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<Review>

Conjugated Linoleic Acid (CLA) Production in the Rumen - Roles of *Butyrivibrio fibrisolvens* A38

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Abstract

Conjugated linoleic acid (CLA) is currently under intensive investigation due to its health benefits. A great deal of interest has been paid to the possible health-promoting roles of CLA, but there are not many studies available on the mechanism of CLA production by ruminal microorganisms. CLA is produced as an intermediate of the characteristic biohydrogenation process of linoleic acid(LA) in the rumen and its production has direct relationship to numerous environmental factors including particle association, substrate concentration, forage-to-grain ratio, pH, ionopore, bacterial cell density, etc. Some of these factors were known to affect hydrogenating activities of *Butyrivibrio fibrisolvens* A38 which is an active rumen bacterium in CLA production. Dairy cow is a main source of CLA, and its level could be increased by dietary manipulation changing the physiological environment of rumen bacteria such as *B. fibrisolvens* A38. Therefore, the effects of various factors on ruminal biohydrogenation should be carefully considered to optimize not only CLA production, but also other fatty acid metabolism, both of which are directly affecting nutritional quality and functionality of dairy products. In this review, the relationship between various environmental factors and ruminal CLA production is discussed focusing on the CLA production of *B. fibrisolvens* A38.

Key words : biohydrogenation, *Butyrivibrio fibrisolvens*, conjugated linoleic acid, fatty acid, rumen

Introduction

There is a great deal of evidence that some natural fatty acids are effective in health promotion. Among these fatty acids, conjugated linoleic acid (CLA) is currently under intensive investigation due to its health-promoting potential. The antitumor activity of CLA is of special interest since 1987 when Ha et al. (1987) showed its inhibitory effects against multistage carcinogenesis at relatively low dietary levels. Many studies using *in vivo* and *in vitro* models have demonstrated that CLA suppresses the development of multistage carcinogenesis in a variety of tissues (Ip and Scimeca, 1997). The research to date on CLA has provided a vast amount of information on chemopreventive roles of CLA (Belury, 1995). Moreover, CLA showed a wide scope of preventive roles against other degenerative diseases (Fig. 1). CLA also reduced catabolic effects of immune stimulation (Cook et al., 1993), prevented atherosclerosis (Lee et al., 1995) and improved the protein to fat ratio(Dougan et al., 1997) in a variety of animal models. Some isomers of CLA have also been linked to the enhancement of growth of lean body mass (Park et al., 1999).

CLA is a collective term referring to a mixture of positional and geometric isomers of linoleic acid(*cis*-9, *cis*-12-octadecadienoic acid; LA) each with a conjugated double bond arrangement at 7 and 9, 8 and 10, 9 and 11, 10 and 12, 11 and 13 or 12 and 14. (Table 1) (Sehat et al.,

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Table 1. Positional and geometric isomers of conjugated linoleic acid (CLA) from linoleic acid(*cis*-9, *cis*-12-octadecadienoic acid)

	Isomers					
Positions	7-, 9-	8-, 10-	9-, 11-	10-, 12-	11-, 13-	12-, 14-
Types	*trans, trans* *trans, cis*	*trans, trans* *trans, cis*	*trans, trans* *trans, cis* *cis, trans* *cis, cis*	*trans, trans* *trans, cis* *cis, trans* *cis, cis*	*trans, trans* *trans, cis* *cis, cis*	*trans, trans* *trans, cis*

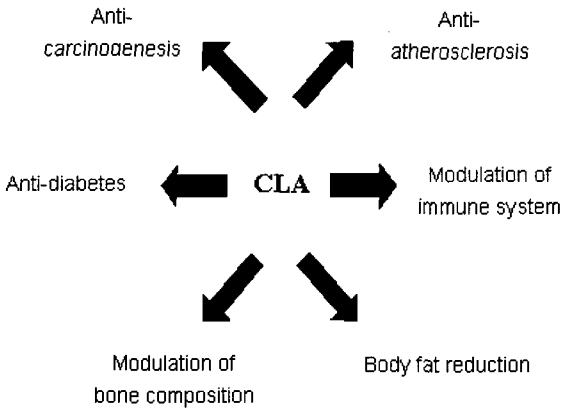

Fig. 1. Various physiological roles of conjugated linoleic acid.

