PRODUCTION, PURIFICATION AND CHARACTERIZATION OF A CLA-FORMING ENZYME FROM *LACTOBACILLUS ACIDOPHILUS*

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ABSTRACT

Conjugated linoleic acid (CLA) has gained much attention recently due to its beneficial health and biological effects on animals and humans. However, the CLA-forming enzyme system has not been studied in details. Six strains of *Lactobacillus acidophilus* L11, L12, L14, L15, *Lactobacillus fermentum* and *Lactobacillus reuteri* were used to study the growth conditions and the production of CLA-forming enzyme in MRS media containing linoleic acid concentrations at 37°C. The maximum growth rates for all strains were between 18 and 24 h. Linoleic acid inhibited the growths. The studies on the disrupted cells from *Lactobacillus acidophilus* L11, *Lactobacillus fermentum* and *Lactobacillus reuteri* producing high levels of CLA showed that the enzyme systems for CLA production were induced by linoleic acid. *Lactobacillus acidophilus* L11 produced the highest enzymatic activity of CLA-forming enzyme on MRS broth supplemented with linoleic acid. The purification and characterization of a CLA-forming enzyme were reported for the first time. A CLA-forming enzyme from *Lactobacillus acidophilus* L11 was purified to homogeneity by ion-exchange (MonoQ) and gel filtration (Superose12) using Fast Protein Liquid Chromatography (FPLC) system. The purified enzyme appeared as a single band on native PAGE and SDS–PAGE. The results showed that this enzyme has a molecular mass of 72 kDa, and is composed of two subunits. The optimal pH and temperature were 7.0 and 37°C, respectively. Kinetic study indicated that the enzyme has a high affinity for linoleic acid having a Km value of 1.49×10^{-5} M and the Vmax was 17.1 μM/mg/min. The enzyme activity was inhibited by the metal chelators such as Ni^{2+}, Cu^{2+}, Mn^{2+}, and EDTA. To further confirm the production ability of the CLA-forming enzyme from *L. acidophilus* L11, GC analysis was used. Both the crude extract and the purified enzyme produced CLA, but the purified enzyme produced more CLA than the crude extract. Further studies will be necessary to synthesis CLA by this enzyme and to develop the potential commercial value of CLA to enrich diary products and pharmaceutical preparations.

RÉSUMÉ

L'acide linoléique conjugué (CLA) a reçu beaucoup d'attention récemment à cause de ces bénéfices sur la santé et de ces effets biologiques chez les animaux et les humains. Cependant, le système enzyme formant le CLA n'a pas été étudié en détails. Pour ce faire, six souches de *Lactobacillus acidophilus* L11, L12, L14, L15, *Lactobacillus fermentum* et *Lactobacillus reuteri* étaient utilisées afin d'étudier les conditions de croissance et la production de l'enzyme formant le CLA dans les milieux MRS contenant des concentrations d'acide linoléique à 37°C. Les taux de croissance maximales pour toutes les souches se situaient entre 18 et 24 heures. L'acide linoléique inhibait les croissances. Les études sur la lyse des cellules de *Lactobacillus acidophilus* L11, *Lactobacillus fermentum* et *Lactobacillus reuteri* produisant de hauts niveaux de CLA démontrent que les systèmes enzyme pour la production du CLA étaient induits par l'acide linoléique. Parmi les souches bactériennes étudiées, *Lactobacillus acidophilus* L11 produisait la plus grande activité enzymatique de l'enzyme formant le CLA sur bouillon MRS supplémenté d'acide linoléique. La purification et la caractérisation de l'enzyme formant le CLA étaient rapportées pour la première fois. Un enzyme formant le CLA de la souche *Lactobacillus acidophilus* L11 était purifié à homogénéité en utilisant le système FPLC à échange d'ions (MonoQ) et à filtration sur gel (Superose 12). L'enzyme purifié apparaissait comme une bande simple sur PAGE natif et sur SDS-PAGE. Les résultats montrent que cet enzyme possède une masse moléculaire estimée à 72 kDa lequel est composé de deux sous-unités. Le pH optimal ainsi que la température étaient de 7.0 et 37°C, respectivement. L'étude cinétique indique que l'enzyme possède une grande affinité envers l'acide linoléique. La valeur du Km était de 1.49 x 10^5 M et la Vmax était de 17.1 μM/mg/min. L'activité de l'enzyme était inhibée par

les métaux chélateus tels que Ni^{2+}, Cu^{2+}, Mn^{2+}, et EDTA. De plus, pour confirmer la capacité *L. acidophilus* à produire l'enzyme formant le CLA, l'analyse GC était utilisée. L'extrait brut ainsi que l'enzyme purifié produisaient le CLA, mais l'enzyme purifié produisait plus de CLA que l'extrait brut. Ainsi d'autres études seront nécessaires pour étudier la synthèse du CLA par cet enzyme et pour développer une valeur commerciale potentielle pour le CLA afin d'enrichir le produit laitier et la préparation pharmaceutique.

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1. GENERAL INTRODUCTION

Conjugated linoleic acid (CLA) is a general term for positional and geometrical isomers of linoleic acid, *cis-9, cis-12* octadecadienoic acid, in which the double bonds are conjugated instead of being in the typical methylene interrupted configuration (Dugan et al., 1997). The two double bonds in CLA are in positions 9 and 11, 10 and 12, or 11 and 13 along the carbon chain (Parodi, 1994). Each of the bonds can be in the *cis (c) or trans (t)* configuration. Up to nine positional and geometric isomers of CLA have been reported as minor components of a variety of food products (Ha et al., 1989). Of the individual isomers, c-9, t-11 octadecadienoic acid has been implicated as being the most biologically active CLA isomer incorporated in the phospolipids of cell membranes (Ha et al., 1989, 1990) and is the most abundant form amounting to as much as 90% of the total CLA content of dairy products (Chin et al., 1992). Recently, CLA has gained considerable attention because of its anticarcinogenic (Ha et al., 1987; Jiang, 1996; Parodi, 1994, 1996, 1997), antioxidative (Durgan et al., 1997), antiatherosclerotic (Lee et al., 1994; Nicolosi et al., 1997) and immunostimulating properties (Klasing et al., 1987; Cook et al., 1993). In addition, CLA has been reported to decrease body fat while increasing muscle and bone mass in animals and human (Lin, 1999). As a result there is considerable interest in including CLA in animal feed to both improve lean production efficiency and provide value-added healthful meat products for human consumption. Most recently, supplementation with CLA in healthy humans, obese rate and obese humans reduced body fat mass possibly by non-enzymatic and enzymatic lipid peroxidation (Smedan and Vessby, 2001).

Results over the last decade have shown CLA to be anticarcinogenic (both in *vitro* and in *vivo*), to reduce atherosclerosis risk, to affect growth rate favorably and to improve feed efficiency. Of the many naturally occurring substances that have been demonstrated to have anticarcinogenic activity in experimental models, a majority of them are of plant origin. CLA is unique because it is present in food from animal sources such as dairy foods and meats. While there is still a lack of supporting human data, the evidence concerning the promising role and limitations of CLA in the prevention of cancer and other conditions so far is reviewed (Lin et al., 1995; Macdonald, 2000).

The current study provides a general survey of the CLA content of various representative foods normally consumed by humans. Many factors affect the concentration of CLA in foods, i.e., seasonal effects, length and conditions of storage, processing, and preparation. The systematic study of these factors was beyond the scope of the current study.

CLA is produced naturally as a result of microbial isomerizaition of dairy linoleic acid in the rumen, by the action of linoleic acid isomerase of the rumen bacterium, *Butyrivibrio fibrisolvens* to the *cis, trans11*-CLA isomer. The isomer is either absorbed or rapidly converted in the mammary tissue by delta 9 desaturase enzyme (Ogawa et al., 2001). Thus much interests have shown in the enrichment of CLA in milk fat of ruminants or in egg yolks by feeding trials. Our results showed that some of cheese associated *Lactobacillus*, *Lactococcus*, and probiotic *Bifidobacteria* cultures contain linoleate isomerase (LI), but the ability to convert linolerc acid into *cis-9, trans11* CLA was shown to be interstrain and interspecies variation. The

potential commercial value of CLA as a CLA enriched dairy product and a pharmaceutical preparation is significant and its production by microbial enzymes rather than by chemical synthesis ($100/g) should have advantage from the consumer viewpoint, but no LI active cheese starters or probiotic cultures to synthesize CLA are studied and are commercially available yet (Ogawa et al., 2001).

Kepler et al. (1967) identified the *c-9, t-11* CLA isomer as an intermediate in the biohydrogenation of linoleic acid by the rumen microorganism, *B. fibrisolvens.* Parodi (1977) also observed that CLA in milk fat consisted essentially of the *c-9, t-11* CLA isomer (Van et al., 1995). This study demonstrated that the *c-9.t-11* CLA isomer was the major isomer in the cheese and meat fats while the other isomers represent only minor fractions. Since the rumen bacterial linoleate isomerase produces exclusively the *c-9,t-11*CLA isomer, it is reasonable to conclude that microbial metabolism is the source of *c-9,t-11* CLA in meat and dairy products. The amount of conjugated dienoic acids in cow's milk and butter (Parodi, 1977) has been correlated positively with dietary intake of linoleic acid, indicating that CLA formed in the rumen is incorporated into milk fat (Parodi, 1977).

Total CLA content and the *c-9, t-11* CLA isomer were comparable in processed and natural cheeses. This indicated that heat treatment during processing does not alter total CLA content or *c-9, t-11* isomer concentration. Lactalbumin and lactoglobulin are the predominant protein in whey (McDermott, 1987). It has been shown that lactalbumin and lactoglobulin from milk binds long chain fatty acids (Perez et al, 1989). Thus, the stability of CLA during processing may be due to its

binding to lactalbumin or lactoglobulin which then protects against isomerization and oxidation.

This research is designed to produce a CLA-forming enzyme from the selected Lactobacillus species, which converts linoleic acid to CLA, and will provide insight in the CLA-forming enzyme.

The main objectives of this study were:

(1). To investigate the CLA-forming enzymes from several *Lactobacillus species*,

(2). To purify a CLA-forming enzyme from the best producer by FPLC system,

(3). To characterize the enzyme for its kinetics.

2. LITERATURE REVIEW

2.1 Conjugated Linoleic Acid (CLA) Sources

CLA concentrations in various foods have been reported to vary from as low as 0.2 mg/g fat in corn and peanut oil (Chin et al., 1992) to as high as 17 mg/g in beef (Ha et al., 1987) and 30 mg/g in milk fat (Parodi, 1994) of which c-9,t-11 accounts for up to 90% of the total CLA. Factors such as seasonal variations (Parodi, 1997) and diet (Chin et al., 1992) have been suggested to alter CLA content of milk fat. In general, animal sources are richer in CLA than are vegetable sources, and foods of ruminant origin generally contain more CLA than foods from nonruminant origin (Jiang et al., 1996). The exception among nonruminants is turkey, which contains CLA at levels of similar to those of ruminant animals (Chin et al., 1991). CLA content in seafood is negligible (Chin et al., 1992). Since CLA content of foods depends on the type of food, human dietary intakes of CLA may also vary widely depending on food selection.

CLA is widespread in biological materials, including plant and animal tissues normally consumed as food by humans. The current study provides a general survey of CLA content in various representative foods. The CLA content of ground beef and cheese in the present study appears to be several-fold higher than that reported previously (Ha et al., 1989). In the method used earlier (Ha et al., 1989), CLA was first separated with a reversed-phase column and then purified with a semipreparatory normal-phase column with gradient mobile phases before quantifying by GC. The method used in the current study employed a single analytical column with an isocratic mobile phase. Total CLA was quantified within

20 min. This prevents sample loss, oxidation, human error, extensive labor, and prolonged handling times (Chin et al., 1992).

CLA content in meat from nonruminant animals is generally lower than that from ruminant animals. A question is whether all of the CLA found in the tissues of nonruminants is a consequence of dietary intake, or perhaps due to the conversion of linoleic acid to the c-9, t-11 CLA-isomer by bacterial flora. In other experiments to be published elsewhere, diets supplemented with linoleic acid were fed to conventional or gnotobiotic rats; the CLA content in conventional rat tissues increased as a function of linoleic acid feeding. However, no increase of CLA was observed in gnotobiotic rats (Chin et al., 1992). This indicates that microorganisms in the rat gastrointestinal tract are capable of synthesizing CLA from linoleic acid. CLA in natural cheeses was found to be comparable to that in milk. In general, natural cheeses with shorter aging or ripening time (4 to 8 weeks) were found to have a higher CLA content. During aging or ripening of cheese lipolysis by bacterial enzymes occurs which partially splits neutral fat into free fatty acid and glycerol (Kosikowski, 1982). Under such conditions, free fatty acids including CLA become very vulnerable to further oxidation. This might indirectly reduce the CLA concentration in aged cheeses. CLA content in cheese can also depend on its concentration in starving material since CLA in cow's milk is affected by the microbial population in the rumen and by the amount of linoleic and linolenic acids in the diet.

The CLA content of plant oils is far lower than that of animal fat. The presence of CLA in plant oils was thought to be due to oxidation and/or bleaching

effects (Van den Bosch, 1973). However, oil extracted from corn in laboratory was shown to contain amounts of CLA comparable to that in commercial corn oil. This indicated that CLA is naturally present in plant oil. The *c-9, t-11* CLA isomer in commercial plant oil was found to be less than 50% of the total CLA isomer, and the *t-10, c-12* CLA isomer accounted for about 40% of the total isomer. Why plant oils have more *t-10, c-12* and less *c-9, t-11* CLA isomer than animal fat is unknown (Macdonald, 2000).

Infant formula, either soy or milk protein base, was found to contain only small amounts of CLA, this is to be expected since the major fat source is plant oil. The total CLA concentrations of infant foods that contain beef, lamb, or turkey were similar to those of the comparable raw materials. Interestingly, infant food with veal had a CLA concentration that was three-fold higher than that of the unprocessed starting material. The reason for the increased level in total CLA of veal infant food is unclear. Cow's milk and milk replacer are the major sources of feed for veal. CLA in milk replacer might vary given that the variation of CLA in cow's milk ranged from 0.2 to 2.8% (Parodi, 1977). This might indirectly affect CLA concentration in veal.

The *c-9, t-11* CLA isomer in seafood was not detectable due to interfering substances eluting with the *c-9, t-11* isomer from the GC capillary column. Foods that contained relatively little CLA should be purified by HPLC before the individual CLA isomers are analyzed by GC (Lin et al., 1999). When enzymatically prepared *c-9, t-11* CLA isomer was derivative with 14% BF3, more than 50% of the *c-9, t-11* isomer was converted to the *t-9, t-11/t-10, 1-12* isomers. However, when the *c-9-t-11*

CLA isomer was derivative with 4% HCI methanol at 60°, only 5% was converted to *t-9, t-11/t-10, t-12* CLA isomers (Haet al.,1990). The methylation method employed in the current study was found to effectively reduce the transformation of the *c-9, t-11* CLA isomer to *t-9, t-11/t-10, t-12* CLA isomers. It is important to maintain the stability of the *t-9, t-11* CLA isomer during derivativation. Only the *c-9, t-11* CLA isomer, believed to be the biologically active isomer, was found in membrane phospholipid from animals fed CLA preparations containing eight different isomers (Ha et al., 1990).

2.2 CLA Production Methods

CLA production methods include chemical method, extraction method, and biotechnological methods which by fermentation and by enzymatic conversion. Chen et al. (1999) reported that linoleic acid was efficiently converted into the two major components of conjugated linoleic acid, *9Z, 11 E*-ocdecadienoic (1a) and *11E, 12Z*-octadecadienoic (1b) using either the superbase (n-butyllithium/potassium tert-butoxide) or by simple refluxing with KOH in 1-butanol. About the extraction method, the extracted LA from an inexpensive oil (safflower oil) used in synthesis of CLA and produce to enrich the *Δ10t, 12c-18:2* isomer (Ma et al., 1999). The reaction in the biohydrogenation of LA by *Butyrivibrio fibrisolvens* demonstrated that an intermediate was hydrogenated to form a mixture of the two trans-monoenoic acids (Keplter et al., 1966). However, Lin (2000) found that inoculation of *Lactobacillus acidophilus* into 60g l^{-1} sweeteners and 10g l^{-1} sodium chloride-treated skim milk medium under aerobic conditions for 24h incubation was most effective in promoting *c9, t11*-CLA formation.

