Journal of Chromatography, 490 (1989) 33–41
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4669

DETERMINATION OF LIPOYLLYSINE DERIVED FROM ENZYMES BY LIQUID CHROMATOGRAPHY

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(First received August 23rd, 1988; revised manuscript received January 4th, 1989)

SUMMARY

Lipoyllysine was liberated from the commercial enzymes bovine heart α-ketoglutarate dehydrogenase and pyruvate dehydrogenase. After incubation of the enzymes with pronase for 4 h, the lipoyllysine liberated was determined by high-performance liquid chromatography with ultraviolet detection at 340 nm. Standard lipoyllysine was synthesized in our laboratory The specific determination of lipoyllysine with ultraviolet detection only at 340 nm could be utilized for the enzyme hydrolysate samples. Recoveries of lipoyllysine added (5.0 μg) to a reaction mixture containing protease and bovine serum albumin or ovalbumin model proteins (1.0 mg) were 116.8 and 119.5%, respectively. The lipoyllysine content in beef heart α-ketoglutarate dehydrogenase was 0.55 μg/mg of enzyme and 0.83 μg/mg of enzyme protein in beef heart pyruvate dehydrogenase.

INTRODUCTION

Lipoic (thioctic) acid is considered to be covalently bound to the ϵ-amino group of lysine residue of the E2 subunit (acyltransferase subunit; lipoate acyltransferase; EC 2.3.1.12) in the pyruvate dehydrogenase system and α-ketoglutarate dehydrogenase system [1–3]. Another lipoate-containing subunit, protein X, was also recently reported in the pyruvate dehydrogenase system [4,5]. Lipoate is also covalently bound to the ϵ-amino group of lysine residue of H protein (hydrogen carrier protein; lipoic acid-containing protein; aminomethyl carrier protein) in the glycine cleavage system [6].

Analysis of the protein-bound lipoic acid has previously been performed by converting radioactive lipoic acid into 6,8-disulphooctanoic acid by performate

0378-4347/89/$03.50

oxidation and subsequent radioisotope measurement [1–3]. This method is time-consuming and requires the radioisotope of ^{35}S. As suggested by Howard and McCormick [7], high-performance liquid chromatography (HPLC) is considered to be "a good method for the study of lipoate derivatives because of its closed nature and high speed in the analysis of such labile lipoic acid derivatives".

In this paper, we present a simple method for determining lipoyllysine in enzyme hydrolysates. The method utilizes only proteinase K or pronase treatment to liberate lipoyllysine, together with filtration of the sample of lipoate-containing commercial enzymes bovine heart α-ketoglutarate dehydrogenase complex and pyruvate dehydrogenase complex.

EXPERIMENTAL

Chemicals and reagents

D,L-Lipoic acid, thionyl chloride, methanol (HPLC grade) and methylcellosolve (amino acid analysis grade) were purchased from Nacalai Tesque (Kyoto, Japan), benzene (amino acid sequencing grade), chloroform (HPLC grade), 1,4-dioxane (HPLC grade) and L-lysine hydrochloride from Wako (Osaka, Japan), α-ketoglutarate dehydrogenase, pyruvate dehydrogenase (from beef heart), hen egg ovalbumin, bovine serum albumin (BSA) and lipoamide from Sigma (St. Louis, MO, U.S.A.) and proteinase K from *Tritirachium album* and pronase from *Streptomyces griseus* from Boehringer (Mannheim, F.R.G.).

