Lipoamidase*

Kantaro Suzuki† and Lester J. Reed

From the Clayton Foundation Biochemical Institute and the Department of Chemistry, The University of Texas, Austin 12, Texas

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Reed, Kolke, Levitch, and Leach (1, 2) described an enzyme in Streptococcus faecalis (strain 10C1) which releases lipoic acid from the protein-bound form present in Escherichia coli pyruvate and α-ketoglutarate dehydrogenation complexes, yielding the corresponding apoenzymes. They proposed the name “lipoamidase” for this enzyme. An apparently similar enzyme was found by Seaman (3) in bakers’ yeast. Nawa, Brady, Kolke, and Reed (4) showed that the lipoamyl moieties in the E. coli pyruvate and α-ketoglutarate dehydrogenation complexes are bound in amide linkage to the ε-amino groups of lysine residues.

The enzyme has been purified approximately 100-fold. In addition to releasing lipoic acid from the protein-bound form, the enzyme hydrolyzes methyl lipoate, lipoamide, and several N-lipoylaminos and peptides, including ε-N-lipoyl-l-lysine. The enzyme is completely or nearly completely inactive toward biocytin (ε-N-biotinyl-l-lysine), ε-N-acetyl-l-lysine and ε-N-benzoyl-l-lysine. In view of the substrate specificity of the enzyme, it is suggested that the trivial name lipoamidase be substituted for lipoamyl-X hydrolase. The purification and some properties of this enzyme are described in the present paper.

EXPERIMENTAL PROCEDURE

Materials—The lipoic acid derivatives were prepared as described previously (1, 4, 5). We are indebted to Dr. H. Nawa, Takeda Chemical Industries Ltd., Osaka, Japan, for generous gifts of dl-lipoamide and ε-N-(dl-lipoil)-l-lysine. Bioyin was provided by Dr. William Shive of this laboratory. 5,5′-Dithiobis(2-nitrobenzoic acid) was prepared by Mr. Preston K. Martin according to the procedure of Ellman (6). ε-N-Benzoyl-l-lysine was purchased from Cyclo Chemical Corporation.

ε-N-Acetyl-l-lysine was prepared by the method of Neuberger and Sanger (7) from 1.8 g of l-lysine monohydrochloride. The solution obtained after removal of barium and copper ions was passed into a column prepared from 10 g of Dowex 50-X2 (50 to 100 mesh) in the hydrogen cycle. The column was washed thoroughly with water and then eluted with 1 N ammonium hydroxide. The fractions containing ninhydrin-reactive material were combined and evaporated to dryness. The residue was dissolved in a minimal amount of hot water, and ethanol was added until a faint turbidity was observed. The solution was cooled, and the solid was collected and recrystallized from ethanol-water, m.p. 250–255° with decomposition. The product did not contain a detectable amount of free lysine as revealed by colorimetric determination with ninhydrin.

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† Permanent address, Department of Biochemistry, School of Dentistry, Nihon University, Tokyo, Japan.

DEAE-cellulose, type 20, laboratory grade, was purchased from the Brown Company. Before use, it was washed successively with 0.5 N sodium hydroxide, distilled water, 0.5 N hydrochloric acid, and distilled water. DEAE-Sephadex A-50 (medium grade) was obtained from Pharmacia. Acid-hydrolyzed casein (HCl) and enzymatic casein hydrolysate were obtained from the Nutritional Biochemicals Corporation. Before use, the casein hydrolysates were treated with activated carbon (Daro G-60). To a solution of 500 g of acid-hydrolyzed casein and 375 g of enzymatic casein hydrolysate in 9 liters of water were added 100 g of activated carbon. The mixture was stirred for 30 minutes and filtered through a Büchner funnel with the aid of Hyflo Super-Cel obtained from Johns-Manville. To the filtrate were added 225 ml of glacial acetic acid and 100 g of activated carbon. The mixture was stirred for 30 minutes and filtered. The filtrate was diluted with distilled water to a volume of 10 liters. Two hundred milliliters of this solution were used per liter of lipoic acid-deficient medium. Yeast extract and tryptone were purchased from Difco Laboratories, Inc. The sources of other materials are described in previous papers (1, 8, 9).