2.3 CLA Formation

Biohydrogenation of linoleic acid and linolenic acids to stearic acid by enzymes from the rumen microorganism, *Butyrivibrio fibrisolvens*, also contributed to the formation of several monoenoic and dienoic isomers including the biologically active CLA, *c-9, t-11-18:2. B. fibrisolvens* carries out the biohydrogenation of linoleic acid in a two steps process: (i) by isomerization of linoleic acid to CLA and (ii) by hydrogenation of the conjugated acid to form *t-11*-octadecadienoic acid (Haas et al., 1999). Chin et al. (1992) have reported that the intestinal flora of rats are also capable of converting linoleic acid to *c-9, t-11-18:2* (Kepler et al.,1966; Kim et al., 2000). However, germ-free rats fed diets supplemented with linoleic acid were not able to produce CLA, suggesting that the intestinal microflora of non-ruminants also has limited ability to isomerize linoleic acid. CLA has also been detected in serum, bile, and duodenal juice from human (Cawood et al., 1983; Shultz et al., 1992). CLA concentrations in human depot fat ranged from 0.3 to 0.9 y/100 g, and both the *c-9, t-11* and *t-9, t-11* CLA isomers are incorporated from diets into human depot fats. The concentration of CLA in humans diet could be increased by food processing technology, but foods with high levels in CLA are also high in fat, so one must weigh the benefits of dietary CLA with the deleterious effects of fat consumption. Cook et al. (1993) isolated and characterized CLA producing bacteria from the colons of conventional rats (Singh et al., 1994).

One strain, which was considered to be *Lactobacillus fermentum* or *Lactobacillus reuteri* was found to produce the highest amount of CLA, *c-9, t-11*, after a 36-hour incubation at 37°C under anaerobic condition in a medium containing

essential nutrients and free linoleic acid. However, virtually no studies have been carried out to characterize linoleate isomerase, which converts linoleic acid to CLA, from lactic acid bacteria and other sources. Lin et al. (1999) reported that inoculation of *Lactobacillus acidophilus* in 1,000 μg/ml linoleic acid added-skim milk medium for 24h was most effective in promoting CLA formation. Also, Lin (2000) found that the washed cells of *Lactobacillus acidophilus* producing high levels of CLA were obtained in a medium containing linoleic acid, indicating that the enzyme system for CLA production is induced by linoleic acid.

2.4 Linoleate Isomerase

Enzymes are proteins although many are conjugated proteins and are associated with non-protein groups. Enzymes are classified under six main headings depending on the nature of the chemical reaction involved, each class being sub-divided on the basis of the substrate and particular coenzyme or grouping involved in the reaction:

(1). Oxidoreductases catalyse the transfer of hydrogen or oxygen atoms or electrons.

(2). Transferases catalyse the transfer of specific groupings.

(3). Hydrolases catalyse hydrolytic reactions.

(4). Lyases catalyse the cleaving of bonds by reactions other than hydrolysis.

(5). Isomerases catalyse intramolecular rearrangements and involve isomerizations (Armstrong, 1983).

(6). Ligases catalyse the formation of bonds and require ATP.

Linoleate isomerases thus convert a linoleic acid into conjugated linoleic acid. Although few CLA-forming enzyme were mentioned (Kim et al., 2000) no LI

enzyme was purified and characterized, but most recently one patent on the sequences of unknown proteins from *Lactobacillus reuteri* was reported (WO patent: 0100846-A).

2.5 Methods of Enzymatic Activity Assay

2.5.1 Spectrophotometrical Method

The use of spectrophotometry to monitor enzyme-catalyzed reactions is a very convenient and popular method due to the simplicity of the technique and the precision which is possible (Armstrong, 1983).

The common polyunsaturated fatty acids in untreated oils show no absorption peaks in the ultraviolet. If the double bonds in those oils can be rearrange to form a conjugated system, selective absorption will appear in the ultraviolet region, and can be used for analytical purposes. Polyunsaturated fatty acids are converted into conjugated isomers by heating in alkali, and the intensity of the resulting selective absorption is determined. The isomerization is enhanced at elevated temperatures. By using ethylene glycol as a solvent, the temperature can be increased and the assay performed more rapidly (Pitt and Morton, 1957).

Mithchell et al. (1943) first published a detailed method by which both linoleic and linolenic acids can be determined. The procedure involves heating 10 ml of ethylene glycol containing 6.5% potassium hydroxide in a test tube in an oil bath. When the temperature reaches 180°C, 100 mg of fat or fatty acids is added, mixed, heated for 25 min, and cooled rapidly. After dilution with ethanol to a suitable volume, the absorbance is measured at 234 nm and 268 nm. A blank solution in the control cell of the spectrophotometer consists of an alkaline glycol

solution heated and diluted as the analysed sample. The extinction at 268 nm is derived from linolenic acid only; the contribution of the linoleic acid at 234 nm used to calculate the linoleic acid content. The method is empirical and suffers from the limitations of nonstoichiometrical analytical procedures. The ultraviolet method has undergone modifications that permit also a determination of arachidonic acid in a mixture with other unsaturated fatty acids. Oxidation of polyunsaturated fatty acids is accompanied by increased ultraviolet absorption (Holman et al. 1966).

Enzymatic activity is assayed spectrophotometrically by measuring the appearance of the conjugated diene system at 233nm using a molar extinction coefficient of 2.4 x 10^4 $M^{-1}CM^{-1}$. This value was derived from the absorption of conjugated octadecadienoic acids isolated from dehydrated licinoleic acid (Kepler et al., 1967).

2.5.2 Gas Chromatography Method

In gas chromatography, a volatile liquid or gaseous solute is carried by a gaseous mobile phase over a stationary liquid coated on the inside of an open tubular columns or on a solid support. Long, narrow open tubular columns have low capacity but give excellent separation (Jennings et al., 1998).

2.5.3 HPLC Method

High performance liquid chromatography (HPLC) uses high pressure to force eluent through a closed column packed with micro-size particles that provide exquisite separations of picograms to micrograms of analyte. HPLC uses columns that are 5-30 cm in length with inner diameter of 1-5 mm. The smaller the particle size, the more efficient the column, but the greater the resistance to flow. Reverse-

phase chromatography is the more common scheme in which the stationary phase is nonpolar or weakly polar and the solvent is more polar. Elution strength is increased by addition of less polar solvent. (Lindsay et al., 1992).

Sehat et al. (1998) first reported the application of silver-ion impregnated HPLC used to separate complex mixtures of CLA isomers present in commercial CLA sources and foods as well as in biological specimens. This method showed a clear separation of CLA isomers into three groups related to their *trans/trans, cis/trans,* or *trans/cis*, and *cis/cis* configuration of the conjugated double-bond system.

2.6 Fraction and Purification of Enzymes

2.6.1 Ammonium Sulfate Precipitation

Ammonium sulfate is the most frequently used salt for protein separation. One major property of ammonium sulfate as a choice is its relatively high solubility (ca. 700 g/L) allowing the precipitation of a wide variety of protein molecules. Ammonium sulfate is available in sufficiently pure state to be used in large quantities economically. The amount of ammonium sulfate to add to reach a predetermined concentration can be calculated from a simple formula (Scopes, 1984).

One great advantage of ammonium sulfate fractionation over virtually all other techniques is the stabilization of proteins that occurs. A 2-3 M ammonium sulfate suspension of protein precipitate or crystals is often stable for years, and it is the normal packaging method for commercial enzymes. The high salt concentration also prevents proteolysis and bacterial action. The redissolved precipitate contains considerable amounts of ammonium sulfate, and usually this must be removed before

proceeding to next step. Dialysis or gel filtration on a "desalting" column are the normal methods employed (Scopes, 1984).

Concentration of protein solutions and dialysis are closely related. Conventional use of dialysis bags involves the removal of unwanted low-molecular-weight solute from the sample and replacement with buffer present in the dialysate. Osmotic forces are usually opposite. High concentrations of salt or organic solvent in the sample cause water to enter the bag before the salt leaves; consequently there is an increase in volume during the early stages if the solute concentration is high. Dialysis is used both for removing excess low-molecular-weight solute and simultaneously introducing a new buffer solution (it may be just water) to the sample (Scopes, 1984).

2.6.2 Ion Exchange Chromatography

Protein is determined as the type of polymer involving mainly amino acids and it includes many thousands of different molecules. The sequence of amino acids in a polypeptide chains is known as the protein primary structure, and the nature of the amino acid residues is of prime importance in the development and maintenance of protein structure. The residues of those amino acids which are classed as either acidic or basic are capable of accepting or donating a proton and will at any given pH carry a charge of characteristic sign and intensity. The presence of all these ionizable groups results in proteins showing not only acidic and basic features but also the characteristics of an electrolyte. Such substances are known as ampholytes. At low pH values the ionization of the basic groups will be dominant and the protein will

carry a net positive charge while at high PH values the ionization of the anionic groups will be most evident (Holme et al., 1993).

The ion-exchange chromatography (IEC) is a process that allows the separation of ions and polar molecules. IEC is based on the affinity of the ions in solution for appropriate charged ions on the stationary phase. The stationary phase is a resin onto which groups are bonded whereas the mobile phase is usually a buffered aqueous solution containing a counter ion of appropriate charge and in equilibrium with the total charge of stationary phase. In ion-exchange chromatography, the solute is attracted to the stationary phase by coulomebic force. Proteins generally are applied in a low ionic strength buffer and are eluted with higher ionic strength buffer (Grab, 1995). There are two kinds of ion-exchange, one is anion exchange with quaternary ammonium ionogenic groups, the other is cation exchange with sulphonic acid ionodenic groups. In anion IEC the fixed charges are positive and in cation IEC, the fixed charges are negative (Grab, 1995).

Ion exchange separates biomolecules on the basis of differences in charge characteristics (cationic/anionic), it is dependent on the pH of the buffer system and the isoelectric points (pI) of the bimolecules. Normally, when buffer pH is above the pI, an anion (Q, DEAE) exchanger must be selected; when buffer pH is below the pI, a cation (S, SP, CM) exchanger must be selected (Holme et al., 1993).

2.6.3 Gel Filtration Chromatography

Gel filtration is based on physical sieving process and not on chemical phenomenon. The degree of retention is dependant on the size of the solved solute molecular relative to the size of the pore: small molecules will permeate the small

pores; the intermediate sized molecules will permeate some pores and the large molecules will be completely excluded and elute faster. Two main types of gel material may be distinguished: xerogels and aerogels. Xerogels are gels in the classical sense; they consist of cross-linked polymers which swell in contact with the solvent to form a relatively soft porous medium, in which the pores are the spaces between the polymer chains in the matrix. If the liquid is removed the gel structure collapses, although it can sometime be stored by replacing the liquid. Aerogels, on the other hand, are rigid materials which are not really gels at all; they are porous solids which are penetrated by the solvent, and they do not collapse when the solvent is removed; porous glass and porous silica are examples. The original gel chromatography medium, which is still widely used, was Sephadex G, a cross-linked dextran. The individual polysaccharide chains of dextran such as Superose can be cross-linked, with glyceryl bridges between the hydroxyl groups, a reaction under alkaline conditions with a dispersion of epichlorohydrin in an organic medium, limited degradation of the polysaccharide chains may occur under oxidizing conditions (Grab et al., 1995).

2.7 Enzyme Characterization

Enzyme catalytic activity is dependent on the maintenance of their native structure and any slight variations may result in significant changes in his activity. A common feature of enzymes is the presence of a clef or depression in the structure in which is lined with mainly hydrophobic amino acid residues and into which the substrate fits. Characterization of enzymes through their enzymatic properties is quite useful in evaluating their catalytic efficiency as they are exposed to some

environment, such as pH, temperature, and the substrate concentration (Armstrong, 1983).

2.7.1 pH Optimum

Enzymes are very sensitive to changes in pH and function best over a very limited range with a definite pH optimum. The effects of pH are due to changes in the ionic state if the amino acid residues of the enzyme and the substrate molecules, causing varying efficiency in the binding of a substrate. Specific residues are implicated in the catalytic process and any alteration in their charge will significantly affect the rate of reaction. Over a narrow pH range, these effects will be reversible but extremes of acidity or alkalinity often cause serious distortion of protein structure and result in permanent denaturation. Generally, enzymes can reach maximum activity at their optimum pH. In the design of assay it is important to appreciate that an enzyme may show different pH optima for different substrates and quoted values may not necessarily hold true for every assay method (Armstrong, 1983).

2.7.2 Temperature Optimum

The velocity of enzymatic reaction increases with increasing-temperature but once the increasing temperature occurs denaturation of the enzyme, the activity will fall. An increase in temperature increases the rate of denaturation of the enzyme with the loss of secondary and tertiary structure. Denaturation occurs more readily with pure solutions of enzymes than with impure. It is sometime suggested that enzymes show an optimum operating temperature but the most suitable temperature for a particular reaction is a compromise between maximal activity for a short period of time and a falling activity due to denaturation for a longer period of time. Many

assays are performed at 37°C either because it is mistakenly assumed that the human body temperature is the optimum temperature for an enzyme or more realistically, because above this temperature the rate of inactivation of the enzyme becomes far more significant (Scopes et al., 1984).

2.7.3 Substrate Specificity

When increasing concentrations of substrate are used in a series of assays, standardized by the above criteria, a plot of v (velocity or reaction rate) *vs* substrate concentration often produces a hyperbolic curve. Velocity is usually expressed as units (μmoles of product produced or substrate transformed per minute) or specific activity (units per milligram of protein). An analysis of the plot reveals reaction characteristics of the enzyme. The plot shows that the velocity increases with substrate concentration until maximum v (Vmax) is approached asymptotically, after which larger concentrations of substrate do not significantly enhance the reaction rate. The substrate concentration needed for half-maximum velocity (1/2Vmax) is called the Km value (Michealis constant) and is expressed in units of substrate concentration (moles per liter or M). Km may be consider an approximate measure of the affinity of an enzyme for its substrate; the lower the Km, the higher the affinity (Armstrong, 1983).

The linoleate isomerase has very stringent substrate specificity requirements. Only those compounds which posses a free carboxyl group and a *cis-9, cis-12* double bonds system are isomerized. The presence of additional double bonds does not affect substrate utilization, e.g., both the *Δ9,12,15* and *Δ6,9,12* isomers of linolenic acid are active. (Kelper and Tove, 1967).

2.7.4 Electrophoresis

In a strict sense, electrophoresis refers to the movement of charged colloidal particles and micromolecular ions under the influence of an electric field. Electrophoresis in food analysis is, generally, restricted to proteins. The charge on the protein depends on the pH of the solution. Every protein has an isoelectric point (pI) at which the net charge is zero and at which it has a zero mobility. At pH value below pI, the protein migrates as a cation, the mobility increasing with decreasing pH. At pH values above the pI, the protein migrates as an anion, the mobility increasing with increasing pH (Pomeranze, 1987).

Differences in migration velocities provide a sensitive means of separating compounds of a mixture that is otherwise difficult to fractionate. Electrophoresis is often the only method available for the quantitative analysis and fractionation of biological fluids, and for the characterization of their purified components. In addition it is a powerful tool for the detection and characterization of macromolecular interactions (Deutscher, 1990).

Naive-PAGE runs the sample in a buffer at a pH where the proteins remain stable and in their native form. This method is the original procedure, marking use both of differences in charges between proteins and their different sizes. The buffer chosen depends somewhat on the nature of the proteins, but generally it is slightly alkaline, in the pH range 8-9, where most proteins are negatively charged and so move toward the anode. If the bulk of the proteins being observed are known to be basic, then a buffer of somewhat lower pH can be employed, and the system operated with the cathode at the bottom of the gel (Scopes et al., 1984). The gel usually used is

polyacrylamide, made by polymerizing acrylamide with N, N-methylene bis-acrylamide (Bis). The polymerization is catalyzed by riboflavin and/or N, N, N, N-tetramethylenediamine (TEMED) and by light. The pore size can be varied by the ratios of the chemicals used (Pomeranz, 1987).

SDS – PAGE overcomes the limitations of native PAGE by imposing uniform hydrodynamic and charge characteristics on all the proteins in a sample mixture. During sample preparation, proteins are treated with hot SDS. The anionic detergent binds tightly to most proteins at about 1.4 mg of SDS/mg of protein, imparting a negative charge to the resultant complexes. Interaction with SDS disrupts all non-covalent protein bonds, causing the macromolecules to unfold. The electrophoretic mobilities of the resultant detergent-polypeptide complexes all assume the same functional relationship to their molecular weights. SDS-PAGE is routinely used for the estimation of protein subunit molecular weights and for determining the subunit compositions of purified proteins (Deutscher, 1990).

3. MATERIALS AND METHODS

3.1 Microorganisms and Media

Six strains of *Lactobacillus acidophilus* L11, L12, L13, L14, *Lactobacillus fermentum* and *Lactobacillus reuteri* were used in this study. *Lactobacillus acidophilus* L11, L12, L13, L14 and *Lactobacillus reuteri* isolated from this laboratory was identified by API 50 CHL (Merieux, St-Laurent, QC), and *Lactobacillus fermentum* (ATCC 14931) from Sigma. All strains were maintained on MRS pH 6.7 (Difco) agar at 4°C. Stock cultures were kept at –20°C in MRS broth containing 15 % (v/v) glycerol. Cultures were revived by two or three consecutive subcultures in MRS broth before inoculation. These strains were activated in MRS broth for 16 h at 37°C, transferred to the culture medium containing different concentrations of linoleic acid and then incubated at 37°C in incubator with agitation (150 rpm). Growth was arrested when O.D. reached about 1.0 at 600 nm (A_{600}) with the medium pH 4.8.

