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# Purification and Amino Acid Sequence of the Linoleate Isomerase Produced from *Butyrivibrio fibrisolvens* A-38

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**Abstract**

**Molecular weight and partial amino acid sequence of the cis,9-cis,12-octadecadienoate isomerase(linoleate isomerase) of *Butyrivibrio fibrisolvens* A-38 were determined. Linoleate isomerase was isolated from the bacteria cultured anaerobically and purified by ultracentrifugation in conjunction with Sepharose 6B column chromatography, Phenyl sepharose 4B column chromatography and fast performance liquid chromatography(FPLC). The isomerase was a single polypeptide with 19KD of molecular weight, when determined by SDS-PAGE. Fourteen amino acids sequence of N-terminal of the linoleate isomerase was N-GEIDKYPRRIIKQQ determined by Edman method.**

**Key words**: conjugated linoleic acid(CLA), linoleate isomerase, *Butyrivibrio fibrisolvens*

## INTRODUCTION

Conjugated linoleic acid(CLA), originally isolated from grilled ground beef(1) and established as a potent cancer inhibitor in several animal models(1-4), is a collective term which refers to a group of naturally occurring isomers of linoleic acid containing conjugated double bond systems. Currently CLA has been recognized for its ability to reduce the catabolic effects of immune stimulation in mice, rats and chickens without adversely affecting immune function(5), to stimulate the growth and development of rats(6), and to reduce serum cholesterol level in rabbits(7). Furthermore, Belury(8) and Gurr(9) extensively reviewed the biological function of CLA.

The cis,9-trans,11 CLA isomer, recognized as biologically active principle(2), is present in virtually all foods, but the principal dietary sources are dairy products and other foods derived from ruminant animals (10-12). The cis,9-trans,11 CLA isomer is produced as an intermediate in the biohydrogenation of linoleic acid by the anaerobic rumen bacterium *Butyrivibrio fibrisolvens*(Fig. 1). Seemingly, some of the CLA produced in this way escapes conversion to stearic acid, is absorbed from the digestive system, and is subsequently incorporated into tissue lipid.

In 1960s, Tove group in University of North Carolina studied intensively the properties of biohydrogenation of linoleic acid in the rumen by *Butyrivibrio fibrisolvens* (13,14) and established some general enzymatic properties(kinetics, pH stability, heat stability etc.) of the crude linoleate isomerase. Since then, a few reports were published in the 1970s(15,16), but so far the concern of biochemists or molecular biologists was not reached at the isomerase due to no further interest in this microorganism. Discovery anticarcinogenic activity of CLA in 1987 by Ha et al.(1), however, had led many scientists including oncologists, nutritionists, and food chemists to attract the CLA as a pharmatheutical and a health food ingredient use.

Current researches of CLA were principally focused on the mechanism by which how CLA acts as anticarcinogens and, in addition, on the utilization of CLA as food ingredients. However, enzymatic nature of linoleate isomerase and the reductase was not intensively studied, so we are the first to investigate the linoleate isomerase in the point of molecular levels.

Present study describes the purification of linoleate isomerase from *Butyrivibrio fibrisolvens* A-38 and the N-terminal amino acid sequence of the enzyme.

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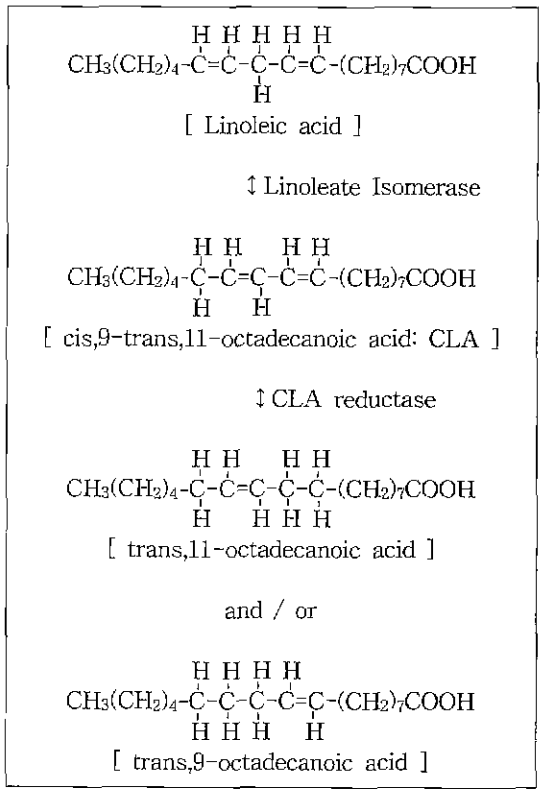