Synthesis of lipollysine

D,L-Lipoyl-L-lysine was synthesized from D,L-lipoic acid and L-lysine hydrochloride essentially according to the procedure described recently [8]. D,L-Lipoic acid (1.06 g, 5 mmol) was neutralized with 50 ml of 0.1 *M* sodium hydroxide solution and lyophilized. Sodium lipoate was then reacted with 3.75 ml of thionyl chloride in 15 ml of benzene. The lipoyl chloride was reacted directly with neutralized lysine in 50 ml of dioxane. The freeze-dried product, which contained residual lipoate and lysine, was then fractionated using an isocratic HPLC system with a volatile mobile phase essentially as described by Howard and McCormick [7]. Lipoyllysine was eluted at a retention time of 4.2 min (Fig. 1). Fractionated lipoyllysine was lyophilized (m.p. 215°C). The lipoyllysine fraction contained lipoic acid moiety (the same UV spectrum as that of lipoic acid was obtained) and the lysine residue, which was assessed by amino acid analysis after acid hydrolysis.

High-performance liquid chromatography

System 1: volatile mobile phase system for lipoyllysine fractionation. Synthesized lipoyllysine was fractionated by HPLC. The apparatus consisted of a Hitachi L-6000 pump, a Rheodyne 7125 injection valve (100-μl loop), a Waters Model 481 LC spectrophotometer (at 240 and/or 340 nm) and a Nucleosil 5 C_{18} column (250 mm$\times$4.6 mm I.D.) (Macherey-Nagel, Düren, F.R.G.). The volatile mobile phase was 50% (v/v) aqueous methanol containing 0.1% acetic acid. The flow-rate was 1.5 ml/min.

System 2: lipoyllysine determination with a phosphate buffer. Because the ion-suppression effect of phosphate ions was superior to that of volatile acetate ions, we used a phosphate-containing mobile phase for the determination of lipoyllysine. The apparatus consisted of a Kontron Model 420 HPLC pump, a Model 430 HPLC detector (dual-wavelength type; 340 and 240 nm) (Kontron Instruments, Zurich, Switzerland), a Model ERC-7512 refractive index detector, a Model ERC-3511 degasser (Erma, Tokyo, Japan), a pulse damper (a trial product from Hitachi), a Rheodyne 7125 injector (100-μl loop) and a Chromatopac C-R6A data processor (Shimadzu, Kyoto, Japan). Determination of lipoyllysine was carried out with a μBondasphere C_8 column (150 mm$\times$3.9 mm I.D., 5μm particle size, 100 Å pore size) (Nihon Waters, Tokyo, Japan). The mobile phase was methanol–methylcellosolve–0.05 *M* sodium phosphate buffer (pH 3.0) (25:6:69, v/v). The flow-rate was 1.0 ml/min. The column inlet pressure was 150 kg/cm^2.

Synthesis of dihydrolipoate and 6,8-disulphooctanoic acid

Dihydrolipoate was synthesized by reduction with sodium borohydride acording to the procedure described by Wagner et al. [9]. 6,8-Disulphooctanoic acid was synthesized by oxidizing lipoate with performic acid according to the procedure described by Nawa et al. [1]. Dihydrolipoic acid was determined using a C_8 column with methanol–methylcellosolve–0.05 *M* sodium phosphate buffer (pH 3.0) (42:5:53, v/v) as the mobile phase.

Amino acid analysis

Amino acid analysis was performed with a Hitachi HPLC amino acid analyser using the *o*-phthalaldehyde derivatization method. The amino acid analyser consisted of a Hitachi L-6200 pump, a Rheodyne 7125 injector (20-μl loop), a Hitachi 655A-13 reaction pump, a Hitachi F-100 fluorescence spectrophotometer (excitation wavelength 360 nm, emission wavelength 440 nm) and a Hitachi D-2000 data processor. The purified lipollysine was hydrolysed in 6 *M* hydrochloric acid at 110°C for 20 h in a vacuum-sealed tube (Pierce, Rockford, IL, U.S.A.).

