Mini Review

Activity assay of Lipoamidase, an expected modulator of metabolic fate of externally administered lipoic acid

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α-Lipoic acid (LA) is a cofactor functioning in four multienzymes: pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, branched-chain α-keto acid dehydrogenase, and the glycine cleavage enzyme systems. In each enzyme, LA is covalently bound to the ε-amino group of lysine residues. LA has been used as a medicine for treating diabetic condition in Europe and is also currently a popular supplement resource, and thus the metabolic fate after orally intake of LA attracts much attention whether externally administered LA is incorporated into protein bound form because large fraction of administered dose is not fully explained yet. Lipoamidase is the enzyme catalyzing formation and breakage of amide bond between LA and lysine ε-amino groups in the protein and thus is expected to play critical role in LA metabolism and function. In order to know how the enzyme lipoamidase is involved in the fate of LA in the body, a simple assay method is requested for determining lipoamidase activity in tissues. The method using simple HPLC detection of newly synthesized fluorescent substrate, dansyl-α-lipoyllysine, is discussed together with other previously reported methods.

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Introduction

α-Lipoic acid (LA) is a naturally occurring disulphide-containing compound known as 6,8-thioctic acid, 5-(1,2-dithiolan-3-yl) pentanoic acid or 1,2-dithiolane-3-valeric acid. It was reported first as the bacterial growth factor presenting in potato extract by Snell and colleagues in 1937[1]. However, the chemical structure was not identified until the factor was isolated and characterized by Reed and colleagues in 1951[2,3]. LA was tentatively classified as a vitamin at the time[2,4,5], but afterward, it was found that animal and human can synthesize it from octanoic acid as precursor and cysteine as sulphur source[4,6]. LA is rather insoluble in water at neutral and acidic pH but soluble in organic solvent such as benzene, ethyl ether, and methanol[4]. There are two enantiomers, R- and S-LA. Synthetic LA is mainly provided as racemic isomer but naturally occurring form is R-isomer[7]. In nature, LA occurs widely in bacterial, plant and animal kingdoms and is functioning as an essential cofactor of mitochondrial multienzyme complex: pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, branched-chain α-keto acid dehydrogenase, and the glycine cleavage enzyme systems. In these enzymes, LA is covalently bound to the protein through the amide linkage to the ε-amino group of lysine residues in the specific subunit with conserved domain called lipoyl domains[8-13]. LA functions as an acyl carrier in the oxidative decarboxylation of the α-keto acids (pyruvate, α-ketoglutarate and branched-chain α-keto acid), and also as an aminomethyl carrier in the glycine cleavage enzyme systems.

LA in tissues

As is expected from the LA status in tissues, LA contents in dietary resources were higher in the tissues with higher energy metabolism such as heart, liver and kidney in animal[14], that is, those tissues with high mitochondrial contents. In plants, spinach recorded the highest content but LA distributes widely in other fruits and vegetables including broccoli, tomato, garden pea, brussel sprouts and rice bran[15]. However, it is not yet fully understood how much LA is nutritionally supplied daily from these dietary resources in human beings.

Antioxidant and physiological activities

Although it is yet remained to be clarified whether the protein bound LA is active as antioxidant, the unique antioxidant property of free LA attracts much attention because of its unique redox potential and the metal chelating functions. Both LA and its reduced form dihydrolipoic acid (DHLA) have high potential of scavenging various types of ROS, free radicals and other oxidants. Moreover, they can act synergistically with other antioxidants[4].

Since the redox potential of LA/DHLA couple (-0.32 V) is low compared to other antioxidants such as GSH/GSSG couple (-0.24 V) or tocopherol/tocopheryl radical, DHLA allows to regenerate these antioxidants used up such as ascorbate, glutathione and vitamin E[16]. Therefore, they are capable of playing central role in anti-oxidant network. This antioxidant property of LA has already been suggested by Rosenberg and his colleagues[5] in 1959 and also by Pruijn and his colleagues[17] in 1991 who reported Vitamin E efficacy was enhanced by a combination of lipoic acid and tocopherol. Based on these facts and the dual solubility of LA in aqueous and non-aqueous media, LA/DHLA is called as "universal antioxidants"[4].