**Fig. 1. Biohydrogenation of linoleic acid by *Butyrivibrio fibrisolvens*.**
It was carried out in a two-step reaction. In the first step, linoleic acid is converted to the cis,9-trans, 11-octadecanoic acid(a CLA isomer) by linoleate isomerase. In the second step, the CLA is converted to the trans, 11-octadecanoic acid and/or trans,9-octadecanoic acid by CLA reductase.

## MATERIALS AND METHODS

### Reagents

Tryptic soy broth and yeast extract(Difco, Detroit, MI, USA), resazurine, L-cysteine · HCl, 1,3-propanediol, acrylamide, N,N'-methylene-bisacrylamide, tris[hydroxylmethyl]aminomethane, sodium dodecyl sulfate, sepharose 6B, and phenyl sepharose 4B(Sigma Chemical Company, St. Louis, MO, USA) and Bradford reagent (Bio-rad, Hercules, CA, USA) were used. Polyethylene glycol(#20,000) was purchased from Shinyo Pure Chemicals(Minoo, Osaka, Japan). Acetonitrile, dichloromethane and methanol from Merk(Darmstadt, Germany) were all HPLC grade. All other chemicals used were the reagent grade.

### Cell culture

*Butyrivibrio fibrisolvens* A-38(ATCC 27208) from the American-type culture collection(Rockville, ML, USA) was anaerobically cultured according to the method of Kepler et al.(14). The culture medium was degassed with a gas mixture(95% $CO_2$ and 5% $H_2$) until the medium color turned into yellow, followed by inoculating the starter of *Butyrivibrio fibrisolvens* A-38 grown for 18 hr at 37°C. The large-scale culture (20L) was achieved by the scale-up procedure, which was a successive four-step-cultures using 10% inoculum each : first 20ml culture, 200ml culture, 2L culture, and finally 20L culture.

### Cell-free preparation

The cells, harvested by centrifugation at 8,000g for 10min and washed twice with 2~3 volumes of 0.1M potassium phosphate buffer(pH 7.0), were ruptured using the Bead-beater(Biospec Products, Bartlesvile, OK, USA). The cell lysate, separated from beads by centrifugation at 8,000g for 20min, was further centrifuged according to the procedure described in Fig. 2. The pellets derived from the last ultracentrifugation(133,000g for 3 hours) was suspended with 0.1M potassium phosphate buffer(pH 7.0) and it is designated as a crude linoleate isomerase. The crude enzyme was divided into a 2ml cryogenic vial(Corning, New York, NY, USA) and stored frozen at -70°C until use.

### Purification of linoleate isomerase

The crude enzyme was further purified at 5°C by a series of column chromatography, using the Econo

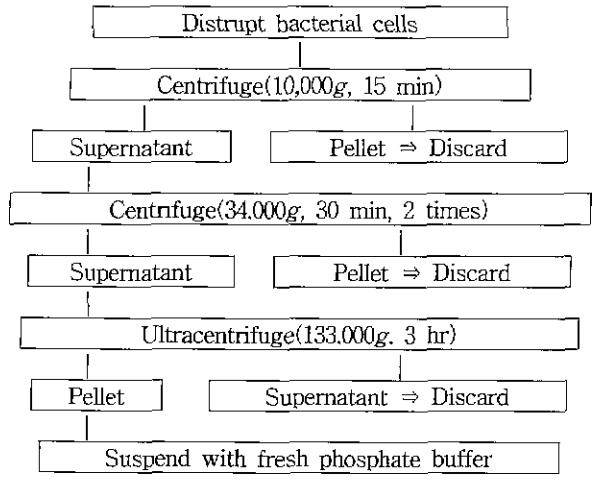


**Fig. 2. Procedure for the preparation of crude linoleate isomerase enzyme. All operations were performed at 0~4°C. The crude enzyme subdivided into a 2 ml aliquot was stored frozen at -70°C.**