Preparation of lipollysine-containing hydrolysate from α-ketoglutarate dehydrogenase by pronase and/or proteinase K

Pyruvate dehydrogenase (4.7 mg of protein) and/or α-ketoglutarate dehydrogenase (4.6 mg of protein) were extensively dialysed three times against 5 l of distilled water, and both were lyophilized. To the lyophilized enzyme (2.0 mg) was added 0.5 mg of protease (pronase or proteinase K) in 0.5 ml of the reaction mixture (0.1 *M* sodium phosphate buffer, pH 7.0). The reaction was started by incubating the mixture at 37°C for 4 h under nitrogen. After reaction for 4 h, the reaction mixture was filtered and lyophilized. The lyophilized sample was dissolved in 0.10 ml of alkaline methanol solution [methanol–0.02 *M* sodium hydroxide solution (1:1, v/v)]. After neutralization with 0.05 ml of 0.02 *M* hydrochloric acid, 90-μl samples of the solution were injected into the HPLC system (system 2). A blank reaction mixture containing only protease was also analysed.

Recovery tests

The recoveries of lipoyllysine from the reaction mixture containing proteinase K and commercial BSA or ovalbumin model protein were measured. Enzymatic hydrolysis was performed as described above.

The recovery of lipoic acid from the solution containing BSA or ovalbumin model protein was also measured by a chemical hydrolysis method, i.e., acid hydrolysis with 6 *M* hydrochloric acid or alkaline hydrolysis with 0.1 *M* sodium hydroxide solution under nitrogen [10].

RESULTS AND DISCUSSION

We developed a direct method for the determination of the content of lipoyllysine from lipoate-containing enzymes. The protease hydrolysis liberates lipoyllysine as a final product under mild conditions (pH 7.0, 37°C). It was necessary to synthesize lipoyllysine for use as an external standard. The synthesized lipollysine was fractionated by HPLC using a volatile mobile phase essentially according to the method of Howard and McCormick [7] with a minor modification, as described under Experimental (system 1). A typical chromatogram is shown in Fig. 1. Lipoyllysine was eluted with a retention time of 4.2 min, and unreacted lysine and lipoic acid were eluted at 2.0 and 14.5 min, respectively. Repeated fractionation and evaporation of the mobile phase gave a sufficient amount of authentic lipoyllysine for use as an external standard. The synthesis using copper(II)-chelated lysine was unsuccessful because of the high affinity of Cu^{2+} ions for the sulphur in lipoyllysine, which interfered with the purification process.

The synthesized standard lipoyllysine was directly analysed with an amino acid analyser. The purity was satisfactory because no lysine peak was detected. After acid hydrolysis (6 *M* hydrochloric acid at 110°C for 20 h) of the synthe-

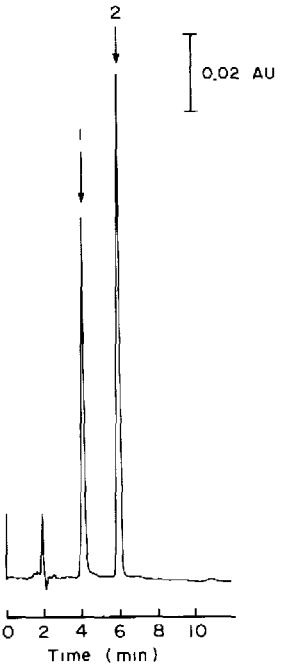

Fig. 1. Isocratic separation of lipoyllysine for fractionation (system 1). Volatile mobile phase, 50% (v/v) aqueous methanol containing 0.1% acetic acid. Column, Nucleosil 5 C_{18} (250 mm × 4.6 mm I.D.). Flow-rate, 1.5 ml/min (column inlet pressure, 260 kg/cm^2). UV detection at 240 nm. Peak 1 = 20 μg of lipoyllysine; peak 2 = 3.0 μg of lipoamide.

sized lipoyllysine, it was again analysed with an amino acid analyser; this time only lysine was detected. Lipoate and lipoyllysine showed identical and characteristic UV absorption spectra. These results indicate that our synthetic method was mild enough to preserve the labile dithiolane ring structure in lipoyllysine.

