol and Mn(II)-bacitracin were dissolved in methanol.

Mechanism of LDH inactivation by metal-mediated Fenton's reaction

To explore the mechanism of LDH inactivation by metal-mediated Fenton's reaction, effect of radicals on protein degradation was examined by conventional SDS-PAGE. Our results strongly indicated that inactivation of LDH catalytic activity was attributed to the degradation of protein molecules (as depicted in Figure 1A). Notification had to be made that the degradation level corresponded to the time of exposure (Lanes 1-4) and metal concentration (Lanes 5-7). By varying incubation time, increasing of enzyme degradation was more pronounced at 4 and 6 minutes (Lanes 3 and 4). Detailed explanation on the metal-dependent degradation was realized upon supplementaion of 10 mM EDTA into the enzyme solution. Pretreatment with EDTA revealed a complete protection of LDH enzyme (Lane 8). This was in contrast to that observed from the post-treatment with EDTA (Lane 9). For comparison, degradation of bovine serum albumin (BSA) was also shown to be potentiated in a similar fashion (Figure 1B).

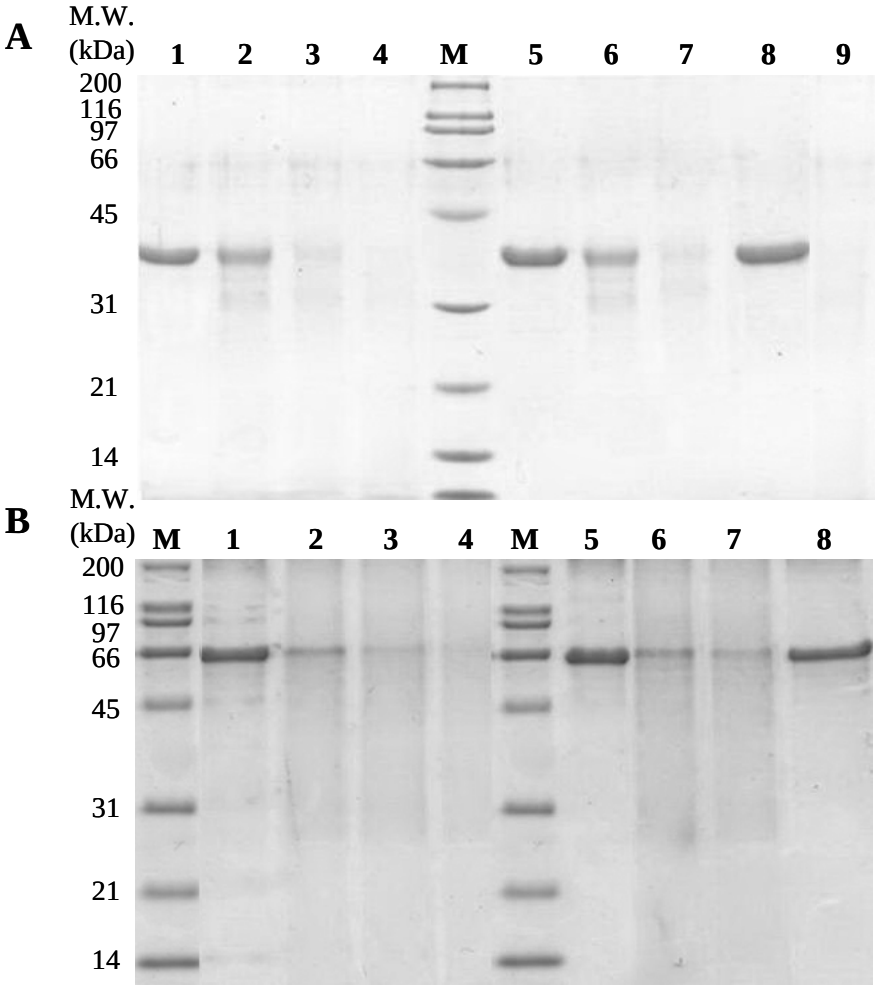

Figure 1: Time- and dose-dependent inactivation of native LDH (panel A) and BSA (panel B). Lane M: Molecular weight markers; Lane 1: LDH or BSA control; Lanes 2-4: LDH or BSA treated with 100 µM Cu^{2+} and 0.525 % H_2O_2 for 2, 4 and 6 minutes; Lane 5: LDH or BSA control; Lanes 6 and 7: LDH or BSA treated with 0.525 % H_2O_2 in the presence of 50 and 100 µM Cu^{2+}; Lane 8: Pretreated LDH or BSA with EDTA prior to exposure to 100 µM Cu^{2+} and 0.525 % H_2O_2; Lane 9: Addition of EDTA after protein inactivation by 100 µM Cu^{2+} and 0.525 % H_2O_2.