3.2 Growth Conditions

Six strains were activated in MRS broth without linoleic acid for 16 h at 37°C. The activated cultures were transferred to the 12 ml culture medium with different concentration of linoleic acid in screw-capped tubes and incubated for defining growth times at 37°C in shaking incubator (Fisher) with agitation (150 rpm). The absorbance of the samples was read spectrophotometrically at 600 nm. In the case of the optical density higher than 1.0, the sample was diluted to fall in the range of 0.2 to 0.8.

Growth curve of *Lactobacillus acidophilus* L11 was prepared at 3 h intervals during the growth cycle.

3.3 Chemicals, and Reagents

Otherwise specified, all chemicals, and reagents used were of analytical grade and were obtained from Sigma Chemicals (St. Louis, USA).

3.4 Equipments

3.4.1. GC System

The instrumentation used for CLA qualification analyses was: a Perkin Elmer GC (Auto system United States) equipped with Supelcowax-10 fused silica capillary column (30m x 0.32mm i.d., 0.25 μm thickness, Supelco Inc., Bellefonte, PA, USA); a flame ionization detector; and a Hewlett-Packard 3395 integrator (Palo Alto, CA, USA). The injection volume was 3.0 μl. The temperature of the GC oven was programmed at 50 and from 50 to 200 °C at the rate of 20°C/min and held for 10 min. The injector and detector temperatures were 250°C (Jiang et al., 1998). Helium was used as carrier gas. The split ratio was set at 1:50. The *c9, t11*-CLA methyl ester eluted out is identified by comparing the retention rime with that of methylated CLA standard (Sigma Chemical Co.). The area of CLA peak was calculated as mg c9, t11-CLA per 20 ml medium using heptadecanoic acid as internal standard (Lin, 2000).

3.4.2 HPLC System

A Waters HPLC system equipped with two 110A pumps were used for the quantification of the total CLA methyl ester in fat. The CLA methyl ester in methanol (40-100 μl) is directly injected into the HPLC. Separations are performed

on a reserved-phase analytical column (Dalta Pak C_{18} Column 100A, 30 x 150 mm, Waters). The isocratic mobile phase (85 % acetonitrile: 15 % water) was delivered at a flow rate of 2 ml per min. Eluents were monitored at 245 nm using a Beckman Model 163 UV detector and recorded by a Spectra Physics Model 4270 integrator (Chin et al., 1992).

3.4.3 Spectrophotometer

A Beckman spectrophotometer (Model DU-7; Beckman Ins., Irvine, State) was used for all the spectrophotometric assays during the whole course of the research.

3.4.4 Fast Protein Liquid Chromatography (FPLC) System

A FPLC system (Pharmacia) was used for the enzyme purification. It consisted of two p-500 pumps; a controller, LCC-501 PLUS equipped with a recorder for peak integration; a fraction collector, FRAC-200; a monitor UV-M II; a gradient mixer and a pH control; a recorder, REC-102; a peristatic pump P-1 for automatic sample injection. Pre-packed chromatography columns of Mono Q HR 10/10 (10 x 1.0 cm i.d.) and 12 HR 10/30 (30 x 1.0 cm i.d.) were used for ion exchange and gel filtration, respectively.

3.5 Enzyme Production

The cells grown in MRS were harvested by centrifugation (5000 x *g*) for 15 min at 4°C. As the extracellular activity of CLA forming-enzyme were minimal than the intracellular one, the supernatants were discarded. The cell pellets were washed twice with 50 mM potassium phosphate buffer (pH 7.0), suspended in the same

buffer, and disrupted by ultrasonic disintegrater (Sonicater 450, Branson, Danbury, CN, USA) for 10 min at 30 sec intervals. The crude extracts were separated from cell debris by centrifugation at (12,000 x *g*) for 15 min at 4°C and the supernatants were tested for the intracellular enzyme activity.

3.6 Protein Determination

Protein concentrations were estimated by the method of Bradford (1976) using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as a standard. This method is based on the observation that Coomassie Brilliant Blue G-250 exists in two different color forms, red and blue. The red form is converted to the blue form upon binding of the dye to protein. The protein-dye complex has a high extinction coefficient thus leading to great sensitivity in measurement of the protein. The binding of the dye to protein is a very rapid process (approximately 2 min), and the protein-dye complex remains dispersed in solution for a relatively long time (approximately 1 h), thus making the procedure very rapid and yet not requiring critical timing for the assay. Figure 1 shows the calibration curve for protein determination.

3.7 Enzymatic Activity Assay

3.7.1 Spectrophotometric Assay

CLA forming enzyme activity in the crude extracts and the purified enzymes were measured by using linoleic acid as the specific substrate. An optically clear solution of 24 μM LA was prepared by mixing 0.1 ml of the LA substrate solution with 2.7 ml of phosphate buffer and 0.2 ml of 1,3-propanediol in a silica cuvette. After preincubation at 35□C for 5 min, the reaction was initiated by the addition of

0.01 ml of enzyme, and the optical density at 233 nm is recorded. A rapid reaction reached an equilibrium in a few minutes. The rate of the reaction was obtained from the initial linear proportion of the curve.

A unit of enzyme was the amount that catalyzes the isomerization of 1 nanomole of LA per min, equivalent to a change in optical density of 0.008 per minute in a 1 cm cuvette. Specific activity is expressed as unit of activity per milligram of protein, as measured by the method of Bradford.

3.7.2 GC Assay

3.7.2.1. Preparation of Washed Cells

After *Lactobacillus acidophilus* was grown in 150 ml MRS broth containing 5 mg/ml linoleic acid at 37°C for 24 h, the cells were harvested by centrifugation (5,000 x *g*, 15 min, 4°C), washed twice with potassium phosphate buffer (pH 7.0), centrifugated again, and then used as the washed cells.

3.7.2.2. Reaction Conditions

Reactions were carried out for 105 h at 37°C with gentle shaking (120 rpm) in screw-capped tubes filled with nitrogen gas. The reaction mixture 10 ml of 100 mM potassium phosphate buffer (pH 6.5), 50 mg of linoleic acid in a complex with BSA (bovine serum albumin) (0.2 mg of BSA/ mg of linoleic acid) and the washed cells (500 mg wet wt) from 150 ml of culture broth.

3.7.2.3. Lipid Extraction

Lipids were extracted by adding 15 ml of isopropanol, mixing vigorously and then adding 10 ml hexane and shaking for 3 min. The mixture solution was centrifuged (3000 x *g*; 5 min) at room temperature. Extraction was carried out with 10 ml hexane twice and the organic layers were collected.

3.7.2.4. Lipid Hydrolysis and Preparation of Fatty Acid Methyl Esters

The collected organic solutions were saponified with 12 ml of 2 M potassium hydroxide in methanol in a screw-capped tube at 100°C for 15 min, and cooled at room temperature for 10 min. The free fatty acids were methylated with 36 ml of 4% hydrochloric acid in methanol at 60°C for 20 min (Chin et al., 1992). The methylated samples were mixed with 6 ml hexane/water (1:1, v /v) and centrifuged (5000 x *g*; 15min; 4°C). The organic layers were dried under a stream of nitrogen at room temperature and the residues were redissolved in 1ml hexane for GC analysis.

3.7.3 HPLC Assay

After the preparation of washed cells, lipid extraction, and lipid hydrolysis and preparation of fatty acid methyl esters (method same as GC), the organic layers were dried under a stream of nitrogen at room temperature and the residues were redissolved in 1ml methanol for HPLC analysis of total CLA.

3.8 French Pressure Cell

Various methods have been reported for cell disruption including sonication, osmotic shock, freeze-thaw, enzymatic treatments, French pressure cell and MSK

homogenizer. The most commonly methods used for cell disruption are French pressure cell and MSK (Scopes et al., 1984).

The French pressure cell is a dispersion unit for disintegrating material, blood cells, unicellular organisms and other biological particles. The French pressure cell (French pressure) achieves cell lysis by placing the sample under high pressure followed by a sudden release to atmospheric pressure (Scopes et al., 1984). In this case, the cells of *Lactobacillus acidophilus* L11 were disrupted by French pressure cell with ice water in large amount sample.

3.9 Phase System

Sodium dodecyl sulfate gel electrophoresis (SDS-PAGE) and native polyacrylamide gel electrophoresis (Native-PAGE) were completed on the protein samples by using Phase System from Pharmacia. Phase System consisted of a separation and control unit and a development unit.

3.9.1 SDS-PAGE

SDS-PAGE kits are designed for use in analyzing proteins by discontinuous denaturing gel electrophoresis. Each kit contains three components: a 450 ml bottle of SDS-PSGE running gel concentrate, a 50 ml bottle of SDS-PAGE running gel buffer and a 50 ml bottle of 4 % stacking gel solution.

Firstly, 100 µl of 10 % ammonium persulfate solution was added for each 10 ml SDS-PAGE running gel used; and then 10 µl of TEMED was added to each 10 ml of SDS-PAGE running gel that was gently swirled to mix. The gel solution was poured into a prepared plate glass assembly; finally if desired, a small amount of isobutanol was slowly added to the top of the separating gel to make a straight

surface before pouring the stacking gel. The gel was allowed to fully polymerized before use.

If isobutanol was layered on the top of the separating gel, it was removed by gentle aspiration and any left over isobutanol was removed by washing with a small amount of water and aspirate off. 20 µl ammonium persulfate (10 %) and 2 µl TEMED were added to each 2 ml stacking gel solution. After gently mixing the stacking gel was carefully laid on the top of the running gel and appropriate comb was inserted and let the gel fully polymerization before use. The buffer system used in the making gel was 25 mM Tris, 192 mM acetic acid and 0.1 % SDS solution.

Tris-glycine electrophoresis buffer was used, which was consisted of 25 mM Tris, 250 mM glycine (electrophoresis grade) pH 8.3 and 0.1 % SDS.

3.9.2. Native-PAGE

Ready to use gels: 8-25 % (Pharmacia) were used for native PAGE. The gels were about 0.45 mm thick having 13 mm stacking gel zone (4.5 % T, 3 % C) and 32 mm gradient gel zone. Gradient 8-25 has a continued 8 % to 25 % gradient gel zone with 2 % cross-linking. The electrophoresis buffer system used in the gel was of 25 mM Tris, 192 mM acetic acid and 0.5 M EDTA (pH 8.0).

3.9.3. Staining and Destaining the Gels

Polypeptides separated by SDS-PAGE and native-PAGE gels were fixed with methanol:glacial acetic acid and stained with Coomassie Brilliant Blue R250, a triphenylmethane textile dye also known as Acid Blue 83. The gel was immersed for several hours in a concentrated methanol/acetic acid solution of the dye, and excess dye was then allowed to diffuse from the gel during a prolong period of destaining.

Firstly, dissolved 0.25 g of Coomassie Brilliant Blue R250 was dissovled in 90 ml of methanol:H_2O (1:1 V/V) and 10ml of glacial acetic acid and filtered the solution through a Whatman No. 1 filter to remove any particulate matter; then immersed the gels in at least 5 volumes of staining solution and placed on a slowly rotating platform for a minimum of 4 h at room temperature and removed the stain and saved it for future use; About the destaining, the gels were soaked in the methanol/acetic acid solution without the dye on a slowly rocking platform for 4-8 h, changing the destaining solution three or four times. The more thoroughly the gels were destained, the smaller the amount of protein that could be detected by staining with Coomassie Brilliant Blue. Destaining for 24 h usually allowed as little as 0.1 μg of protein to be detected in a single band. After destaining, gels were stored in water in a sealed plastic bag or photographed.

3.10 Enzyme Purification

The enzyme purification scheme is shown in Figure 2. All purification steps were done at 4°C to preserve the enzymatic activity.

3.10.1 Ammonium Sulfate Precipitation

The crude enzyme solution were precipitated with ammonium sulfate between 30 % and 65 % saturated with gentle stirring at 4°C. Pellet were collected by centrifugation (5,000 x *g*; 15 min; Model J221, Beckman) and redissolved in minimum volume of 0.1 M potassium phosphate buffer (pH 7.0). Crude samples were desalted by passing through a disposable Sephadex G-25 column (Pharmacia) which was previously equilibrated with 25 ml of 0.1 M potassium phosphate buffer (pH 7.0).

3.10.2 Ion-exchange Chromatography

The ion-exchange column (Mono Q HR 10/10) was pre-equilibrated with 0.1 M potassium phosphate buffer, pH 7.0 (Buffer A). Portions (2 ml) of desalted crude sample (100-150mg of protein) precipitated by ammonium sulfate were injected onto the column, and the column was eluted with 48ml of 0.1M potassium phosphate buffer containing 1 M NaCl pH 6.55 (Buffer B). Fractions (2.0 ml) were collected and tested spectrophotometrically for CLA-forming enzyme activity at 233 nm, and for protein content at 595 nm. Pooled fractions with enzyme active of several chromatographic runs were combined, concentrated by ultrafiltration using CX-10 Centriprep membrane (exclusion limit 10 kDa; Amicon Corp., Toronto, ON, Canada), desalted with the G-25 column, and assayed for protein and enzyme activity.

The concentrated enzyme was further subjected to a second ion-exchange chromatography procedure in which used a linear NaCl gradient from 0.15 M to 0.25 M and 0.25 M to 0.35 M at a flow rate of 4.0 ml/min. Active fractions were pooled and concentrated again by ultrafiltration using the same type Centriprep membrane as first ion-exchange step. The concentrated sample was further subjected to gel filtration chromatography.

3.10.3 Gel Filtration Chromatography

Molecular sieve chromatography was performed with a Superose-12 column (30 x 1.0 cm), which separates protein with a molecular weight between 40 to 330 kDa under a pressure of 15 bars. Portions (100 to 200 µl) of concentrated enzyme sample were injected onto the column and eluted with 0.1M potassium phosphate

buffer (pH 7.0) with 0.05 M NaCl at a flow rate of 0.2 ml/min; sensitivity is 0.05 and total elution time of 120 min. The enzyme was located by CLA-forming enzyme activity assay using linoleic acid as substrate and spectrophotometrically measured at absorbance 233 nm and 595 nm. The collected active fractions from several injections were then concentrated by ultrafiltration using CX-10 membrane and assayed for protein content and CLA-forming enzyme activity.

3.11 Purity and Molecular Weight Determination

The purity of the enzyme at each purification step was examined by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) using ready gels (Bio-Rad Lab., Hercules, CA). The samples were prepared by heating them to 100 °C for 3-5 min in 1X SDS gel-loading buffer to denature the proteins. 1X SDS gel-loading buffer is composed of 50 mM Tris.HCl (pH 6.8), 100 mM dithiothreitol (DTT), 2 % SDS (electrophoresis grade), 0.1% bromophenol blue, and 10% glycerol. The minimum amount of protein content for loading samples is 5 μg for each well. After heating, the whole mixture was centrifugated for 1-3 min (Sorvall, Speed Vac, Savant Inc., Farmingdale, NY.) to precipitate out insoluble materials. The separating and stacking gels and SDS-buffer strips as described previously were used. The gels were stained by using stain solution with Coomassie Blue R-250. Standard proteins was used as markers to plot a standard curve for determination of CLA-forming enzyme molecular weight. To verify whether the CLA-forming enzyme is composed of monomeric or subunits, native-PAGE of the protein sample was similarly run on ready gels.

3.12 Enzymatic Properties and Kinetic Studies

3.12.1. Determination of pH Optimum

The effect of pH on the activity of CLA-forming enzyme was measured in 0.05 M acetate buffer pH (3.0 to 5.0), 0.05M phosphate buffer pH (6.0 to 8.0) and 0.05 M glycine-NaOH buffer pH (9.0 to 10.0). The reaction mixture was composed of 900 µl of the 24 µM substrate solution (0.1 ml of 0.714 mM linoleic acid solution) and 0.2 ml 1,3-propanediol suspended in different pH buffer (2.7 ml) that was incubated with 100 µl of CLA-forming enzyme at 37°C for 10 min. The absorbance of product released was read at 233 nm.

3.12.2 Determination of Temperature Optimum

The effect of temperature on the activity of the CLA-forming enzyme was performed by incubating 100 µl of the purified enzyme plus 900 µl of the substrate solution (0.1 ml of 0.714 mM linoleic acid solution) and 0.2 ml 1,3-propanediol of suspended in 2.7 ml phosphate buffer (pH7.0) for 10 min at temperature ranges from 20°C to 50°C. The amount of product released was read at absorbance 233 nm.

3.12.3 Effect of Substrate Concentration

The effect of substrate concentration was performed with the purified enzyme. Final concentration of the substrate ranged from 2.4 µM to 120 µM. The reaction mixture composed of 900 µl of the substrate solution with 100 µl of the purified enzyme was incubated at 37°C for 10 min. The enzymatic activity was spectrophotometrically measured at 233 nm.

The kinetic constants (Km and Vmax) of the enzyme were determined. A Lineweaver-Burke plot was established by plotting reciprocals of the initial velocity

against substrate concentration, and the values of Km and Vmax were computed from the slope and intercept of the regression line.