system(Bio-Rad, Herculus, CA, USA). First, Sepharose 6B column chromatography was adopted to fractionate the crude enzyme with an isocratic elution, using potassium phosphate buffer(0.1M, pH 7.0). Phenyl sepharose 4B column chromatography was also used by a gradient system using 1M $(NH_4)_2SO_4$ and 0.1M potassium phosphate buffer(pH 7.0) with an Econo pump (Bio-Rad, Herculus, CA, USA). In both cases, eluent was monitored at 280nm with Econo system. Finally, fast performance liquid chromatography(FPLC) packed with Superose 6 was used to purify the enzyme using phosphate buffer(0.1M, pH 7.0) using a Pharmacia FPLC Model LKB LCC-500 PLUS(Uppsala, Sweden).

The fraction exhibited activity was concentrated in a 15ml centrifuge tube by speed vacuum concentrator (Bondiro, IL SIN engineering, Korea) or in a molecular porous membrane(M.W=6,000~8,000; Spectrum, Laguna Hills, CA, USA) by polyethylene glycol(#20,000).

### Electrophoresis

Electrophoresis was performed on a 12% SDS-PAGE, using upper buffer(0.5M Tris-HCl, pH 8.8) and low buffer(1.5M Tris-HCl, pH 6.8), using Bio-Rad mini-PROTEAN II electrophoresis model equipped with Hoefer Scientific Instrument power supplier(San Francisco, USA). Sample loaded on the gel was prepared by heating in a mixture of 1 volume protein solution and 4 volume buffer(62.5mM Tris-HCl, pH 6.8+10% glycerol +2% SDS+5% β-mercaptoethanol) for 4 min at 95°C. Molecular markers used were phosphorylase B(106,000), bovine serum albumin(80,000), ovalbumin(49,500), carbonic anhydrase(32,500), soybean trypsin inhibitor(27,500), lysozyme(18,500). The protein bands separated was stained by coomassie blue.

### *Assay of linoleate isomerase activity*

Enzyme solution(100μl) was added to the reaction mixture(1,3-propanediol 0.3ml+0.1M potassium phosphate buffer, pH 7.0, 3.7ml+linoleic acid 200μg) preincubated at 35°C for 5 min and then reacted for 30min at 35°C(15). CLA produced was extracted with hexane, washed with water and monitored at 233nm with UV spectrophotometer(Beckman DU-600, Harbor Blvd-Fullerton, CA, USA).

HPLC analysis of CLA produced was also performed to predict enzyme activity. For HPLC analysis, CLA produced was derivatized with 4% $H_2SO_4$/methanol by boiling in a boiling water bath for 10min. The CLA methylester was extracted with hexane and washed with water. After removing the remaining water under $Na_2SO_4$, the hexane solution was dried under vacuum and resuspended with methanol for HPLC analysis(2). Enzyme activity from the reaction of 30min at 35°C was represented as absorbance unit/mg protein for the case of UV absorption analysis and CLAmg/mg protein for the HPLC analysis.

### Determination of protein

Protein concentration was determined by the method of Bradford(17). Bovine serum albumin was used as a standard protein.

### Determination of amino acid sequence

Protein band separated by SDS-PAGE without staining was blotted by PVDF membrane(Milipore, Bedford, MA, U.S.A.). Blotting was performed by electrophoretic transfer kit(LKB, Miliford, MA, U.S.A). On the electrophoretic transfer kit, were placed in the order by the 3MM filter paper(Whatman, Maidstone, England) soaked in an anode buffer I(0.3M Tris+10% MeOH, pH 10.4), the 3MM filter paper soaked in an anode buffer II(25 mM Tris+10% MeOH. pH 10.4), a PVDF membrane, the gel containing protein separated without staining, and 3MM filter paper soaked in a cathode buffer(25mM Tris+40mM glycine+10% MeOH, pH 9.4). Blotting to PVDF membrane was initiated by connecting the power supply. Amino acid sequence of the blotted protein was determined by automated amino acid analyzer(Hisashi Hirano, National Institute of Agrobiological Resources, Ibaraki, Japan)(18).