We developed a method for the determination of lipoyllysine using a refractive index detector. The volatile mobile phase system (system 1, Fig. 1) was not suitable in this assay owing to overloading; this suggested incomplete ion suppression by the acetate ions. A mobile phase containing acidic phosphate buffer and methylcellosolve and a C_8 column were found to be a sufficiently reproducible and stable assay system for lipoyllysine (system 2). A typical chromatogram is shown in Fig. 2. The phosphate buffer affords a stronger ion-suppressing effect, which gives a wider determination range (0.10–25 μg of lipollysine). Inclusion of methylcellosolve in the buffer gave symmetrical peak shapes for both lipoyllysine and lipoamide. As shown in Fig. 2, both detectors (refractive index and UV at 340 nm) gave symmetrical peaks. The use of an organic solvent in the eluent, however, caused a drift in refractive index detection. Hence both detectors demonstrated approximately the same sensitivity

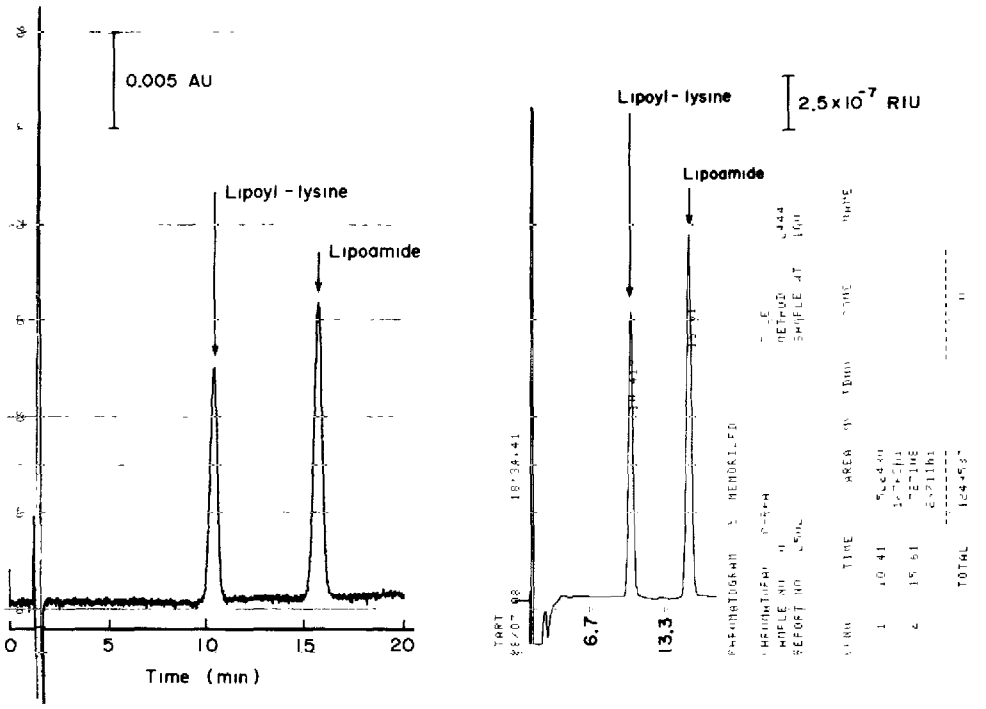

Fig. 2. Typical chromatogram of lipoyllysine for lipoyllysine determination (system 2): 5.0 μg of lipoyllysine and lipoamide were separated. Simultaneous determination of standards was performed [refractive index (right panel) and UV detection (left panel)] UV detection was performed at 340 nm. Column, μBondasphere C_8 (150 mm×3.9 mm I.D.). Mobile phase, methanol–methylcellosolve–0 05 *M* sodium phosphate buffer (pH 3.0) (25·6·69, v/v). Flow-rate, 1.0 ml/min.

for both lipoyllysine and lipoamide. In this system (system 2), lipoyllysine was eluted at 10.4 min. After establishing the conditions for the assay of lipoyllysine, we applied this method with specific UV detection at 340 nm to the measurement of the lipoyllysine in two commercially available lipoate-containing enzymes, α-ketoglutarate dehydrogenase and pyruvate dehydrogenase.

