ENZYME CATALYZED SYNTHESIS OF STRUCTURED PHOSPHOLIPIDS WITH CONJUGATED LINOLEIC ACID AND PLANT STEROLS

A Dissertation

by

MD. MONJUR HOSSEN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2005

Major Subject: Food Science and Technology

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Approved as to style and content by:

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May 2005

Major Subject: Food Science and Technology

ABSTRACT

Enzyme Catalyzed Synthesis of Structured Phospholipids with Conjugated Linoleic Acid and Plant Sterols. (May 2005)

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Structured phospholipids with functional ingredients like conjugated linoleic acid (CLA) and plant sterols to deliver their physiological effects in different food formulations were synthesized. The lipase and phospholipase A_2 catalyzed enzymatic acidolysis reaction between phospholipids (PLs) and CLA was used for fatty acid modification, while the phospholipase D catalyzed transphosphatidylation reaction between PLs and sterol was used for head group modification. Enzymatic processes were an effective way to produce structured phospholipids. Screening of four lipases and immobilized phospholipase A_2 and combination of lipase and phospholipase showed that only Lipozyme RM IM and Lipozyme TL IM were effective in incorporation of CLA into PLs. The maximum incorporation achieved by the latter enzyme was 16% with soy PLs in 72 h. The class of phospholipids had a significant effect on the rate of incorporation of CLA compare to source of PLs. A method capable of predicting the rate of incorporation of CLA into phospholipids was developed using response surface methodology. A three-level four-factor Central Composite Rotatable Design (CCRD) was used. The four factors selected were lipase dosage (E_d, wt.% of substrate), substrate

ratio (S_r,mol%), reaction time (t_i, h) and reaction temperature (T_e,oC). The enzyme load and substrate ratio had a greater effect on the rate of incorporation than did reaction time and temperature. A polynomial regression equation was developed to predict the reaction rate. The new phosphatidyl derivative, phosphatidyl-sitosterol, was found to be synthesized by the transfer reaction of phosphatidyl residue from phosphatidylcholine to β-sitosterol by phospholipase D from *Streptomyces* sp. in biphasic medium. The novel phosphatidyl –sitosterol derivative was identified by MALDI-TOF mass spectrometry. Plant sterols were modified to a more polar lipid class by synthesizing phospholipid derivatives of them. When these structured phospholipids were added to a whey protein based oil-in-water emulsion, the CLA incorporated structured phospholipids (CLA-PL) had higher heat stability and oxidative stability compared to the controls.

To God, who urged me to seek knowledge.

To my parents, who love me more than anything.

To my wife, Sheuli, and daughter, Fahima, for their love, support, and sacrifice.

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CHAPTER I

INTRODUCTION

There is an increasing need to develop functional and nutraceutical foods with special characteristics that improve, treat or prevent disease. The global conventional food sector has an expected growth rate of 1-3%, but functional food has an anticipated growth rate of 7-8% through 2010 (Timmermann 2002). Functional attributes of different components such as carotenoids, dietary fiber, omega-3 fatty acids, medium chain triglycerides (MCT), conjugated linoleic acid (CLA), polyphenolics, phytosterols, and tocotrienols are documented. Food supplements are less desirable than food as a source of nutrients, nutraceuticals, and phytochemicals.

Far better approach would be to design functional and nutritional foods enriched with these functional ingredients. Now researchers are trying to establish the most effective but versatile delivery system of these functional ingredients to increase their bioavailability and chemopreventive effect.

Phospholipids may be efficient dietary fractions to deliver these functional components. There is an increasing interest in the use of phospholipids as natural emulsifiers, wetting agents, and dispersal agents in foods, cosmetics, and pharmaceuticals. Phospholipids with specific fatty acids and/or polar head groups are desirable. We incorporated CLA fatty acids and plant sterols into phospholipid to

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This dissertation follows the style and format of the Journal of Food Science.

produce novel phospholipid-conjugates suitable for use as nutaceuticals or as functional food ingredients.

There have been many reports on phospholipid acyl exchange and polar head group modification (Chamiel and others 1999, Mustranta and others 1994a, Aura and others 1995). Fatty acids might be more easily digested as phospholipids than as triacylglycerols or esters, especially when the fatty acids are situated on the sn-2 position of phospholipids (Hallberg and Härröd 1994). Carnielli and others (1998) reported greater absorption of long chain polyunsaturated fatty acids from phospholipids compared to triacylglycerols for infant formula.

1.1 Enzyme catalyzed synthesis of structured phospholipids with CLA

Conjugated linoleic acid (CLA) is a generic term for geometric and positional isomers of linoleic acid. It is a family of fatty acids rather than a single molecular entity. CLA has been reported to have effects in diverse biological functions including: anticarcinogenesis, antiatherogenicity, fatty acid metabolism, immune function, and bone metabolism (Doyle 1998, Reany and others 2002, Scimeca and others 1994). CLAs in foods are quantitatively minor; hence, their consumption by humans is only 0.5-1 g/d/person (McGuire and others 1999). This current intake is insufficient to meet potential biological needs. Ip and others (1994) estimated, on the basis of a rat model, that a 70 kg human should consume 3.0 g CLA/day to obtain its beneficial effects. An alternative method is to increase CLA content in eggs, milk, and meat by feeding CLA to chickens and cows. But Chouinard and others (1999) reported that cows receiving

exogenous CLA increased its content in milk but had reduced milk fat yield with a change in fatty acid composition.

The use of CLA as free fatty acids in foods and pharmaceutical products is limited. So, CLA incorporated into triacylglycerols and phospholipids may offer advantages. Incorporation of CLA into triacylglycerols has been done (Torres and others 2002, Nagao and others 2002, Won-Seck and others 2000, Garcia and others 1998, Arcos and others 1998, McNeil and others 1999). But no reports have been published on incorporation of CLA into phospholipids for different types of food and pharmaceutical products. Also, the mechanism of CLA function in anticarcinogenesis has not been confirmed. This is possibly due to antioxidant activity (Ha and others 1990, Ip and others 1991). Ip and others (1991) reported that mammary tumors in rats and mouse forestomach neoplasia were suppressed by CLA isomers. They suggested that CLA incorporated into phospholipids were active form of CLA isomers. They also postulated that the preferential incorporation of *cis*-9, *trans*-11 isomer of CLA into membrane phospholipid was related to the signal transduction pathway on cell proliferation. Pariza and Ha (1991) in their US patent claimed CLA was an effective antioxidant and mold growth inhibitor. CLA esterified in phospholipid was more effective as an antioxidant. However, several studies (Banni and others 1998, Van den Berg and others 1995) were undertaken to reinvestigate the antioxidant property of CLA related to the efficacy in cancer prevention and the authors believed that the anticarcinogenesis of CLA was not due to antioxidant activity. Since, there are no clinical studies with CLA containing phospholipids; our newly produced dietary form of CLA containing phospholipids might

explain the mechanism of bioactivity of CLA isomers. This part of the study was focused on production of structured phospholipids with modified fatty acid composition by enzyme-catalyzed synthesis of CLA containing phospholipids.

Specific objectives were:

1) Determine the enzyme or combination of enzymes required to produce CLA containing structured phospholipids.
2) Incorporation of CLA into phospholipids from two different sources namely soybean and egg yolk.
3) Optimize reaction parameters such as enzyme load, reaction time, substrate molar ratio and temperature for the production of CLA containing phospholipids.
4) Study the physiological properties of CLA containing phospholipids and determining their applications in foods.

1.2 Enzyme catalyzed synthesis of structured phospholipids with plant sterols

Phytosterols, which are structurally related to cholesterol but differ in their nuclear and/or side chain configuration or polar groups, are claimed to be effective in lowering plasma total and LDL cholesterol. The cholesterol lowering effect of plant sterols (phytosterols) has been studied since the 1950s (Pollak 1953). But, because of poor solubility and bioavailability, doses as high as 25g/day were required for efficacy. The crystalline nature and poor solubility of free phytosterols limit their application in foods. The enrichment of margarine with plant sterol or stanol ester is a recent development in functional foods. The spread supplemented with 8-10% plant sterol lowers serum total cholesterol and LDL cholesterol by 8-13% (Weststrate and Meijer 1998; Hendrinks and others 1999). This is equivalent to the consumption of 1.6-2.0 g of plant sterols per day. In an efficacy study on plant stanols, daily consumption of 24 g of spread containing 2-3 g of plant stanol esters lowered total serum cholesterol and LDL cholesterol by 6.4% and 10.1% respectively (Nguyen and others 1999). However, their intestinal bioavailability was still limited. Phytosterols are highly hydrophobic and do not dissolve in the micelles of the digestive tract. Therefore, they are incapable of efficiently blocking cholesterol absorption. Richard and others found that sitostanol reduces cholesterol absorption at doses much lower than reported previously when administered in lecithin micelles (1999). So, it is desirable to develop biologically effective phytosterols that are effective at lowering plasma cholesterol at lower doses and that can also be added to a variety of foods. A hydrophilic phytosterol composed of

sitosterol- and campesterol-ascorbyl-phosphate had 15-fold greater cholesterol inhibition activity than free sterol (Ramaswamy and others 2002). An aqueous dispersible sterol product was invented for beverage applications (Stevens and Schmelzer 2003) and it has been reported that orange juice fortified with these plant sterols were effective in reducing LDL cholesterol (Devaraj and others 2004). In our present study, synthesis of phospholipid derivative of plant sterols is proposed based on these findings. We attempted to produce amphiphilic lipids by synthesizing phospholipid containing plant sterol amenable to formulation as component of different functional foods. The targeted health claim is therapeutic cholesterol lowering.

This part of the study was focused on production of structured phospholipids with modified polar head group by enzyme-catalyzed synthesis of phospholipd-phytosterol conjugates.

Specific objective was:

1) Study the enzymatic transphosphatidyl reaction by phospholipase D (PLD) for production of phospholipid-phytosterol conjugate.

CHAPTER II

LITERATURE REVIEW: SYNTHESIS OF STRUCTURED PHOSPHOLIPIDS WITH CLA

2.1 Phospholipids

More than one hundred fifty years have passed since lecithin was identified by Mauric Gobley in egg yolk. “Lecithin” is a term derived from the Greek word lekithos (egg yolk) (Wendel 2000). Today, lecithin is described as mixtures of several phosphorus containing substances collectively known as phospholipids-present in cell membranes of animals and plants. Crude lecithins are mixture of oil and phospholipids with minor amounts of other substances and usually prepared from the oil de-gumming process. Phospholipids are functional ingredients of lecithin. They have amphiphillic molecular structure with a lipophillic part from two fatty acids attached and a hydrophilic group from phosphoric acid ester. Phospholipids are determined by the type of substance linked to the phosphate group (Figure 1).

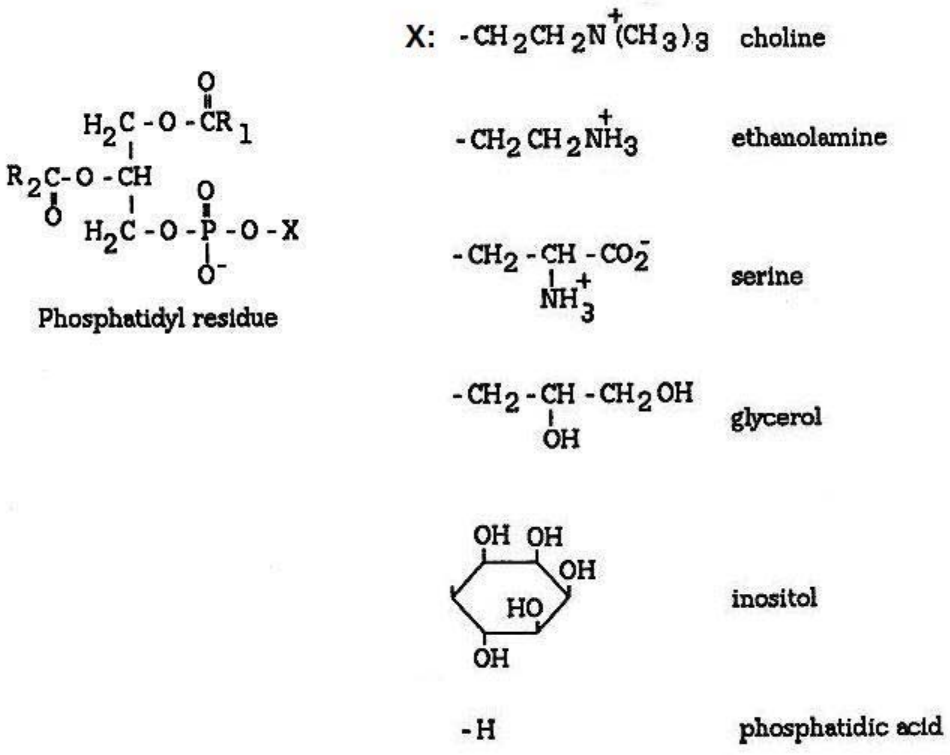

Figure 1- Structure of glycerophospholipids. R_1 and R_2 are fatty acids attached to phospholipids.

2.1.1 Modifications of phospholipids

The compositional variety of phospholipids in native lecithin is limited. But, it is possible to produce structurally divergent phospholipids with modified head group and fatty acids compositions. These structured phospholipids can be tested for their functional properties and uses in food products. As part of the modification techniques, methods containing physical, chemical and enzymatic processes were used for phospholipid modification (Doig and Diks 2003a). Enzymatic modification of phospholipids had a clear advantage over chemical and physical techniques. Several enzymatic pathways were described (Figure 2) by D'Arrigo and Servi (1997) for preparation of natural and synthetic phospholipids for applications in pharmaceuticals, food additives, cosmetics, in liposome technology and in gene therapy.

2.1.1.1 Fatty acids modification of phospholipids

The use of lipase and phospholipase enzymes allow the specific removal/replacement of the acyl chains at positions *sn*-1 and *sn*-2 of phospholipids via hydrolysis and subsequent reesterification or through direct interesterification with an acyl donor (Mustranta and others 1994b). Lipases can be used to selectively exchange fatty acids in the *sn*-1 position and phospholipase A_2 can be used for the corresponding selective exchange reaction at *sn*-2 position of phospholipids. Phospholipase A_1 is also specific for the *sn*-1 position but it has not been used much in phospholipid modifications. Phospholipase A_2 has been used for the synthesis and modification of phospholipids through esterification (Hosokawa and others 1995) or acidolysis

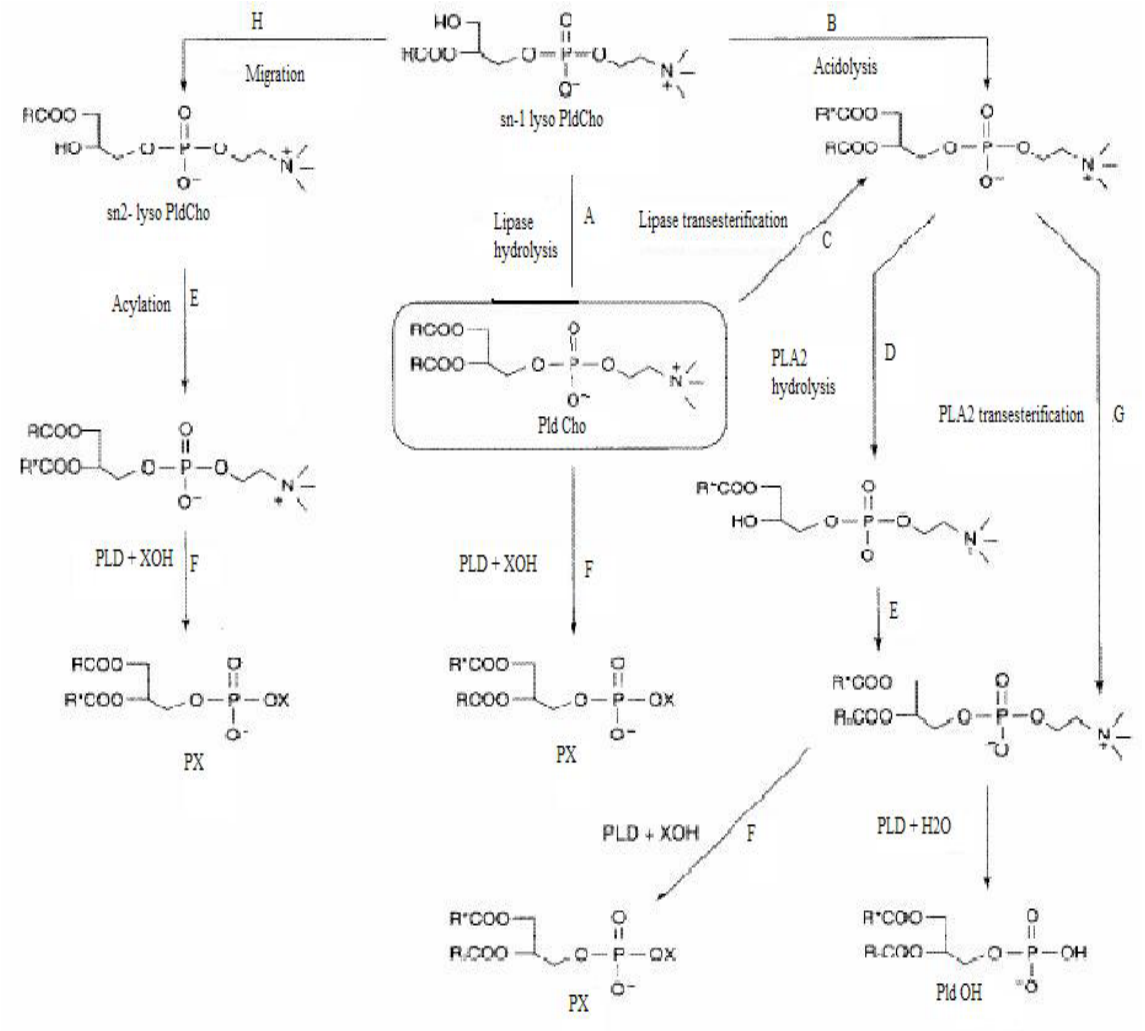

Figure 2- Modification of phospholipids by combined use of lipases, phospholipase A_2 (PLA_2), and phospholipase D (PLD). (D'Arrigo and Servi 1997)

(Hosokawa and others 1998). A few reviews have also been published recently regarding the applications of phospholipases for phospholipid modification (Servi 1999; Ulbrich-Hofmann 2000; Adlercreutz and others 2002). Lipases were used to modify the existing acyl chain of phospholipids (Yagi and others 1990; Mutua and Akoh 1993; Haraldsson and Thorarensen 1999; Svensson and others 1990). But lipases have found their most extensive use in fatty acid exchange at the *sn*-1 position of phospholipids (Adlercreutz and others 2002). Good selectivity for this position has been observed for several lipases, which are specific for the *sn*-1 and *sn*-3 positions of triacylglycerols (Yagi and others 1990; Svensson and others 1990). Commercial availability of immobilized lipases makes the production of structured phospholipids with novel and/or defined fatty acids more feasible. Most studies have used organic solvents as reaction medium (Mustranta and others 1994a; Svensson and others 1990).

Recently, Adlercreutz and others (2002) reviewed the use of lipases for fatty acid exchange in the *sn*-1 position and of phospholipases A_2 for *sn*-2 position exchange. Modified tables obtained from that reviewer are shown in Table 1 and Table 2. Table 1 shows the published results of lipase-catalyzed incorporation of fatty acids into the *sn*-1 position of phospholipids and Table 2 shows that of phospholipase A_2-catalyzed incorporation of fatty acids into the *sn*-2 position. To increase the incorporation of fatty acids into the phospholipids, combination of lipase and phospholipase has been used for phospholipid modification (Chamiel and others 1999; Aura and others 1995). Considerable efforts are being made to incorporate health promoting fatty acids into phospholipids. The primary targets of such efforts are medium chain fatty acids, n-3

polyunsaturated fatty acids, mainly eicopentaenoic acid (EPA), docosahexaenoic acid (DHA) and conjugated linoleic acid (CLA). Incorporation of DHA and EPA into Phospholipids was reported by several authors (Haraldsson and Thorarensen 1999; Mutua and Akoh 1993; Totani and Hara 1991; Na and others 1990). Reports on incorporation of medium chain fatty acids and CLA are limited. Recently, Peng and others (2002) reported the incorporation of caprylic (C8:0) and CLA into soybean phospholipids. Unlike all the above mentioned processes, where substrate acyl chains were free fatty acids or fatty acid methyl esters form, recently Hara and others (2002) reported the enzymatic transesterification of phospholipids with monoacylglycerol (MG) or diacylglycerol (DG) having different acyl groups (C4-C18:3).