Enzyme Assay—The assay is a modification of that described previously (1), and is based on the loss of pyruvate dismutation activity (Equation 1) accompanying release of protein-bound lipoic acid from a purified preparation of the E. coli pyruvate dehydrogenation complex.

2 Pyruvate + P i → acetyl-P + CO2 + lactate

(1)

The reaction mixture contained 5 units of E. coli pyruvate dehydrogenation complex (specific activity, 800 to 1000) (9) and enzyme in a total volume of 0.25 ml of 0.02 M potassium phosphate buffer, pH 7.0. The mixture was incubated for 1 hour at 30°, then cooled in ice during addition of components necessary to complete the pyruvate dismutation assay system. These components included 100 μmoles of potassium phosphate buffer, pH 7.0, 50 μmoles of potassium pyruvate, 0.2 μmole of thiamine pyrophosphate, 0.1 μmole of CoA, 0.23 μmole of DPN, 4 μmoles of MgSO4, 6.4 μmoles of l-cysteine, 12 μmoles of phosphotransacetylase, and 2000 units of lactate dehydrogenase in a final volume of 1.0 ml. The mixture was incubated for 30 minutes at 30° and then assayed for acetyl phosphate by the hydroxamic acid method of Lipmann and Tuttle (10). One unit of pyruvate dismutation activity corresponds to the production of 1 μmole of acetyl phosphate per hour. One unit of lipoamidase is defined as the amount of enzyme which produces a loss of 1 unit of pyruvate dismutation activity under the conditions described above. The loss of pyruvate dismutation activity was proportional to the amount of lipoamidase up to 4 units (Fig. 1A).
For determination of lipoamidase activity in samples which might contain substances that would interfere with the determination of pyruvate dismutation activity, e.g. acetyl phosphatase in crude extracts, the assay was based on the rate of release of radioactive lipoic acid from a preparation of the E. coli pyruvate dehydrogenation complex containing bound lipoic acid-35S (9). The sample was incubated for 1 hour at 30° with 115 µg (90 units) of pyruvate dehydrogenation complex containing 0.24 µg (1.18 mmoles) of bound radioactive lipoic acid-35S (13,000 c.p.m. per µg) in a total volume of 0.5 ml of 0.02 M potassium phosphate buffer, pH 7.0. At the end of the incubation period, 20 µg of nonradioactive D,L-lipoic acid were added. The reaction mixture was cooled, acidified carefully with 0.05 ml of concentrated hydrochloric acid, and extracted with two 1.5-ml portions of benzene. The benzene extracts were combined, evaporated to dryness on a stainless steel planchet, and the radioactivity was measured with a Nuclear-Chicago gas flow counter. Preliminary experiments showed that recovery of an internal standard of D,L-lipoic acid (5 to 25 mmoles) was quantitative under these conditions.

**Determination of Protein-bound Lipoic Acid**—Lipoic acid content of protein fractions was determined by radioactivity measurements and by microbiological assay with lipoic acid-deficient S. faecalis as described previously (9). A procedure utilizing lipoamidase and the colorimetric method of Ellman (6) was also developed. An amount of protein containing approximately 10 mmoles of bound lipoic acid was incubated with 400 units of lipoamidase in 0.5 ml of 0.02 M phosphate buffer, pH 7.0, for 1 hour at 30°. The incubation mixture was allowed to stand at 30° for 15 minutes and then was adjusted to pH 7.0 with 0.03 N hydrochloric acid (approximately 0.07 ml) to destroy unreacted sodium borohydride. To the solution were added 0.9 ml of 0.04 M phosphate buffer, pH 8.0, and 0.01 ml of a 0.396% solution of 5,5'-dithiobis(2-nitrobenzoic acid) in 0.04 M phosphate buffer, pH 7.0. The reference cuvette contained the latter reagent and buffer. The absorbance was measured at 412 mµ in a Beckman spectrophotometer and the content of sulfhydryl groups was calculated with a molar extinction coefficient of 13,000 M⁻¹ cm⁻¹ (6). Preliminary experiments showed that recovery of an internal standard of lipoic acid (5 to 25 mmoles) was quantitative under these conditions.