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Metal-binding peptide: a protective role against metal-mediated Fenton's reaction

To investigate whether engineering of metal-binding peptide played any protective role against metal-catalyzed enzyme inactivation, two and four Cadmium-binding peptides were fused in-frame with the LDH and applied for comparison. It was noteworthy that addition of 50-100 μM Cu^{2+} together with H_2O_2 to the $CdBP_2LDH$ led to the protein degradation (Figure 2) as those observed in the native LDH and BSA (Figure 1).

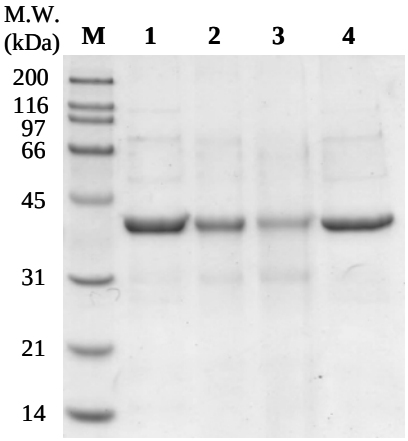

Figure 2: Effect of Cu^{2+} on the Fenton's reaction catalyzed oxidation of $CdBP_2LDH$. Lane M: Molecular weight markers; Lane 1: $CdBP_2LDH$ control; Lanes 2 and 3: $CdBP_2LDH$ treated with 0.525 % H_2O_2 in the presence of 50 and 100 μM Cu^{2+}; Lane 4: Pretreated $CdBP_2LDH$ with EDTA prior to exposure to 100 μM Cu^{2+} and 0.525 % H_2O_2.

However, the presence of cadmium-binding peptide provided toleration against metal-mediated Fenton's reaction upon interaction with low dose (10-50 μM) copper ions. As shown in Figure 3A, the remaining activity of $CdBP_2LDH$ was found to be approximately 20-30 % higher than those of the native LDH. Much emphasis could also be taken when the $CdBP_4LDH$ was subjected to the assay reaction. The remaining activity of up to 90 % was achieved in the presence of 100 μM Cu^{2+} and 0.525 % H_2O_2 (Figure 3B). Meanwhile, only 10 % activity was retained in the cases of native LDH and $CdBP_2LDH$. However, it was worth to note that such tolerance activity of the $CdBP_4LDH$ could no longer protect the enzyme inactivation once the copper ions were raised up to 1 mM or in the presence of 5 mM ascorbic acid (data not shown).

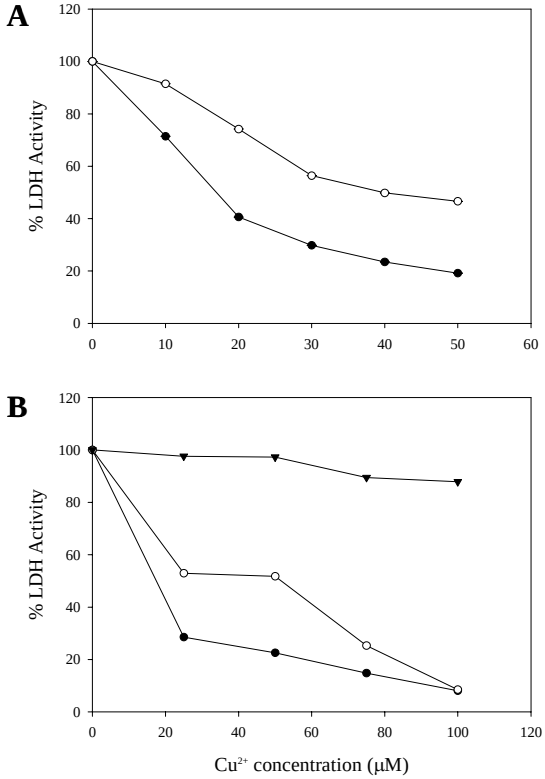

Figure 3: Remaining activity of LDHs after inactivation with 0.525 % H_2O_2 in the presence of low (A) and high (B) doses of Cu^{2+} ions. Symbols are represented as LDH (close circle), $CdBP_2LDH$ (open circle) and $CdBP_4LDH$ (close triangle).