A method of liberating or hydrolysing lipoyllysine using protease and model proteins (BSA and ovalbumin) and standard lipoyllysine was studied. Protein hydrolysis was assessed by reversed-phase HPLC as described previously [11]. The elimination of the BSA and ovalbumin peaks using reversed-phase HPLC was complete after incubation with pronase and/or proteinase K at 37°C for 4 h (data not shown). The recovery of lipoyllysine from the incubation mixture (BSA) was also studied, and the results are given in Table I. The recovery of 5 μg of added lipoyllysine from the model incubation mixture system was 116.8±9.5% and the within-day coefficient of variation (C.V.) was 8.13%. For ovalbumin, a recovery of 119.5±6.61% (C.V.=5.5%, n=4) was obtained. The

TABLE I

RECOVERY OF LIPOYLLYSINE FROM THE MODEL REACTION MIXTURE

Lipoyllysine (5 μg) was added to the reaction mixture and the recovery was measured as described under Experimental.

Bovine serum albumin (mg)	Proteinase K (mg)	Lipollysine found (μg)	Recovery (%)
1.0	0.25	6.2	124
		6.0	120
		6.0	120
		6.0	120
		5.0	100
		Average	117
		C.V.	8.1
0.1	0.025	5.2	104
		5.5	110
		Average	107
		C.V.	3.9

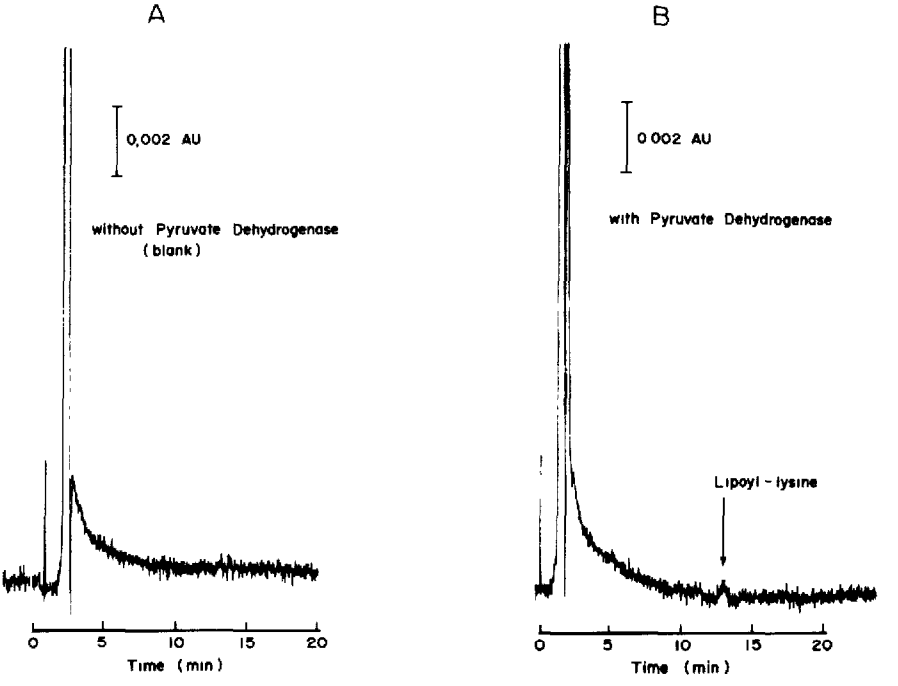

Fig. 3. Typical analysis of liberated lipoyllysine in an enzymatic hydrolysate (system 2). Sample preparation as described under Experimental. Pronase was used in this instance, 0.09 ml of the reaction mixture was injected. Detection, UV at 340 nm. Flow-rate, 0.8 ml/min. (A) Blank sample which contained only pronase; (B) sample hydrolysate which contained substrate of pyruvate dehydrogenase and pronase.