1998). Among individual isomers, *cis*-9, *trans*-11 isomer is the major isomer commonly found in nature, and is mainly produced by rumen bacteria such as *Butyrivibrio fibrisolvens* A38 as a by-product in the biohydrogenation process (Kepler and Tove, 1967) (Fig. 2).

Ruminants rely on a unique host-microbial symbiosis in the rumen in which microorganisms metabolize feed material into utilizable energy sources. Among feed components, polyunsaturated fatty acids such as LA(C18:2) and linolenic acids (LNA, C18:3) are abundant, which are toxic to the rumen microorganisms. Growth of gram positive bacteria was significantly inhibited by long chain fatty acids, and biohydrogenation was thought to be an efficient detoxification mechanism (Galbraith et al., 1971; Galbraith and Miler, 1973; Henderson, 1973).

Discovery of Ruminal Biohydrogenation

Booth et al. (1935) firstly indicated the presence of conjugated unsaturated fatty acids in butter fat, and found that summer butter fat had higher absorbance at 230 nm where the conjugated double bond showed its maximum absorbance. The saturation of unsaturated fatty acids occurring in the gut of

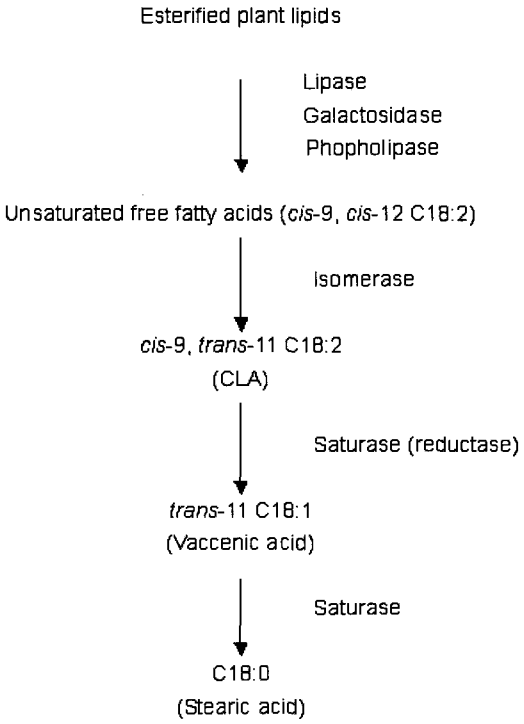

Fig. 2. Key steps in the conversion of esterified plant lipids to saturated fatty acids by lipolysis and biohydrogenation in rumen.

some animals has long been recognized since Reiser (1951), who first showed the function of rumen in fatty acid unsaturation, observed that LNA present in linseed oil was converted *in vitro* into LA by the action of sheep-rumen contents *in vitro*. Reiser also suggested that the high content of stearic acid in the depot fat of ruminants might be due to the hydrogenation of dietary unsaturated fatty acids in the rumen. Later, diets rich in unsaturated acids was found to modify the depot fats of most animals, but ruminant depot fats were relatively unaffected indicating that unsaturated fatty acids are mostly hydrogenated in the rumen (Shorland and Weenink, 1955).

Major bovine dietary sources, pasture grasses and grains,

are rich source of unsaturated fatty acids such as LA and LNA, most of which are eventually hydrogenated to stearic acid in the rumen (Garton, 1960). Thus, milk produced from ruminants is higher in stearic acid content than in LA or LNA, since the fatty acids modified by rumen bacteria are absorbed and deposited into the body fat. This finding was further supported by the studies of Tove and Mochrie (1963). They found a dramatic increase in LA levels in the milk of cows which were intravenously infused with cottonseed oil emulsion, whereas LA levels in cow's milk fed cottonseed oil was slightly increased. This indicated the presence of fatty acid conversion process in the rumen.