Table 1- Example of lipase-catalyzed incorporation of new acids in phospholipids

Entry No.	Reaction type	Lipase used	Solvent	Acid	Incorp. (%)
1	Acidolysis	*R. oryzae*	toluene	C17:0	49.3
2	Acidolysis	*R. miehei* Lipozyme IM-60	hexane	C20:5	42
3	Acidolysis	*R. miehei* Lipozyme IM-60	no	C20:5	53
4	Acidolysis	*R. miehei* Lipozyme TL IM	no	C8:0	38
5	Esterification	*R. oryzae*	toluene	C10:0	49.6

Table 2-Examples of phospholipase A_2-catalyzed incorporation of new acids in the sn-2 position of phospholipids

Entry No.	Reaction type	PLA_2 used	Solvent	Acid	Incorp. (%)
1	Esterification	pancreatic	isooctane	C22:6	35.0
2	Esterification	pancreatic	glycerol	C20:5	46.5
3	Acidolysis	pancreatic	glycerol	C20:5	36.0
4	Acidolysis	pancreatic	toluene	C6:0	41.0
5	Esterification	pancreatic	toluene	C6:0	50.0

2.1.1.2 Polar head group modification of phospholipids

To produce structurally divergent phospholipids with different functional properties, several methods (physical, chemical, and enzymatic techniques) have been developed to modify the polar head group distribution of the native phospholipids (Doig and Diks 2003b). The polar head group of phospholipids can be best modified by using

enzymatic reactions catalyzed by phospholipase D (PLD). PLD catalyzes the hydrolysis of the terminal phosphodiester bond, yielding phosphatidic acid (PA), as well as the transphosphatidyl reaction (Frohman and Morris 1999). Phospholipase D from either cabbage or microbial sources such as *Stretomyces* sp. have been used to incorporate a wide range of novel head groups, including both primary and secondary alcohols into phosphatidylcholine (D'Arrigo and others 1996; Juneja and others 1987; Takami and others 1994). Figure 3 shows the list of different head groups introduced by PLD into phospholipids (Ulbrich-Hofmann 2000). These reactions are usually carried out in aqueous/organic two-phase systems, where organic solvent is required to dissolve phospholipids and serve as substrate reservoir. The enzymes reside in the aqueous environment. But the biphasic reaction system results in the undesirable enzyme-catalyzed hydrolysis of phospholipids (D'Arrigo and others 1996). So, a new reaction system suitable for phospholipase D-catalyzed transphosphatidylation of alcohols with phosphatidylcholine (PC) in anhydrous organic solvents was reported (Rich and Khmelnitsky 2001).

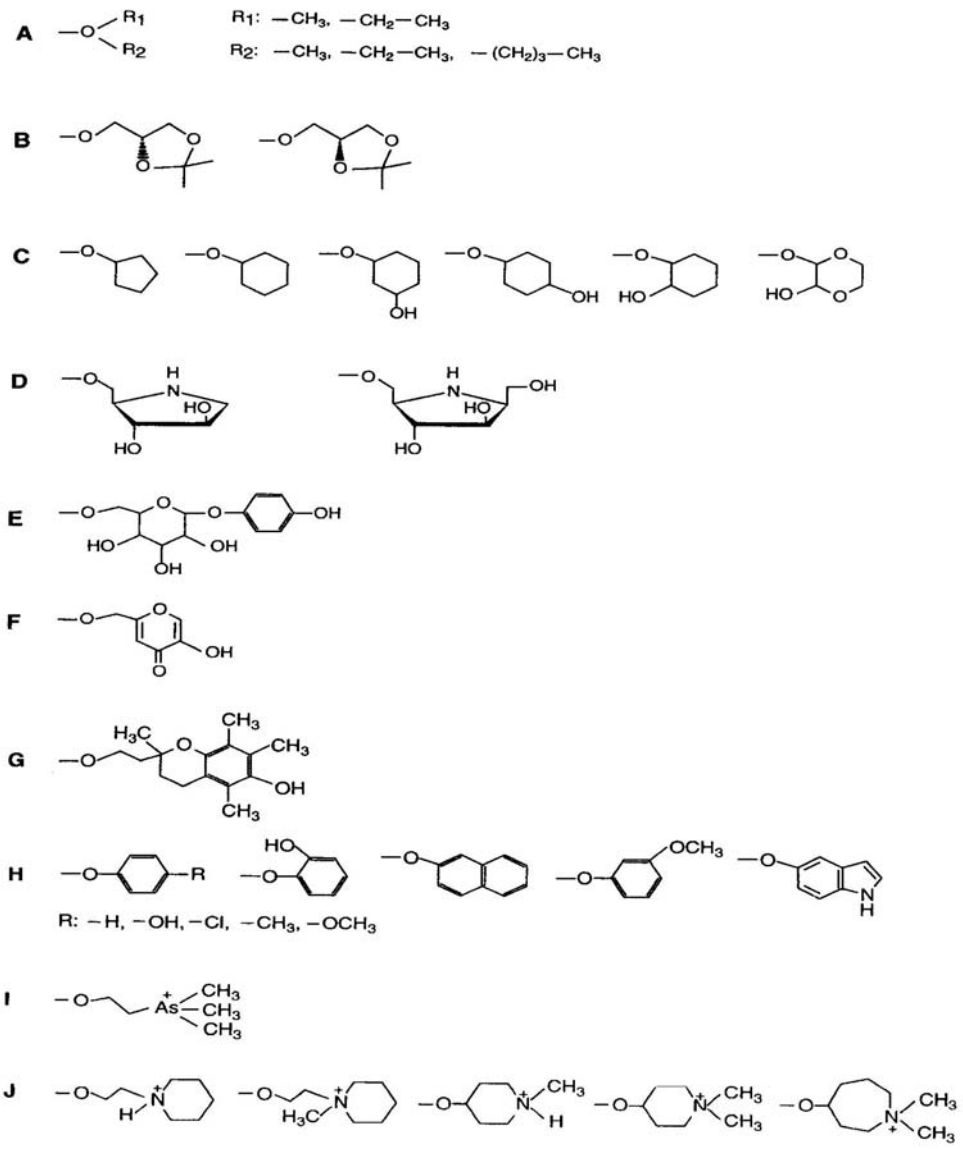

Figure 3-Head group introduced by phospholipase D into glycerophospholipids. (Ulbrich-Hofmann 2000)

2.1.2 Phospholipids for nutraceuticals and functional foods

Phospholipids exhibit a wide range of nutritional and even preventive or therapeutic activities (Schneider 2001). Kidd (2002) also reported phospholipids as versatile nutraceutical ingredients for functional foods and reviewed their clinical efficacy and safety. Mastellone and others (2000) clearly established the beneficial metabolic effects of dietary soybean lecithin on lipid metabolism. The intestinal absorption of cholesterol was decreased by soybean phosphatidylcholine-enriched diet and resulted in a cholesterol-lowering effect. In another report, soy phosphatidylcholine showed beneficial effect on liver detoxification and repair of damaged liver tissue (Leiber and others 1990) Phosphatidylcholine and other egg phospholipids added to infant formula markedly reduced life-threatening necrotizing enterocolitis in hospitalized preterm babies (Carlson and others 1998). The efficacy of phosphatidylserine (PS) for cognition and other higher brain functions was established (Pepeu and others 1996, Jorissen and others 2001). Because of the natural tendency of phospholipids to form ultra fine molecular dispersions, they can be used to improve the bioavailability of lipophilic nutrients. The right type of phospholipids combined with the active substance enhanced their bioavailability dramatically (Leigh and Leigh 1999).

2.1.3 Therapeutic phospholipids

Phospholipids combined with certain nutrients through modification of natural phospholipids have further health benefits. Polyunsaturated fatty acids, mainly n-3 fatty acids-containing phospholipids are currently receiving attention because of their novel

physiological functions. Hosokawa and others (1995) reported weight decrease in some major organ adipose tissue after the administration of eicosapentaenoic acid (EPA C20:5) containing phospholipids. Hosokawa and others (1995) mentioned that docosahexaenoic acid (DHA C22:6) containing phosphatidylcholine (DHA-PC) induced differentiation of murine tumor cells. Polyunsaturated fatty acid containing phospholipids (PUFA-PL) produced by enterobacteria in certain fishes reduce levels of neutral fat or cholesterol (Totani and Hara 1991). Phosphatidylcholine (PC) with eicosapentaenoic (EPA C20:5) and docosahexaenoic acid (DHA C22:6) at the *sn*-2 position could be digested more easily in the body and might be of value in nutritional and medical applications.

2.2 Conjugated linoleic acid

Conjugated linoleic acid, commonly known as CLA, is mixture of positional (C8, C10; C9, C11; C10, C12; and C11, C13) and geometric (*cis, cis*; *cis, trans*; *trans, cis*; and *trans, trans*) isomers of linoleic acid (C18:2 n-6) (Ha and others 1987). In the native food system CLA is mainly formed during the biohydrogenation of linoleates by rumen bacteria. So, food products derived from ruminants are the major sources of CLA. Virtually all *cis*- and *trans*- isomeric combinations of CLA have been identified in food. However, the *cis*-9*, trans*-11 isomer of CLA is predominant in natural food systems (McGuire and others 1999). In milk fat, this isomer is about 85% of total CLA isomers (Chin and others; Shanta and others 1995). The *cis*-9*, trans*-11 isomer and *trans*-10, *cis*-12 isomer of CLA (Figure 4) are considered to be the major biologically active isomers

(Gnädig and others 2001). Reaney and others 2002 described research on CLA between 1935 and 2002 relating to its discovery, synthesis, and analysis.

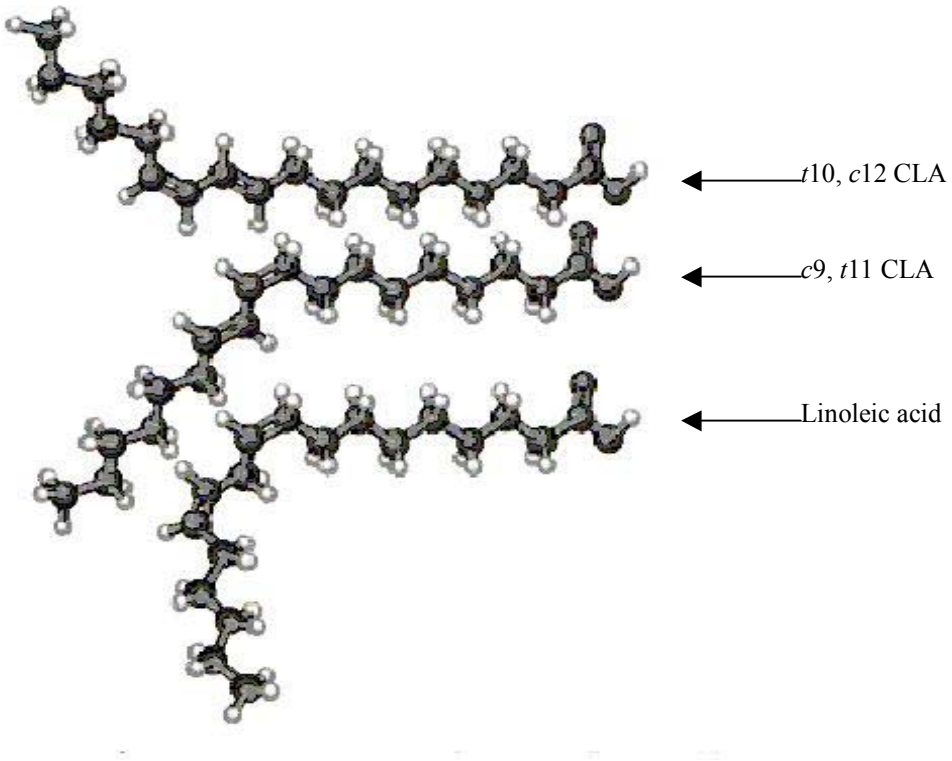

Figure 4- Structures of two major biologically active CLA isomers and linoleic acid (C18:2 n-6). (Pariza 2004)

2.2.1 Sources of CLA

As stated before, ruminant species and their products are rich dietary sources of CLA. Vegetable oils are poor sources of CLA; however, safflower and sunflower oils have 0.7 and 0.4 mg CLA/g of fat respectively (Table 3, Rainer and Heiss 2004). CLA concentrations in dairy products typically range from 2.9 to 8.92 mg / g fat. CLA content of cheeses typically ranges from 3.59 to 7.96 mg/g fat (McDonald 2000). Cultured dairy products usually have 3.82 to 4.66 mg CLA/g fat (Lin and others 1995).

Table 3- Conjugated linoleic acid content of various foods

Food	**mg/g fat**	**Food**	**mg/g fat**
Dairy Products		**Meats/Fish**	
Homogenized milk	5.5	Fresh ground beef	4.3
2% milk	4.1	Veal	2.7
Butter fat	6.1	Lamb	5.8
Condensed milk	7.0	Pork	0.6
Cultured buttermilk	5.4	Chicken	0.9
Butter	4.7	Fresh ground turkey	2.6
Sour cream	4.6	Salmon	0.3
Ice cream	3.6	Egg yolk	0.6
Low-fat yogurt	4.4		
Custard style yogurt	4.8	Vegetable Oils	
Plain yogurt	4.8	Safflower oil	0.7
Frozen yogurt	2.8	Sunflower oil	0.4
Medium chedder	4.1		
American processed	5.0		

Variation of CLA content in foods is affected by the ruminant's diet (type of feed, feeding regimen, grass quantity, dietary restriction), ruminant age or breed, and seasonal factors (O'Shea and others 1998). Cows from Australia and New Zealand have 2-3 times higher CLA concentrations than cows in North America and Northern Europe (O'Shea and others 1998). CLA isomers can also be commercially produced by alkali isomerization or by partial hydrogenation of linoleic acid (Banni 2002). Production of free CLA by lactic acid bacteria *Lactoacillus acidophilus* and *Lactobacillus casei* in medium supplemented with linoleic acid was reported by Alonso and others (2003).

2.2.2 Health properties of CLA

Numerous physiological properties have been attributed to CLA mostly in experimental animal models including antiadipogenic, antidiabetogenic, anticarcinogenic, and antiatherosclerotic activities. In addition, CLA affects on bone formation, immune system, lipid metabolism, and gene expression in numerous tissues. Some of these physiological properties and their effects on the models are summarized in Table 4 (Belury 2002).

Table 4- Physiological properties of conjugated linoleic acid

Major Function	Physiological Model
Body	↓ Adiposity in chicks, mice, and rats
	↑ Adiposity in obese Zucker rats; ↓ Adiposity in ZDF
	↓ Adiposity is isomer specific (*trans*-10, *cis*-12 CLA)
	↓ Adiposity in human subjects; ↔ Adiposity in human
Diabetes	↓Onset of diabetes in ZDF male rats
	Aids in the management of metabolic parameters in
	subjects with type 2 diabetes
	↓ Insulin sensitivity in mice
Carcinogenesis	↓ Chemically induced mammary carcinogenesis in rats
	↓ Chemically induced mammary carcinogenesis in rats
	cis-9, *trans*-11 CLA or synthetic CLA
	↓ Chemically induced mammary carcinogenesis in rats
	of level of fat or esterification of CLA (in triglyceride)
	↓ Growth of transplantable breast cancer tumor cells in
	↓ Growth of transplantable prostate cancer tumor cells in
	↓ Stages of chemically induced skin tumorigenesis in
	↓ Chemically induced colon carcinogenesis in rats
	↔ Carcinogenesis in Min mice
	↓ Chemically induced forestomach
Atherosclerosis	↓ Atherosclerotic plaque formation in hamsters
Bone formation	↓ Eicosanoid production
Immune system	↓ Eicosanoid and histamine production
	↑ Onset of lupus in mouse model
↓, decrease; ↑, increase; ↔, no effect; ZDF, Zucker diabetic fatty.	

2.2.2.1 Anticarcinogenic properties of CLA

Anticarcinogenic effect of CLA was first observed by Pariza and Hargraves (1985) in cooked beef. They established that anticarcinogenic effect was associated with conjugated dienoic isomers of linoleic acid, termed CLA. In the 1990s, several other studies reported anticarcinogenic activity of CLA against chemical carcinogens (Ip and others 1991; Ip and others 1994). CLA also displayed anticarcinogenic effects against breast, colon, and prostrate cancer cell lines (Cesano and others 1998; Park and others 2001). But, not all studies consistently demonstrated that CLA inhibited carcinogenesis. CLA was unable to alter the growth of transplanted prostate (Scimeca 1999) and breast (Wong and others 1997) cancer cells and did not reduce tumorigenesis in an intestinal model of colon carcinogenesis using specific mouse model (Petrick and others 2000). A number of processes are thought to be responsible for mediating anticancer effects of CLA. Park and others (2001) reported that CLA may reduce incidence of cancer by increasing apoptotic cell death of cancer cells. A study with CD2F1Cr mice showed the isomer specific adipose apoptotic effect. The *trans*-10, *cis*-12 isomer, not *cis*-9*, trans*-11 isomer of CLA rapidly induced apoptosis of the white and brown adipocytes (Masso-Welch and others 2004). Park and others (2001) reported that dietary CLA inhibited 1,2-dimethylhydrazine-induced colon carcinogenesis by mechanisms probably involving increased apoptosis. Studies involving the evaluation of anticarcinogenic effect of CLA in humans have shown limited evidence for a direct association between CLA intake and cancer reduction in humans. However, a recent case-control study in Finnish women suggested that dietary CLA may be protective against breast cancer (Aro and others

2000). In their review article, Ip and others (2003) suggested that CLA may be an excellent candidate for prevention of breast cancer. Thus, the role of CLA in prevention or treatment of cancer in humans requires more research.

2.2.2.2 Antiatherosclerotic effects of CLA

Antiatherosclerotic effects of CLA have been reported in experiments with hamsters and rabbits fed high-cholesterol diets, as measured by improved blood lipid profiles (Lee and others 1998; Nicolosi and others 1997). CLA also causes regression of established atherosclerosis in rabbits (Kritchevsky and others 2000). Truitt and others reported the inhibitory effects of platelet aggregation by mixtures and individual isomers of *trans*-10, *cis*-12 CLA and *cis*-9*, trans*-11 CLA. However, CLA does not appear to produce identical results in all animal models of atherosclerosis. Diets with 1% CLA resulted in increasing levels of triglycerides, VLDL, and LDL cholesterol in adult female pigs (Stangl and others 1999).

2.2.2.3 Conjugated linoleic acid reduces adipose tissue

Wang and Jones (2004) reviewed numerous studies in various animals and cell cultures that demonstrated the ability of CLA to reduce fat tissue deposition as well as cellular and whole-body lipid content. The inhibitory effect of CLA on adiposity was likely due to *trans*-10, *cis*-12 isomer of CLA (Park and others 1999; Ostrowska and others 1999). CLA accelerates the decomposition of storage lipids in white adipose tissue (WAT) and clearance of serum nonesterified fatty acid (NEFA) levels, resulting in lipid peroxidation and a morphological change in the rat liver (Yamasaki and others 2000). Unlike growing rats, adult male rats did not respond to the fat-to-lean partitioning effect of CLA (Mirand and others 2004). In humans, although it is indicated that *trans*-10, *cis*-12 isomer of CLA is the antiadipogenic isomer (Blankson and others 2000; Thom and others 2001), the effects of CLA on fat deposition are less significant as compared to results observed in animals. Possible mechanisms through which dietary CLA reduce body fat deposition has been summarized in Figure 5.