### Determination of reducing sugar

Reducing sugar content of samples was determined by the method of Somogyi-Nelson(19). Sample(1ml) was taken in a screw-capped test tube containing 1ml of solution A(420mM $Na_2CO_3$+120mM Rochelle salt+240mM $NaHCO_3$+1.41M $Na_2SO_4$ : 30g $CuSO_4$+200ml distilled water containing 4 drops of c-$H_2SO_4$=25 : 1, v/v) and heated in a boiling water bath for 20min. 20min after cooling, 1ml of solution B [43mM $(NH_4)_6Mo_7O_{24}$] was added and vortexed. Total volume was adjusted to 25ml using water and then the absorbance was measured at 520nm by UV/VIS spectrophotometer(Beckman DU-600, Harbor Blvd-Fullerton, CA, USA). Glucose was used as a stan-

dard substance.

## RESULTS

### Purification of the linoleate isomerase

The crude linoleate isomerase was purified by Sepharose 6B column chromatography(Fig. 3). The linoleate isomerase activities of the three fractions(PK I , PK II, PK III) separated were found to be 1.48unit/mg, 0.31 unit/mg, and 0.19unit/mg. respectively, the facts that the most enzymatic activity was found in PK I .

The PK I was further purified by Phenyl sepharose 4B column chromatography and was subfractionated into four fractions(PK I-1, PK I-2, PK I-3, PK I-4) as shown in Fig. 4. Linoleate isomerase activity of four fraction(PK I-1, PK I-2, PK I-3, and PK I-4) separated was 3.2unit/mg, 2.6unit/mg, 2.9unit/mg and 6.4unit/mg, respectively, as shown in Fig. 5. These results indicate that PK I-4 is the major linoleate isomerase, so that the

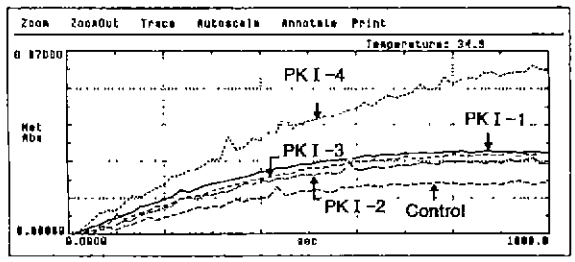


**Fig. 5. Absorbance at 233nm due to the formation of CLA from linoleic acid by PK I-4.**
The peaks seperated by Phenyl sepharose 4B column chromatography shown Fig. 4 were reacted with linoleic acid for 30min at 35°C.

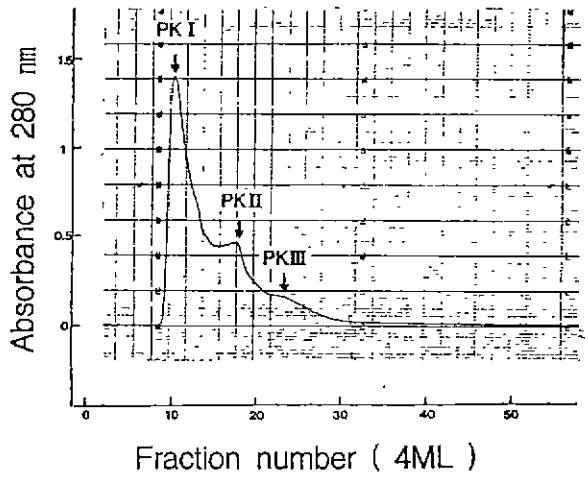


**Fig. 3. Sepharose 6B column chromatography of the crude linoleate isomerase.**
Sepharose column was equilibrated and isocratically eluted with 0.1M potassium phosphate buffer(pH 7.0).