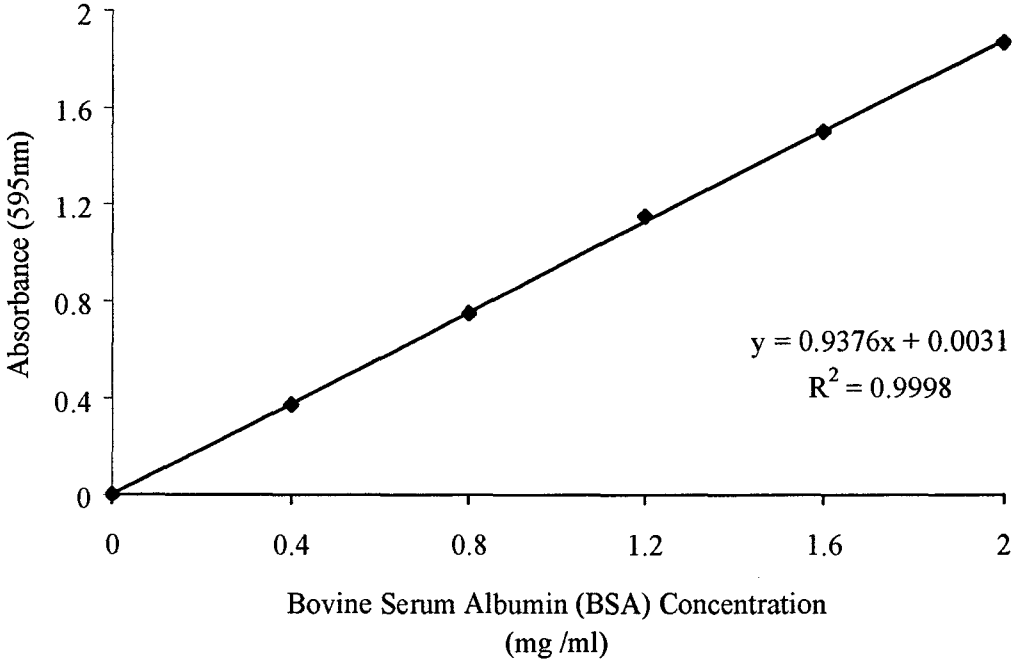

Figure 1. Calibration curve for protein determination

CRUDE EXTRACT OF ENZYME
[Centrifugation 5,000 x *g*; 15 min; 4 °C]

↓

AMMONIUM SULFATE PRECIPITATION
[30% TO 65% Saturated]
[Desalt with Sephadex G-25, PD-10 Column]

↓

FIRST ION-EXCHANGE CHROMATOGRAPHY
[Mono Q HR 10/10]
[Concentration by Ultrafiltration]

↓

SECOND ION-EXCHANGE CHROMATOGRAPHY
[Mono Q HR 10 /10]
[Concentration by Ultrafiltration]

↓

GEL FILTRATION CHROMATOGRAPHY
[Superose 12 HR 10/30]
[Concentration by Ultrafiltration]

↓

PURITY AND MOLECULAR WEIGHT DETERMINATION
[SDS-PAGE]
[Native-PAGE]

Figure 2. The enzyme purification scheme

4. RESULTS AND DISCUSSION

4.1 Production of a CLA-forming enzyme from *Lactobacillus species*

The six strains were grown in MRS broth without linoleic acid at 37°C from 3 to 42 h until the late logarithmic phase when optical density reached about 1.0 at 600 nm. The maximum growth rates of the four strains: *Lactobacillus acidophilus* L11, L12, L14, L15, one *Lactobacillus fermentum*, and one *Lactobacillus reuteri* on MRS broth without linoleic acid were obtained at 24 h, 24 h, 12 h, 21 h, 18 h and 24 h, respectively. Among four strains of *Lactobacillus acidophilus*, *Lactobacillus acidophilus* L11 produced the highest enzymatic activity from the cell grown in MRS broth containing 5 mg/ml linoleic acid (table 2). L12 was maximal with 1 mg/ml linoleic acid. The *Lactobacillus fermentum* and *reuteri* were maximal with 5 mg/ml and 7 mg/ml, respectively (figure 3).

Although *Lactobacillus reuteri* appeared to be better than others in terms of CLA production at 7 mg/ml, *L. reuteri* work has been protected by a patent (Peng at al., 2001 but not found in reference), a probiotic strains, *Lactobacillus acidophilus* L11 was randomly chosen to characterize the CLA forming enzyme in this work.

4.1.1 *Growth and Enzyme Production of* Lactobacillus acidophilus *L11*

Figure 4 shows the growth curve of *Lactobacillus acidophilus* L11 in MRS without linoleic acid in which its maximum cell growth was obtained after 24 h of growth. Figure 3 showed CLA production rates by three strains of *Lactobacillus acidophilus* L11, *Lactobacillus fermentum* and *Lactobacillus reuteri*.

When linoleic acid was supplemented in MRS broth medium, it inhibited the growth. However, after incubation in MRS medium with linoleic acid at 37°C for 24 h, the optimal concentration of linoleic acid on the production of CLA forming enzyme from disrupted cells of *Lactobacillus acidophilus* L11 was found to be in the range of 5 mg/ml (Table 1) and at this concentration the specific activity was highest (1.20 nM/mg). The results indicate that the enzyme systems for CLA production appeared to be induced by linoleic acid.

4.1.2 *GC and HPLC Analysis*

CLA concentration was tested mostly by using GC and HPLC after incubation of *L. acidophilus* in specific media (Lin et al., 1999; Jiang et al., 1998). Instead of using the incubated media solution, the washed cells were reacted in LA solution to observe the CLA production, similar to the experiments of Ogawa et al. (2001). When washed cells of *Lactobacillus acidophilus* were grown in MRS broth with or without 5 mg/ml LA at 37°C for 24 h, CLA was produced after 105 h of reaction with substrate solution. The CLA forming ability was higher with 5 mg/ml LA (300mg CLA) than that of control without LA (250mg CLA) in Figures 6 and 7. However, the level of LA in experimental sample were not decreased apparently as compared with control, probably due to the solvent extraction efficiency.

The retention time of CLA methyl ester observed in the gas chromatogram of either standard fatty acid methyl esters or fatty acid methyl esters of reaction solution from *L. acidophilus* L11 was from 15 min to 15.8 min, as shown in Figures 5, 6, and 7, respectively. In HPLC chromatograms, the retention time for both standard CLA methyl esters and fatty acid methyl esters of the reaction solution from *L. acidophilus*

L11 was 7.43 min., as shown in Figures 8, 9, and 10, respectively. These results were in agreement with those of spectrophotometer analysis, but not with GC data.

The same procedure was used for *L. acidophilus* for GC, but the analysis for GC, cells were grown in 150 ml MRS and for HPLC, cells were grown in 12 ml MRS media. The washed cells from *L. fermentum* and *L. reuteri* were also tried and showed their abilities to form CLA. *L. reuteri* appeared to induce more CLA-forming enzyme and produced more CLA than *L. acidophilus* (Figure 11and 12), but sequence data of *L. reuteri* linoleate isomerase was recently patented (Peng et al., 2001).

4.2 Purification of CLA-forming enzyme from *Lactobacillus acidophilus* L11

4.2.1 Enzyme Purification

Following ammonium sulfate precipitation the crude enzyme was subjected to three column chromatography steps. The yield and activity of CLA-forming enzyme during purification are summarized in Table 3. The enzyme was purified approximately 122-fold over the crude extract with a recovery of 16 %. Although the purified enzyme showed a high specific activity, its lower yield was partially due to deactivation of the enzyme during the purification steps. Initial studies showed that 85 % of enzyme activity was salted out between 30 and 65 %.

Elution profile of the CLA-forming enzyme in first ion-exchange chromatography is shown in Figure 13. After elution with NaCl gradient, the highest enzyme activity was obtained from 0.15 to 0.25 M NaCl, the corresponding elution volume of 34 to 41 ml. The major activity fractions after concentration were applied again to the second ion-exchange chromatography and eluted with a NaCl gradient of

0.15 to 0.25 M and 0.25 to 0.35 M (Figure 14). In the second ion-exchange chromatography, the highest enzyme activity was eluted at 0.195 M NaCl with elution volume of 26 ml, and only one peak showed a significant activity. Two steps of ion-exchange chromatographies removed about 98 % crude protein with a good recovery.

The active fraction from the second ion-exchange chromatography was injected onto gel filtration column. Figure 15 shows the gel filtration elution profile of CLA-forming enzyme. The enzyme was eluted at 16 ml volume.

Purity determination and estimation of the molecular weight were determined on SDS-PAGE. The electropherograms of the crude cell extract, partially purified and purified forms of the enzyme are shown in Figure 16. Both SDS-PAGE and Native-PAGE revealed one protein band after staining with Coomassie blue.

4.2.2 Molecular Mass of the Enzyme

A CLA-forming enzyme of *L. acidophilus* L11 showed an apparent molecular mass of about 72 kDa (Figure 17).

However, the results of the native-PAGE when plotted (Figure 18) showed that a CLA-forming enzyme is composed of two subunits with total molecular mass of 134 kDa.

4.3 Characterization of CLA-forming Enzyme from *L. acidophilus* L11

4.3.1 pH Optimum

Effect of pH on the CLA-forming enzyme activity was examined at pH ranges from 4 to 10. The maximal activity was obtained at pH 7.0 but the enzyme was stable in pH ranges from 4.5 to 8 (Figure 19). In general, higher activities were

observed when enzyme was stored in slightly acid medium (pH 6.0 to 7.0). However, there was a sharp decrease in enzyme activity when stored at pH 8.0. The optimal pH of CLA-forming was similar to the result of linoleate isomerase (pH 7.0 to 7.2) from rumen bacterium *Butyrivibrio fibrisolvens* (Kepler and Tove, 1967).

4.3.2 Temperature Optimum

The CLA-forming enzyme showed the highest activity at 37°C (Figure 20). Considering *L. acidophilus* L11 residues in human intestine, the optimal temperature appear to be in normal range of 37°C and is agreement with those of *Lactobacillus* species, but the activity decreased sharply over 40°C, probably because of its thermal inactivation.

4.3.3 Effect of Substrate Concentration

The affinity of substrate (linoleic acid) used in the assay is shown in Figure 21. As the enzyme concentration increased, the isomerization increased linearly up to a saturation point. However, the maximal isomerization was found only in a narrow concentration range. When the substrate concentration increased more than 50 μM, the reaction decreased rapidly due to the substrate inhibition to enzyme reaction. The substrate inhibition to the linoleate *Δ12-cis, Δ11-trans*-isomerase was reported (Kepler and Tove, 1967). Kim et al. (2000) found that the LA isomerase from *Butyrivibrio fibrisolvens* A38 did not recycle like a normal enzyme to catalyze more substrate, and the CLA production was highly cell density dependent. The CLA production increased if more LA was added, but only if the LA concentration was low, because CLA was as toxic as LA, this was no advantage in releasing large amounts of free CLA.

Figure 22 showed the Lineweaver-Burke plot on the CLA-forming enzyme. The Km and Vmax values were 14.9 μM and 17.1 μmol/mg/min. The Km is an approximate measure of the affinity of an enzyme for its substrate; the lower the Km, the higher the affinity. From the results, we can say that the CLA-forming enzyme had a strong affinity in the substrate, linoleic acid. The plot also showed that the velocity increased linearly with the substrate concentration until Vmax = 17.1 (data not shown) and then the high concentrations did not significantly enhance the reaction rate.

4.3.4 Effect of divalent cations and inhibitors

The activity of CLA-forming enzyme from *L. acidophilus* L11 was investigated in the presence of a variety of cations and other possible enzyme inhibitors (Table 4). The reaction mixture composed of 100 μl of CLA-forming enzyme and 100 μl of the cations or inhibitors (final concentration: 0.1 or 1.0 mM) with 800 μl of the substrate solution (0.714mM CLA-forming enzyme in 0.1M phosphate buffer, pH 7.0) incubated at 37°C for 30min. The enzyme activity was assayed under the standard conditions described for the enzyme assay procedure. Enzyme activity were inhibited by Ni^{2+}, Cu^{2+}, and Mn^{2+}, and only slight inactivation was observed in the presence of Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, and Fe^{3+}, but Co^{2+} had no inhibitory effect. The activity was strongly inhibited by EDTA. These results were in agreement with the results of reversible inhibition by the metal chelators and EDTA (Kepler and Tove, 1967).

4.3.5 The CLA production ability by crude enzyme and the purified enzyme

Figures 23 and 24 showed the GC chromatograms on CLA generated by crude and purified enzymes of *L. acidophilus* L11, respectively. Both enzymes produced CLA, but CLA production ability was much higher in the purified enzyme than in the crude extract. The results from GC analysis are in agreement with those of spectrophotomeric method, confirming that the purified enzyme is a CLA–forming enzyme from *L. acidophilus*.

4.4 Conclusions

Growth of *L. acidophilus* L11 into MRS broth containing 5.0 mg/ml LA at 37°C for 24 h was most effective in promoting CLA formation, indicating that the enzyme system for CLA production was induced by the addition of LA. After enzymatic reaction of the washed cells in the presence of linoleic acid for 105 h, the results of both GC and HPLC analysis showed that CLA was produced by the CLA-forming enzyme from *L. acidophilus* L11. However, the result of GC data was not clearly demonstrated than that of HPLC. The results of the CLA production from *L. acidophilus* and the enzyme system for CLA production induced by LA are in agreement with those of Lin et al. (1999) and Ogawa et al. (2001).

A CLA-forming enzyme was purified rapidly by FPLC system. The enzyme molecular mass evaluated from SDS-PAGE and native-PAGE demonstrated that this enzyme might be composed of two subunits having a single unit of 72 kDa. This enzyme differed substantially from the isomerase isolated from *Lactobacillus reuteri* which was patented (Peng et al., 2001), whose analytical molecular mass was 32 kDa.

The CLA-forming enzyme showed optimal isomerizatation at mild conditions, pH 7.0 and 37°C. The Km and Vmax values were 14.9μM and 17.1 μM/mg/ml. The enzyme activity was inhibited by the metal chelators, and EDTA. One of the future challenges will be the molecular cloning of this enzyme for overproduction of this enzyme to investigated the utility.

Table 1. CLA forming activity with *L. acidophilus* L11 grown different Concentrations of LA in MRS at 37°C for 24 h.

Concentration of LA in media (mg/ml)	Protein content OD (595)	Total protein (mg)	Total enzyme activity (units) *	Specific activity (units/mg)**
0.0	1.331	2.1244	1.8270	0.86
1.0	1.244	1.9852	1.6279	0.82
3.0	0.984	1.5693	1.5379	0.98
5.0	0.978	1.5589	1.8707	1.20
7.0	0.972	1.5501	1.4571	0.94
9.0	0.823	1.3117	1.0887	0.83
11.0	0.750	1.1949	0.8962	0.75

* One unit of enzyme activity is defined as the amount of enzyme required to catalyze the isomerization of 1 n mole of linoleic acid per minute.

** Specific activity is defined as the enzyme units per mg protein.

Table 2. CLA-forming activity with *L. acidophilus* L11, L12, L14, L15 grown 5 LA mg/ml in MRS at 37°C for 24h.

L. acidophilus \ Projects	Total protein (mg)	Total enzyme activity (units) *	Specific activity (units/mg) **
L11	1.5589	1.8707	1.20
L12	1.2473	1.0672	0.86
L14	0.8256	0.4824	0.58
L15	0.3271	0.0835	0.26

* One unit of enzyme activity is defined as the amount of enzyme required to catalyze the isomerization of 1 n Mole of linoleic acid per minute.

** Specific activity is defined as the enzyme units per mg protein.

Table 3. Purification steps of a CLA-forming Enzyme from *L. acidophilus* L11

Purification steps	Total protein (mg)	Total activity* (units)	Specific activity** (units/mg)	Purification (-fold)	Yield (%)
1. Crude extract	131.94	113.47	0.86	1.00	100
2. Ammonium sulfate precipitation	112.15	109.64	0.98	1.14	96.60
3. Ion-exchange I chromatography	1.6	75.00	46.88	42.60	66.10
4. Ion-exchange II chromatography	0.8	72.10	90.13	104.80	63.54
5. Gel filtration	0.18	17.82	104.83	121.87	15.70

* One unit of enzyme activity is defined as the amount of enzyme required to catalyze 1 n Mole of linoleic acid per minute.

** Specific activity is defined as the enzyme units per mg protein.

Table 4. The effect of metals ions and reducing agents on the CLA-forming enzyme activity.

Effector	Concentration		Specific Activity *	
	A (mM)	B (mM)	A	B
Control **	--	--	14.79	14.79
Ca^{2+}	0.1	1.0	11.83	11.83
Mg^{2+}	0.1	1.0	10.55	10.53
Fe^{2+}	0.1	1.0	11.78	11.72
Fe^{3+}	0.1	1.0	12.07	11.98
Zn^{2+}	0.1	1.0	12.61	12.60
Co^{2+}	0.1	1.0	14.58	14.53
Ni^{2+}	0.1	1.0	9.54	9.36
Cu^{2+}	0.1	1.0	8.87	8.76
Mn^{2+}	0.1	1.0	8.26	8.13
EDTA	0.1	1.0	6.34	6.25

* Specific activity is defined as the amount of the enzyme required to catalyze the isomerization of 1 n Mole of linoleic acid per min per mg protein.