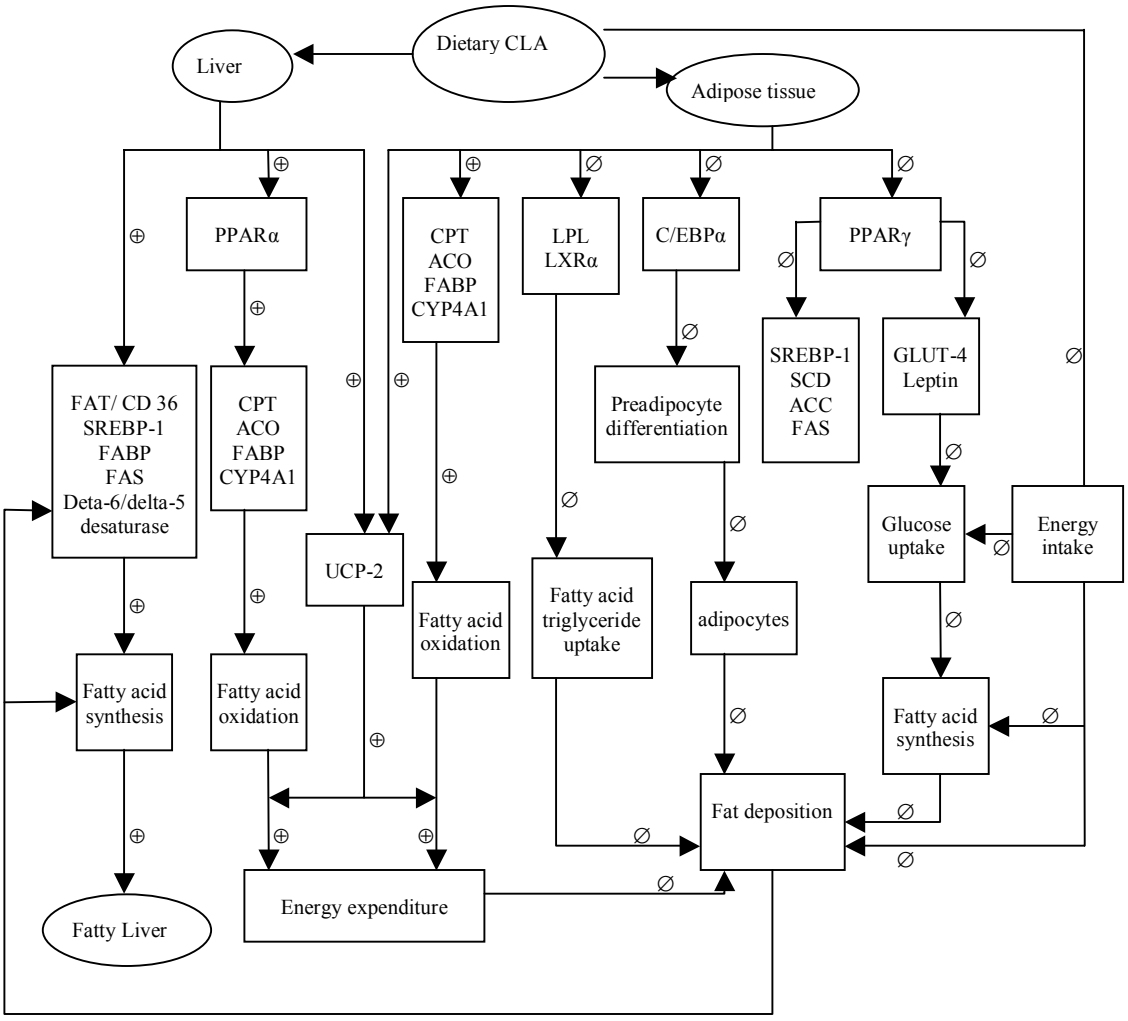

Figure 5-Possible mechanisms of antiadiposity action of conjugated linoleic acid. ⊕, enhance; ∅, suppress. (Wang and Jones 2004)

2.2.2.4 Antidiabetogenic properties of CLA

Houseknecht and others (1998) reported that CLA can improve blood glucose control and prevent the development of elevated blood sugar levels in diabetic animal models by acting as an insulin-sensitizer. In another study with Zucker diabetic fatty fa/fa rat, dietary CLA restored insulin sensitivity what led to the proposal that CLA might be useful in treating type-2 diabetes (Belury and Vanden 1999). Recently, it was noticed that diabetic rats fed either a 50:50 mixture of *trans*-10, *cis*-12 CLA and *cis*-9, *trans*-11 CLA isomers or a 90% *cis*-9, *trans*-11 CLA isomer experienced stimulated insulin action in fat and muscle cells (Ryder and others 2001). The findings suggest that CLA can prevent and/or delay the onset of diabetes and that the *cis*-9, *trans*-11 isomer may contribute much of this activity.

2.2.2.5 Conjugated linoleic acid modulates lipid metabolism

Various studies have shown that CLA affects lipid metabolism. A comprehensive review by Belury (2002) reported that dietary CLA alters the levels of other fatty acids in phospholipids and neutral lipids in the liver. Park and others (2000) observed the decrease in palmitoleic and oleic acids in hepatic neural lipids by *trans*-10, *cis*-12 CLA isomer in mice. Banni and others (2001) also mentioned that it is possible that the altered levels of monounsaturated fatty acids such as palmitoleate and oleate may result from displacement of monounsaturated fatty acids by CLA. In addition, CLA may alter enzymatic pathways responsible for altering fatty acid composition of lipid fractions. In fact, CLA has been shown to reduce the Δ9 desaturase in mouse liver (Lee and others

1998). Supplementation of CLA in the diet of laying hens decreased the concentration of oleic acid (18:1 n-9), arachidonic acid (20:4 n-6), and docosahexaenoic acid (22:6 n-3) but increased that of linolenic acid (18:3 n-3), stearic acid (18:0), and palmitic acid (16:0) in the egg yolk, suggesting that CLA may inhibit Δ6 and Δ9 desaturases (Yang and others 2002). In another study, exogenous conjugated linoleic isomers reduced bovine milk fat concentration and yield by inhibiting De Novo fatty acid synthesis (Loor and Herbein 1998).The schematic pathway how CLA modulates metabolism of nonconjugated fatty acids via enzymatic systems such as Δ6 desaturase-elongase-Δ5 desaturase and alters eicosanoid formation is shown in Figure 6. A recent study showed that the *cis*-9, *trans*-11 CLA isomer inhibited arachidonic acid conversion to prostaglin E_2 by 20-30% in human breast cancer cell line (Miller and others 2001). In addition, CLA was shown to inhibit the activity of Δ6 desaturase, a rate limiting enzyme for the conversion of eicosanoid precursors (Chuang and others 2001). In particular, Belury (2002) proposed that CLA modulates lipid metabolism, in part, by a mechanism dependent on the activation of the group of nuclear transcription factors, peroxisome proliferators-activated receptors (PPARs).

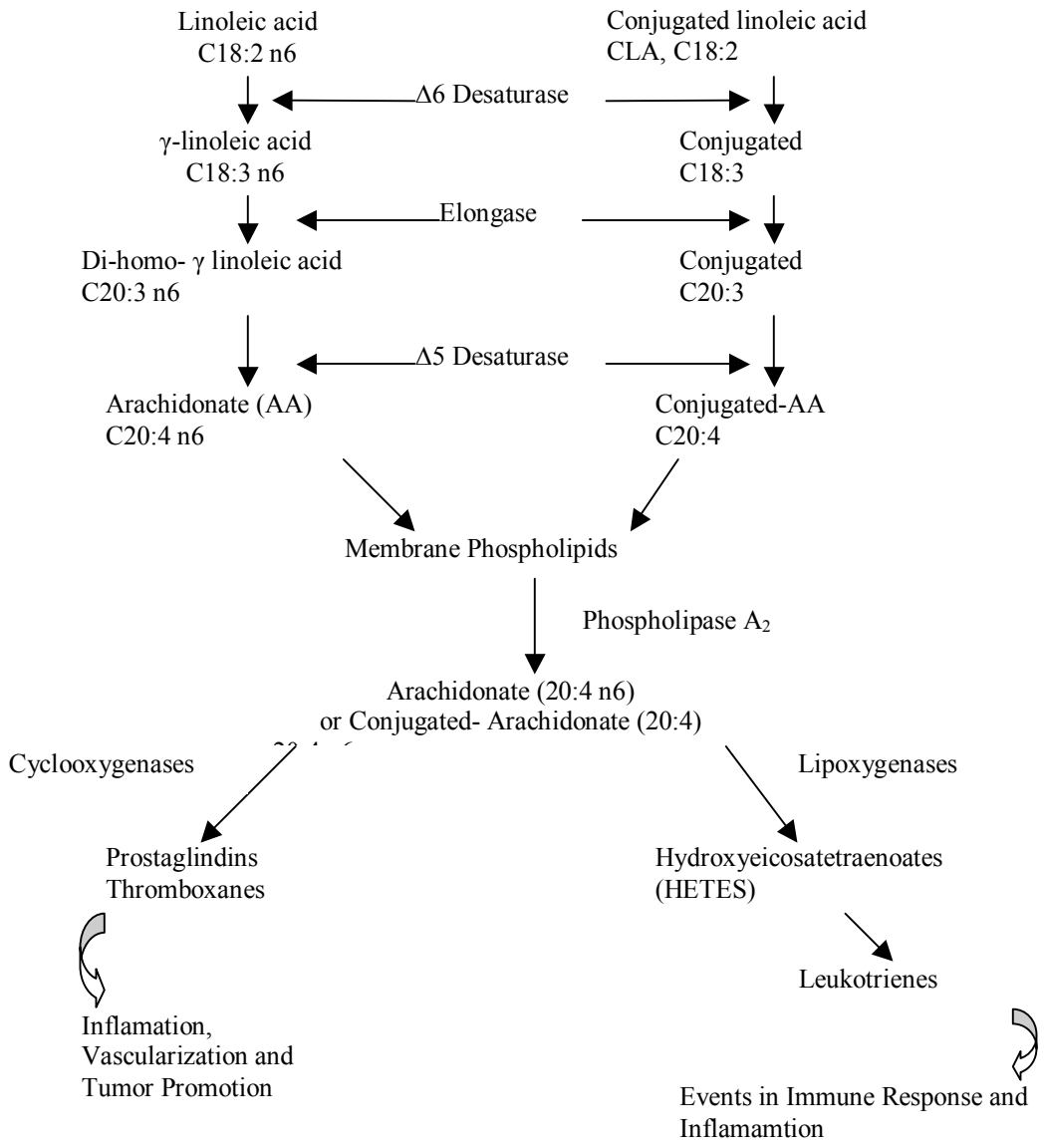

Figure 6- Pathway for desaturation and elongation of CLA and schematic pathway for eicosanoid synthesis from arachidonate.

2.2.2.6 CLA effects on immune system

Hayek and others (1999) reported that dietary CLA influenced immune response in mice. In an earlier study, Cook and others (1993) found that CLA-fed rats exhibited enhanced immune function. CLA administration enhanced cellular immunity in pigs by modulating white blood cell types that control adaptive and innate immunity (Bassaganya-Riera and others 2001). According to Pariza (1999), CLA is the only known dietary factor that both enhances the immune system and at the same time protects against the catabolic effects of immune stimulation, suggesting potential further applications of CLA to the improvement of health. However, no effects were observed on several measures of immune function including delayed-type hypersensitivity response, number of circulating white blood cells, and antibodies to vaccine following the feeding of CLA to young healthy women (Kelley and others 2000). Difference in immune responses may be due to the selection of a young, healthy population with optimal immune function, species difference, or dietary CLA isomer composition (Kapoor and others 2003).

2.3 CLA in human studies

Numerous scientific reviews on biological effects of CLA in animal models and cell culture experiments indicated that CLA could be useful in improving human health. More research is required in humans to confirm these beneficial effects before CLA supplementation can be used as nutraceutical or chronic disease prevention. Several studies were reported with human subjects. A study with 60 overweight men and women

showed that consumption of 1.8 or 3.6 gm CLA/day for 13-week affected the regain of fat-free mass dose independently and increased the resting metabolic rate without improving the body weight maintenance after weight loss (Kamphuis and others 2003a). In another study, Kamphuis and others (2003b) observed that 13-week supplementation with CLA in 60 overweight subjects after body weight loss did not affect body-weight maintenance, but favorably affected the subjective parameters of appetite (satiety, fullness, hunger). A study by Noone and others (2002) confirmed that some of the cardio-protective effects of CLA that were shown in animal studies are relevant to men. They observed that 50:50 CLA isomer blend (*cis*-9, *trans*-11, *trans*-10, *cis*-12) significantly reduced fasting plasma TAG concentration in human subjects whereas 80:20 CLA isomer blend significantly reduced VLDL-cholesterol concentration. CLA supplementation had no significant effect on LDL-cholesterol, HDL-lipid-protein composition or reverse cholesterol transport. CLA supplementation for 4 weeks in twenty-five obese men with metabolic syndrome decreased abdominal fat without concomitant effects on overall obesity or other cardiovascular risk factors (Risérus and others 2001). Another study indicated that supplementation with 2.1 g of CLA daily for 45 days increased CLA concentration in blood but had no effect on body composition or lipid profile of non obese women (Petridou and others 2003). Brown and Mclntosh (2003) reviewed CLA isomer-specific regulation of adiposity and insulin sensitivity in humans. A recent study with 6 healthy women showed no effect of dietary CLA on FA metabolism. But CLA positional isomers were metabolically different and conversion of CLA isomers to desaturated and elongated metabolites was low (Emken and others

2002). Recently, Risérus and others (2003) reported that CLA may slightly decrease abdominal fat without simultaneous reduction of body weight or improvement of lipid or glucose metabolism. Rather, they indicated that the *trans*-10, *cis*-12 isomer unexpectedly caused significant impairment of peripheral insulin sensitivity as well as blood glucose and serum lipid levels. In addition, CLA markedly elevated lipid peroxidation. Albers and others (2003) suggested that CLA may beneficially affect the initiation of a specific response to a hepatitis B vaccination. They observed that 60% subjects who consumed 50:50 (*cis*-9*, trans*-11 and *trans*-10, *cis*-12) CLA isomers had protective antibody levels to hepatitis B compared to 33% subjects who had reference diet and 36% subjects who consumed 80:20 CLA isomers. Most of the studies stated above used CLA mixtures containing two major isomers. A recent study by Risérus and others (2000) with pure *trans*-10, *cis*-12 CLA isomer revealed unexpected metabolic actions by this isomer in humans. This *trans*-10, *cis*-12 CLA isomer, but not a CLA mixture (with isomers *cis*-9*, trans*-11 and *trans*-10, *cis*-12), significantly increased insulin resistance, fasting glucose, and dyslipidemia in abdominally obese men. Recently, Risérus and others (2004) suggested that human supplementation with high doses of the *trans*-10, *cis*-12 CLA isomer should be avoided while awaiting further information on possible effects and side effects.

2.4 Safety aspects of conjugated linoleic acid

Regarding a possible application of CLA in functional food, it is important to know about possible toxicological activities of CLA. Until today, only few studies investigated toxicological aspects of CLA supplementation. Scimeca (1998) conducted a 36-week rat feeding trial with 1.5% CLA supplemented diet, a level about 30% greater than humans would ingest at 3g CLA/day. Food disappearance, body weights, cage side examinations, and hematologic and histopathologic analysis of 15 major organs were conducted. No adverse effects were observed. Recently, a 5-month mice feeding study with CLA observed a reduction of white adipose tissue, while tumor necrosis factor α increased 12-fold and (UCP)-2 mRNA-levels were up regulated 6-fold. The author mentioned that CLA supplementation resulted in something similar to lipoathrophic diabetes (Tsuboyama-kasaoka and others 2000). A human double-blind placebo-controlled study was carried out on overweight volunteers to test the safety of CLA supplementation and effect on body composition was investigated (Berven and others 2000). No differences between the CLA-supplemented and the control group were found on blood parameters such as blood lipids, hematology, liver enzymes, blood electrolytes, creatinine and lactate dehydrogenase or clinical vital signs, such as blood pressure or heart rate. A number of other human clinical trials also reported that high-quality CLA, when consumed at 3-6 g/d, did not appear to induce adverse effects in humans (Noone and others 2002, Albers and others 2003; Kamphuis and others 2003a). Despite these studies, some researchers have recently raised concerns about the potential safety of CLA for humans (Larsen and others 2003; Risérus and others 2004). The reported

concerns are the induction of fatty liver, insulin resistance, and lipodystrophy in mice fed CLA supplemented diets and enhanced C-reactive protein, lipid peroxidation, unfavorable changes in serum lipids, and reduced milk fat in human trials. Recently, Pariza (2004) discussed all these concerns considering overall scientific literature database on CLA. So, more research is needed to confirm the safety of CLA isomers individually or collectively.

CHAPTER III

MATERIALS AND METHODS: SYNTHESIS OF STRUCTURED PHOSPHOLIPIDS WITH CLA

3.1 Materials

Soybean phospholipids, Lipoid S100 (phosphatidylcholine ≈ 94%) and Lipoid S20 (phospatidylcholine 20-24%) and egg yolk phospholipids, Lipoid E PC S (phophatidylcholine ≈ 98%) and Lipoid E 80 (phosphatidylcholine ≈ 80%) were provided by Lipoid GmBH (Ludwigshafen, Germany). Phospholipid from fresh egg yolk was isolated according to the procedure descried by Singleton and others (1965). Safflower oil used for the synthesis of CLA was purchased from a local super market.

3.1.1 Enzymes

Immobilized lipases from *Mucor miechei* (Lipozyme RM IM), *Thermomyces lanuginose* (Lipozyme TL IM) and *Candida Antarctica* (Novozym 435), and phospholipase A_2 from *Porcine pancreas* (Lecitase 10 L) were donated by Novozym es North America (Franklinton, NC). Lipase from *Rhizopus oryzae* (Lipase F-AP15) in powder form was provided by Amano Enzyme USA Co. Ltd. (Elgin, IL).

3.1.2 Chemicals

Soybean and egg yolk phosphatidylcholine (99% purity), other individual phospholipid standards , a mixture of 37 fatty acid methyl esters to identify the different fatty acids , and other chemicals and reagents were purchased from Sigma Chemical Company (St. Louis, MO). CLA reference standards (+98% pure) of *cis*-9, *trans*-11, and

trans-10, *cis*-12 CLA and 1,2-dipalmitoyl-sn-glycero-3-phosphorylcholine (DPPC, MW 734.1) obtained from Matreya, Inc. (PA, USA). All solvents used were of HPLC grade and purchased from VWR-Scientific Products (McGaw Park, IL).

3.2 Methods

3.2.1 Synthesis of CLA

CLA as free fatty acids were produced by alkali isomerization of safflower oil with7% NaOH in propylene glycol at 180^{o}C for 2h according to Rocha-Uribe and Hernandez (2004). Propylene glycol was used to produce high yields of the two major active isomers (i.e. *cis*-9, *trans*-11, and *trans*-10, *cis*-12 CLA isomers). 500g of safflower oil was added to a 5-liter, 3-neck flask containing 218g of NaOH dissolved in 2900 g of propylene glycol. The flask was equipped with a mechanical stirrer, a thermometer, a reflux condenser and a nitrogen (N_2) inlet. The mixture was flushed with N_2 and heated to180oC for 2h. The resulting isomerized mixtures (CLA-containing mixtures) were collected after neutralizing with HCl followed by hexane extraction and water washing according to Rocha-Uribe (2002).

3.2.2 Enzyme catalyzed acidolysis reaction

Deoiled phospholipids from different sources (soybean, egg yolk) with different phosphatidylcholine (PC) content and isomerized CLA mixture were interesterified by different lipases, by phospholipase A_2, and by combination of one the four lipases and phospholipase A_2 according to the reaction scheme 1 (Figure 7).