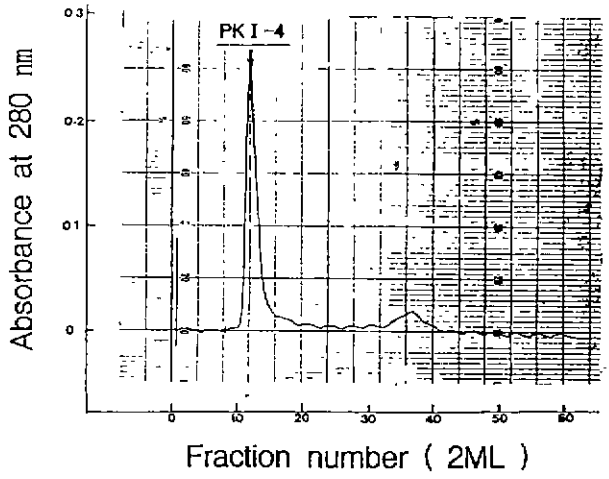


**Fig. 6. Purification of linoleate isomerase(PK I-4) by FPLC.**
FPLC column packed with superose 6 was equilibrated and isocratically eluted with 0.1M potassium phosphate buffer(pH 7.0).

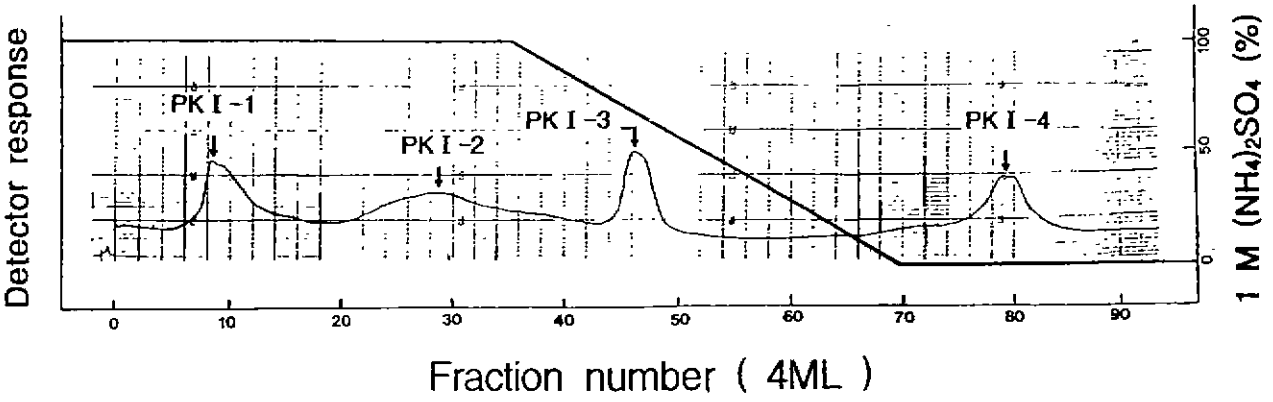


**Fig. 4. Fractionation of PK I shown in Fig. 3 by Phenyl sepharose 4B column chromatography.**
Phenyl sepharose 4B column was equilibrated with 1M $(NH_4)_2SO_4$. Sample was eluted by a gradient with 1M $(NH_4)_2SO_4$ and 0.1M potassium phosphate(pH 7.0) as shown.