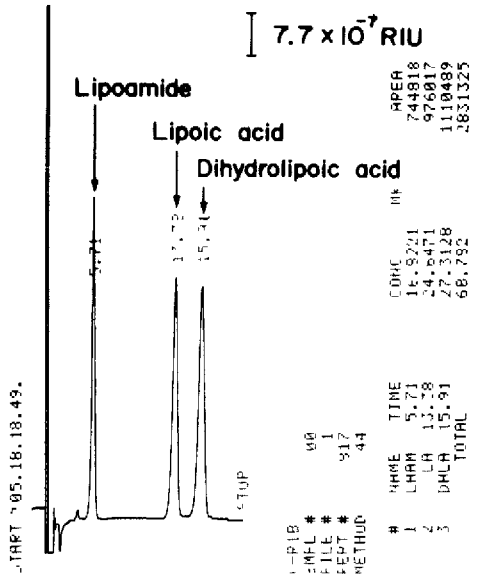

Fig. 4. Typical chromatogram of standard lipoamide, lipoic acid and dihydrolipoic acid. Column, μBondasphere C_8 (150 mm×3.9 mm I.D.). Mobile phase, methanol–methylcellosolve–0.05 *M* sodium phosphate buffer (pH 3.0) (42·5 53, v/v). Flow-rate, 1.0 ml/min. Detection, refractive index. Amount of standard mixture injected, 1.0 μg.

recovery above 100% might have been due to interfering compounds (at 340 nm) in the commercial BSA and ovalbumin.

The determination of lipoyllysine using pronase or proteinase K was carried out with the lipoate-containing enzymes at a reduced flow-rate (0.8 ml/min), because these samples showed some interfering peaks. Only specific UV detection at 340 nm was found to be applicable to this system, as expected, and typical chromatograms are shown in Fig. 3. In this system, the standard lipoyllysine was eluted with a retention time of 13.5 min. The hydrolysed sample solution contained a single peak exhibiting UV absorption at 340 nm. We manually fractionated this peak and lyophilized the sample. This material was acid-hydrolysed and analysed with the amino acid analyser; only lysine was detected. Hence the peak at 13.5 min was considered to be lipoyllysine. The height of the lipoyllysine peak was measured manually and the lipoyllysine content was calculated. The result was 0.55 μg/mg for α-ketoglutarate dehydrogenase enzyme and 0.83 μg/mg for pyruvate dehydrogenase enzyme. The lipoate content for *Escherichia coli* α-ketoglutarate dehydrogenase enzyme is reported to be 1.12 μg/mg of enzyme [12]; the content for beef heart enzyme was not available in the literature. However, a consistently higher lipoate content was observed in the bovine heart pyruvate dehydrogenase complex than in the α-ketoglutarate dehydrogenase complex. This finding might be due to the pres-

ence of other lipoate-containing components (protein X) in the pyruvate dehydrogenase system [4,5].

The acid or alkaline hydrolysis method (6 *M* hydrochloric acid or 0.1 *M* sodium hydroxide solution) for liberating lipoic acid was studied using the lipoic acid determination system (Fig. 4). The recovery of lipoate with drastic chemical methods was low (not higher than 70%).

The derivatization method, which involves the oxidation of lipoate to 6,8-disulphooctanoic acid and then acid hydrolysis, was found to have good potential [1]. 6,8-Disulphooctanoic acid was separated using an ion-pairing method with PIC-A reagent [13] and detected with a refractive index detector. However, this required a time-consuming peroxidation process.

ACKNOWLEDGEMENTS

The authors are grateful to Fumiyo Hara for technical assistance. This work was supported by a grant from the Ministry of Health and Welfare of Japan.

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