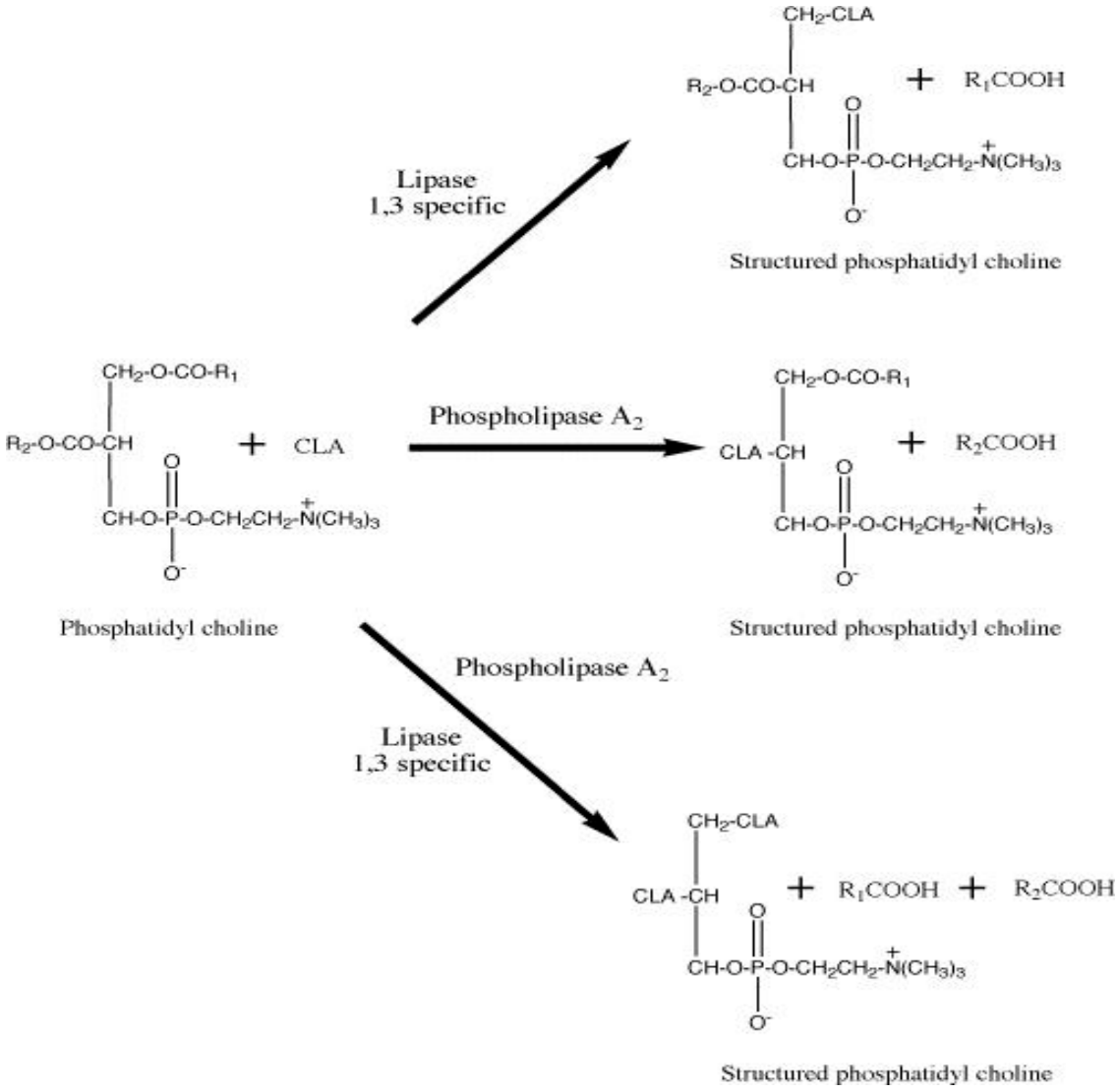

Figure 7- Reaction scheme 1 of lipase and phospholipase mediated acidolysis reaction. R_1 and R_2 are natural alkyl groups.

3.2.2.1 Lipase catalyzed acidolysis reaction

In a typical reaction, 100 mg of phospholipid was mixed with 185 mg of isomerized CLA mixtures (1:5 mol phospholipids/mol CLA) in 1 ml of hexane in screw cap vial. 20% of lipase enzyme (wt.% of phospholipids) was added to the reaction mixture. About 2 µl of water was sprayed to the immobilized lipases only and conditioned at 4^{o}C before adding to the reaction mixture. The reaction was carried out at 40^{o}C for enzymes Lipozyme RM IM and Lipase F-AP15. When enzymes Novozym 435 and Lipozyme TL IM were used, the reaction was carried out at 57^{o}C. The reaction was started in a water bath with magnetic stirring at 300 rpm. From the reaction mixture, 50 µl of aliquot was collected in vials over different time intervals. The solvent was evaporated by N_2 blowing and 300 µl of chloroform: methanol (2:1) was added into the residue and centrifuged for 5 min using a mini-centrifuge (Phenix Rsearch System, Hayward, CA). The solvent layer was carefully withdrawn and kept in the freezer (<-20^{o}C) for analysis.

3.2.2.2 Phospholipase A_2 (PLA_2) catalyzed acidolysis reaction

The acidolysis reaction between phospholipids and CLA mixtures was also carried out by PLA_2 (Lecitase 10L) after immobilization. The Lecitase 10L was immobilized on diatomaceous earth (Celite) by adsorption according to the procedure described by Aura and others (1995). In the reaction catalyzed by immobilized PLA_2, 20 mg of immobilized enzyme was added into the reaction mixture of 100 mg phospholipids and 185 mg CLA mixtures dissolved in 1 ml of hexane. 8µl of Tris-HCL

buffer (pH 8) containing 20 mM $CaCl_2$ was sprayed over immobilized PLA_2 before adding into the reaction mixture. The reaction was carried out at 50^oC in a screw cap vial placed in a water bath with magnetic stirring at 300 rpm. The samples were collected for analysis as mentioned earlier.

3.2.2.3 Lipase and phospholipase A_2 catalyzed acidolysis reaction

In this reaction, both enzymes lipase and phospholipase A_2 were used together. In a typical reaction, 100 mg of phospholipid was mixed with 185 mg of isomerized CLA mixtures in 1 ml of hexane. 20 mg of lipase from either Lipozyme RM IM or Lipozyme TL IM along with 10 mg of immobilized Lecitase 10L was added into the reaction mixture. No buffer was added but 2µl of water was sprayed over lipase enzymes before they were added into the reaction mixture. The temperatures of the reaction were 40^oC and 57^oC when Lipozyme RM IM and Lipozyme TL IM were used respectively.

3.2.3 Phospholipids analysis by TLC and HPLC

TLC analysis. TLC was used to monitor the acidolysis reaction and isolate the reaction products. Aliquots collected in chloroform: methanol (described above) at different time intervals were applied to TLC plates (silica gel 60 F254, E. Merck Co., Darmstadt, Germany). The plates were developed with chloroform/methanol/water (65:25:4 by vol.). Eluted compounds were detected by spraying 5% phosphomolybdic acid in ethanol followed by heating.

HPLC analysis. To measure the degree of hydrolysis of phospholipids during acidolysis reaction, a Beckman System Gold Module HPLC System (Beckman Instrument Inc., Fullerton, CA) with 508 auto sampler module (20µl injection loop) , 126 pump module and 168 detector module was used. A normal-phase silica column (Lichrosorb , Phenomenex) was used at room temperature for this analysis. The mobile phase was acetonitrile/methanol/phosphoric acid (130:5:1.5, vol/vol) run isocratically, and flow rate was 1.5 ml/min. The component phospholipids were detected at 205 nm.

3.2.4 Fatty acid analysis of structured phospholipids

Fatty acid composition of phospholipids classes in acidolysis reaction was determined by using preparative TLC followed by gas chromatographic (GC) analysis. Reaction products were first separated on preparative TLC plates (10cm × 10cm) by developing with mobile phase chloroform/methanol/water (65:25:4 by vol.). Part of the plate was carefully cut and eluted compounds were detected by spraying 5% phosphomolybdic acid in ethanol followed by heating. This was used as template to identify the phospholipids classes which were then scraped off from the TLC plates and put into a test tube for fatty acid methyl ester preparation of individual class. Fatty acid methyl esters were prepared by adding 1.0 ml of 0.25 M sodium methoxide in methanol/diethylether (1:1) to the scrapings in test tubes from the TLC plates. After incubation for 5 min in a water bath shaker at 45^{o}C, 500 µl of hexane was added followed by 3 ml of saturated NaCl solution. After vortexing and centrifugation, another 500µl of hexane was added and methyl esters extracted in hexane were collected from

upper layer for GC analysis. A Varian Model 3400 GC system equipped with a split injector, a flame ionization detector, and a fused silica capillary column Supelco SP 2560 (100 m, 0.25 mm I.D., 0.20 μm film, Bellefonte, PA) was used for fatty acid methyl esters analysis. The initial column oven temperature was 150^{o}C for 3 min and then raised to 200^{o}C at 10^{o}C/min, finally it was raised to 230^{o}C at rate 3^{o}C/min and held for 10 min. The injector and detector temperatures were 250^{o}C and 300^{o}C respectively. The hydrogen was used as carrier gas. Fatty acids were identified by comparing their retention times with those of standards.

3.2.5 Optimization of acidolysis reaction

Experimental design. A three-level four-factor Central Composite Rotatable Design (CCRD) was used according to the principle of Response Surface Methodology (RSM) to optimize the acidolysis reaction conditions for maximum incorporation of CLA into phospholipids. The four factors chosen as independent variables were, enzyme load (E_d, wt.% of phospholipids), substrate ratio (S_r, CLA/PL, mol/mol), temperature (T_e, ^{o}C), and reaction time (t_i, h). The incorporation of CLA into phospholipids was the response. The ranges of the four factors (E_d, 15-25%, S_r, 3.5-6.5%, T_e, 50-60 ^{o}C, t_i, 30-70 h) were chosen based on the primary experiments with single factor and variable ranges published in the literature (Peng and others 2002). The variables with actual and coded ranges are presented in Table 5.

Table 5- Experimental setting of three-level, four factor response surface design for optimization of incorporation of CLA into phospholipids

Experiment	Variable (coded)				Variable (actual)			
	E_d	S_r	T_e	t_i	E_d	S_r	T_e	t_i
1	-1	-1	-1	-1	15	3.5	50	30
2	1	-1	-1	-1	25	3.5	50	30
3	-1	1	-1	-1	15	6.5	50	30
4	1	1	-1	-1	25	6.5	50	30
5	-1	-1	1	-1	15	3.5	60	30
6	1	-1	1	-1	25	3.5	60	30
7	-1	1	1	-1	15	6.5	60	30
8	1	1	1	-1	25	6.5	60	30
9	-1	-1	-1	1	15	3.5	50	70
10	1	-1	-1	1	25	3.5	50	70
11	-1	1	-1	1	15	6.5	50	70
12	1	1	-1	1	25	6.5	50	70
13	-1	-1	1	1	15	3.5	60	70
14	1	-1	1	1	25	3.5	60	70
15	-1	1	1	1	15	6.5	60	70
16	1	1	1	1	25	6.5	60	70
17	-2	0	0	0	10	5.0	55	50
18	2	0	0	0	30	5.0	55	50
19	0	-2	0	0	20	2.0	55	50
20	0	2	0	0	20	8.0	55	50
21	0	0	-2	0	20	5.0	45	50
22	0	0	2	0	20	5.0	65	50
23	0	0	0	-2	20	5.0	55	10
24	0	0	0	2	20	5.0	55	90
25	0	0	0	0	20	5.0	55	50
26	0	0	0	0	20	5.0	55	50
27	0	0	0	0	20	5.0	55	50
28	0	0	0	0	20	5.0	55	50

Abbreviations: E_d, lipase dosage (wt.% of phospholipids); S_r, substrate ratio (CLA/PL, mol/mol); *Te*, reaction temperature (^{o}C); t_i, reaction time (h).

Statistical design. The data were analyzed using the response surface regression (RSREG) procedure of SAS statistical software (SAS Institute). The incorporation of conjugated linoleic acid (CLA), which is the most important factor, was used as main responses for the model evaluation. The degree of hydrolysis of phospholipids was also fitted into the model for evaluation. Response was first fitted to the factors by multiple regression, and then the models generated were used to evaluate the effects of various factors. The goodness of fit was evaluated by the coefficients of determination (R^2) and the analysis of variances (ANOVA). The first- or second- order coefficients were generated by regression analysis with backward elimination method. The insignificant coefficients were eliminated after evaluating the coefficients and the model was refined. The quadratic response surface model was fitted to the following equation:

$$Y = \beta_o + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \sum \beta_{ij} x_i x_j \qquad (1)$$

where Y is the response variable, β_o is the intercept, β_i is the first-order model coefficient, β_{ii} is the quadratic coefficient for the ith variable, β_{ij} is the interaction coefficient for the interaction of variables i and j, and x_i and x_j are ith and jth independent variables respectively. Response surfaces were developed using the fitted quadratic polynomial equations obtained from RSREG analysis and holding the independent variables with the least effect on the response at a constant value and changing the levels of the other two variables.

3.2.6 Stability of CLA-PL containing emulsions

Whey protein (1%) based oil-in-water emulsions with 10% oil (commercial soybean oil) and 0.5% phospholipids were prepared according to the method of Hernandez (2001). Samples were homogenized at 20 MPa using an APV Rannie Lab 2000 Homogenizer (Albertslund, Denmark). Particle size distribution was measured by Coulter LS 130 light scattering apparatus (Coulter Corporation, Miami, FL) after diluting the samples with water and using real refractive index of 1.33 for water. Peroxide value and *p*-anisidine value of oil extracted from emulsions were measured by AOCS official methods - Cd 8-53 and Cd 18-90 respectively. Oil was extracted from emulsions according to Osborn-Barnes and Akoh (2003).

3.2.7 Physiological study of CLA containing structured phospholipids

This study was done in collaboration with the Department of Nutrition and Food Science, Texas Woman University, Denton, TX. The impact of the structured phospholipids with CLA on the proliferation of tumor and colon cancer cells was evaluated. The cultures, seeded with 3.3×104 cells/ml were first incubated and then the samples dissolved in ethanol were added and incubated for 72 h according to the procedure of McAnally and others (2003). The viable cells were harvested, counted and expressed as % of control growth.

CHAPTER IV

RESULTS, DISCUSSIONS, AND SUMMARY: SYNTHESIS OF STRUCTURED PHOSPHOLIPIDS WITH CLA

4.1 Synthesis of CLA

The fatty acid composition of starting safflower oil and that of alkali isomerized CLA mixture is shown in Table 6. Mainly the linoleic acid was converted to conjugated linoleic acid (CLA). About 97.7% of linoleic acid of safflower oil was converted to CLA. The two major isomers *cis*-9, *trans*-11 and *trans*-10, *cis*-12 of CLA were well resolved during fatty acid profile analysis by our gas chromatographic (GC) system with 100 m capillary column. These two major isomers are 97.4% of total isomers synthesized by alkali isomerization and are approximately equal in amount, 48.2% of *cis*-9, *trans*-11 isomer and 49.2% of *trans*-10, *cis*-12 isomer. The composition of other fatty acids (palmitic, C16; stearic, C18:0; oleic, C18:1) present in starting safflower oil was similar after alkali isomerization.

Table 6. Fatty acid composition of safflower oil before and after alkali isomerization

	Fatty acid composition (% area)	
Fatty acid	Safflower oil[a]	Isomerized mixture[b]
C16:0	6.7 ± 0.14	6.7 ± 0.32
C18:0	2.6 ± 0.18	2.6 ± 0.22
C18:1	15.4 ± 0.54	15.4 ± 0.61
C18:2	74.5 ± 1.3	1.7 ± 0.11
CLA[c]		72.8 ± 2.1
cis-9 , trans-11- CLA		35.1 ± 1.1
trans-10, cis-12- CLA		35.8 ± 1.3
Others	0.7 ± 0.03	2.6 ± 0.04

[a] Starting safflower oil. Mean ± standard deviation of three analyses.
[b] Fatty acids after alkali isomerization. Mean ± standard deviation of three analyses.
[c] Total conjugated linoleic acid isomers.

4.2 Enzyme catalyzed CLA incorporation in phospholipids

Enzyme catalyzed acidolysis reaction between phospholipids and free fatty acids is an effective way to modify phospholipids with exchanged acyl chains for specific needs. In this study we tried to incorporate CLA into phospholipids. In the series of experiments with four different lipases and one phospholipase A_2, we found that rate of incorporation of CLA depends on enzyme or combination of enzymes used.

4.2.1 Lipase catalyzed acidolysis reaction

Triacylglycerols are known substrate for lipase enzymes. Like many other reports mentioned in chapter II, our results also showed that lipases have phospholipase (phospholipids are known substrate) activity as well. Among the four different lipases (Lipozyme RM IM, Lipozyme TL IM, Novozym 435, and Lipase F-AP15) used, only Lipozyme RM IM and Lipozyme TL IM were effective in incorporation of two major CLA isomers into phospholipids (Tables 7 through 10). The two major ismomers- *cis*-9, *trans*-11 and *trans*-10, *cis*-12 of CLA were equally incorporated into the soybean phosphatidylcholine (PS 100) used for these set of experiments. The maximum incorporation of these two isomers was 16%, obtained by Lipozyme RM IM after 72 h of reaction. Whereas, similar incorporation (about 15.5%) was obtained by Lipozyme TL IM after 48 h which then decreased to 11.6% after 72 h. Only 1.5% of total CLA isomers were incorporated by Novozym 435 even after 72 h of reaction. No isomers were detected in phosphatidylcholine catalyzed by Lipase F-AP15. Unlike other enzymes which were immobilized, Lipase F-AP15 was used without immobilization.

Table 7- Fatty acid composition of transesterified soy PC (PS 100) at different reaction times catalyzed by Lipozyme RM IM

	Fatty acid composition (% area)[a]					
	Substrate		Modified PC			
Fatty acid	PC	Isomerized CLA	12 h	24 h	48 h	72 h
C16:0	12.8 ± 0.21	6.7 ± 0.32	13.7 ± 0.26	11.5 ± 0.42	10.8 ± 0.28	9.5 ± 0.18
C18:0	3.5 ± 0.14	2.5 ± 0.22	3.9 ± 0.19	3.6 ± 0.16	3.2 0.08	3.2 ± 0.11
C18:1	10.7 ± 0.34	15.4 ± 0.61	10.5 ± 0.14	11.3 ± 0.21	13.4 ± 0.31	13.6 ± 0.16
C18:2	66.4 ± 1.06	1.7 ± 0.11	63.0 ± 0.77	58.5 ± 0.91	55.3 ± 0.56	53.0 ± 0.47
C18:3	6.6 ± 0.31	-	6.8 ± 0.15	6.7 ± 0.08	4.9 ± 0.32	4.4 ± 0.13
*c*9,*t*11-CLA	-	35.1 ± 1.1	1.1 0.07	4.0 ± 0.14	5.7 ± 0.09	8.0 ± 0.12
*t*10,*c*12-CLA	-	35.8 ± 1.3	1.1 ± 0.02	4.4 ± 0.13	6.6 ± 0.04	8.0 ± 0.09

[a]Mean ± standard deviation of three analyses.

Table 8- Fatty acid composition of transsterfied soy PC (PS 100) at different reaction times catalyzed by Lipozyme TL IM

	Fatty acid composition (% area)[a]					
	Substrate		Modified PC			
Fatty acid	PC	Isomerized CLA	12 h	24 h	48 h	72 h
C16:0	12.8 ± 0.21	6.7 ± 0.32	12.9 ± 0.11	11.9 ± 0.09	9.4 ± 0.22	12.1 ± 0.18
C18:0	3.5 ± 0.14	2.5 ± 0.22	3.8 ± 0.16	3.5 ± 0.10	3.8 ± 0.10	3.7 ± 0.17
C18:1	10.7 ± 0.34	15.4 ± 0.61	10.5 ± 0.17	11.8 ± 0.09	12.6 ± 0.08	11.3 ± 0.13
C18:2	66.4 ± 1.06	1.7 ± 0.11	62.6 ± 0.61	58.5 ± 0.44	49.4 ± 0.72	55.9 ± 0.39
C18:3	6.6 ± 0.31	-	5.9 ± 0.13	6.1 ± 0.07	9.2 ± 0.14	5.3 ± 0.11
*c*9,*t*11-CLA	-	35.1 ± 1.1	1.8 ± 0.02	4.7 ± 0.04	7.9 ± 0.09	5.1 ± 0.11
*t*10,*c*12-CLA	-	35.8 ± 1.3	2.4 ± 0.04	3.5 ± 0.07	7.6 ± 0.06	6.5 ± 0.06

[a]Mean ± standard deviation of three analyses.

Table 9- Fatty acid composition of transsterfied soy PC (PS 100) at different reaction times catalyzed by Novozym 435

	Fatty acid composition (% area)					
	Substrate[a]		Modified PC[b]			
Fatty acid	PC	Isomerized CLA	12 h	24 h	48 h	72 h
C16:0	12.8 ± 0.21	6.7 ± 0.32	13.4	12.6	12.6	12.8
C18:0	3.5 ± 0.14	2.5 ± 0.22	3.6	3.5	3.4	3.6
C18:1	10.7 ± 0.34	15.4 ± 0.61	9.3	9.8	10.1	10.5
C18:2	66.4 ± 1.06	1.7 ± 0.11	64.2	66.4	66.0	65.6
C18:3	6.6 ± 0.31	-	8.8	6.7	6.5	6.0
c9,t11-CLA	-	35.1 ± 1.1	0.3	0.24	0.5	0.4
t10,c12-CLA	-	35.8 ± 1.3	0.25	0.7	0.8	1.1

[a]Mean ± standard deviation of three analyses
[b]Average of two analyses.