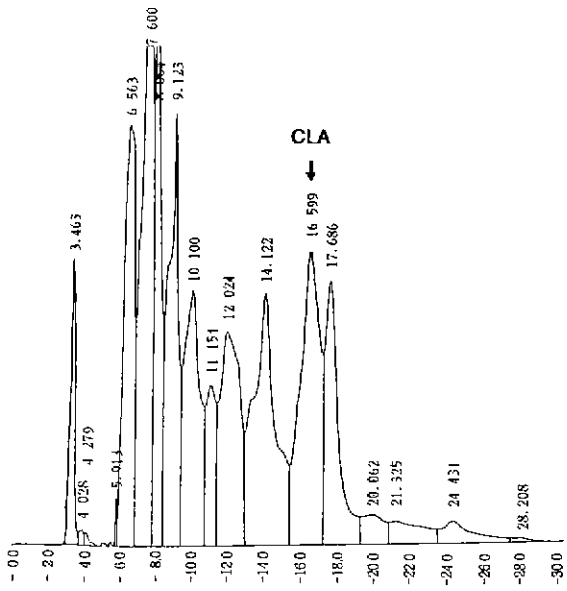


**Fig. 7. HPLC chromatogram of CLA methylester derived from linoleic acid by the reaction with PK I-4.**
Reaction mixture contained 0.2mg of linoleic acid, 0.3ml of 1,3-propanediol, 3.8ml of 0.1M potassium phosphate buffer and 0.1mg of PK I-4. Analytical conditions: μBondapak $C_{18}$ column(3.9×300mm); mobile phase(AcCN:water=80:20, v/v); and flow rate, 0.5ml/min..

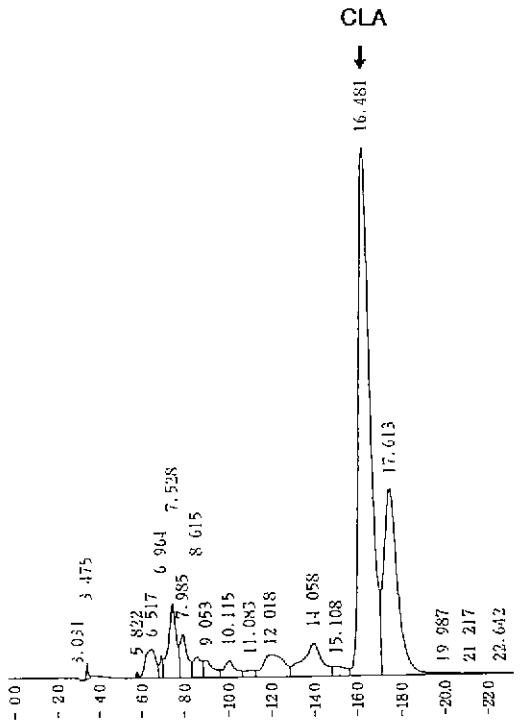


**Fig. 8. HPLC chromatogram of the CLA methylester standard.**
Analytical condition was the same as shown in Fig. 7.

PK I-4 was subjected to FPLC analysis. Fig. 6 showed the chromatogram of the PK I-4 by FPLC. Only one sharp peak was observed from the PK I-4, indicating highly pure protein.

Linoleate isomerase activity of PK I-4 was determined by HPLC analysis. Fig. 7 showed a HPLC chromatogram of CLA formed by PK I-4. The peak with retention time of 16.599 was identified as CLA, compared to standard CLA chromatogram shown in Fig. 8. CLA amount produced by PK I-4 and PK I was found to be 658μg/mg and 188μg/mg, respectively, indicating that PK I-4 has 4 times higher linoleate isomerase activity than PK I(Table 1).

## Electrophoresis of linoleate isomerase

Electrophoresis of PK I-4 and PK I fractions were performed on a 12% SDS-PAGE(Fig. 9). For comparison, crude linoleate isomerase was used as a positive control. PK I-4 contained only a single band with 19KD(Fig. 10), but the crude enzyme exhibited so many bands. Nondenaturating gel electrophoresis revealed that the PK I-4 was a single polypeptide chain(data not shown).

## Amino acid sequencing of linoleate isomerase

Fig. 11 showed amino acid sequence of linoleate isomerase(PK I-4) starting from N-terminal to 14th amino acid position, determined by automated Edman de-

**Table 1. Comparision of CLA formation[1] from linoleic acid by PK I and PK I-4.**

| Fraction[2] | CLA (μg/mg protein) |
|---|---|
| PK I | 188 |
| PK I-4 | 658 |

[1]CLA formation was determined by HPLC. Each fraction was reacted with linoleic acid in the conditions shown in Fig. 7
[2]PK I and PK I-4 were from Fig. 3 and 4, respectively

**Table 2. Reducing sugar concentration in crude enzyme and PK I-4**

| Fraction[1] | Reducing sugar(μg/mg protein) |
|---|---|
| Crude enzyme | 84 |
| PK I -4 | -[2] |

[1]Fraction reacted must be filtered with watman filter paper for removing the precipitate suspended in Somogyi-Nelson's reaction mixture
[2]No reducing sugar detected from PK I-4

gradation. Two amino acids identity was uncertain: 11th amino acid(isoleucine or lysine) and 14th amino acid (glutamate or lysine).