Kepler and Tove (1967) firstly showed that conjugated fatty acids are occurred in the biohydrogenation process and that they are not normal constituents of ruminant feed. Conjugated fatty acids were found to arise as a transit by-product in the rumen bacterial biohydrogenation of LA or LNA. They showed that CLA is the first intermediate during biohydrogenation, but its distinct biological effects were recognized two decades later (Ha et al., 1987). Ingested feed materials of cows are subjected to rumen microbial conversion, and long chain unsaturated fatty acids are hydrogenated by some rumen bacteria producing a mixture of isomers which could be accumulated at certain conditions (Kim et al., 2000).

Factors Affecting CLA Concentration in Dairy Products

CLA is found in many foods, but principal dietary sources are animal products from ruminants as it is a by-product in biohydrogenation process (Kepler and Tove, 1967). Isomer profile of CLA in dairy foods varies, but the *cis*-9, *trans*-11-isomer was known to be the predominant form (as much as 90% of the total CLA). The presence of the CLA in dairy products is mainly due to isomerization of LA by rumen microorganisms and endogenous production in the various tissues from *trans*-monoenoic acids by desaturase reaction. Ruminal CLA production could be influenced by various dietary factors such as the source and the level of lipid substrates(Dhiman et al., 2000; Kelly et al., 1998; Kim et al., 2003), forage-to-grain ratio (Dewhurst et al., 2001) and the presence of feed particles (Harfoot et al., 1973, Kim et al., 2003). Based on these findings, it has been attempted to increase the CLA concentration of milk through dietary modification. The fatty acid profiles of milk have also been shown to vary with by lactating season and diet compositions (Dhiman et al., 1996; Galbraith et al., 1971, Kim et al., 2003). Indeed, the population of main CLA producing rumen bacterium such as *B. fibrisolvens* decreased when cows were fed high-grain diets in the winter (Latham et al., 1972). On the other hand, Stanton et al. (1997) reported the stage of lactation did not affect levels of milk fat. This discrepancy may be due to the regional variation in grass quality in the late lactation periods. Many quantitative data also have shown that the content of CLA in various dairy products was influenced by production condition, especially fermented dairy products (Chin et al., 1992; Kim and Liu, 2002). It has been known that some lactic acid bacteria have CLA producing ability and the production can be enhanced by buffering growth medium (Kim and Liu, 2002). Cheese processing parameters such as aeration (Ha et al., 1989), temperature (Shantha et al., 1992), milling pH, additives and ripening (Lin et al., 1995) have been found to affect CLA content.

CLA Production of *B. fibrisolvens* A38

B. fibrisolvens is a commonly found rumen bacterium which is capable of hydrogenating LA to monoenoic acids (C18:1) at a strictly anaerobic condition. When cows were fed low-roughage diets, these bacteria decreased in numbers followed by ruminal biohydrogenation whereas the number of lactic and propionic acid-producing bacteria increased in the rumen (Latham et al., 1972). Biohydrogenation is preceded by lipolysis of dietary lipids in the overall metabolism of dietary lipids in the rumen, and a variety of fatty acid metabolites become available to ruminants for the incorporation into tissue and milk of ruminant. Thus, supplemented LA has been shown to elevate CLA content in the cow's milk (Kelly et al., 1998). Biohydrogenation of LA by *B. fibrisolvens* A38 is known to be a two step process, isomerization to *cis-trans* (*trans-cis*) octadecadienoic acid (C18:2) and hydrogenation of the isomers to *trans*(*cis*)-monoenoic acids (C18:1) and eventually to stearic acid (C18:0)(Fig. 2). The isomerization process is also thought to depend on specific enzymes generated by certain rumen bacteria, but little attempt was made to optimize conditions for CLA accumulation by the manipulation of a series of enzymatic processes.