** The enzyme assay was carried out in absence of effectorfs.

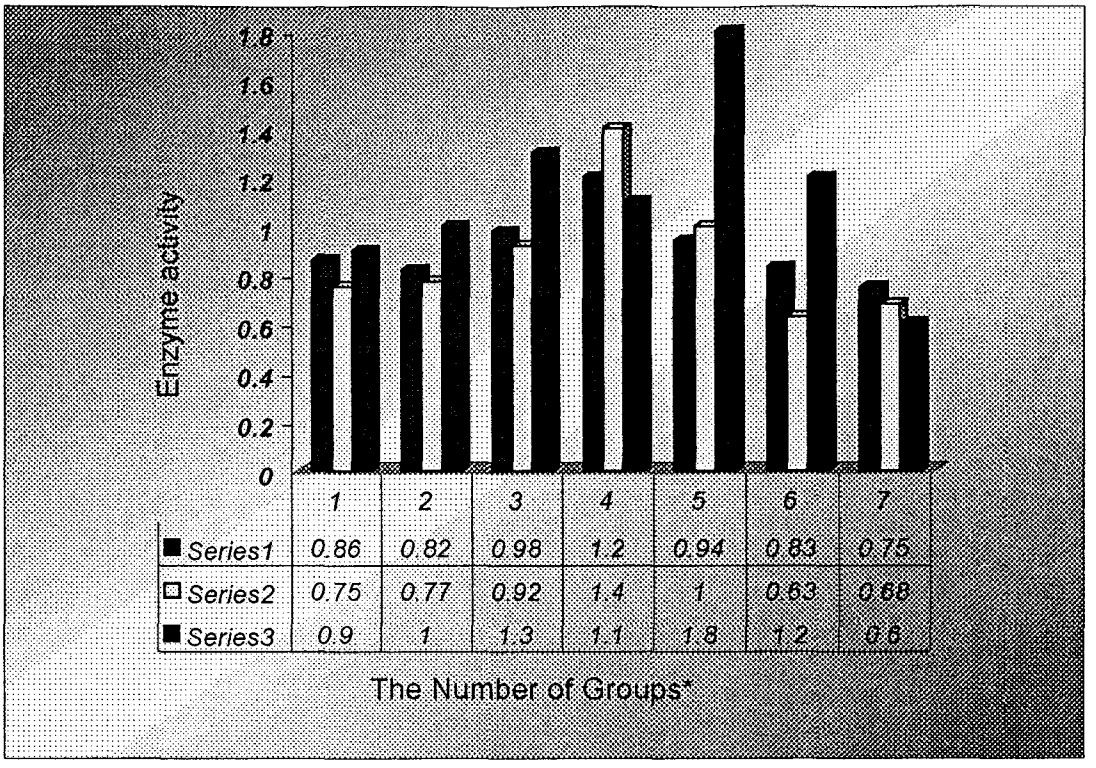

Figure 3. The CLA forming abilities by three strains (*L. acidophilus, L. fermentum* and *L. reuteri*).

Series 1: The enzyme activity levels of *L. acidophilus* L11 in MRS broth with different LA concentrations

Series 2: The enzyme activity levels of *L. fermentum* in MRS broth with different LA concentrations

Series 3: The enzyme activity levels of *L. reuteri* in MRS broth with different LA concentrations

*The number of Groups to:
*LA concentrations in MRS media,
1: no LA/ml MRS; 2: 1.0mg /ml; 3: 3.0 mg /ml; 4: 5.0 mg /ml;
5: 7.0 mg /ml; 6: 9.0 mg /ml; 7: 11.0 mg /ml.

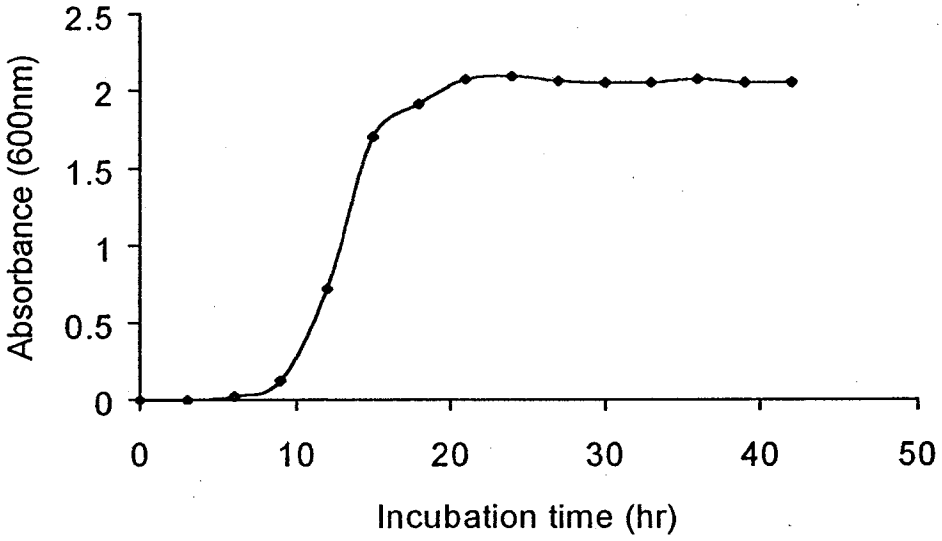

Figure 4. Calibration curve of *L. acidophilus* L11without linoleic acid in MRS media.

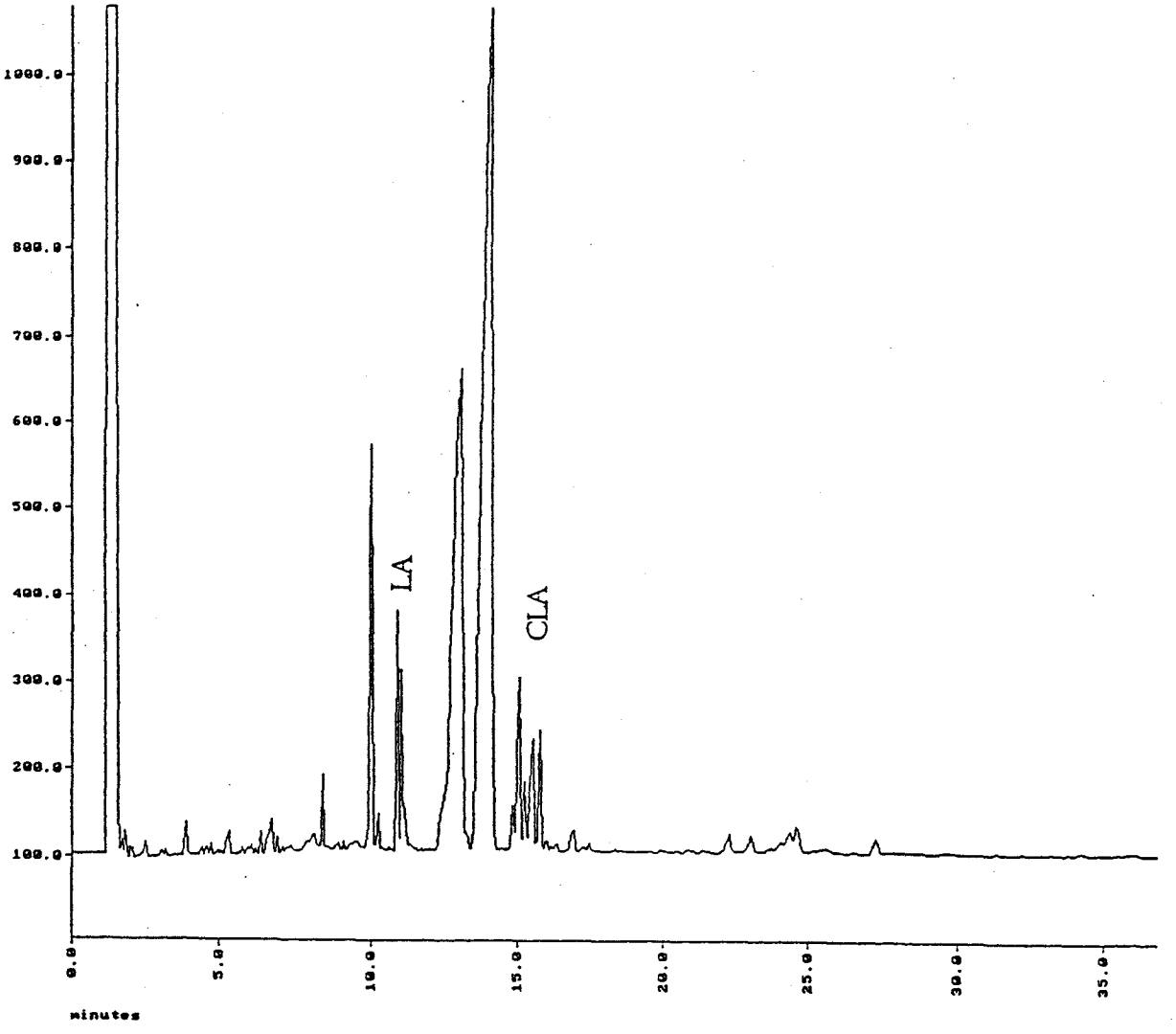

Figure 5. GC chromatogram of standards of LA and CLA.

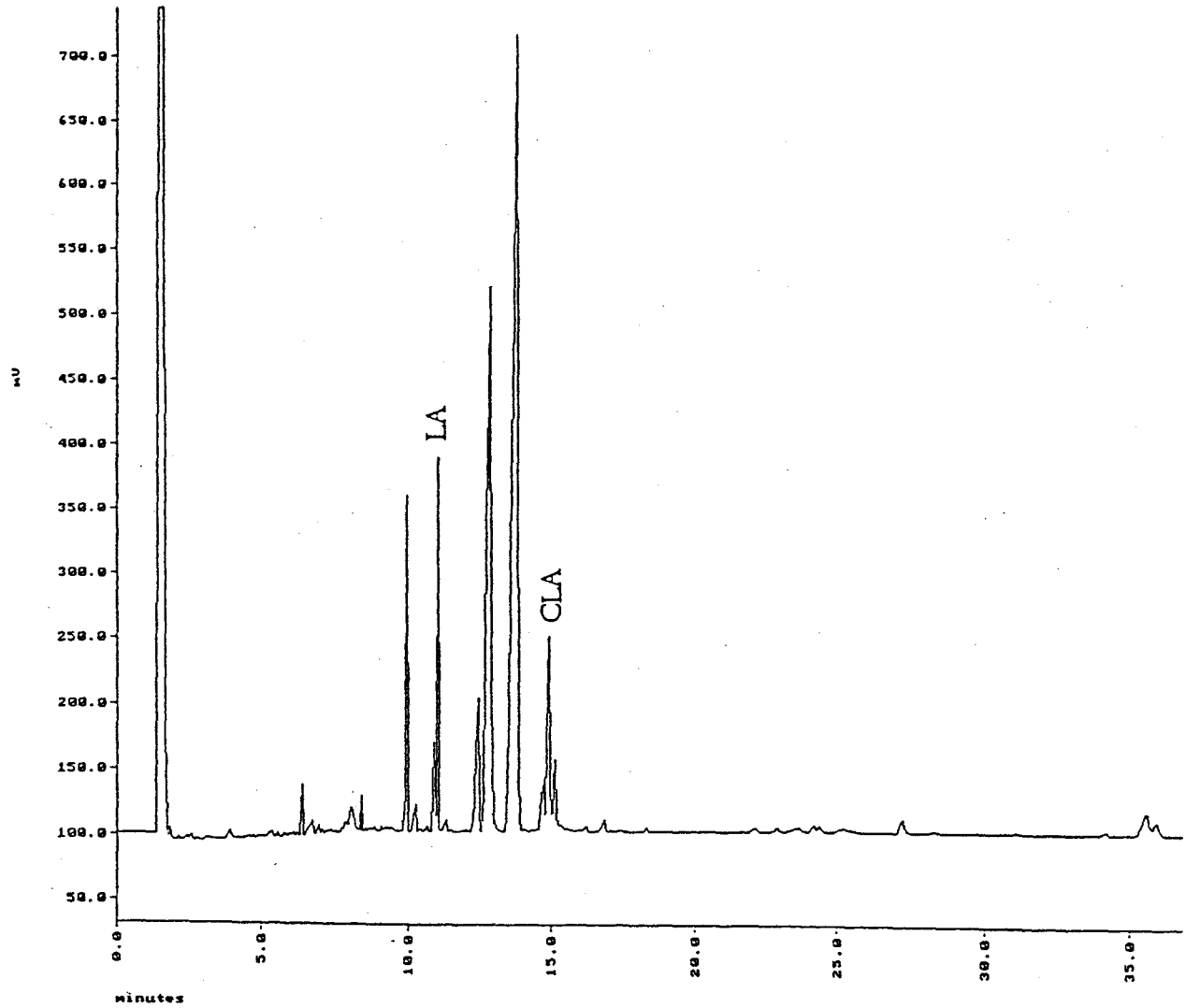

Figure 6. GC chromatogram from *L. acidophilus* L11 grown without LA in MRS media.

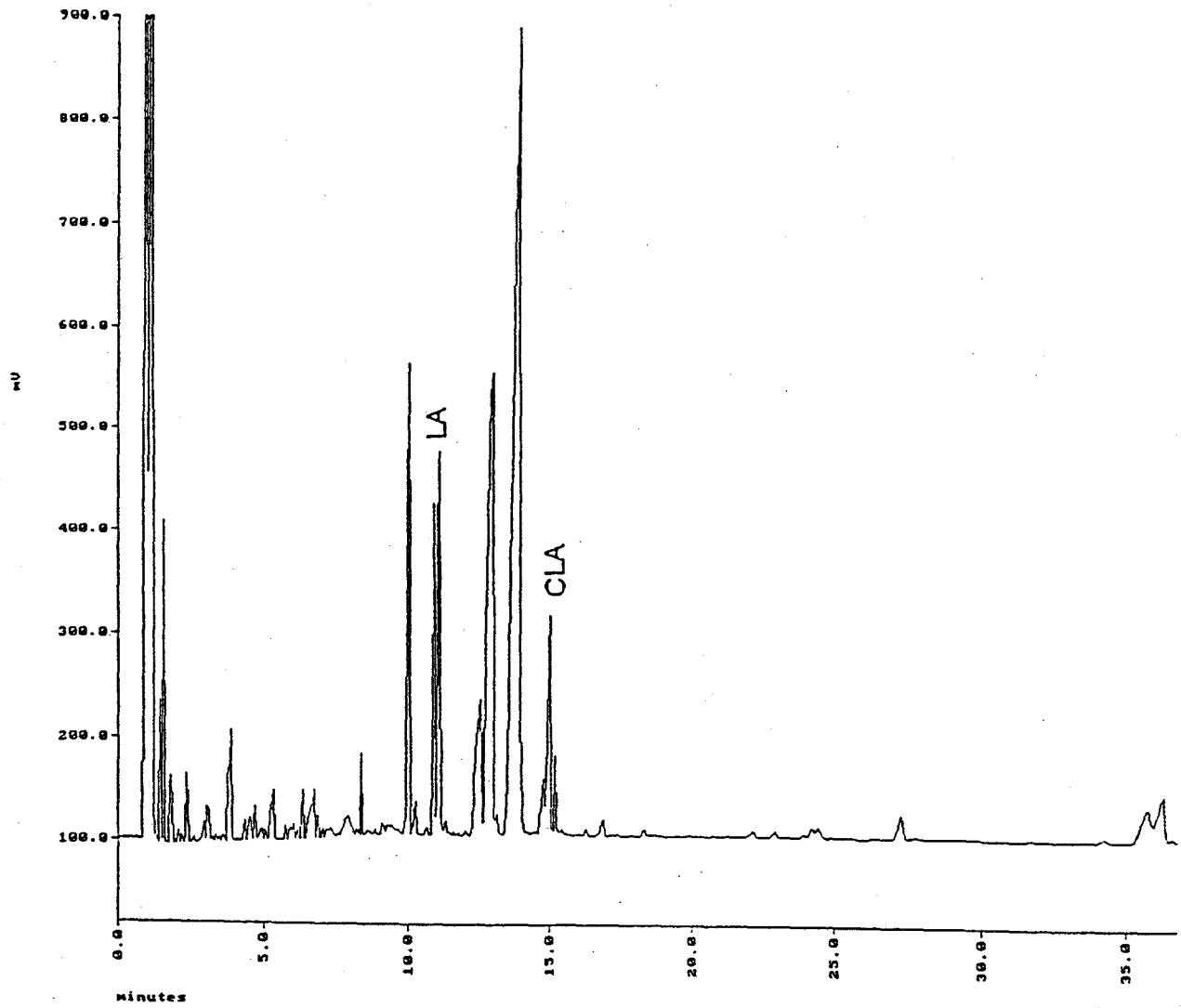

Figure 7. GC chromatogram from *L. acidophilus* L11 grown with 5 mg/ml LA in MRS.