### Sugar content in purified linoleate isomerase

Reducing sugar content in PK I-4 and crude linoleate isomerase was determined by Somogyi-Nelson method (19). Reducing sugar content in crude enzyme was found to be 84μg/mg protein, but not found in PK I-4(Table 2).

## DISCUSSION

Linoleate isomerase from anaerobic rumen bacterium *Butyrivibrio fibrisolvens* converts linoleic acid to the cis,9-trans,11 CLA during biohydrogenation of linoleic acid in the rumen. In 1970s, Tove and his colleagues

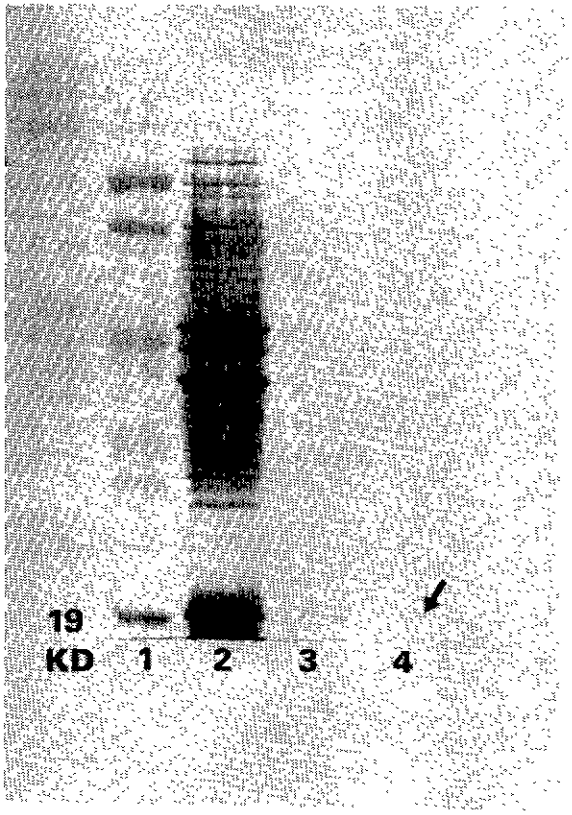


**Fig. 9. SDS-PAGE of linoleate isomerase(PK I-4).**
Shown is a 12% SDS-PAGE and stained by coomassie blue. Lane 1, molecular weight markers: phosphorylase B(106,000), bovine serum albumin(80,000), ovalbumin(49,500), carbonic anhydrase(32,500), soybean trypsin inhibitor(27,500) and lysozyme(18,500); lane 2, crude enzyme; lane 3, protein(PK I) from sepharose 6B chromatography(Fig. 3); and lane 4, protein(PK I-4) from phenyl sepharose 4B chromatography(Fig. 4)