Table 10- Fatty acid composition of transsterfied soy PC (PS 100) at different reaction times catalyzed by Lipase F-AP15

	Fatty acid composition (% area)					
	Substrate[a]		Modified PC[b]			
Fatty acid	PC	Isomerized CLA	24 h	48 h	24 h*	48* h
C16:0	12.8 ± 0.21	6.7 ± 0.32	13.5	14.0	14.4	14.0
C18:0	3.5 ± 0.14	2.5 ± 0.22	3.7	3.8	3.9	3.9
C18:1	10.7 ± 0.34	15.4 ± 0.61	9.2	9.4	9.5	9.7
C18:2	66.4 ± 1.06	1.7 ± 0.11	64.8	65.9	65.6	65.7
C18:3	6.6 ± 0.31	-	8.8	6.7	6.7	6.6
*c*9,*t*11-CLA	-	35.1 ± 1.1	0.1	ND	ND	ND
*t*10,*c*12-CLA	-	35.8 ± 1.3	ND	ND	ND	ND

[a]Mean ± standard deviation of three analyses
[b]Average of two analyses.
ND- Not detected

Tables 7 and 8 shows that CLA isomers were incorporated into phosphatidylcholine mainly with the exchange of linoleic acid (C18:2) and also with linoleneic acid (C18:3). Fatty acids other than these acids were randomly increased and decreased with time. This may be due to the complex reaction kinetics consisting of interesterification as well as hydrolysis of phosphatidylcholine. So, when CLA esterified into PC with exchange of existing acyl chains, lyso phsphatidylcholine (LPC, PC with one acyl chain cleaved off) was formed with time. The liberated fatty acids then acted as substrate for esterification reaction. The formation of lyso-PLs during acidolysis reaction of PLs was also reported by other authors (Peng and others 2002; Haraldsson and Thorarensen 1999). In our study, the qualitative TLC analysis of acidolysis reaction products between PC and CLA confirmed the formation of LPC with time (Figure 8). The reaction products were migrated on TLC plates as FA (fatty acids) > PC > LPC. No LPC spots were visualized in reaction products catalyzed by Lipase F-AP15. Also, we did not have any CLA incorporation into PC by this enzyme. Quantitative data of LPC was obtained from HPLC analysis of reaction products with time and %hydrolysis was calculated based on the relative amount of LPC present in the reaction products. Figure 9 shows the hydrolysis ratio of two reactions catalyzed by Lipozyme RM IM and Lipozyme TL IM. The pattern of hydrolysis was similar for both the enzymes. The rate of hydrolysis was slow at the beginning and was almost constant at about 8% between 24 and 48 h. After 48 h, the ratio increased rapidly to 19% at 72 h of reaction time.

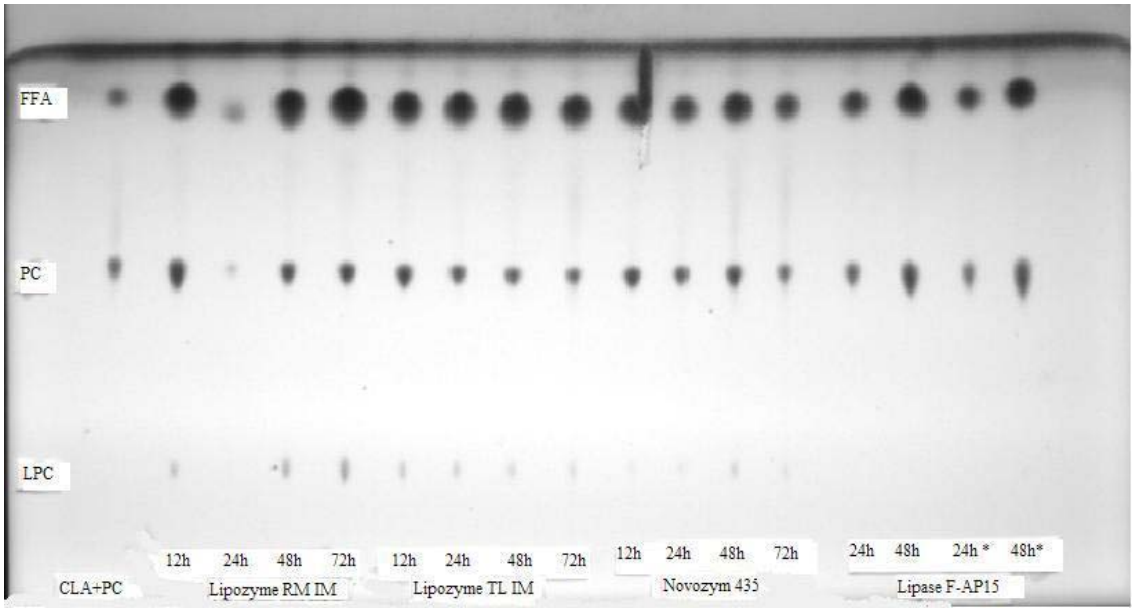

Figure 8- TLC analysis of acidolysis reaction products between PC and CLA isomers by different enzymes at different times.

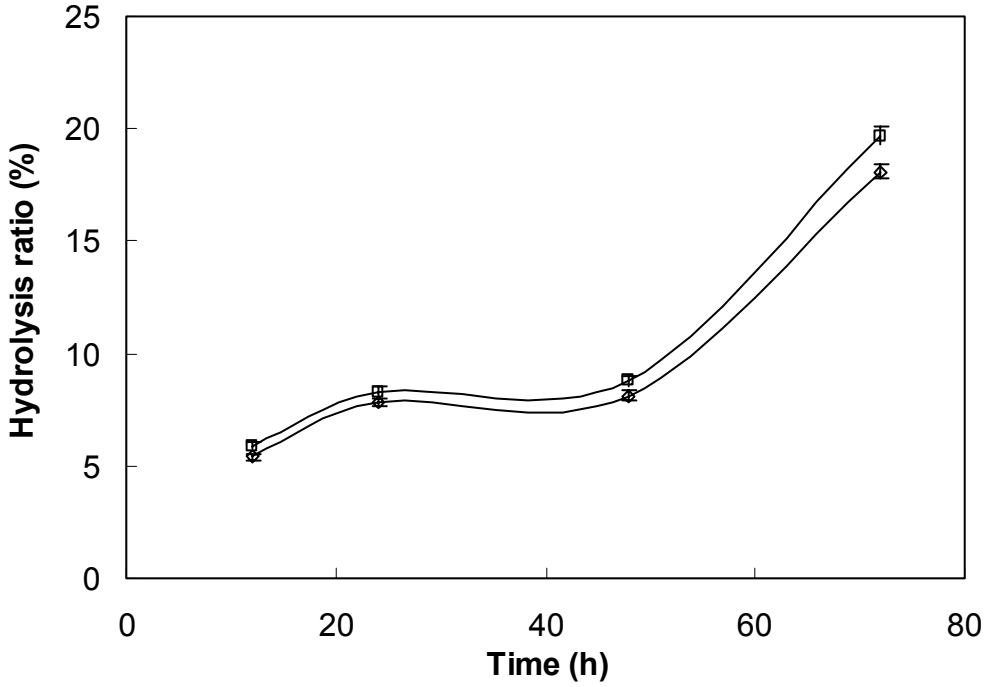

Figure 9- Degree of hydrolysis during acidolysis reaction. ■ –catalyzed by Lipozyme RM IM; ♦-catalyzed by Lipozyme TL IM.

To monitor the reaction kinetics, we measured the fatty acid composition of LPC of reaction products at different times by preparative TLC. Tables 11 through 13 shows the fatty acid composition of lyso phosphatidylcholine. About 12.6% of total CLA isomers were incorporated into LPC after 48 h of reaction catalyzed by Lipozyme TL IM, which then decreased to 8.1% after 72 h. The composition of total CLA isomers in LPC catalyzed by this enzyme was higher than that of LPC catalyzed by other lipase enzymes (Lipozyme RM IM and Novozym 435). Fatty acid composition of palmitic acid (C16:0) and stearic acid (C18:0) decreased with reaction time. Lipozyme TL IM and Lipozyme RM IM preferentially cleaved acyl chain attached at position *sn*-1 of PC. According to the supplier, Lipozyme TL IM and Lipozyme RM IM are 1,3- position specific lipases and Novozym 435 is non-specific lipase. Unlike triacylglycerols where these 1,3 -position specific enzymes can exchange or cleave fatty acids from *sn*-1 and *sn*-3 positions, these enzymes can exchange or cleave fatty acids from position *sn*-1 only of phospholipids (PLs).

Table 11- Fatty acid composition of lyso-PC formed due to the hydrolysis of soy PC (PS 100) at different reaction times catalyzed by Lipozyme RM IM

	Fatty acid composition (% area)				
	Substrate[a]		Lyso-PC		
Fatty acid	PC	Isomerized CLA	12 h	48 h	72 h
C16:0	12.8 ± 0.21	6.7 ± 0.32	7.1	2.7	5.6
C18:0	3.5 ± 0.14	2.5 ± 0.22	5.6	0.7	1.4
C18:1	10.7 ± 0.34	15.4 ± 0.61	19.7	11.2	15.3
C18:2	66.4 ± 1.06	1.7 ± 0.11	57	74	67.4
C18:3	6.6 ± 0.31	-	7.1	7.7	7.4
*c*9,*t*11-CLA	-	35.1 ± 1.1	0.3	1.3	0.9
*t*10,*c*12-CLA	-	35.8 ± 1.3	0.4	2.0	2.1

[a]Mean ± standard deviation of three analyses
[b]Average of two analyses.

Table 12- Fatty acid composition of lyso-PC formed due to the hydrolysis of soy PC (PS 100) at different reaction times catalyzed by Lipozyme TL IM

	Fatty acid composition (% area)					
	Substrate		Lyso-PC			
Fatty acid	PC	Isomerized CLA	12 h	24 h	48 h	72 h
C16:0	12.8 ± 0.21	6.7 ± 0.32	4.0	3.9	4.0	4.9
C18:0	3.5 ± 0.14	2.5 ± 0.22	1.7	1.1	1.4	1.4
C18:1	10.7 ± 0.34	15.4 ± 0.61	10.8	11.4	11.1	13.4
C18:2	66.4 ± 1.06	1.7 ± 0.11	71.7	71.6	64.0	66.5
C18:3	6.6 ± 0.31	-	8.1	7.0	6.8	5.6
*c*9,*t*11-CLA	-	35.1 ± 1.1	1.0	1.6	5.0	3.0
*t*10,*c*12-CLA	-	35.8 ± 1.3	2.5	3.3	7.6	5.1

[a]Mean ± standard deviation of three analyses
[b]Average of two analyses

Table 13- Fatty acid composition of lyso-PC formed due to the hydrolysis of soy PC (PS 100) at different reaction times catalyzed by Novozym 435

	Fatty acid composition(% area)					
	Substrate[a]		Lyso-PC[b]			
Fatty acid	PC	Isomerized CLA	12 h	24 h	48 h	72 h
C16:0	12.8 ± 0.21	6.7 ± 0.32	4.2	3.6	3.9	4.7
C18:0	3.5 ± 0.14	2.5 ± 0.22	4.2	1.3	1.2	1.4
C18:1	10.7 ± 0.34	15.4 ± 0.61	9.4	10.0	11.3	11.5
C18:2	66.4 ± 1.06	1.7 ± 0.11	71.4	72.9	73.4	72.1
C18:3	6.6 ± 0.31	-	7.9	11.3	7.4	6.7
*c*9,*t*11-CLA	-	35.1 ± 1.1	ND	ND	0.6	1.6
*t*10,*c*12-CLA	-	35.8 ± 1.3	0.2	0.8	1.7	2.0

[a]Mean ± standard deviation of three analyses
[b]Average of two analyses
ND-not detected

4.2.2 Phospholipase A_2 (PLA_2) catalyzed acidolysis reaction

Phospholipase A_2 (PLA_2) has specificity for *sn*-2 position of PLs and it has been applied for incorporating highly unsaturated fatty acid into *sn*-2 position of PC by transesterification reaction (Hosokawa and others 1998). They reported about 26% incorporation of eicosapentaenoic acid (EPA) into soy PC after 72 h. In our study we have only 4.2% incorporation (Table 14) of CLA into PC in a similar reaction between PC and CLA catalyzed by PLA_2 in 72 h. In that earlier study, they used freeze-dried PLA_2 after dialysis. Also, the reaction was carried out in glycerol medium. But, in our study we used immobilized (Celite was used as carrier) PLA_2 in hexane. Surprisingly, we have noticed an increase in palmitic (C16:0) acid in transesterified PC with time. The palmitic acid concentration was 18.3% in PC after 72 h. Similarly, the fatty acid composition of stearic (C18:0) and oleic (C18:1) acids increased with the decrease of linoleic (C18:2) and lioleneic (C18:3) acids with time. However, Svensson and others (1990) showed that immobilized (Celite) PLA_2 did not catalyze the interesterification reaction between PC and heptadecanoic acid in toluene. But, they did not report about the change in other fatty acids composition with time. More research is needed to identify whether the presence of organic solvent changes the *sn*-2 positional specificity of PLA_2.

Table 14- Fatty acid composition of transsterfied soy PC (PS 100) at different reaction times catalyzed by phospholipase A_2 enzyme (Lecitase 10 L)

	Fatty acid composition (% area)[a]					
	Substrate		Modified PC			
Fatty acid	PC	Isomerized CLA	12 h	24 h	48 h	72 h
C16:0	12.8 ± 0.21	6.7 ± 0.32	15.2 ± 0.26	16.4 ± 0.42	17.9 ± 0.28	18.3 ± 0.83
C18:0	3.5 ± 0.14	2.5 ± 0.22	4.1 ± 0.19	4.3 ± 0.16	4.6 ± 0.21	5.7 ± 0.19
C18:1	10.7 ± 0.34	15.4 ± 0.61	12.1 ± 0.15	13.3 ± 0.12	13.9 ± 0.36	14.2 ± 0.15
C18:2	66.4 ± 1.06	1.7 ± 0.11	59.4 ± 0.22	58.7 ± 0.55	55.8 ± 0.94	53.1 ± 1.15
C18:3	6.6 ± 0.31	-	5.7 ± 0.08	5.7 ± 0.23	5.2 ± 0.12	3.5 ± 0.32
*c*9,*t*11-CLA	-	35.1 ± 1.1	ND	0.9 ± 0.07	1.3 ± 0.08	2.2 ± 0.06
*t*10,*c*12-CLA	-	35.8 ± 1.3	ND	0.8 ± 0.04	1.2 ± 0.07	2.0 ± 0.08

[a]Mean ± standard deviation of three analyses.

4.2.3 Lipase and phospholipase A_2 catalyzed acidolysis reaction

As part of our effort for enzyme screening to get maximum incorporation of CLA into phospholipids with higher yield, we investigated the acidolysis reaction between CLA and soy PC catalyzed by mixture of lipase and phospholipase A_2 enzymes. Results are shown in Figures 10 and 11 in terms of % incorporation of CLA into PC and lyso PC with time. For comparison, we included the rate of incorporation of CLA by lipases and phospholipase A_2 alone. Though it was expected, Figure10 shows that the rate of incorporation did not increase by the enzyme mixture. The rate was higher by Lipozyme RM IM and Lipozyme TL IM than by their mixture with phospholipase A_2. But the mixture of Lipozyme TL IM and Lipozyme RM IM with phospholipase A_2 (Lecitase 10 L) had higher rate of incorporation than it was by Lecitase 10 L and Novozym 435 alone. Among the mixture of two lipases and phospholipase A_2, the rate of incorporation was much higher by Lipozyme TL IM and Lecitase 10 L than that by Lipozyme RM IM and Lecitase 10 L at early hours. But after 72 h both the mixture had similar incorporation rate. Aura and others (1995) reported that the mixture of lipase and phospholpase A_2 incorporated lauric acid (C12:0) to the same extent as 1,3-specific lipase (Lipozyme IM-60) incorporated alone. Figure 11 shows the rate of incorporation of CLA into lyso PC by these enzymes. The rate of incorporation was higher by mixture of Lipozyme TL IM and Lecitase 10 L compared to others for the first 24 h but then the rate increased with time in the case of Lipozyme TL IM alone. However, the rate was higher by the mixture of lipases and phospolipase A_2 than it was by Lipozyme RM IM and Novozym 435 alone. Several factors, including the interaction of different carriers

used for enzyme immobilization, relative enzyme specificity in presence of others, and water availability, might be responsible for this. More details study of reaction kinetics is needed to address this behavior which is not tried in this study.

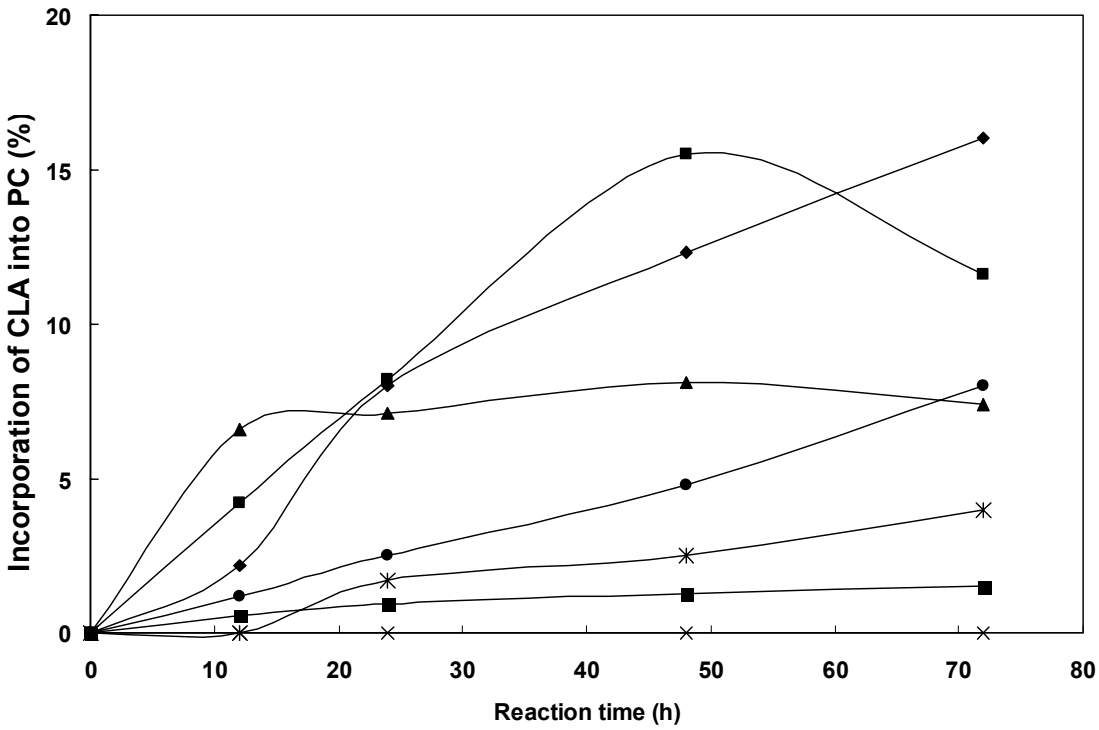

Figure 10- Comparison of enzymes in terms of rate of incorporation of CLA into PC. ■, Lipozyme TL IM; ♦, Lipozyme RM IM; ▲, Lipozyme TL IM and Lecitase 10 L; ●, Lipozyme RM IM and Lecitase 10 L; ∗, Novozym 435; ×, Lipase F-AP15.

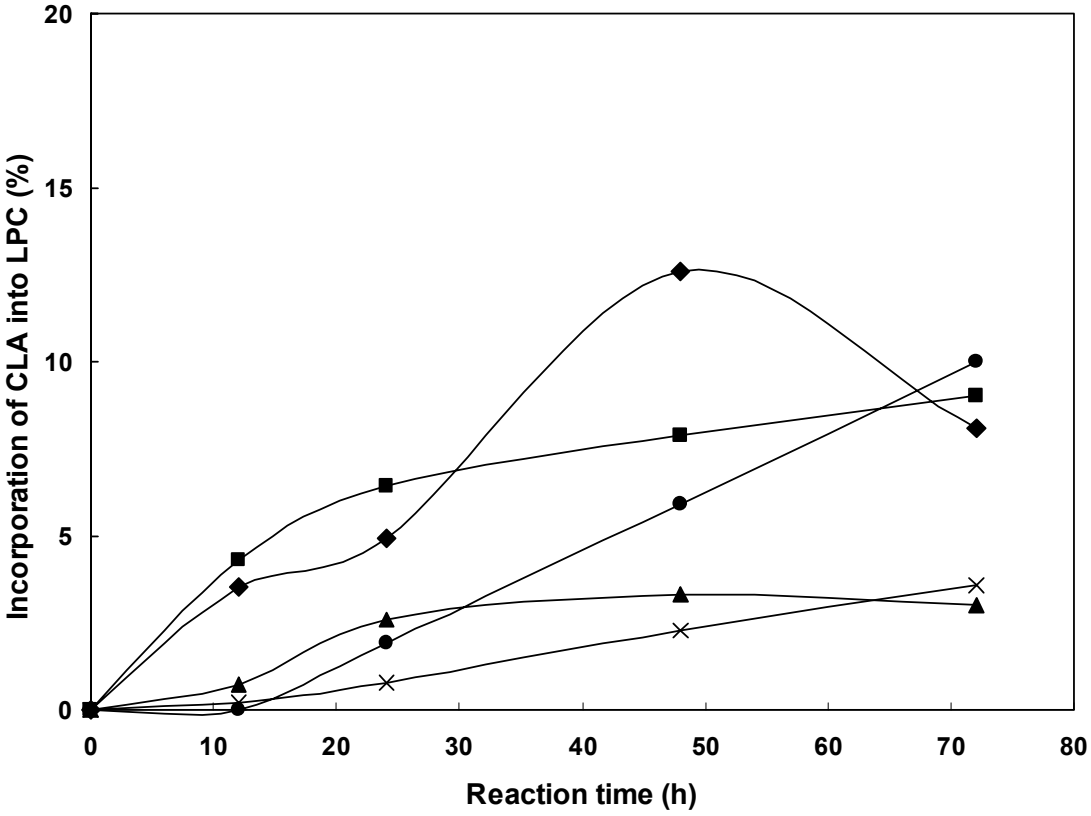

Figure 11- Comparison of enzymes in terms of rate of incorporation of CLA into lyso PC. ♦, Lipozyme TL IM; ■, Lipozyme TL IM and Lecitase 10 L; ●, Lipozyme RM IM and Lecitase 10 L;▲,Lipozyme RM IM; ×, Novozym 435.