When lipoyl derivatives were used which were extractable by benzene from the acidified reaction mixture, i.e. lipoamide and methyl lipoate, the extraction procedure was modified as described below. The reaction mixture was adjusted to pH 9 to 10 with dilute potassium hydroxide and then extracted with two 1.5 ml portions of benzene to remove unhydrolyzed lipoamide or methyl lipoate. The aqueous phase was then acidified with concentrated hydrochloric acid and free lipoic acid was extracted with benzene as described above. Control experiments showed that lipoic acid could be separated quantitatively from lipoamide and methyl lipoate in this manner.

**Lipoic Acid Assay**—Lipoic acid released from lipoyl derivatives by lipoamidase was reduced with sodium borohydride, and the dithiol produced was determined by the colorimetric method of Ellman (6). The reaction mixture (0.5 ml) was cooled in ice, acidified carefully with 0.05 ml of concentrated hydrochloric acid, and then extracted with two 1.5-ml portions of benzene. The benzene extracts were combined and evaporated by means of a stream of nitrogen. The residue was dissolved in a small volume of 0.04 M phosphate buffer, pH 8.0, to give a solution containing 5 to 25 mmoles of lipoic acid per 0.05 ml. To the 0.05-ml aliquot was added 0.05 ml of a 0.2% aqueous solution of sodium borohydride. The solution was allowed to stand at 30° for 15 minutes and then was adjusted to pH 7.0 with 0.03 N hydrochloric acid (approximately 0.07 ml) to destroy unreacted sodium borohydride. To the solution were added 0.9 ml of 0.04 M phosphate buffer, pH 8.0, and 0.01 ml of a 0.396% solution of 5,5'-dithiobis(2-nitrobenzoic acid) in 0.04 M phosphate buffer, pH 7.0. The reference cuvette contained the latter reagent and buffer. The absorbance was measured at 412 mµ in a Beckman spectrophotometer and the content of sulfhydryl groups was calculated with a molar extinction coefficient of 13,000 M⁻¹ cm⁻¹ (6). Preliminary experiments showed that recovery of an internal standard of lipoic acid (5 to 25 mmoles) was quantitative under these conditions.

1 Recovery of lipoic acid was low when the amount of bound lipoic acid present was less than 5 mmoles.
cooled in ice, acidified carefully with 0.05 ml of concentrated hydrochloric acid, and extracted with two 1.5-ml portions of benzene. The benzene extracts were combined and evaporated by means of a stream of nitrogen. The residue was treated with sodium borohydride and thiol groups were determined colorimetrically as described above. Good correlation of the results with those obtained by radioactivity determinations was observed. Thus, a highly purified preparation of the E. coli pyruvate dehydrogenation complex (specific activity, 1000) was found to contain 12.3 mmol of lipoic acid per mg of protein by radioactivity measurement and 11.3 mmol by the procedure described above.

Other Methods-Protein was determined by the method of Lowry et al. (11). Crystalline bovine serum albumin was used as standard. Biotin was determined by microbiological assay with Lactobacillus arabinosus (strain 17-5) (12). Lysine was determined colorimetrically with ninhydrin (13). A number of experiments were performed to determine the most suitable conditions for the differential analysis of L-lysine and l-5-lipoyl-L-lysine. A solution containing 0.5 ml of glacial acetic acid, 0.5 ml of the unknown sample, and 0.5 ml of ninhydrin reagent was heated in a boiling water bath for 5 minutes. The solution was cooled rapidly to room temperature and 1.5 ml of glacial acetic acid was added. The absorbance was measured at 440 nm in a Beckman spectrophotometer. Usually, 1.0 µmol of lysine gave an absorbance of 0.46. The ninhydrin reagent was prepared by dissolving 250 mg of ninhydrin in 6 ml of glacial acetic acid followed by addition of 4 ml of 0.6 M aqueous phosphoric acid.

Distribution—Lipoamidase activity was detectable neither in homogenates of bovine heart, liver, or kidney, nor in E. coli (Crookes strain) extract. Seaman (3) had described the presence of an apparently similar enzyme in bakers' yeast extract. Further distribution studies were not attempted since S. faecalis appeared to be a suitable source material for purification of the enzyme. Moreover, S. faecalis also served as a source material for a lipoic acid-activating enzyme and an apopyruvate dehydrogenation complex (8).