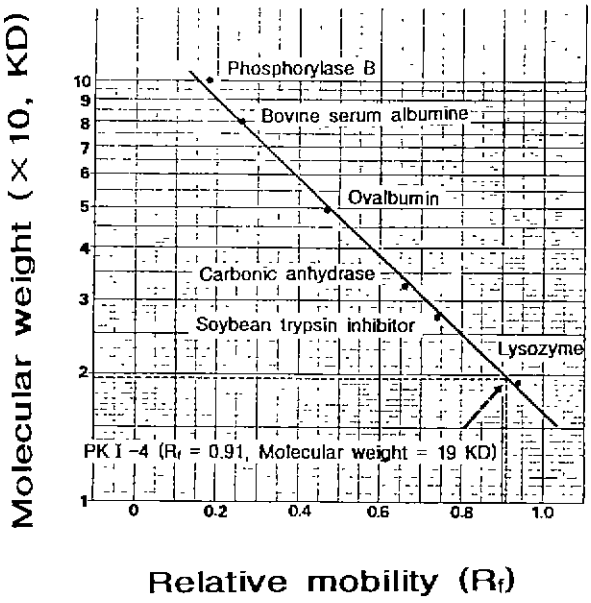


**Fig. 10. Calibration curve for the determination of molecular weight of linoleate isomerase(PK I-4), using the proteins from the SDS-PAGE(Fig. 9).**
Molecular weight markers used were phosphorylase B(106,000), bovine serum albumin(80,000), ovalbumin (49,5000), carbonic anhydrase(32,5000), soybean trypsin inhibitor(27,500) and lysozyme(18,500).

N-GEIDKYPRRIIKQQ

**Fig. 11. Amino acid sequence from N-terminal segment of linoleate isomerase(PK I-4) from *Butyrivibrio fibrisolvens* A-38.**
Amino acid sequence was determined by Edman degradation. The 11th and 14th amino acid identities from N-terminal were uncertain and both might be identified as KS.

(13-16) partially purified linoleate isomerase by the ultracentrifugation and studied general enzymatic properties such as temperature stability, optimum pH, cofactor requirements, reaction kinetics, substrate specificity and inhibitors. However, they did not study further purification and partial or full amino acid sequence of the enzyme. Thus, protein and molecular natures of this enzyme are not completely understood so far.

We are the first to purify and determine the amino acid sequence of the linoleate isomerase from *Butyrivibrio fibrisolvens* A-38. Linoleate isomerase activity was dramatically elevated by chromatographic purification as shown in Table 3. Activity of the linoleate

**Table 3. Purification of linoleate isomerase from *Butyrivibrio fibrisolvens* A-38[1)]**

| Step | Total proteins(mg) | Total activity[2)] (unit) | Specific activity[2)] (unit/mg) | Yield(%) | Purification fold |
|---|---|---|---|---|---|
| Crude extract[3)] | 1067 | 117 | 0.11 | 100 | 1 |
| Crude enzyme[4)] | 87.30 | 49 | 0.56 | 51.5 | 5.1 |
| Sepharose 6B[5)] | 30.40 | 45 | 1.48 | 16.2 | 13.5 |
| Phenyl sepharose 4B[6)] | 0.780 | 5 | 6.40 | 3.00 | 58.2 |

[1)]*Butyrivibrio fibrisolvens* A-38(ATCC 27208)
[2)]Absorbance from the reaction mixture of protein and linoleic acid
[3)]Crude extract from 20L of culture
[4)]Protein from the crude extract by ultracentrifugation(133,000*g*, 3hr)
[5)]PK I from Sepharose 6B column chrom atographic
[6)]PK I-4 from Phenyl sepharose 4B column purification

isomerase purified by Phenyl sepharose 4B increased to a 58.2-fold, compared the activity of crude extract. We found the linoleate isomerase was found to be single polypeptide chain with 19KD when determined by SDS-PAGE(Fig. 9 and 10). Amino acid sequence from N-terminal of this enzyme was found to be the GEIDKYPRRII(K)KQQ(K)(Fig. 11). Of the 14 amino acid sequences, 11th amino acid isoleucine and 14th amino acid glutamate were not clearly identified, and so both might be lysine. This amino acid sequence did not show homology with any other protein amino acid sequences known, when searched by Blast, indicating that molecular level of the enzyme was not studied yet.

Purified linoleate isomerase(PK I-4) did not contain any reducing sugars, but crude enzyme contained substantial amount of reducing sugar(84mg/mg protein) (Table 2). These results were not agreement with the observation of by Kepler and Tove(14) who observed that linoleate isomerase contained 44% reducing sugar. Hence, the sugars found in the linoeate isomerase purified by Kepler and Tove(14) must be impurities.

Currently, using the 14 amino acid sequence, we are cloning the linoleate isomerase from *Butyrivibrio fibrisolvens* A-38 and studying the expression of the clone in an appropriate hosts, like *E. coli*, or lactic bacteria.

## ACKNOWLEDGEMENTS

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