In addition to antioxidant activity, LA is known to have anti-inflammatory and immune activities[7]. It has been clinically applied for treating many diseases or pathological conditions[4,7,17,18] such as diabetes ischemia-reperfusion injury, liver diseases-such as mushroom poisoning and alcoholic liver diseases, HIV and AIDS, neurodegenerative diseases, radiation injury, cigarette smoke damages, heavy-metal poisoning, Chagas' disease, tumors and heart disease.

Recently, unique role of LA was reported by Dan et al in plants, in which LA modulates tissue transformation processes[19]. It enhanced plant transformation efficiency across four different crop species, through functioning at four different steps in the developing process which are tissue browning, transient gene expression, transformation efficiency, and shoot escapes. Any other antioxidant has not been reported to have all these functions in plant transformation.

Determination of protein bound LA

When orally administered, LA is absorbed from gastrointestinal tract and rapidly appears in plasma. Plasma clearance is rather high but only 50 to 70% of administered dose is recovered in urine as the metabolite[20]. Since plasma level of free LA is known to be quite low, significant amount of administered dose of LA could be stored in tissue as is in the physiological enzyme complex. To understand physiological functions of externally administered LA, it is a prerequisite to know the tissue levels of LA.

Several methods have been developed to determine the tissue LA as reviewed by Kataoka et al.[21] such as gas chromatography, capillary electrophoresis and high-performance liquid chromatography. However, it was found the tissue LA is essentially protein bound, and thus complicated sample preparation steps including protease digestion and solvent extraction are required until liberated free LA is detected by high sensitive methods even at the picomole range such as HPLC with various detection systems such as ultraviolet (UV), fluorescence (FL) and electrochemical (EC) detectors[22-23]. For example, Durrani et al[24] developed a method for the determination of LA in dietary supplements based on high performance liquid chromatography coupled with a coulometric electrode array detector (CEAD) and an electrospray ionization mass spectrometer (ESI-MS). In this method, LA was first extracted with methanol by sonication, following chromatographic separation by isocratic elution using an ACE 3-C-18 column, and then LA was detected by means of a CEAD against palladium reference electrodes. For ESI-MS detection, the mobile phase and flow rate had to be further changed.

Another reported approach to detect LA levels in tissue is enzyme recycling method which does not require any extraction or purification process after protease digestion and thus was advantageous for evaluating total amount of LA in tissues[16].

Recently, Satoh and his colleagues[25] developed a direct determination method of lipoyl-N-ε-lysine using reversed-phase HPLC with fluorescence detection. In this study, proteins containing LA were first hydrolyzed with enzymes including pronase E and subtilisin A. The disulfide bond (-S-S-) in lipoyl-N-ε-lysine liberated from the enzyme digestion was reduced with tris (2-carboxyethyl) phosphine to the thiol form (-SH). The reduced lipoyl-N-ε-lysine was then labeled with ammonium 4-fluoro-2, 1, 3-benzoxadiazole-7-sulfonate, and was separated by reversed-phase HPLC and fluorometrically detected. This method was used to determine LA levels in animal tissues and spinach. The values of lipoyllysine per 1g wet tissues obtained were 3.67 μg in kidney, 1.97 μg in heart, 0.59 μg in brain, 0.30 μg in lung, 0.38 μg in pancreas and 0.20 μg in spleen of rat tissue homogenate and 4.31 μg in spinach. This tissue distribution trend was almost same as the pattern previously reported[15,16,25] although the levels of LA were fluctuated. However, more simple methods or alternate approaches are necessary to be developed for evaluating the change of LA level in tissues after free LA intake to elucidate the physiological role of externally administered LA.

Lipoamidase assay

As is described above, the tissue LA is essentially protein bound. It is thus suggested that the considerable fraction of administered free LA is incorporated into protein bound form as in the physiological enzyme complex. A question arose is whether the protein bound LA is still active as an antioxidant, or functions as an antioxidant buffer from which free LA is liberated under a certain oxidative stress condition of host. We thus focused on the role of enzyme, lipoamidase as depicted in Figure 1. Lipoamidase is the enzyme involving in lipoamide bond formation and breakage and thus its activity might reflect the physiological status of LA or oxidative stress condition in tissues. Dissociation of lipoic acid from lipoylated proteins catalyzed by lipoamidase from Enterococcus faecalis was described many years ago[8,26-27], but the reaction has not been discussed in relation to the metabolic fate of lipoic acid.