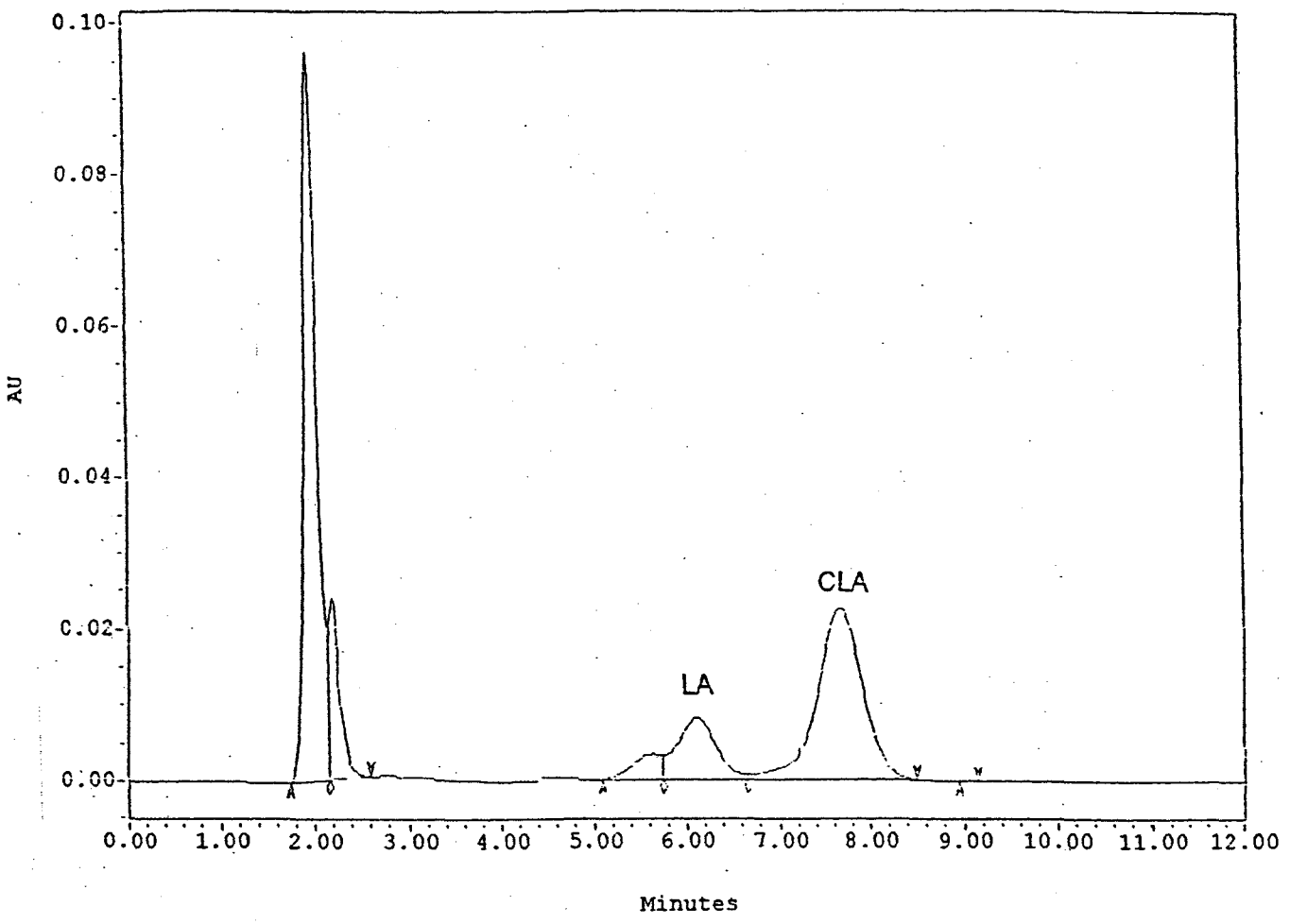

Figure 8. HPLC chromatogram of standards of LA and CLA.

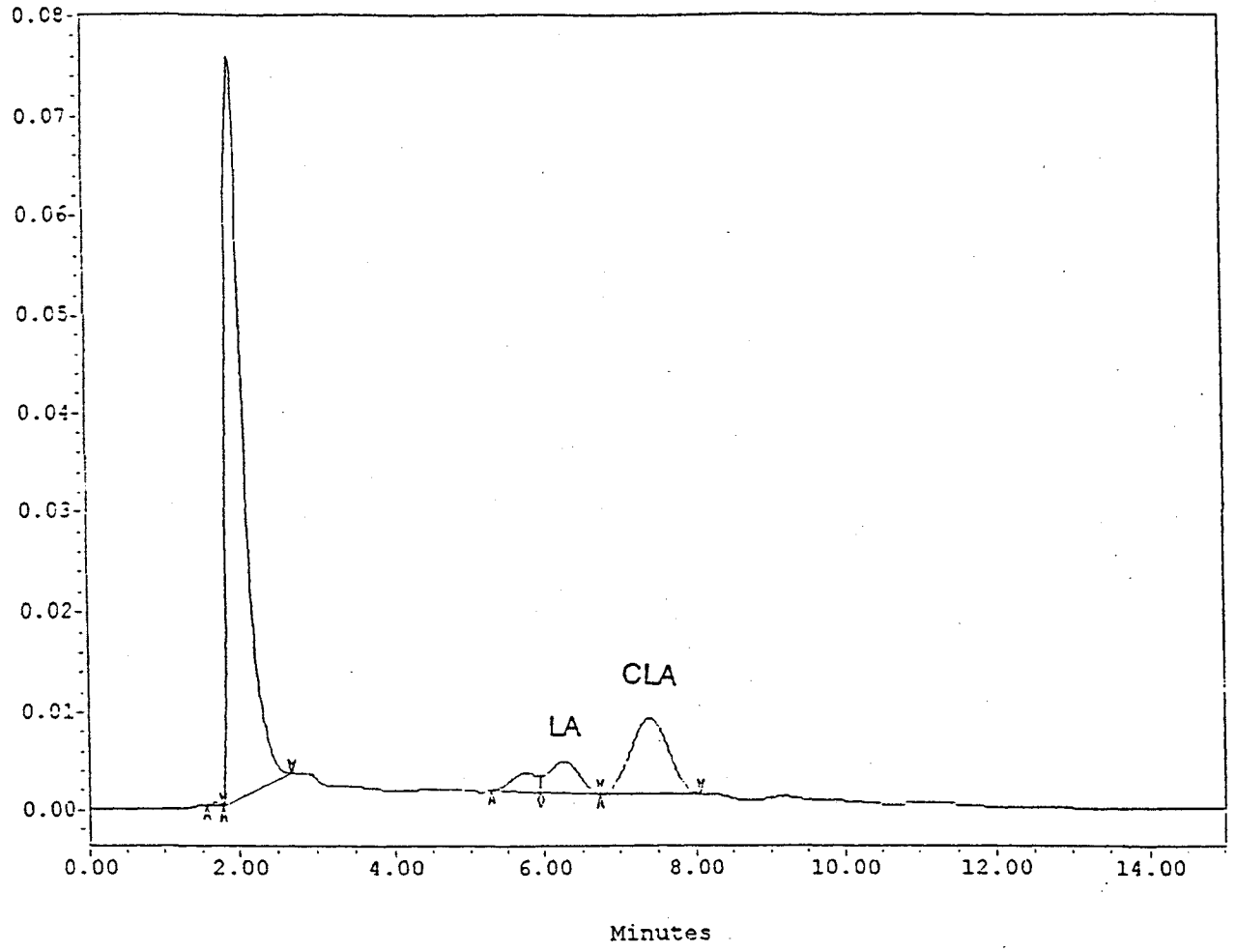

Figure 9. HPLC chromatogram of cells of *L. acidophilus* L11 grown without LA in MRS (12 ml).

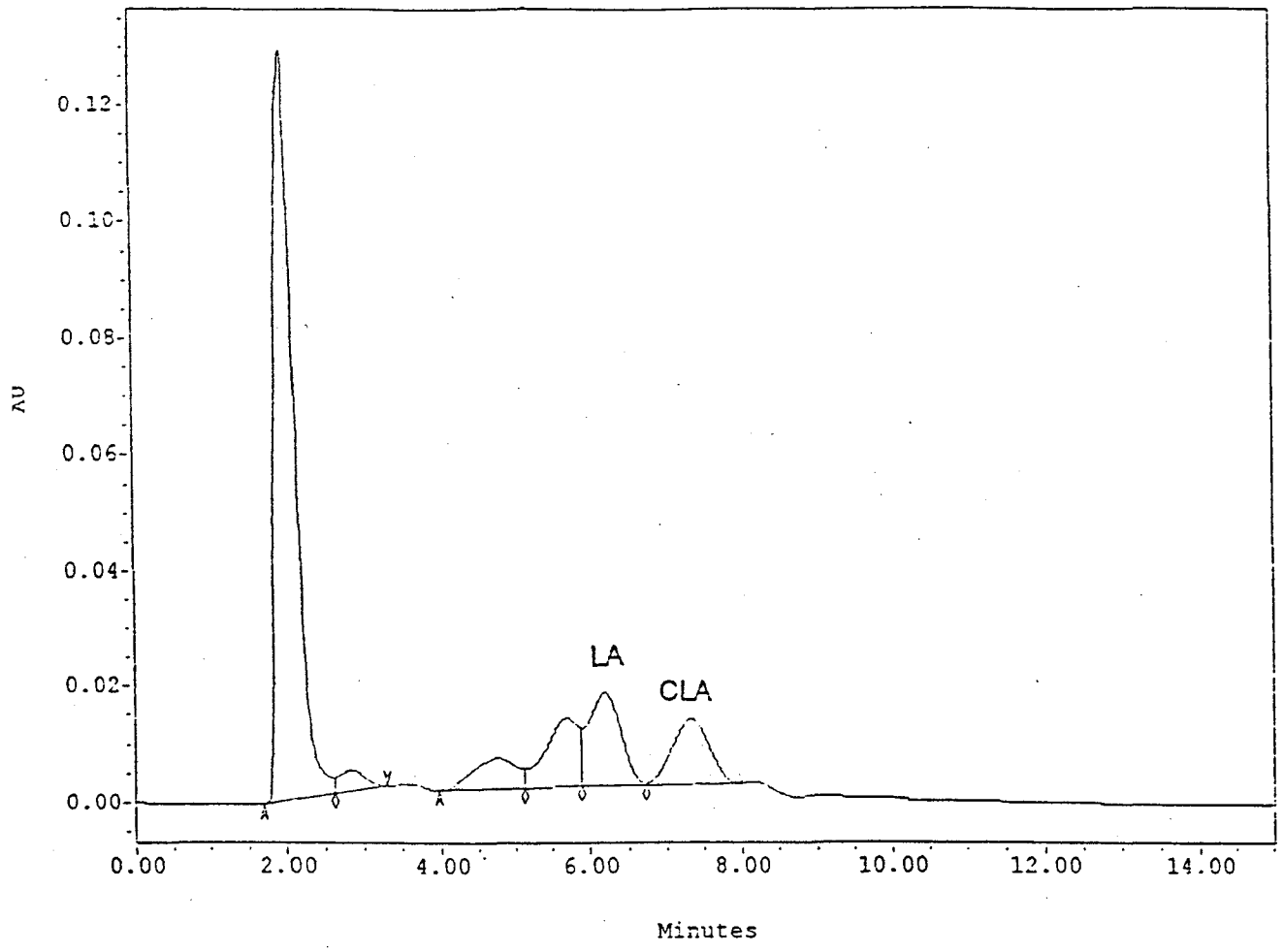

Figure 10. HPLC chromatogram of cells of *L. acidophilus* L11 grown with 5mg LA /ml MRS (12 ml).

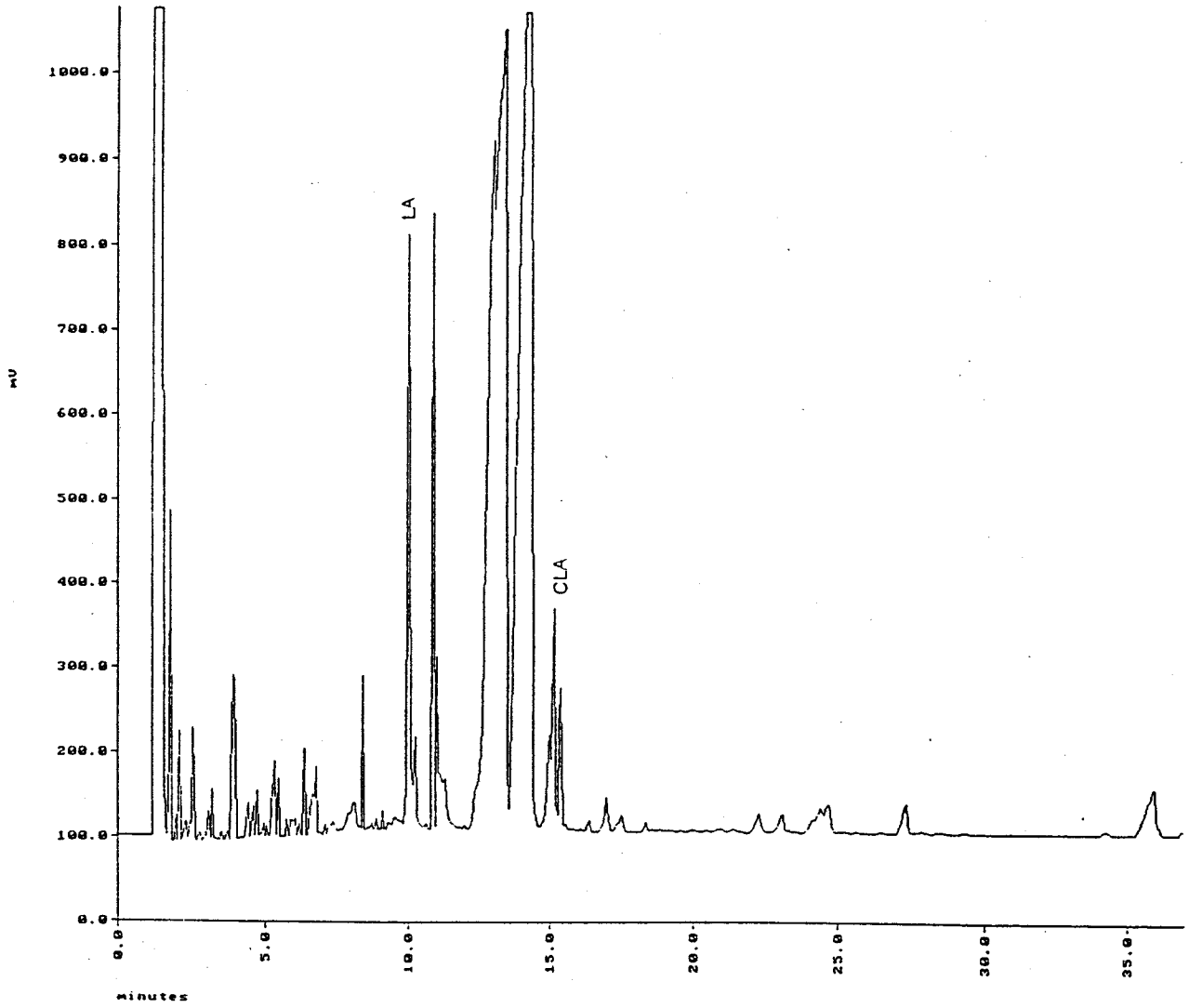

Figure 11. GC chromatogram from *L. reuteri* grown without LA in MRS.