4.3 Synthesis of structured phospholipids with CLA from different sources

Phospholipids from different sources with different fatty acids composition and classes of phospholipids have very specific functional and physiological properties. The effect of phospholipid sources and different pospholipid classes on the rate of incorporation of CLA into phospholipids by enzyme catalyzed acidolysis reaction was investiated. Soybean and egg yolk phospholipids with varying content of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were used for this purpose. Figure 12 shows the effects of source and class of phospholipids on the rate of incorporation of CLA. The content of PC in individual lipid class has higher effect on rate of incorporation. The rate is directly proportional to PC content. The soybean phospholipids (soy-PL) with more than 94% PC content had 16% total CLA isomers incorporated into PLs. Whereas, the soyPL with about 20% PC content had only 3.5% incorporation. Similarly, egg phospholipids (egg-PL) with more than 98% PC content had about 13.6% CLA isomers incorporated compare to 7% in egg-PL with 80% PC content. Egg phospholipids prepared from fresh egg yolk in our facilities had similar rate of incorporation of total isomers (7.4%) like commercial egg-PL with 80% PC. The amount of PC in our prepared egg-PL was about 78%. The soy PC has higher rate of CLA incorporation than egg PC (Figure 12). This may be due to the difference in inter - molecular packing because of their difference in fatty acids composition. Further research is needed before an explanation is made. Table 15 shows the incorporation of CLA in different classes of phospholipids. PC has higher rate of incorporation than PE with time. Similar results were reported by Peng and others (2002).

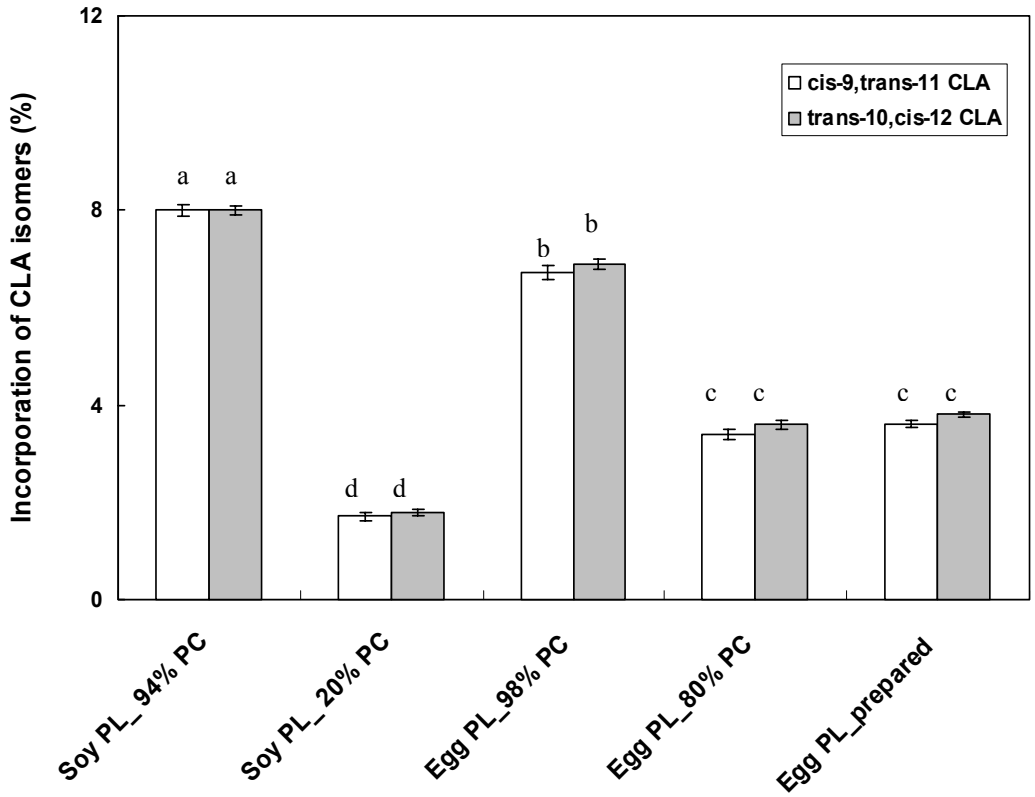

Figure 12- Effect of different phospholipids on the incorporation of CLA. Bars with similar letter are not statistically significant ($\alpha = 0.05$). SoyPL_94% PC, soybean phosphatidylcholne w/ 94% PC. SoyPL_20% PC, Regular deoiled soybean lecithins. EggPL_98%PC, egg phosphatidylcholine w/ 98% PC. EggPL_80%, deoiled egg lecithin. EggPL-prepared, Deoiled egg yolk lecithin prepared in the lab. These reactions were catalyzed by Lipozyme RM IM for 72 h.

Table 15- Incorporation of CLA isomers in different classes of phospholipids. Deoiled egg yolk lecithin catalyzed by Lipozyme RM IM was used for this study. Phospholipid classes separated on TLC plate was methylated for fatty acid composition

	Incorporation of CLA isomers (%)			
	PC		PE	
Reaction time (h)	*c*9,*t*11-CLA	*t*10,*c*12-CLA	*c*9,*t*11-CLA	*t*10,*c*12-CLA
24	1.7	1.4	0.4	0.3
48	2.9	2.7	1.2	1.3
72	6.3	6.4	2.9	3.0

4.4 Optimization of incorporation of CLA into phospholipids

4.4.1 Model fitting

The primary objective of this study was to maximize the incorporation of CLA into phospholipids. The central composite rotatable design, the best design for response surface optimization, was selected with four parameters, i.e. lipase dosage, substrate ratio, reaction time and temperature. Table 16 lists the experimental parameter settings and results of incorporation of CLA into phospholipids based on the experimental design. The best fitting model was determined by regression and backward elimination. The model coefficients (β) and probability (P) values are given in Table 17. Among all the possible regression models i.e. linear, quadratic, cross product, and combination of all, linear model was highly significant (P-value 0.0004) with coefficient of determination (R^2) 0.68. Though the quadratic and cross product P-values were 0.125 and 0.703 respectively, the total model (equation 1) was highly significant (P-value 0.0080) with R^2 value 0.8526. According to the analysis of variance, the P-value for lack of fit was 0.0869. The observed values and predicted values of incorporation of CLA into PC based on this model were sufficiently correlated (Figure 13). As formation of lyso PC (LPC) because of hydrolysis of PC was observed along with the interesterification reaction, we applied response surface methodology to identify the reaction parameters to optimize the CLA incorporation as well as yield. The values of LPC are listed in the Table 15. The observed values and predicted values of LPC based on this model were also correlated (Figure 14). The model that is best suited for these selected ranges can not be extrapolated.

Table 16- Experimental setup for four-factor, three-level response surface design and the response after analysis

Experiment	Variable (actual)				Incorporation of CLA into PC	Formation of lyso PC (LPC) (%)
	E_d	S_r	T_e	t_i		
1	15	3.5	50	30	4.10	12.32
2	25	3.5	50	30	7.20	10.53
3	15	6.5	50	30	7.60	5.44
4	25	6.5	50	30	10.20	10.89
5	15	3.5	60	30	5.15	11.78
6	25	3.5	60	30	8.30	8.91
7	15	6.5	60	30	9.40	7.74
8	25	6.5	60	30	9.70	12.13
9	15	3.5	50	70	7.85	16.69
10	25	3.5	50	70	8.55	14.28
11	15	6.5	50	70	6.25	9.11
12	25	6.5	50	70	12.85	13.94
13	15	3.5	60	70	6.20	18.38
14	25	3.5	60	70	7.30	14.88
15	15	6.5	60	70	6.95	13.65
16	25	6.5	60	70	11.40	17.40
17	10	5.0	55	50	6.40	11.12
18	30	5.0	55	50	13.10	13.09
19	20	2.0	55	50	4.85	18.04
20	20	8.0	55	50	11.80	13.67
21	20	5.0	45	50	5.35	7.85
22	20	5.0	65	50	6.05	10.76
23	20	5.0	55	10	3.35	7.43
24	20	5.0	55	90	10.25	17.08
25	20	5.0	55	50	9.10	15.70
26	20	5.0	55	50	8.85	15.70
27	20	5.0	55	50	8.45	14.67
28	20	5.0	55	50	8.13	14.01

Abbreviations: E_d, lipase dosage (wt.% of phospholipids); S_r, substrate ratio (CLA/PL, mol/mol); *Te*, reaction temperature (^oC); t_i, reaction time (h).

Table 17- Regression coefficient (β) and significance (*P*-value) of the polynomial model of RSM for incorporation of CLA into PC

Parameters	Coefficient (β)	*P*-value
Intercept	8.975000	<.0001
E_d	1.475000	0.0006
S_r	1.400000	0.0008
T_e	0.050000	0.8739
t_i	0.812500	0.0230
E_d*E_d	0.263542	0.4804
S_r*E_d	0.368750	0.3489
S_r*S_r	-0.092708	0.8020
T_e*E_d	-0.250000	0.5208
T_e*S_r	0.081250	0.8332
T_e*T_e	-0.748958	0.0622
t_i*E_d	0.231250	0.5519
t_i*S_r	-0.287500	0.4616
t_i*T_e	-0.443750	0.2639
t_i*t_i	-0.473958	0.2157

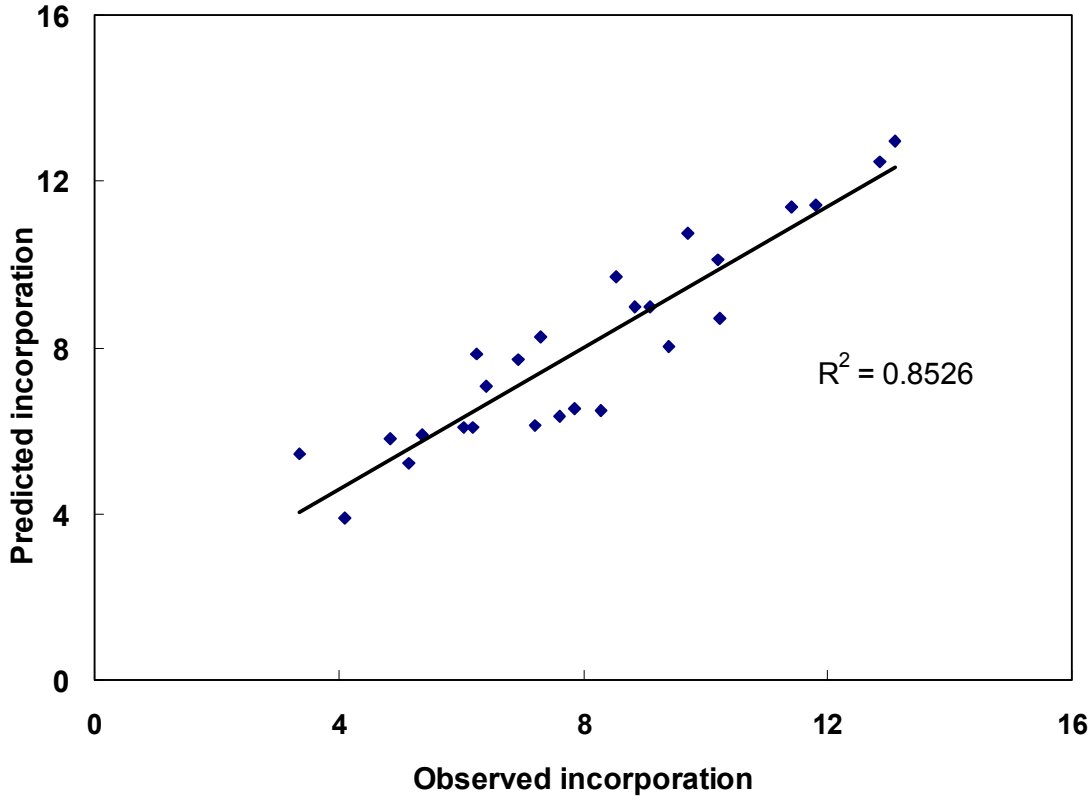

Figure 13- Relationship between the observed and predicted incorporation of CLA into phospholipids catalyzed by Lipozyme TL IM. Values inside the figure are from experimental settings.

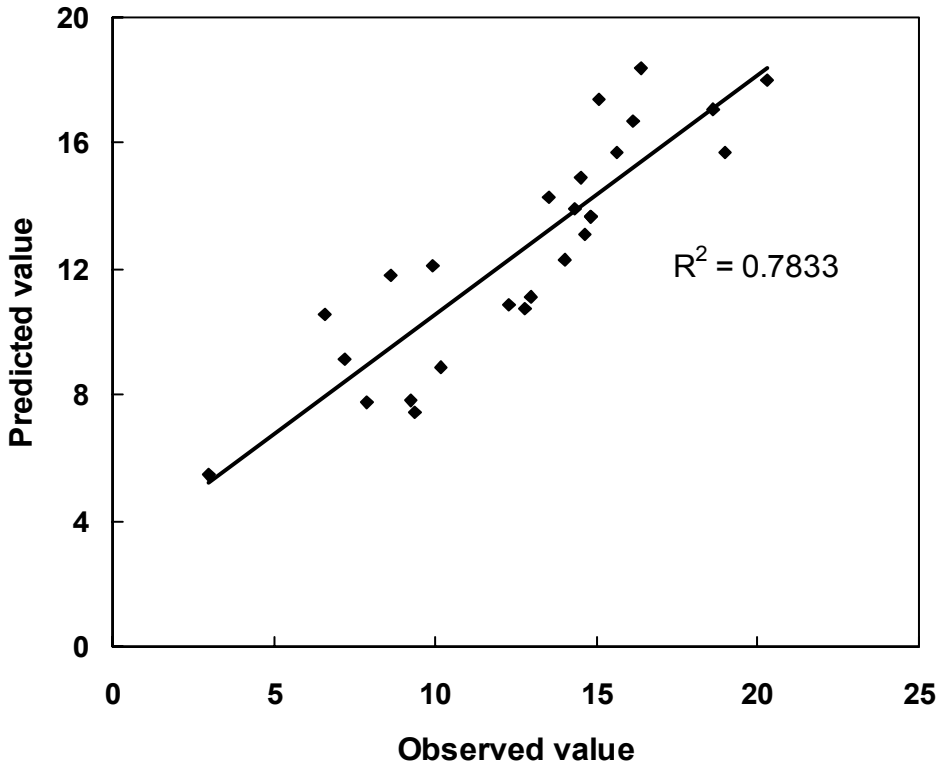

Figure 14- Relationship between the observed and predicted values of lyso PC formation (LPC) because of hydrolysis during acidolysis reaction catalyzed by Lipozyme TL IM.

4.4.2 Effects of different parameters on CLA incorporation

The effects of all parameters on the incorporation of CLA and their significance are shown in Figure 15. All first-order coefficients have positive effects on the incorporation. Some second-order coefficients have positive effects and some have negative effects. So, the relationship between the factors and the response was not linear. The enzyme load (E_d) had the most significant effect followed by substrate ratio (S_r) and reaction time (t_i) respectively. The temperature (T_e) had smaller effect but square of T_e had highest negative effects. The individual effect of four factors on the incorporation is given in Figure 16 through 19. As expected, incorporation was improved by longer reaction time (Figure 16). The incorporation was maximum around 60 h but in the single study catalyzed by this enzyme, we had higher incorporation at 48h compare to 72 h (Table 8). Lipase dosage caused a positive linear increase in the extent of CLA incorporation (Figure 17). The incorporation of CLA increased with increasing substrate ratio up to a ratio of around 6 (Figure 18). Excessive free fatty acids may result in enzyme inhibition. The effect of temperature on incorporation had showed highly bell-shaped curve (Figure 19). The optimum reaction temperature was 55^{o}C after which the rate decreased very fast. Enzymes might be deactivated at higher temperature.

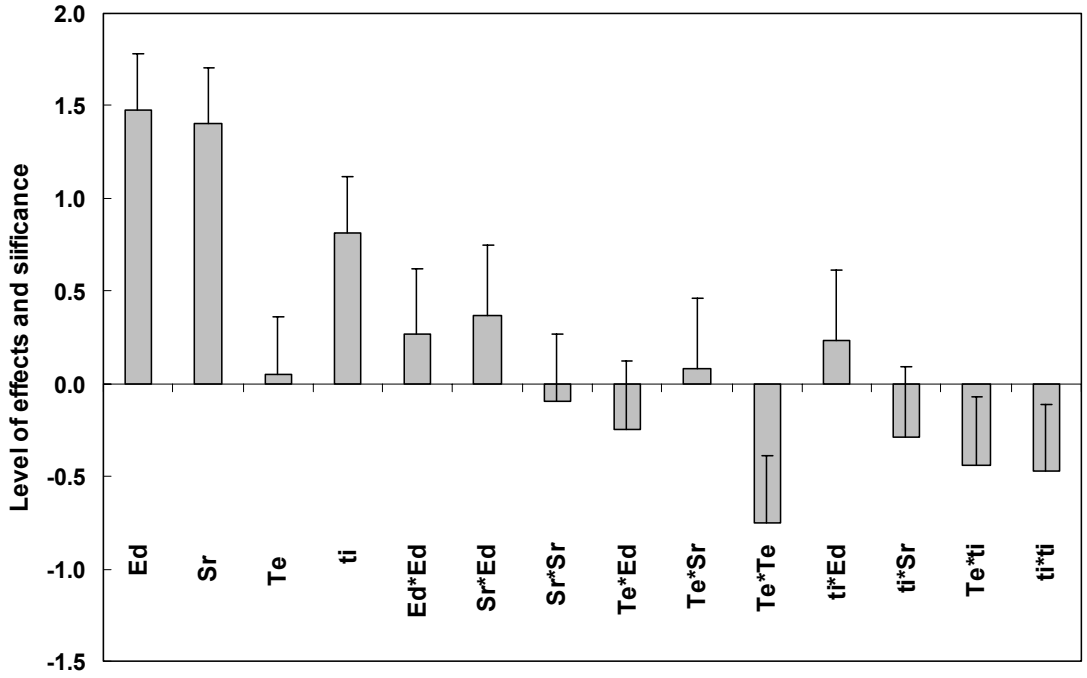

Figure 15- Factor effects and their significance on the incorporation of CLA into phospholipids catalyzed by Lipozyme TL IM. For abbreviation, see Table 5.