Biotin is covalently attached to the ε-amino group of a specific lysine in the biotin-dependent carboxylases. Biocytin (D-biotinyl-N-ε-lysine) is produced during proteolytic degradation of the carboxylases and is hydrolyzed by biotinidase[28]. The role of biotinidase in biotin recycling suggested that a specific lipoamidase may be responsible for recycling lipoic acid during the turnover of lipoic acid-dependent enzymes.

The structure and function of lipoamidase were studied by several researchers[29-31]. Lipoamidase cleaves the amide bond linking lipoic acid to ε-amino group of lysine in the protein to release free LA from the protein. The enzyme was completely or nearly completely inactive toward ε-N-acetyl-L-lysine, ε-N-benzoyl-L-lysine, and ε-N-biotinyl-L-lysine (biocytin)[27] and thus specific for Lipoyl-N-ε-lysine[32]. Therefore, lipoyl-N-ε-lysine is accepted as a reliable substrate for lipoamidase activity assay.

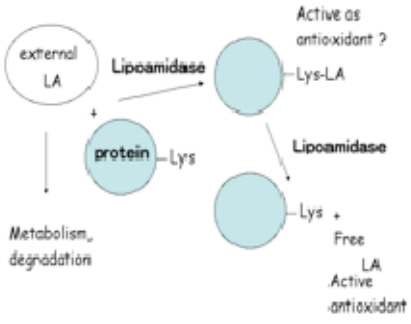

Fig. 1 Working of hypothesis of the metabolic fate of externally administered lipoic acid

A few methods have been reported to quantify lipoamidase mediated hydrolysis of lipoyl-N-ε-lysine. For example, liberated lysine was determined after the reaction with 1, 2-diacetylbenzene but the method requires removal of endogenous lysine from the sample[32]. Other methods reported for the colorimetric determination of thiols and disulfides are not suitable for the detection of small amounts of sulfhydryl compounds like DHLA originated from LA in biological samples[33-34]. Garganta and Wolf developed a colorimetric assay for quantifying LA liberated from lipoyl-N-ε-lysine by lipoamidase action using 2,6-dibromoquinone-4-chlorimide. This method involved acidification of the assay mixture with HCl and separation of LA from lipoyl-N-ε-lysine by ethyl acetate extraction before treating with 2, 6-dibromoquinone-4-chlorimide[35]. Quantification of liberated LA indirectly using labeling reagent may not be feasible because LA is readily converted to its reduced form, DHLA, in mammalian cells by mitochondrial lipoamide dehydrogenase[36-37].

Instead, direct evaluation of lipoamidase activity has been trialed using several artificial substrates, for example lipoyl-N-p-aminobenzoic acid (LPAB)[38-39]. This substrate was thought to be acceptable, because the biotin analog, biotinyl-N-p-aminobenzoic acid is a specific substrate for biotinidase. Interestingly, lipoamidase (LPAB hydrolase) activity in human serum showed strong correlation to biotinidase activity[40-41]. Hayakawa and Oizumi[42-44] purified proteins from guinea pig liver, pig brain, and human breast milk, which hydrolyze LPAB. The proteins in liver and breast milk were shown later to be identical with bile salt-stimulated lipase. In addition, when bile salt-stimulated lipase was modified by site-directed mutagenesis, LPAB hydrolysis activity was retained but the esterase activity was disappeared[45]. Thus, LPAB is not suitable for the substrate of lipoamidase in tissue samples that contain either biotinidase or bile salt-stimulated lipase activities. Biotinidase assay with biotinyl-6-aminoquinoline was superior to biotinyl-4-aminobenzoate, since there were less interfering peaks appeared in the HPLC chromatogram of 6-aminoquinoline than p-aminobenzoic acid. Therefore, lipoyl-6-aminoquinoline was devised as alternate synthesized substrate for lipoamidase activity assay[46-47]. The liberated 6-aminoquinoline was separated by HPLC and measured at an excitation wavelength of 350 nm and an emission wavelength of 550 nm by a fluorescence detector. However, the occurrence of the interference peaks limited the sensitivity of the assay.