Preparation of Enzyme

Growth of Cells—Streptococcus faecalis (strain 10C1) was carried as a stab culture in medium containing 1% yeast extract, 1% tryptone, 0.5% K2HPO4, 0.1% glucose, and 2% agar, and was transferred monthly. Inoculation from the stab culture was made in 10 ml of the same medium minus agar. The culture was incubated for approximately 24 hours at 37° and then used to inoculate 1 liter of lipoic acid-deficient medium or yeast extract-tryptone medium (2 ml per liter). The cells were grown for 15 hours at 37°, and the culture was transferred to 9 liters of medium. The cells were grown at 37° for 8 hours and harvested at 5° in a Sharples centrifuge; yield, 1.7 to 2.3 g of wet cells per liter.

The lipoic acid-deficient medium was similar to that described by Gunsalus, Dolin, and Struglia (14) with the exceptions that the acid-hydrated casein and enzymatic casein hydrolysate were commercial products (see "Materials"), and ascorbic acid was omitted from the Salts B solution. The hydrolysate were commercial products (see "Materials"); yield, 1.7 to 2.3 g of wet cells per liter.  

* We are indebted to Dr. Joanne M. Ravel of this laboratory for the biotin assay.
pH 7.0. Fractions, 5 ml, were collected at 0.1 M and 0.15 M potassium chloride in 0.02 M buffer, pH 7.0, and then eluted stepwise with solutions containing the column was washed with about 80 ml of 0.02 M phosphate buffer, pH 7.0. The solution was dialyzed for 8 hours against two changes of the same buffer. The dialyzed solution (13.2 ml) was dissolved in a minimal volume of 0.02 M phosphate buffer, pH 7.0. The precipitate was collected by centrifugation and the supernatant fluid was added 25.2 ml of saturated ammonium sulfate solution, and the precipitate was discarded. To the supernatant fluid enzyme solution from the previous step were added slowly with stirring 29.8 ml of a saturated solution of ammonium sulfate, adjusted to pH 7.0 with concentrated ammonium hydroxide. The mixture was centrifuged for 20 minutes at 10,000 × g, and the precipitate was discarded. To the supernatant fluid were added 25.2 ml of saturated ammonium sulfate solution, pH 7.0. The precipitate was collected by centrifugation and was dissolved in a minimal volume of 0.02 M phosphate buffer, pH 7.0. The solution was dialyzed for 8 hours against two changes of the same buffer. The dialyzed solution (13.2 ml) contained 26.2 mg of protein per ml.

Step 4. Second Ammonium Sulfate Fractionation—To the enzyme solution from the previous step were added slowly with stirring 29.8 ml of a saturated solution of ammonium sulfate, adjusted to pH 7.0 with concentrated ammonium hydroxide. The mixture was centrifuged for 20 minutes at 10,000 × g, and the precipitate was discarded. To the supernatant fluid were added 25.2 ml of saturated ammonium sulfate solution, pH 7.0. The precipitate was collected by centrifugation and was dissolved in a minimal volume of 0.02 M phosphate buffer, pH 7.0. The solution was dialyzed for 8 hours against two changes of the same buffer. The dialyzed solution (13.2 ml) contained 26.2 mg of protein per ml.

Step 5. DEAE-cellulose Column Chromatography—The enzyme solution from the previous step was passed through a DEAE-cellulose column (2.5 × 15 cm) which had been equilibrated against 0.02 M potassium phosphate buffer, pH 7.0. The column was washed with about 80 ml of 0.02 M phosphate buffer, pH 7.0, and then eluted stepwise with solutions containing 0.1 M and 0.15 M potassium chloride in 0.02 M phosphate buffer, pH 7.0. Fractions, 5 ml, were collected at a flow rate of 30 ml per hour. The elution was followed by ultraviolet absorption at 280 nm and by measurement of enzyme activity. Some inactive protein was eluted with 0.1 M potassium chloride. The enzyme was eluted with 0.15 M potassium chloride. Most of the inactive protein remained on the column. The active fractions were combined and dialyzed for 7 hours against 2 liters of 0.02 M phosphate buffer, pH 7.0. The dialyzed solution (35 ml) contained 0.78 mg of protein per ml. A summary of the purification is presented in Table II. Dilute solutions of the enzyme were concentrated approximately 10-fold, with essentially quantitative recovery of activity, by adsorption on a small column (0.5 × 1.5 cm) of DEAE-Sephadex, followed by elution with a solution of 0.4 M potassium chloride in 0.02 M phosphate buffer, pH 7.0.