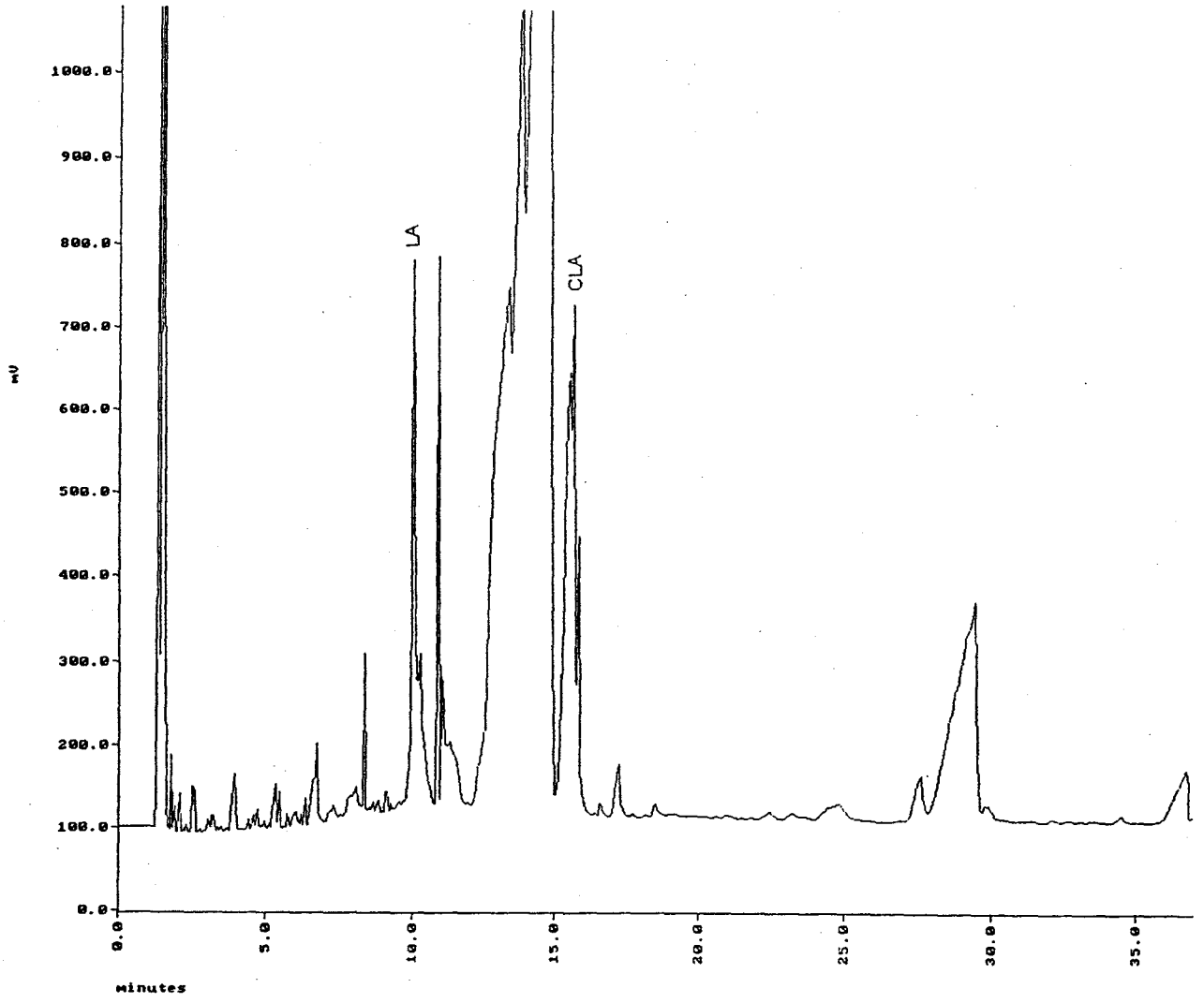

Figure 12. GC chromatogram from *L. reuteri* grown with 7mg LA/ml MRS.

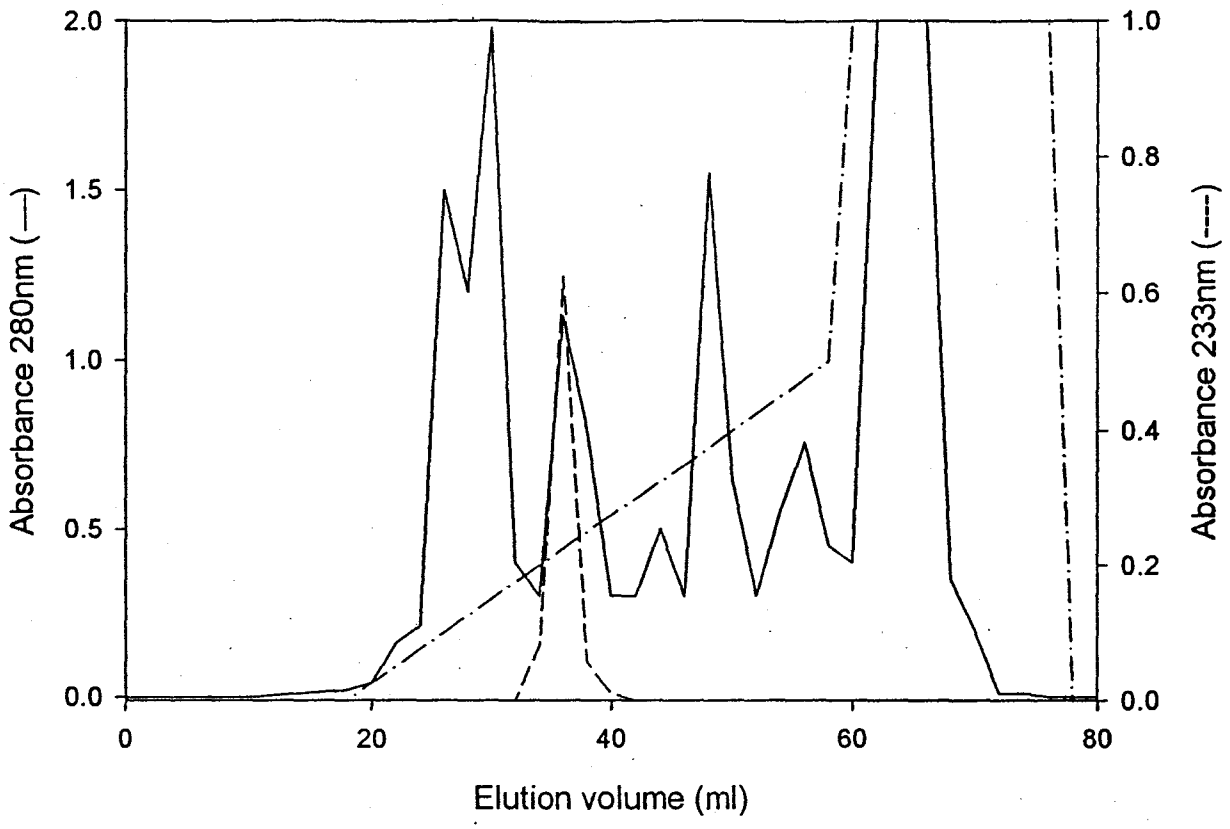

Figure 13. First ion-exchange elution profile of crude extract of *L. acidophilus* L11 in MRS media after ammonium sulfate precipitate.

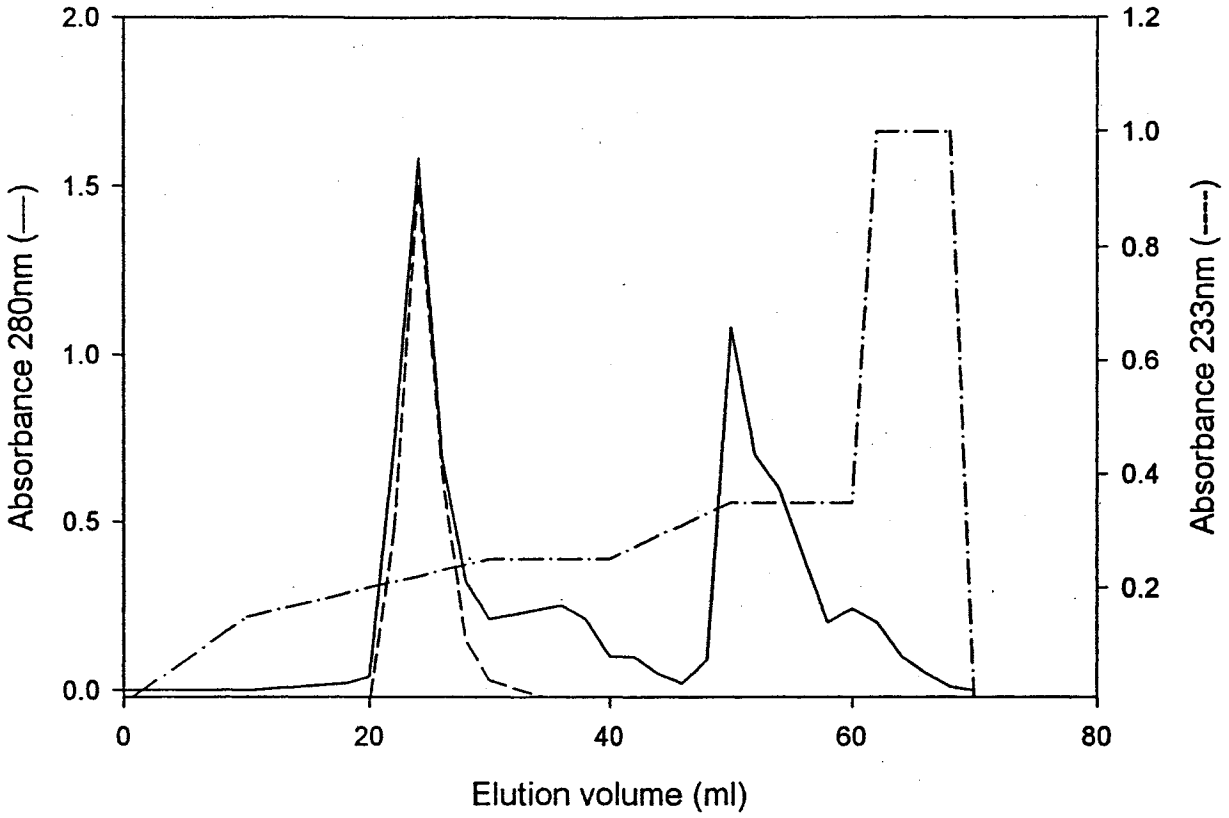

Figure 14. Second ion-exchange elution profile of CLA-forming enzyme fraction after first ion-exchange chromatography.

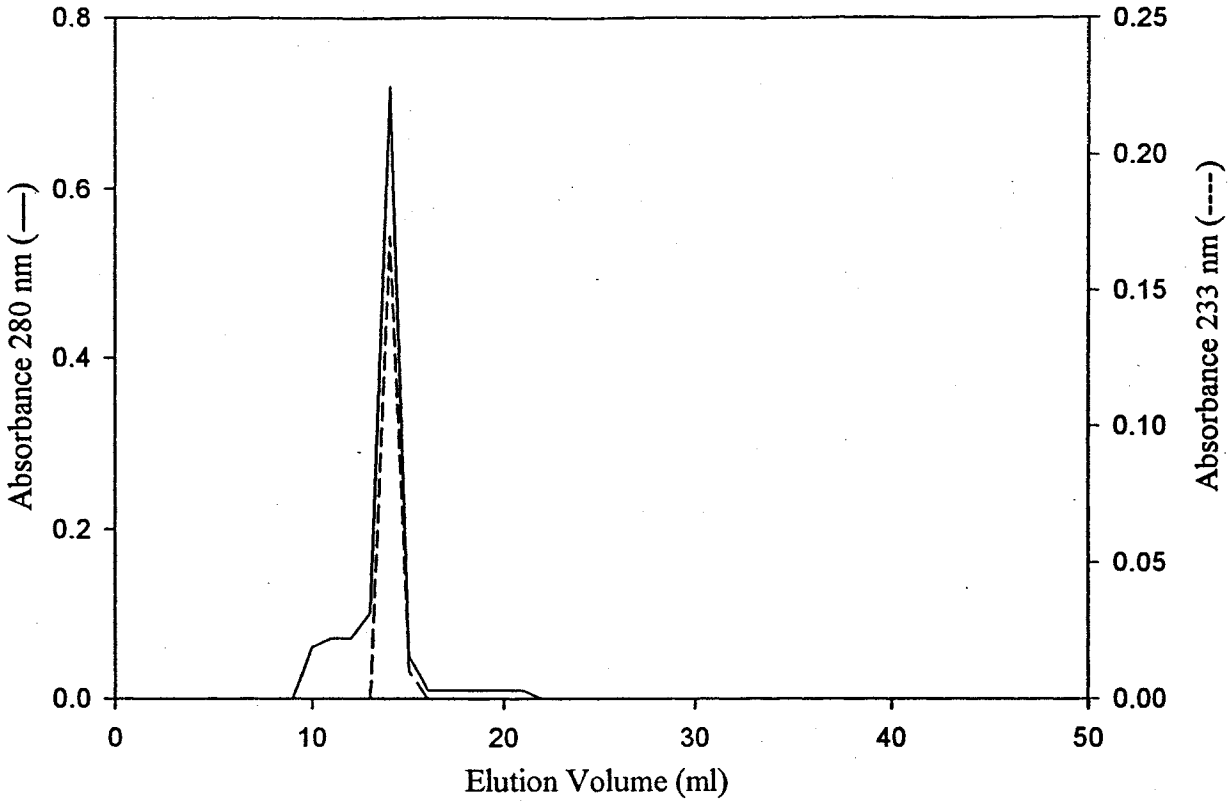

Figure 15. Gel filtration elution profile of the active CLA enzyme fraction after second ion-exchange chromatogrphy.

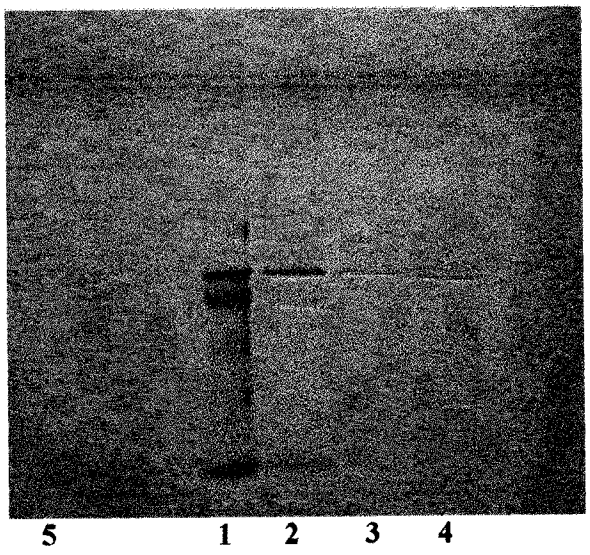

Figure 16. SDS-PAGE patterns of crude extract and CLA-forming enzyme fractions obtained from different purification steps.

Lane 1: Molecular weight markers of seven protein standards (from15,000 to 150,000)

Lane 2: Crude extract

Lane 3: After first ion-exchange chromatography

Lane 4: After second ion-exchange chromatography

Lane 5: After gel filtration chromatography

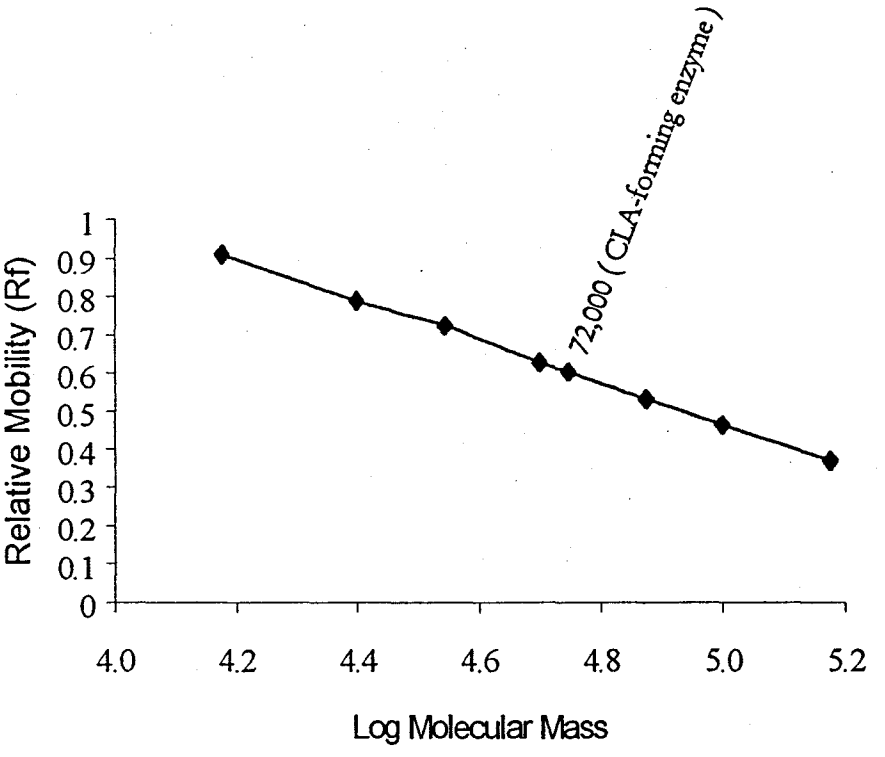

Figure 17. A molecular mass calibration curve for the CLA enzyme on SDS-PAGE.