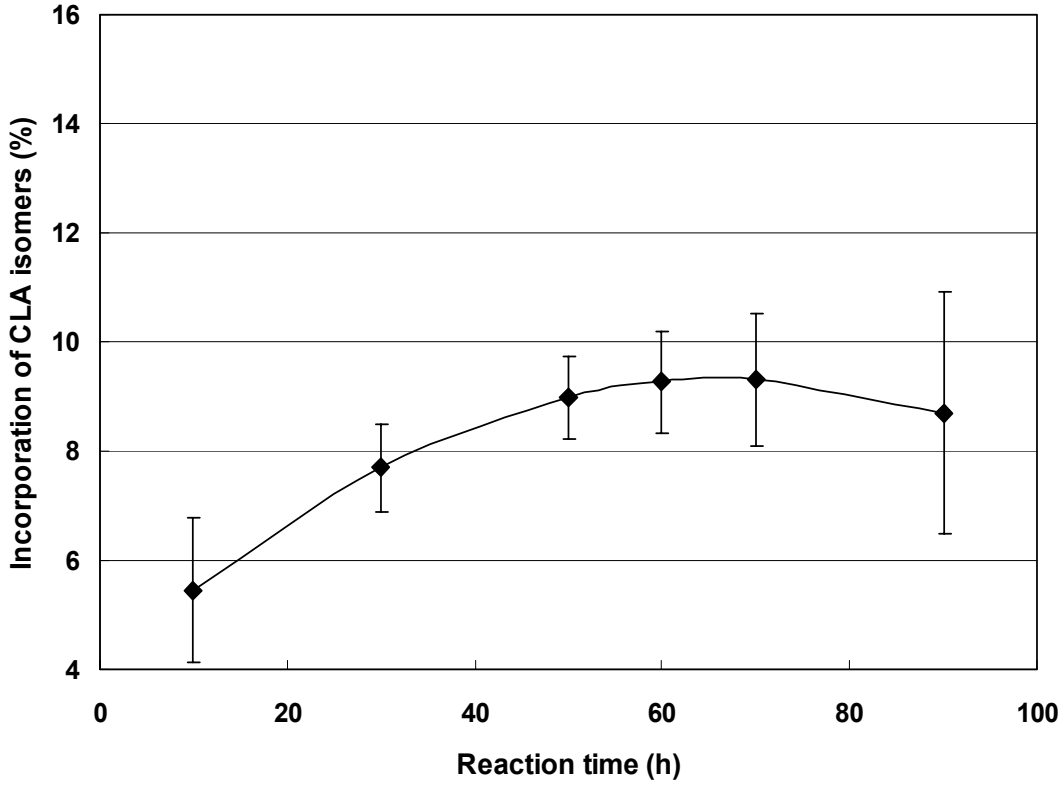

Figure 16- Effect of reaction time on the incorporation of CLA into phospholipids catalyzed by Lipozyme TL IM.

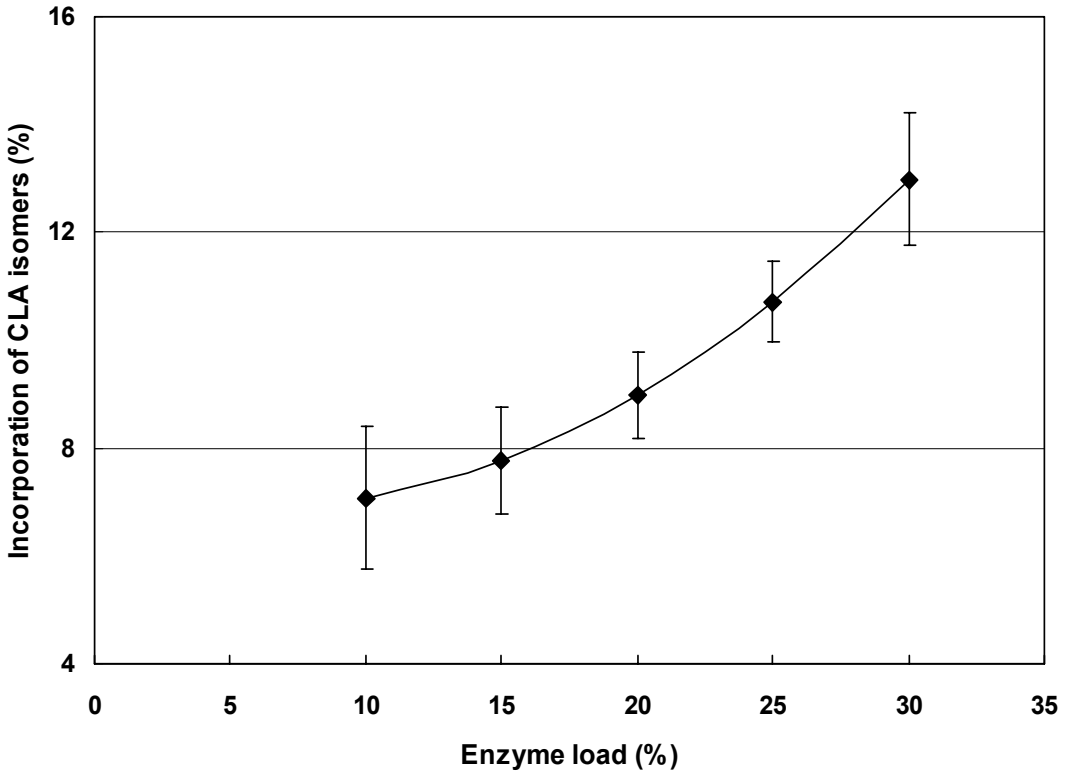

Figure 17- Effect of enzyme load on the incorporation of CLA into phospholipids catalyzed by Lipozyme TL IM.

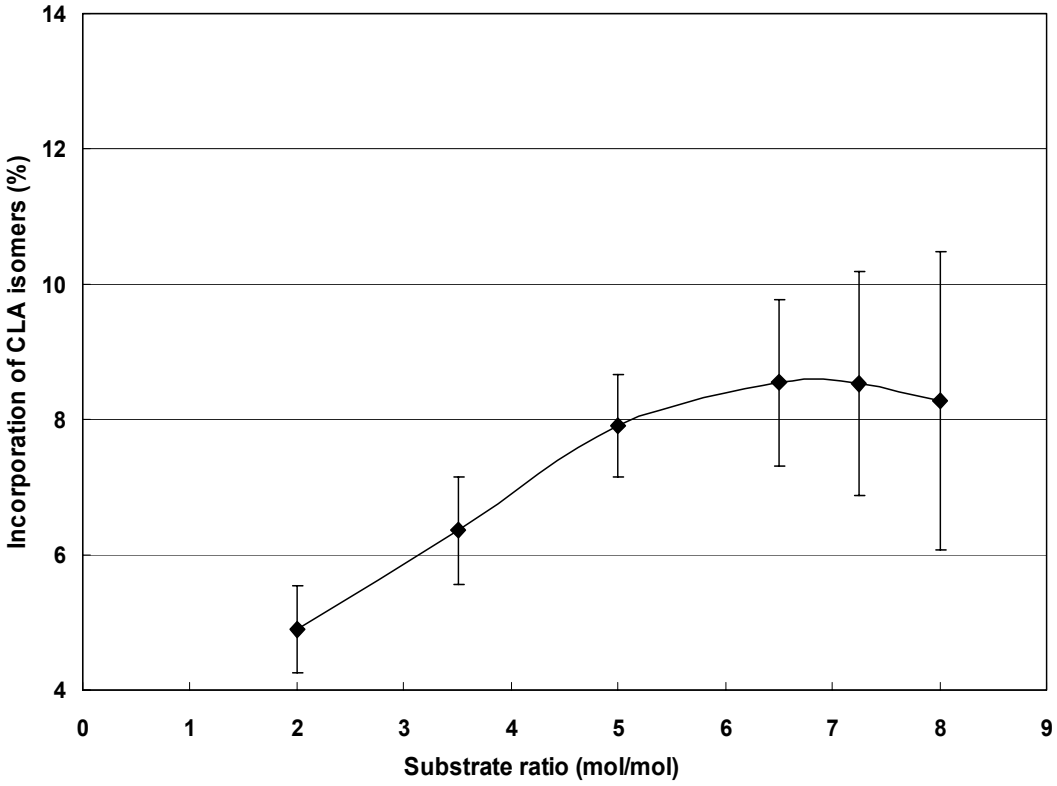

Figure 18- Effect of substrate ratio on the incorporation of CLA into phospholipids catalyzed by Lipozyme TL IM.

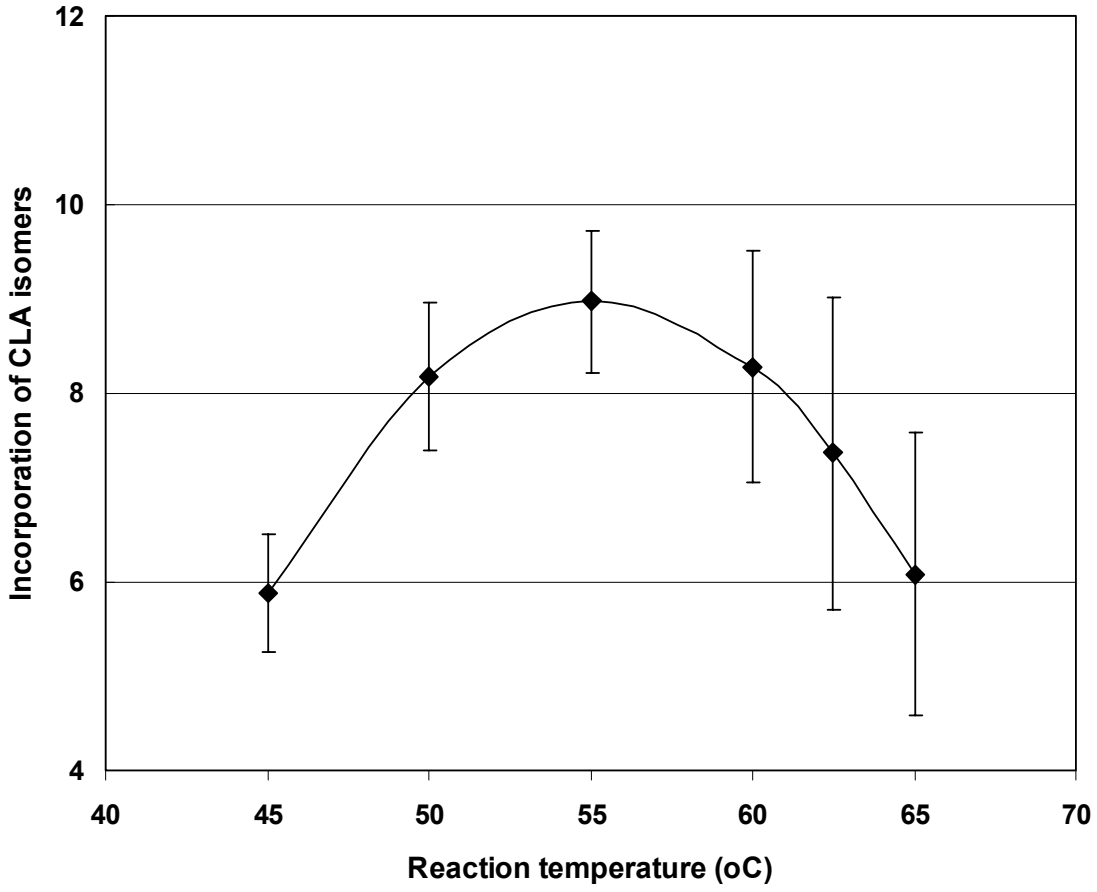

Figure 19- Effect of reaction temperature on the incorporation of CLA into phospholipids catalyzed by Lipozyme TL IM.

4.4.3 Optimization of reaction system

From the economic point of view, the most efficient conditions for this reaction would be to use the lowest amount of enzyme load and at the lowest temperature with the lowest substrate ratio to achieve the highest incorporation of CLA. Though the single factor plot shows the value or trend to get the maximum incorporation, the three dimensional response surfaces generated by using linear, quadratic and cross-product terms in the second order polynomial give the desirable combination of variables that can be selected to obtain the highest incorporation at the least cost. The response surfaces generated for our study are shown in Figures 20 through 25. Figure 20 shows that the rate of incorporation could be increased by increasing time and enzyme load while keeping the temperature and substrate ratio constant. Figures 21 and 22 show that change in temperature in any direction from center point would decrease the rate of incorporation of CLA. But increase of substrate ratio will increase the rate at any temperature while keeping time and enzyme load constant (Figure 21). Similarly, Figure 23 shows that the maximum rate at center value of temperature and time which are 55^{o}C and 50 h. Figure 24 shows that rate can be increased by increasing enzyme load and substrate ratio while keeping time and temperature constant. Figure 25 shows that substrate ratio increased the rate with time up to certain value after which it started decreasing. The overall shape of the response surfaces and the canonical analysis of SAS software identified the stationary points of this model as saddle points which are neither maximum nor minimum.

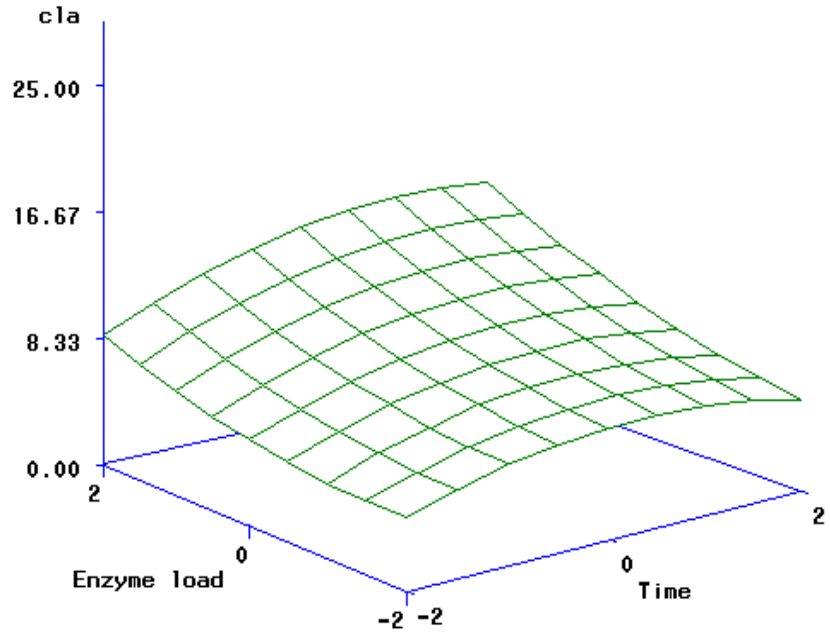

Figure 20- Response surface showing the effect of enzyme load and time with temperature and substrate ratio held constant at 55^{o}C and 5.0 respectively.

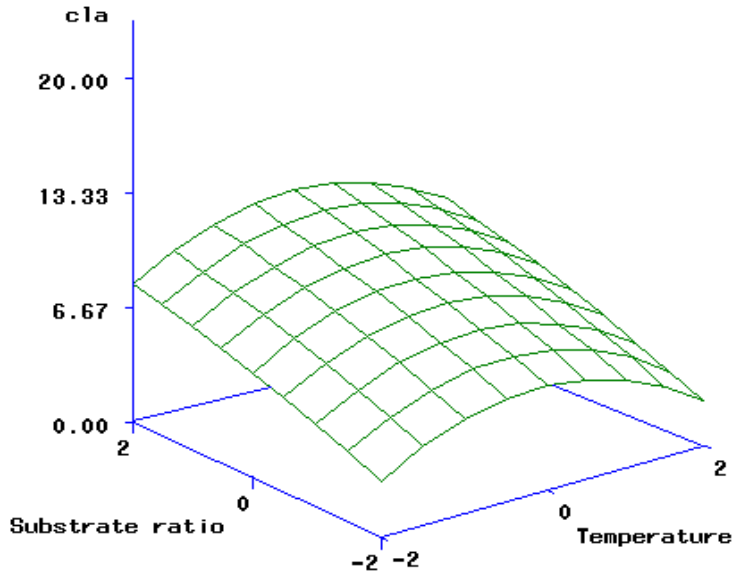

Figure 21- Response surface showing the effect of substrate ratio and temperature with enzyme load and time held constant at 20 (wt.%) and 50 h respectively.

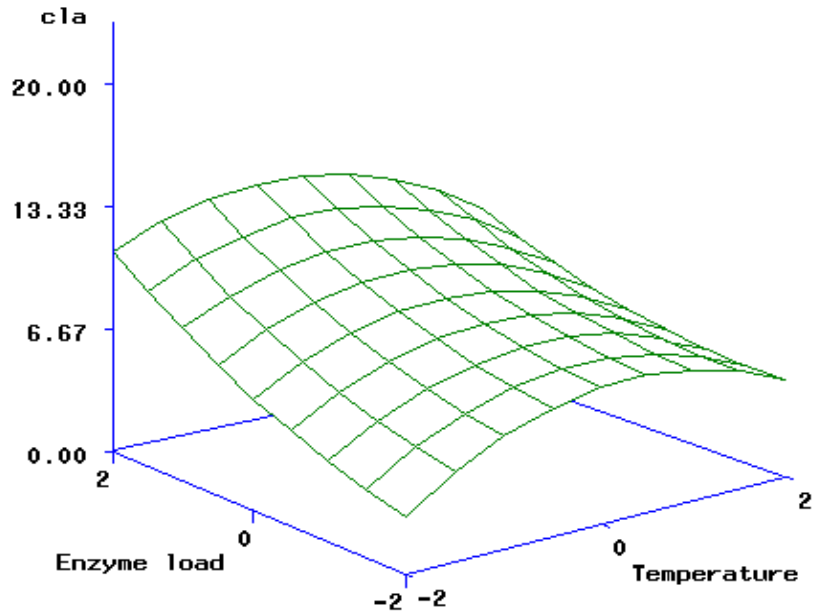

Figure 22- Response surface showing the effect of enzyme load and temperature with substrate ratio and time held constant at 5 and 50 h respectively.

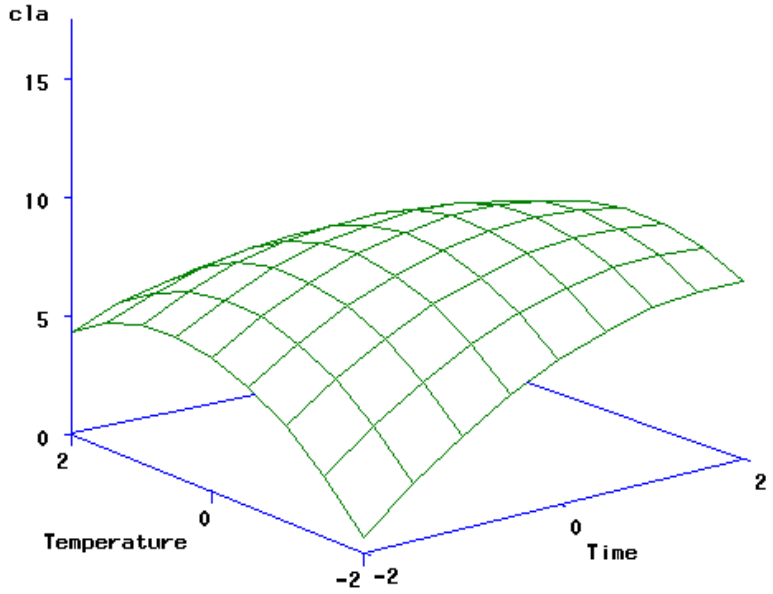

Figure 23- Response surface showing the effect of temperature and time with substrate ratio and enzyme load held constant at 5 and 20 (wt.%) respectively.

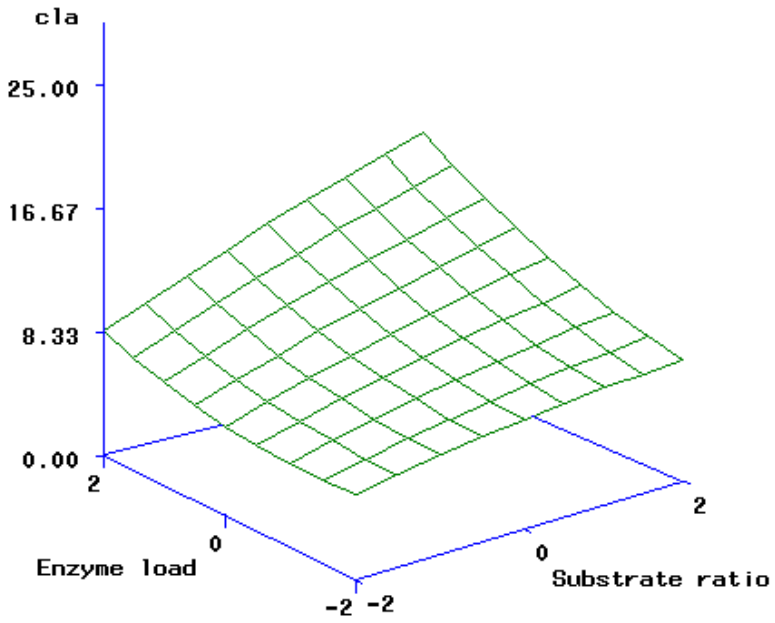

Figure 24- Response surface showing the effect of enzyme load and substrate ratio with time and temperature held constant at 50 h and 55^{o}C respectively.