DISCUSSION

Effective tool for rapid screening of lipophilic and hydrophilic antioxidant compounds

Herein, a simple and reliable method for screening of potential antioxidant compounds has successfully been developed. Inactivation of LDH activity as a consequence of Cu^{2+}-mediated Fenton's reaction can be spectrophotometrically monitored by determining NADH reduction rate at 340 nm. Compounds those possessed protective activity against enzyme inactivation can be referred to as the antioxidants. This method is easy to handle and provides several advantages over the others using bio-

logical probes, i. e. β-phycoerythrin (Cao et al., 1993), deoxyribonucleic acid (Srinivasan et al., 2002), and alkaline phosphatase (Mordente et al., 1987). These include the simplicity of protein purification, durability of storage, and protein stability in solvent-buffer milieu (Holmberg et al., 1999; Mejare et al., 1998b). The latter is particularly important since discovery of novel antioxidant compounds possessing ability to pass through lipid bilayer membrane can provide high positive impacts in pharmaceutical, cosmetics, and supplementary food products (Jin and Yoshioka, 2005; Silva et al., 2006). Therefore, our system opens up perspectives for high throughput screening of both lipophilic and hydrophilic antioxidant compounds in single assay (Tables 3 and 4).

Mechanism of enzyme inactivation and protective role of metal-binding region

Action of free radicals on protein structure can usually be taken place in three ways; i) attacking on and cleavage of peptide bond, ii) oxidation of amino acid side chain, and iii) formation of covalent protein-protein cross-linked derivatives (Kowalik-Jankowska et al., 2008). Our findings indicate that inactivation of LDH activity by hydroxyl radicals generated from Fenton's reaction are attributable to the protein degradation. Breaking of LDH structure is found to be metal- and time-dependent mechanisms (Figure 1). In previous report, LDH inactivation by radicals is accompanied by a loss of sulphydryl group and subsequent conformational changes (Buchanan and Armstrong, 1976). Other dehydrogenase enzymes, such as yeast alcohol dehydrogenase (Kittridge and Willson, 1984) and NADP-isocitrate dehydrogenase (Murakami et al., 2006), are also inactivated upon attacking by hydroxyl radicals. Especially, inactivation of NADP-isocitrate dehydrogenase by iron/ascorbate results in protein breakdown at the supposed metal binding sites or specific residues of radical attack (Murakami et al., 2006). A similar degradation pattern can also be observed on BSA (Jazzar and Naseem, 1994) and Cu-Zn superoxide dismutase (Ookawara et al., 1992). Pretreatment the enzyme solution with EDTA can completely neutralize the harmful effect. Meanwhile, no protection has been observed upon addition of EDTA after inactivation process (Figure 1A). All these findings support the critical role of metal in protein oxidation, degradation and loss of their functionalities.

More importantly, extension of metal-binding peptides at the N-terminus of LDH provides a strong protective activity against metal-mediated Fenton's reaction (Figure 3). Taken together with the results from inactivation of $CdBP_4LDH$ by adding ascorbic acid or increasing of Cu^{2+} concentration up to 1 mM, it can be suggested that the cadmium-binding peptide may play two major roles. First, the reduction potential of Cu^{2+} has been changed upon binding to the Cd-binding peptide. This may subsequently affect the reduction of Cu^{2+} to Cu^{+} by H_2O_2, a critical step in hydroxyl radical generation (Rael et al., 2007). However, production of hydroxyl radical can still be existed only in the presence of biological reductants (Ueda et al., 1995). Therefore, addition of 5 mM ascorbic acid together with the other components can rapidly inactivate the $CdBP_4LDH$. Second, Cu^{2+} ions are saturated around the Cd-binding peptide rather than at the critical site of attack, which can reduce the harmful effect of hydroxyl radical particularly at low concentrations (in micromolar) of copper ions. Efficiently capturing of zinc ions from the environment of cells expressing intracellular chimeric green fluorescent protein carrying polyhistidine has previously been reported (Isarankura-Na-Ayudhya et al., 2005). More supportive evidence can be drawn on the direct transfer of copper ions from the N-terminal histidine-rich region to the active site of the Cu/Zn SOD (Battistoni et al., 2001).

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