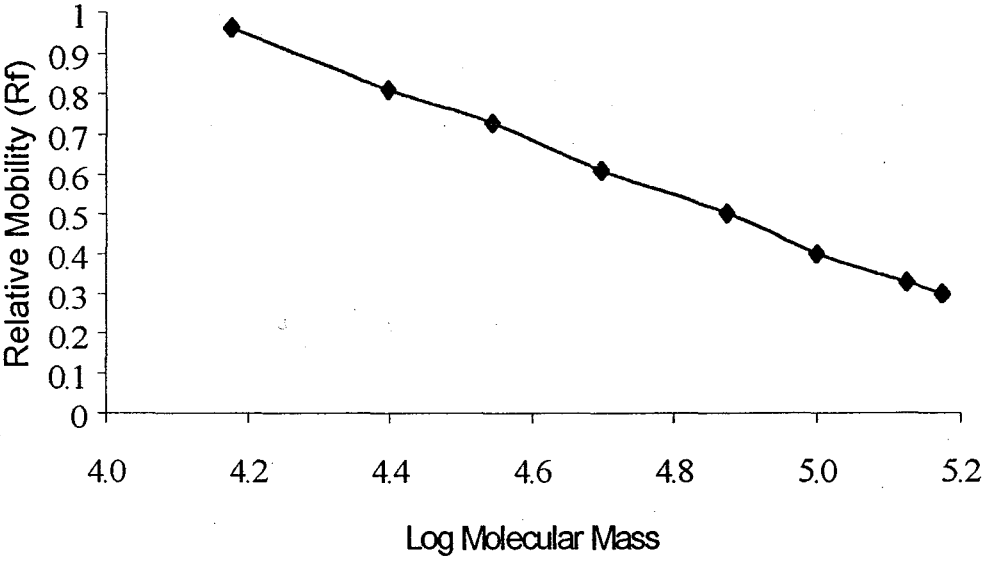

Figure 18. A molecular mass calibration curve for the CLA enzyme on Native - PAGE

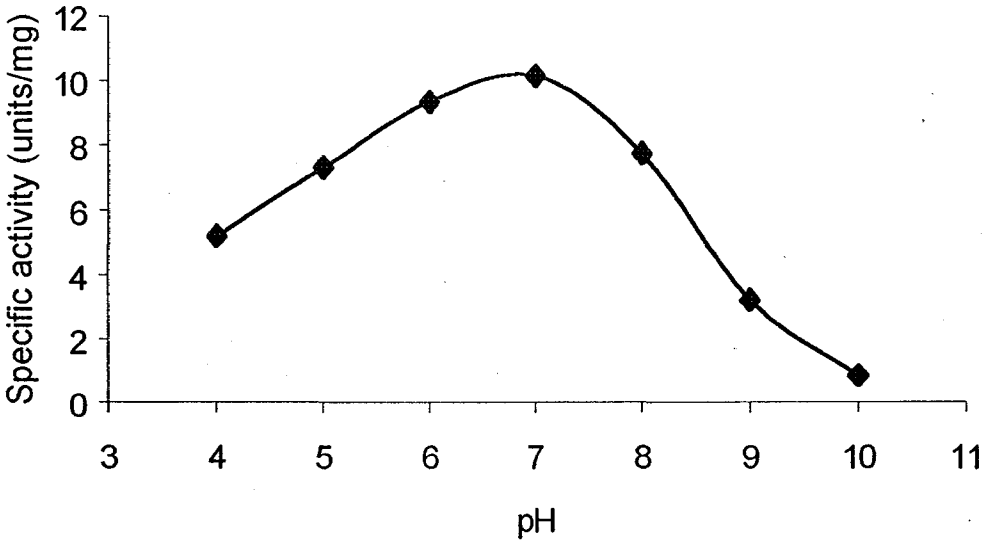

Figure19. pH optimum of a CLA-forming enzyme from *L. acidophilus* L11 at 37°C.

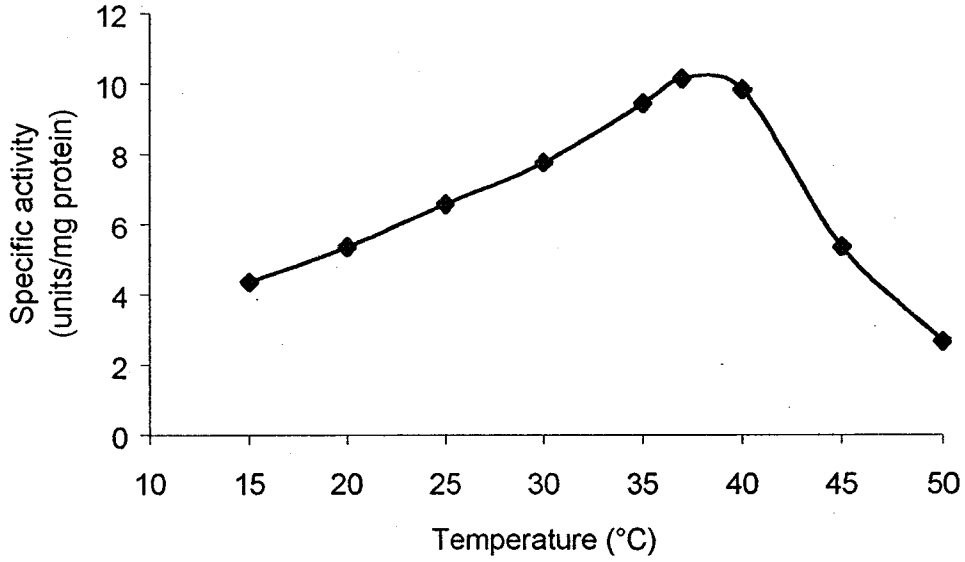

Figure 20. Temperature optimum of a CLA-forming enzyme from *L. acidophilus* L11 at pH7.0.

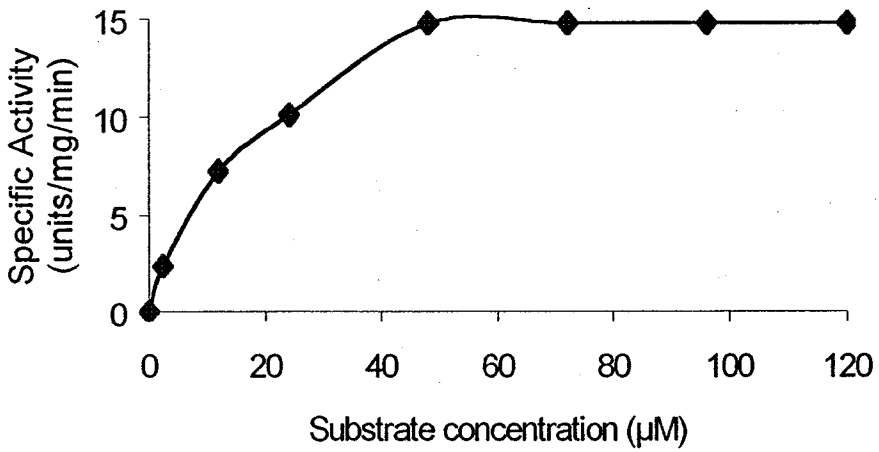

Figure 21. Effect of substrate concentration on the reaction rate by the CLA-forming enzyme.

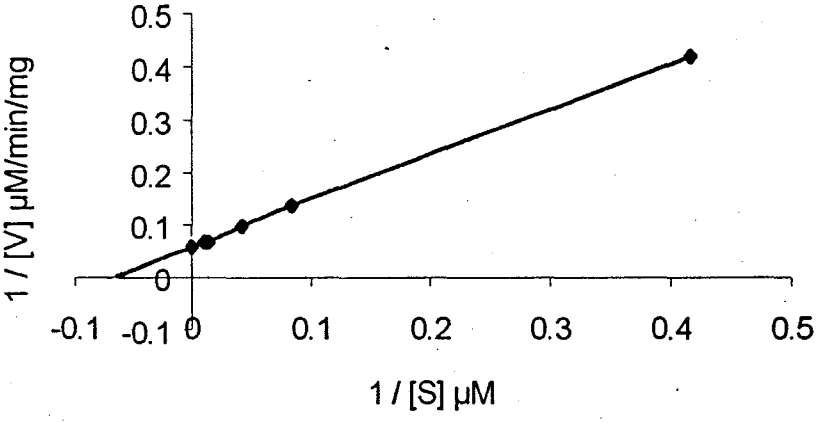

Figure 22. A Lineweaver-Burke plot of the CLA-forming enzyme with LA as substrate.

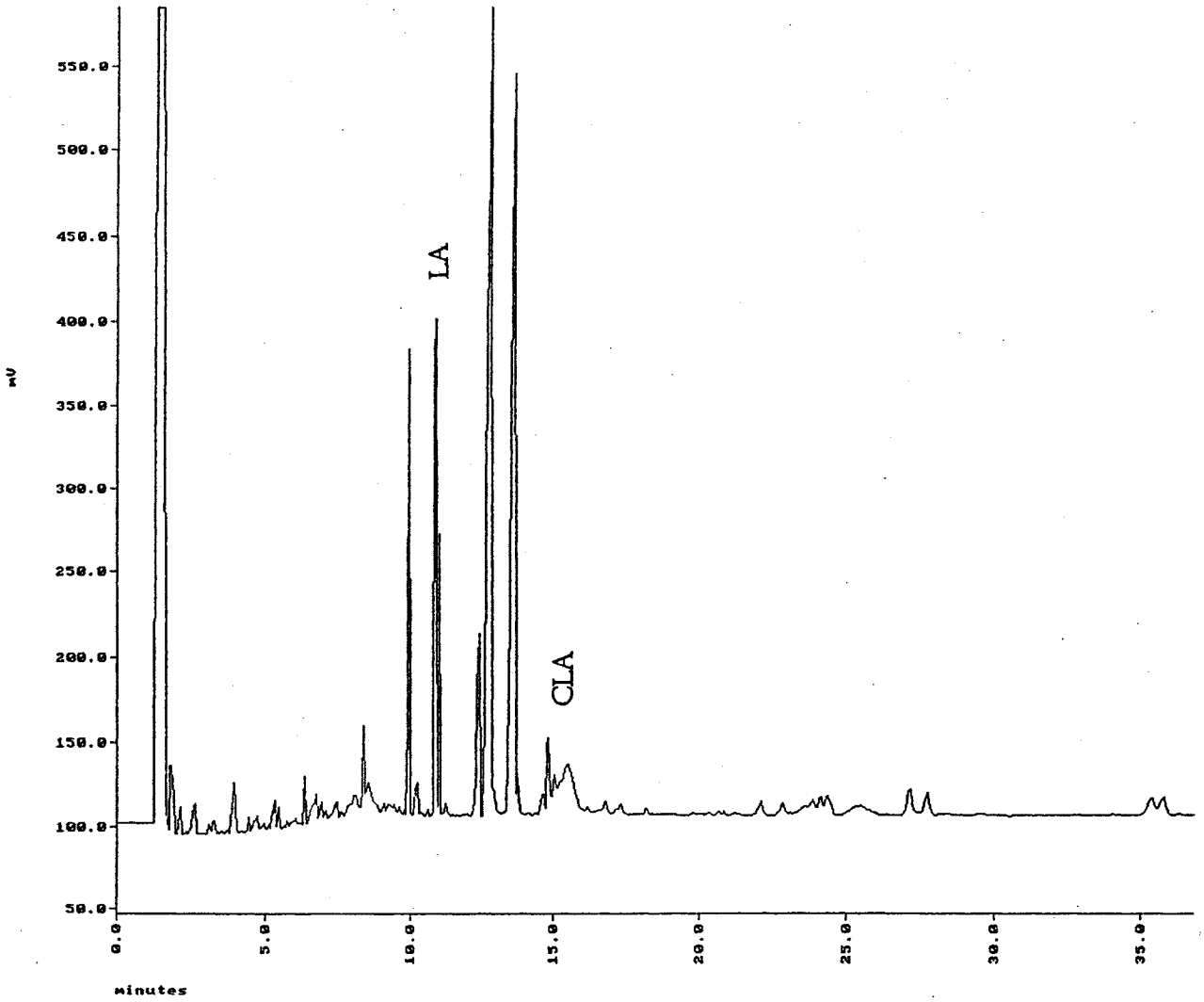

Figure 23. A GC chromatogram showing CLA formation by the crude extract after ammonium sulfate precipitation.

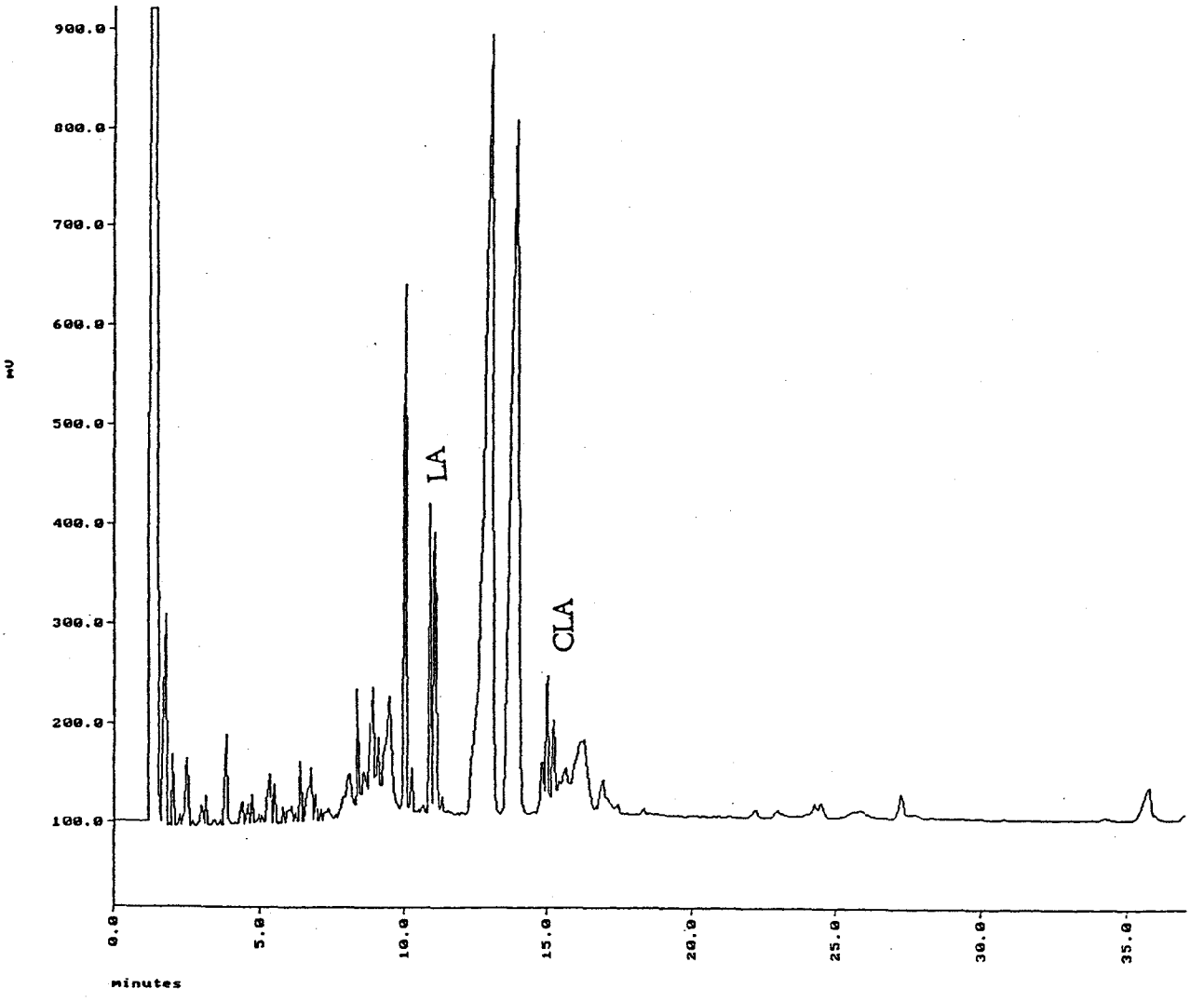

Figure 24. A GC chromatogram showing CLA formation by the purified enzyme after gel filtration.

5. GENERAL CONCLUSIONS

The potential commercial value of CLA as a pharmaceutical and nutraceutical preparation and veterinary composition is significant and its production by microbial enzymes rather than by chemical synthesis should have advantage from the consumer point of view. To investigate the possibility of this type of enzyme that can produce CLA for the industry application, detailed researches were carried out to study: (1) the growth and enzyme production, (2) purification, (3) characterization of a CLA-forming enzyme and further to confirm the function of this enzyme by GC analyses.

Six strains of *Lactobacillus* species were tested for their abilities to produce CLA. The maximal growth and enzyme production was performed after 24 h at 37°C in MRS broth with or without different concentration of linoleic acid. Linoleic acid induced the enzyme system for CLA production. However, high concentration of linoleic acid in media inhibited the cell growth.

The CLA-forming enzyme produced from *Lactobacillus acidophilus* L11 was studied in details. The enzyme was purified with ion-exchange and gel filtration chromatography using FPLC system. The molecular mass of this enzyme was estimated to be 72 kDa by SDS-PAGE and this enzyme has two identical subunits with total mass of 144 kDa on native-PAGE as well as gel filtration.

The pH and temperature optima were 7.0 and 37°C, respectively. The Km and Vmax for linoleic acid were 14.9 μM and 17.1 μM/mg/min, respectively.

Although some studies on the production of CLA by growing culture in the presence of LA, none of the CLA-forming enzyme was purified and characterized so

far. This study should be continued for the molecular characterization to confirm the role of linoleic acid isomerase and to serve as an enzyme for producing CLA.

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