CLA is produced by a wide range of bacteria but, to our knowledge, LA isomerase, the enzyme that converts LA to

CLA, has not been successfully isolated or cloned yet. Earlier studies by Kepler and Tove (1967) indicated the LA isomerase of *B. fibrisolvens* was a membrane associated enzyme, but purification schemes were not successful because the fractions having the highest isomerase activity were contaminated by carbohydrates. *B. fibrisolvens* is a strictly anaerobic bacterium, but we found that the activity of semi-purified isomerase was not inhibited in aerobic condition (Kim et al., 2000). So far, the pure isomerase was neither identified nor cloned. Moreover, the understanding of the environmental factors affecting the isomerization of unsaturated fatty acids by *B. fibrisolvens* A38 is still unclear.

In our previous study, the production mechanism was thoroughly investigated. Exponentially growing cultures of *B. fibrisolvens* A38 produced less CLA than stationary phase cells, and the glycolytic inhibitor, iodoacetate, increased the CLA production of cells when incubated anaerobically. Stationary phase cells that were incubated in an aerobic condition produced more CLA than those incubated anaerobically(Kim et al., 2000). These findings suggested that when the microbial biohydrogenation is active, CLA accumulation is relatively poor. Moreover, CLA was produced very rapidly, but washed cells that were pre-incubated with CLA did not produce additional CLA from LA. Besides, CLA accumulation was a linear function of the cell density. These results indicated that LA isomerase could not continuously recycle to produce more CLA. Cultures that were gradually adapted to LA produced less CLA, and more of the LA was converted to *trans*-vaccenic acid (*trans*-C18:1). These results supported the idea that LA isomerase and △-9 saturase reactions of fatty acid biohydrogenation were obligately linked. Since growing cultures of *B. fibrisolvens* A38 did not produce significant amounts of CLA until the LA concentration was high, biohydrogenation was arrested, and the cell density had declined, it was speculated that the flow of CLA from the rumen was due to LA-dependent bacterial lysis(Kim et al., 2000).

In order to attain high CLA accumulation during the biohydrogenation process, multiple factors should be considered simultaneously because isomerization process is easily overwhelmed by active reduction steps in an aerobic condition. Therefore, both inhibitory and stimulating factors affecting biohydrogenation were to be considered to enhance CLA accumulation of *B. fibrisolvens* A38 and to interrupt following reduction steps simultaneously(Kim, 2003). Since some inhibitory conditions for CLA reduction by *B. fibrisolvens* A38 brought about more CLA accumulation, it was suggested that partial inhibition of biohydrogenation could be a strategy to increase the CLA production. The isomerization step in biohydrogenation, which converts LA to CLA, was not limited in the inhibitory conditions. The presence of LA impeded not only the cell growth, but also the complete hydrogenation of LA and this effect was more evident at high concentrations, and the conversion of CLA to hydrogenated products(*trans*-C18:1 and C18:0) were significantly reduced.

Even more CLA was accumulated during the aerobic incubation when high levels of LA were added with a glycolytic inhibitor, iodoacetate(IAA) to the cells, and this effect was more significant with cells that had been pre-adapted to LA($p<0.05$, Fig. 3). In this study, monensin had more inhibitory effect compared to IAA to the cell growth, but less effective on CLA accumulation. Both IAA and monensin were thought to be inhibitory only to biohydrogenation. On the other hand, the inclusion of rumen fluids appeared to activate whole biohydrogenation even in the aerobic condition resulting in lower CLA level than the control group ($p<0.05$) (Kim, 2003). Since the isomerization and reduction steps are coupled reactions in biohydrogenation of most hydrogenating bacteria including *B. fibrisolvens* A38 cells, any positive or negative factors for the reduction steps could be key determinants in CLA accumulation.

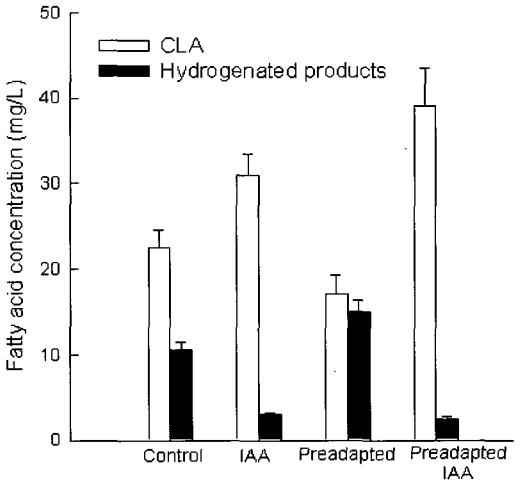

Fig. 3 Effect of iodoacetate (IAA) on conjugated linoleic acid(CLA) production by *Butyrivibrio fibrisolvens* A38 in aerobic and anaerobic conditions. Cells were grown anaerobically for 24 hours and incubated aerobically or anaerobically with 0.1 g/L linoleic acid for 10 min (modified from ref. 20).