**Properties of Enzyme**

Lipoamidase activity, as measured by the rate of release of radioactive lipoic acid from the *E. coli* pyruvate dehydrogenation complex, was not affected by EDTA, MgSO₄, MnSO₄, or CaCl₂ at 1 × 10⁻³ M. The reaction was inhibited slightly (20%) by 1 × 10⁻⁴ M p-chloromercuribenzoate.

**Stability and pH Optimum**—Solutions of the purified enzyme have been stored at -15° for 6 months without significant loss of activity. Optimal activity was observed at pH 7.8 (Fig. 2).

**Substrate Specificity**—Since the amino acid sequence around the lipoyl moieties in the *E. coli* pyruvate dehydrogenation complex is known to be Gly-Asp-(epsilon-lipoyl)Lys-Ala (4, 16), it was of interest to determine whether lipoamidase would hydrolyze epsilon-lipoyl-L-lysine and derivatives thereof. The activity of the enzyme with various lipoic acid derivatives is shown in Table III. At a concentration of the lipoic moiety of

**Table II**

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluted extract*</td>
<td>415</td>
<td>3650</td>
<td>32†</td>
<td>100</td>
</tr>
<tr>
<td>Protamine sulfate fractionation</td>
<td>420</td>
<td>1930</td>
<td>60</td>
<td>102</td>
</tr>
<tr>
<td>First (NH₄)₂SO₄ fractionation</td>
<td>36.5</td>
<td>675</td>
<td>140</td>
<td>82</td>
</tr>
<tr>
<td>Second (NH₄)₂SO₄ fractionation</td>
<td>13.2</td>
<td>346</td>
<td>268</td>
<td>80</td>
</tr>
<tr>
<td>DEAE-cellulose fractionation</td>
<td>35</td>
<td>27.3</td>
<td>1960</td>
<td>46</td>
</tr>
</tbody>
</table>

* From 100 g of cells.
† The specific activity of extracts obtained from different batches of cells varied from 10 to 32.

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**Fig. 2.** Enzyme activity as a function of pH. Conditions were as described in Fig. 1B except for pH and buffer concentration. Lipoamidase (specific activity, 1740) with 2.5 μg of protein.

**Table III**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Lipic acid released</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>e-N-(DL-Lipoil)-L-lysine</td>
<td>0.0043</td>
<td>1</td>
</tr>
<tr>
<td>Pyruvate dehydrogenation complex</td>
<td>0.015</td>
<td>3.5</td>
</tr>
<tr>
<td>e-N-(DL-Dihydrolipoil)-L-lysine</td>
<td>0.11</td>
<td>1</td>
</tr>
<tr>
<td>α-N-Acetyl-e-N-(DL-Lipoyl)-L-lysine</td>
<td>0.09</td>
<td>0.8</td>
</tr>
<tr>
<td>α-N-Acetyl-e-N-(DL-Lipoil)-L-lysaminamide</td>
<td>0.10</td>
<td>0.9</td>
</tr>
<tr>
<td>α-N-(α-L-Asparaginyl)-e-N-(DL-Lipoil)-L-lysine</td>
<td>0.07</td>
<td>0.6</td>
</tr>
<tr>
<td>α-N-(β-L-Asparaginyl)-e-N-(DL-Lipoil)-L-lysine</td>
<td>0.13</td>
<td>1.2</td>
</tr>
<tr>
<td>α-N-(L-Asparaginyl)-e-N-(DL-Lipoil)-L-lysine</td>
<td>0.12</td>
<td>1.1</td>
</tr>
<tr>
<td>N-(DL-Lipoil)-L-alanine</td>
<td>0.04</td>
<td>0.4</td>
</tr>
<tr>
<td>N-(DL-Lipoil)glcyglycine</td>
<td>0.06</td>
<td>0.5</td>
</tr>
<tr>
<td>DL-Methyl lipolate</td>
<td>0.18</td>
<td>1.5</td>
</tr>
<tr>
<td>DL-Methyl lipocate</td>
<td>0.19</td>
<td>1.7</td>
</tr>
</tbody>
</table>