Recently, a new fluorescence substrate for lipoamidase, dansyl-α-lipoyllysine (its structure shown in Fig.2) was synthesized and applied for lipoamidase activity assay in rat tissues by our group[48]. Since dansyl fluorophore has stronger fluorescent character than previously reported probe such as 6-aminoquinoline, more sensitive assay systems was designed using dansyl-α-lipoyllysine as lipoamidase substrate. In addition, the longer excitation wavelength for dansyl probe reduced nonspecific fluorescent peaks originated from contaminants in the extracted sample; the fluorescence peaks due to the substrate DLL and liberated DL were more clearly separated in the chromatogram as shown in Fig. 3. Further, we could skip some of the non-essential tissue preparation steps and reduced the reaction period to increase the sensitivity of the fluorescent sub-

strates. As the typical HPLC chromatogram shown in Fig 3, the effect of D and L isomer of LA was preliminary examined using our established method. It was found that the hydrolytic cleavage rate of dansyl-lipoyllysine was competitively inhibited by free LA and the inhibitory action was greater in R-isomer than S-isomer of LA. It has been reported the R-LA is better absorbed than S-LA and reduced readily to DHLA in tissue[4]. The above data also support R-isomer will be more physiological and thus interactive with lipoamidase. It is thus indicated the method is applicable for further studies to clarify the differential physiological role of externally administered R- and S-isomers. It is indicated that precise analysis of lipoamidase activity in tissue will be another promising approach to understand the physiological function of externally administered LA. We have still a little knowledge on how much dose of LA can be used as supplements in normal persons or as treatment in patients. Tissue level of lipoamidase activity might be one of the marker affected by LA level and also by oxidative stress condition as assumed in Fig 1 and thus a precise analysis of lipoamidase activity in tissue will be another promising approach to understand the physiological function of externally administered LA.

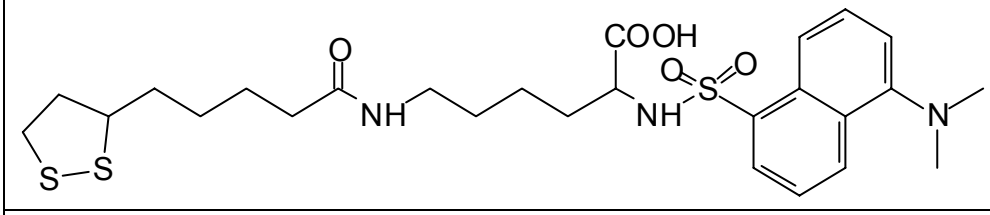

Fig. 2 Structural formula of Dansyl-α-Lipoyllysine

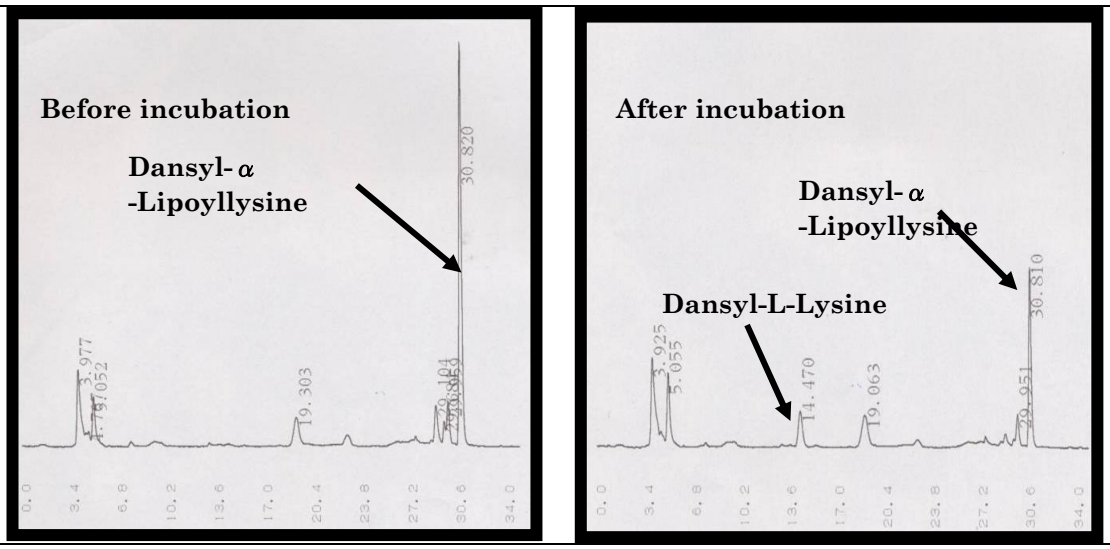

Fig. 3 Representative HPLC chromatograms of samples11

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