CLA Production of Mixed Rumen Bacteria

Based on the studies with single bacterium, *B. fibrisolvens* A38, further studies were performed to establish thestrategy to increase the CLA content in milk and other dairy products by bovine dietary manipulation (Kim et al., 2003). Supplementation of sunflower oil enhanced CLA concentration up to five times, but this increase was not prolonged more than eight weeks (Fig. 4). Dairy cattle are often fed with large amounts of grain to increase ruminal fermentation rate and energy availability. The high-concentrate bovine diet provides more fermentable energy sources to the ruminal microorganisms and has shown to cause a substantial decrease in cellulose digestion, ruminal pH, as well as changes in volatile fatty acids patterns and sometimes causes acidosis (Ben Salem et al., 1993). Ruminal environment, which is significantly influenced by bovine diets, may subsequently cause significant changes not only in the rumen microbial ecosystem, but also in the fatty acid profiles of the rumen content (Latham et al., 1972).

It has been suggested that feed particles in the rumen provide an important site for the adsorption of lipids in the rumen of sheep. The rumen bacteria associated with feed particles are largely responsible for fatty acid hydrogenation (Kim, 2003). Harfoot et al. (1973) found that particle-attached bacteria were responsible for more than 70% of the total ruminal biohydrogenation. The effect of feed particle was partially elucidated in our previous study in which the effects of LA on CLA production by the rumen bacteria obtained from cows on different diets were investigated (Kim et al., 2003). Rumen bacteria from grain-fed cows were more active in biohydrogenation than those from hay-fed cows. Particle-associated bacteria produced more hydrogenated products leaving less CLA than the free-floating bacteria ($p<0.05$). CLA production by free floating bacteria did not always correlate to given amount of LA, and longer incubations generally decreased CLA concentration and increased *cis*-9, *trans*-11/*trans*-10, *cis*-12 ratio, especially at higher LA concentrations. The pre-incubated cells with LA produced more CLA than the unexposed ones and the increase was more evident with *cis*-9, *trans*-11 CLA ($p<0.05$). These investigations provide insight into how cattle diet and LA feedings affect ruminal CLA production.

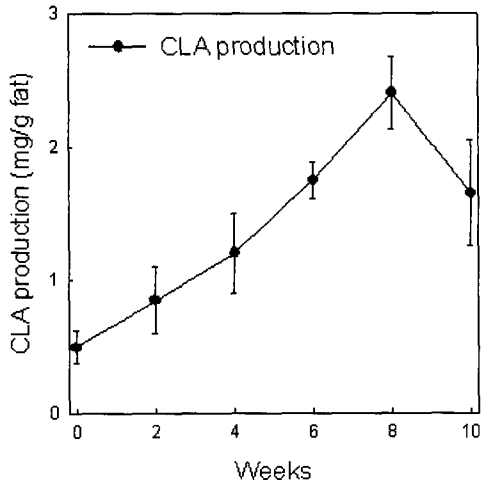

Fig. 4. Increase in conjugated linoleic acid (CLA) concentration by supplementing sunflower oil. CLA level was measured for 12 weeks (n=5) (adopted from ref. 23).

Further Studies

In order to maximize the bacterial CLA production, multidisciplinary investigation considering a variety of extrinsic factors affecting ruminal biohydrogenation should be performed. Further work is also needed to define more clearly the contribution of rumen bacteria such as *B. fibrisolvens*. Moreover, systematic feeding trials should be performed in a various dietary conditions to enhance ruminal CLA production and to increase CLA concentration in dairy products.

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