In Experiment 1, the reaction mixtures contained, in a final volume of 0.5 ml: potassium phosphate buffer, pH 7.0, 30 μmole; substrate, equivalent to 0.02 μmole of lipoyl moiety; and enzyme, 204 units (specific activity, 1600). In Experiment 2, 1.0 μmole of substrate and 529 units of enzyme were used. Incubation was for 2 hours at 30°.
4 x 10^{-4} M, \epsilon N-(DL-lipooyl)-L-lysine was hydrolyzed at approximately 29% of the rate at which lipoic acid was released from the pyruvate dehydrogenation complex. Reduction of the lipoic moiety, or substitution at the \alpha-amino group or the carboxyl group in \epsilon N-(DL-lipooyl)-L-lysine did not affect significantly the rate of hydrolysis. \epsilon N-(DL-lipooyl)-L-lysine and derivatives thereof were hydrolyzed at a faster rate than \epsilon N-(DL-lipooyl)-L-alanine and N-(DL-lipooyl)glycylglycine. Of the synthetic substrates tested, DL-lipoamide and methyl DL-lipoate were hydrolyzed at a faster rate than \epsilon N-(DL-lipooyl)-L-lysine.

The enzyme released equimolar amounts of lipoic acid and lysine from \epsilon N-(DL-lipooyl)-L-lysine. A reaction mixture containing potassium phosphate buffer, pH 7.0, 30 \mu moles; \epsilon N-(DL-lipooyl)-L-lysine, 1.0 \mu mole; and enzyme, 1560 units, in a final volume of 0.5 ml was incubated for 2 hours at 30°. The reaction mixture was cooled in ice. A 0.1-ml aliquot was acidified with 0.05 ml of concentrated hydrochloric acid, and its lipoic acid content was determined calorimetrically as described above. The remainder of the reaction mixture (0.4 ml) was added 0.4 ml of 12% trichloroacetic acid. The mixture was centrifuged, and 0.5 ml of the supernatant fluid was used for colorimetric lysine determination. Lipoic acid, 0.33 \mu mole, and lysine, 0.32 \mu mole, were formed.

Under the conditions specified above, \epsilon N-acetyl-L-lysine, \epsilon N-benzoyl-L-lysine, and \epsilon N-biotinyl-L-lysine were not hydrolyzed, as ascertained by the acid ninhydrin assay for lysine. The reaction mixture containing lipoamide and \epsilon N-biotinyl-L-lysine was also assayed for free biotin by a microbiological method, which is a more sensitive assay than the acid ninhydrin method for lysine. The microbiological assay showed that 0.008 \mu mole of \epsilon N-biotinyl-L-lysine was hydrolyzed. The rate of hydrolysis of \epsilon N-biotinyl-L-lysine was approximately \frac{1}{2} that obtained with \epsilon N-(DL-lipooyl)-L-lysine.

**Discussion**

The substrate specificity exhibited by lipoamidase appears to distinguish it from \epsilon lysine acylase described previously (17-19). Thus, lipoamidase does not hydrolyze \epsilon N-acetyl-L-lysine or \epsilon N-benzoyl-L-lysine. The complete or nearly complete lack of activity of lipoamidase toward biocytin appears to distinguish it from biotinidase, which is also present in *S. faecalis* (strain 10C1) (20). Lipoamidase appears to be specific for the lipoic moiety of protein-bound lipoic acid and of simple lipoic acid esters and amides.

Lipoamidase has proved to be invaluable, in conjunction with a lipoic acid-activating enzyme, in providing unequivocal evidence of the involvement of protein-bound lipoic acid in certain enzyme-catalyzed reactions (1, 2). This evidence comprises a demonstration of inactivation and reactivation of the enzyme accompanying, respectively, release and reincorporation of the lipooyl moiety.

**Summary**

Lipoamidase was purified approximately 100-fold from extracts of lipoic acid-deficient *Streptococcus faecalis* 10C1. The enzyme releases lipoic acid from the \epsilon N-lipooyllysine residues in the *Escherichia coli* \alpha-keto acid dehydrogenation complexes and also hydrolyzes simple lipoic acid amides and esters. The enzyme was completely or nearly completely inactive toward \epsilon N-acetyl-L-lysine, \epsilon N-benzoyl-L-lysine, and \epsilon N-biotinyl-L-lysine (biocytin).

**References**