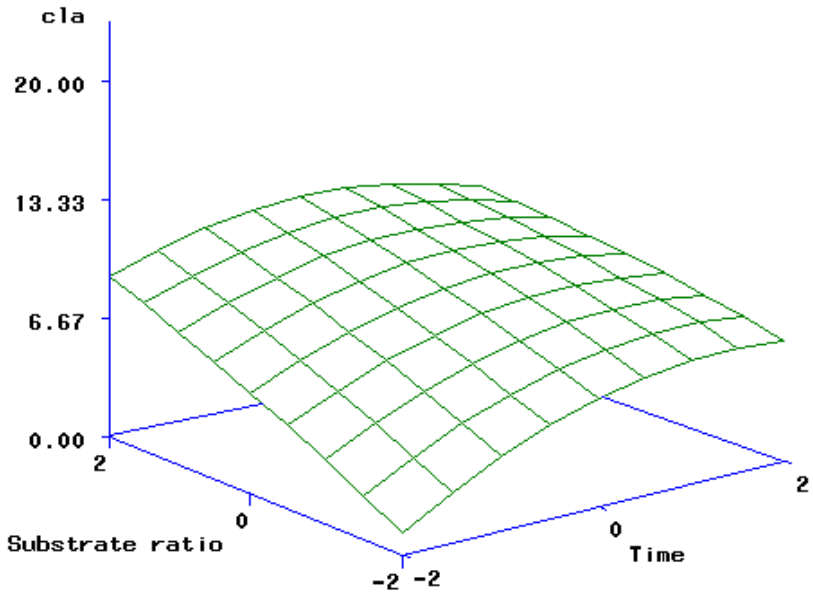

Figure 25- Response surface showing the effect of substrate ratio and time with enzyme load and temperature held constant at 20 (wt.%) and 55^{o}C respectively.

4.5 Applications of newly synthesized structured phospholipids in foods and their physiological properties

The ultimate objective of this study was to produce structured phospholipids for use as nutaceuticals and functional food ingredients. Our initial efforts to use them in food system served to asses whether they can provide functional properties as well as bioactive properties in foods.

4.5.1 Emulsifying properties of structured phospholipids

There is an increasing interest in the use of phospholipids as natural emulsifier. Thus the utility of any structured phospholipids will depend upon its ability to act as an emulsifier. For this reason the emulsifying capacities of CLA containing structured phospholipids was tested. The materials were used in efforts to stabilize whey protein based emulsion during both heat and oxidative stability tests. Whey proteins are usually heat unstable. So, whey protein based emulsions need to be stabilized by a means before any heat treatment like retort processing. Structured phospholipids with two levels of CLA incorporation derived from two sources (egg yolk and soy PC) were compared for their emulsifying properties. Whey protein based emulsion with 16% CLA containing soy PC (PS 100) showed highest heat stability even after heating at 121^{o}C for 15 min (Figures 26 and 27). Heat stability was assessed based on the mean particle size distribution of emulsion before and after heating. Emulsion with CLA-PS 100 had smaller mean particle size distribution compare to others. The particle size distribution was similar before and after heating. But, emulsions with controls and CLA containing

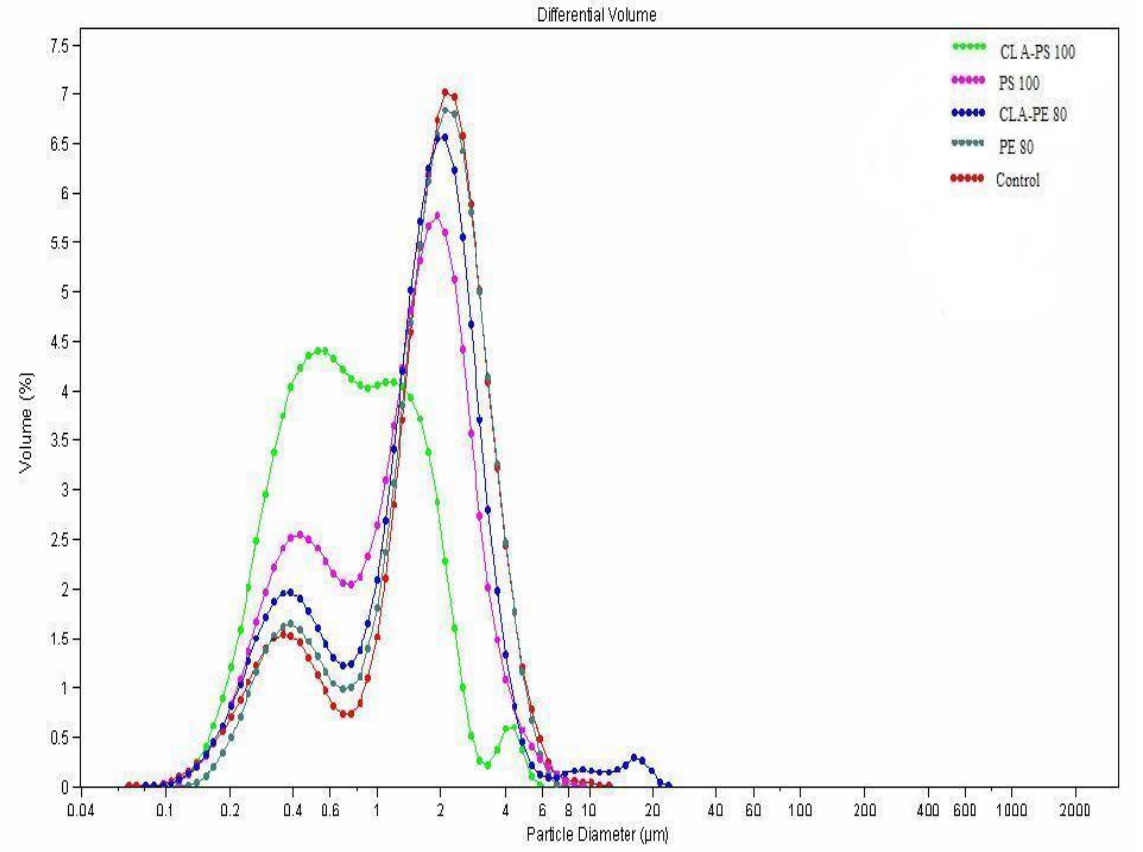

Figure 26- Particle size distribution of emulsions before heating. PE 80 –egg phospholipids (80% PC), PS 100- soy PC (94% PC), CLA-PE 80- CLA containing PE 80 (7% CLA), CLA-PS 100- CLA containing PS 100 (16% CLA), control- emulsion without phospholipids. Control, emulsion without any lecithin.

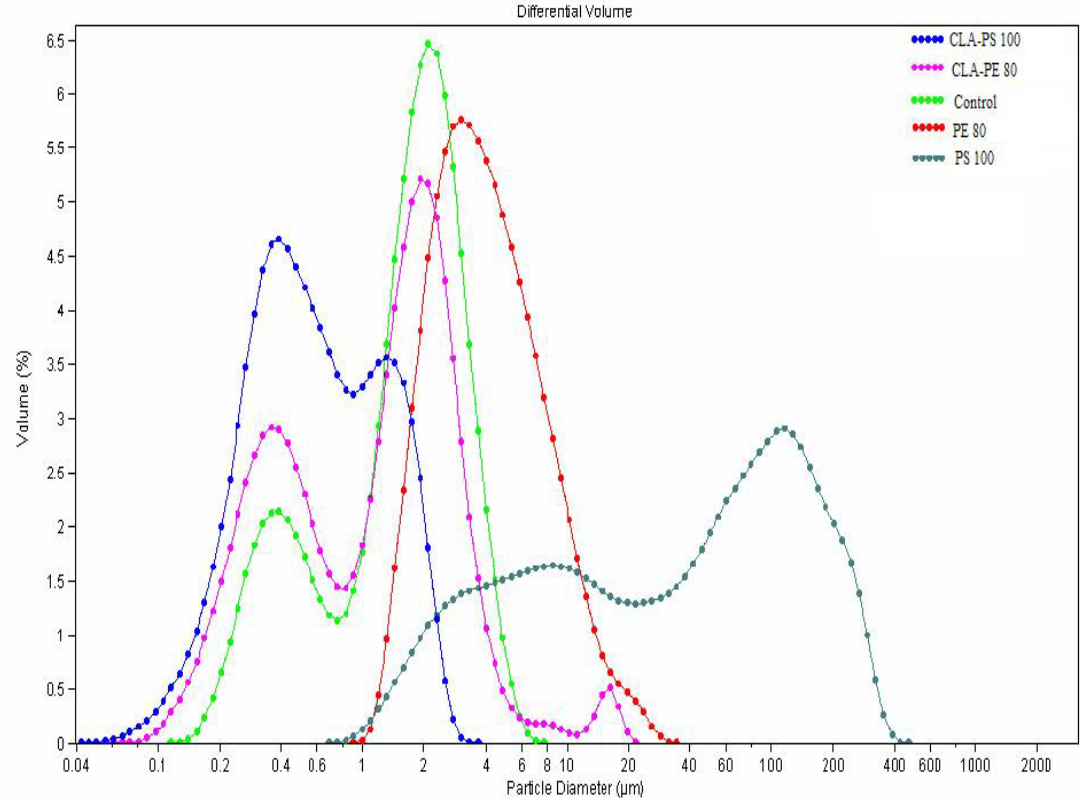

Figure 27- Particle size distribution of emulsions after heating. For abbreviations, see Figure 26.

egg PC (7% CLA) had higher mean particle size distribution which increased after heating because of disintegration of emulsions.

Oxidative stability was determined by measuring peroxide value (PV) and *p*-anisidine (*p*-AV) value of emulsions incubated at 50^{o}C on day 1, day 3 and day 7 after preparation. Emulsions with CLA –phospholipids from both egg yolk and soy PC had lower PV and *p*-AV values even after 7 days compared to their controls (Figures 28 and 29). Values are about 4 times lower than their corresponding controls.

4.5.2 Physiological properties of CLA containing structured phospholipids

As numerous physiological properties have been attributed to CLA, we studied the anticarcinogenic properties of CLA containing structured phospholipids from two sources (egg yolk and soy PC) in tumor cells. Table 18 shows some preliminary results with melanoma skin tumor cells. We compared the CLA isomers as free fatty acids in Caco-2 cancer cells (Table 19). Table 18 shows that level of CLA incorporated into soy phospholipids (94% PC) has direct effect on cell suppression. The cell suppression was higher for the higher rate of CLA incorporation. Source of phospholipid with similar CLA incorporation has some effect in terms of the dose of test sample. More research is needed to confirm this result. The cell suppression was defined as the difference between the final count of viable cells after incubating with test samples and the corresponding seeding population before incubation.

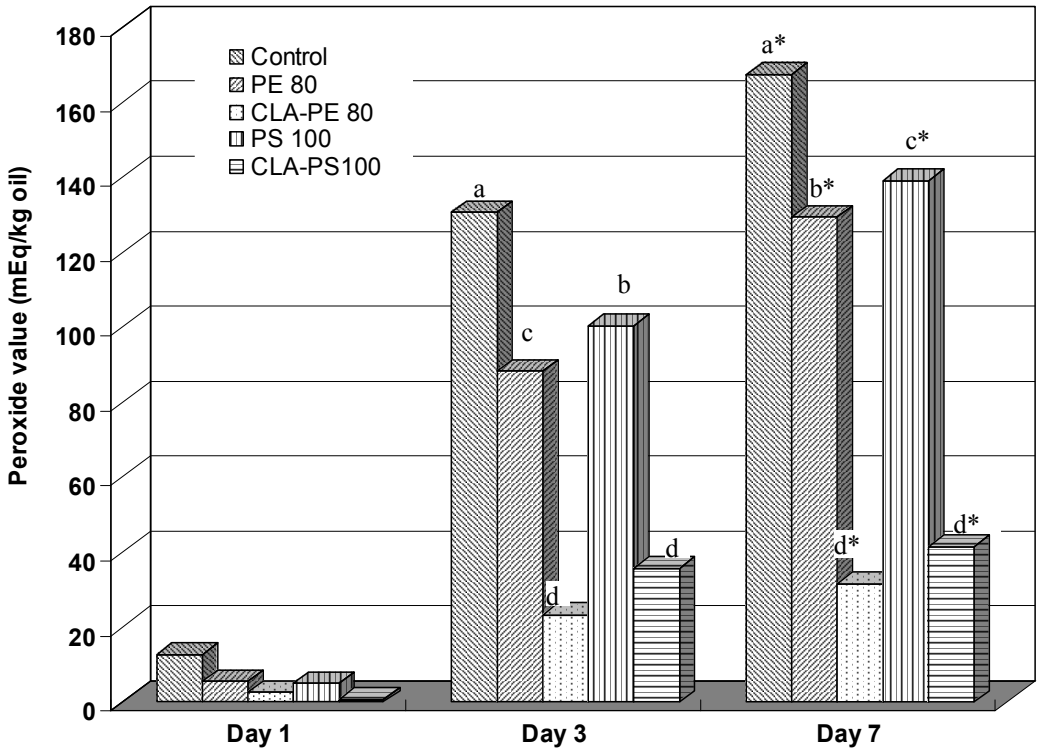

Figure 28- Oxidative stability (peroxide value) of emulsion. Incubation at 50^{o}C for day 1 , day 3, day 7. PE 80 –egg phospholipids (80% PC), PS 100- soy PC (94% PC), CLA-PE 80- CLA containing PE 80 (7% CLA), CLA-PS 100- CLA containing PS 100 (16% CLA), control- emulsion without phospholipids. Bars with similar letter are not statistically significant ($\alpha = 0.05$).

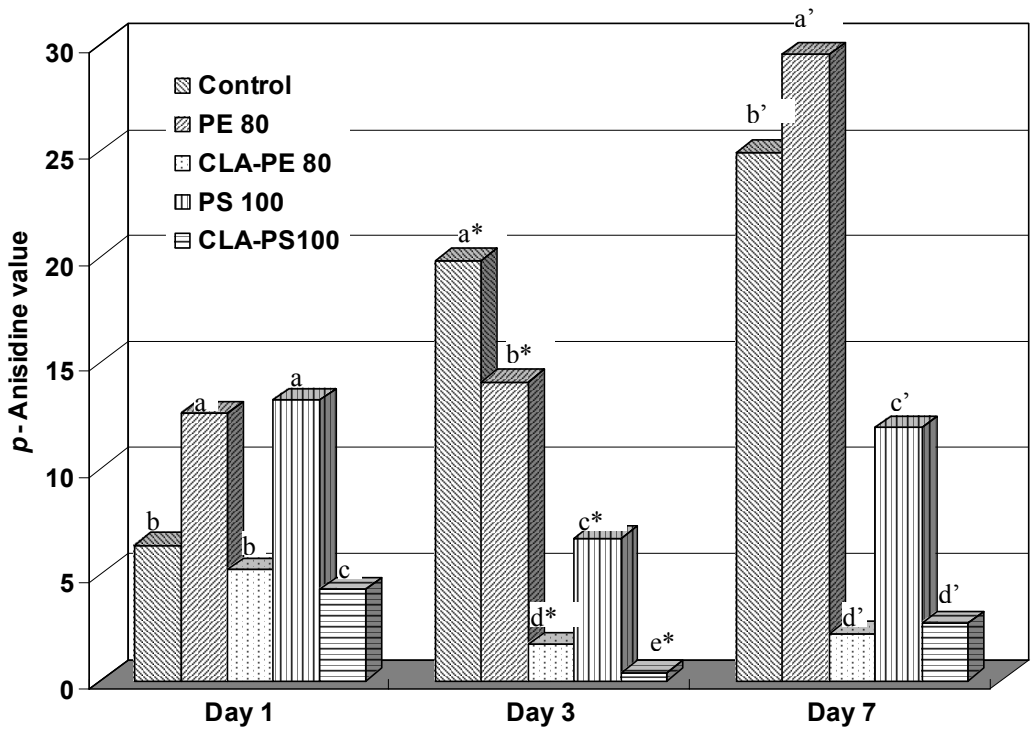

Figure 29- Oxidative stability (*p*-anisidine value) of emulsion. Incubation at 50°C for day 1 , day 3, day 7. PE 80 –egg phospholipids (80% PC), PS 100- soy PC (94% PC), CLA-PE 80- CLA containing PE 80 (7% CLA), CLA-PS 100- CLA containing PS 100 (16% CLA), control- emulsion without phospholipids. Bars with similar letter are not statistically significant ($\alpha = 0.05$). For abbreviations- see Figure 26.

Table 18- Suppression of murine B16 melanoma skin tumor cells by CLA-PL

Samples	Phospholipid source	*c*9,*t*11- CLA + *t*10,*c*12- CLA	% control growth (mean ± SD, n=4)
Solvent		-	100 ± 3
CLA-PS100, 0.25 mM	Soybean PC	4.0%	95 ± 7
CLA-PE80, 0.15 mM	Egg yolk	3.6%	84 ± 5
CLA-PS100, 0.06 mM	Soybean PC	16.0%	45 ± 2

For abbreviations: see Figure 26

Table 19- Suppression of Caco-2 colon cancer cells by CLA isomers

Samples	*c*9,*t*11- CLA + *t*10,*c*12- CLA	*% control growth* (mean ±SD, n=4)
Solvent	-	100 ± 3
CLA isomers, 0.1% (35% c9t11, 35% t10c12)	70%	38 ± 2

For abbreviations: see Figure 26

4.6 Summary

Enzymatic processes proved an effective technique to modify fatty acid composition and head group of phospholipids. Lipase catalyzed acidolysis reaction successfully incorporated CLA into egg yolk and soy phospholipids. Among the four lipases, Lipozyme RM IM, Lipozyme TL IM, Novozym 435, and Lipase F-AP15 and an immobilized phospholipase A_2 tested, only Lipozyme TL IM and Lipozyme RM IM showed higher CLA incorporation. From commercial point of view, Lipozyme TL IM will be the best choice for incorporation of CLA into phospholipids. The cost of Lipozyme TL IM is lower than others as low cost silica based materials are used for immobilization. The different classes of phospholipids had different rates of incorporation. The rate of incorporation is much higher in phospholipids with high level of PC and lowest in phospholipids with lowlevel od PC. CLA containing lyso-phospholipids were formed due to hydrolysis reaction and possibly acyl migration. Positional specificity (1,3 position specific) of lipase was retained as CLA was preferentially incorporated in *sn*-1 position of phospholipids. Two major bioactive isomers of CLA were equally incorporated into phospholipids. Polynomial regression equation using response surface method satisfactorily predicted the rate of incorporation of CLA against the actual experimental values (R^2 0.85) with regard to enzyme dosage, substrate ratio, reaction time and temperature. The best operating conditions to achieve the highest rate of incorporation catalyzed by Lipozyme TL IM are as 30% enzyme load, 6 (mol CLA/mol PL) substrate ratio, 60 h reaction time and temperature 55^{o}C. CLA containing structured phospholipids can provide higher heat stability and oxidative

stability of food emulsion systems. Also, CLA incorporated phospholipids may offer versatile dietary source of CLA with higher chemo preventive effects specially in anticarcinogenesis

CHAPTER V

LITERATURE REVIEW: SYNTHESIS OF STRUCTURED PHOSPHOLIPIDS WITH PLANT STEROLS

5.1 Sterols

Sterols are essential compounds for all eukaryotes. Mammalian cells generally contain one major sterol, cholesterol; but mixtures of sterols are always present in plants (Yeagle 1985; Hartmann 1998). So far, over 200 different plant sterol molecules have been found in nature. Sterols are cell membrane components that regulate the membrane fluidity and permeability and participate in the control of membrane-associated metabolic processes. Sterols are also precursors for several other compounds involved in important cellular and developmental processes in animals and higher plants. Sterol molecules consist of a planar tetracyclic skeleton, a hydroxyl group and a hydrophobic tail. Most of the sterol molecule is hydrophobic, but the polar hydroxyl group gives the molecule an amphiphilic character.

5.1.1 Cholesterol

Although cholesterol is essential for normal cell growth and function, a high concentration of cholesterol in serum and tissues can be harmful. Epidemiological, post mortem, and angiographic studies have established a casual relation between elevated serum cholesterol levels (>5 mmol/1) and genesis of atherosclerosis and thus cardiovascular diseases, which are the major cause of morbidity and mortality in western

societies (Levine and others 1995; Sullivan 2002). In addition to atherosclerotic lesions, elevated cholesterol levels play an important role in the genesis of cholesterol gallstones (Strasberg 1998). Cholesterol is practically insoluble in water and thus it is associated with different vehicles in the human body. Cholesterol is either solubilized in bile salt micelles or associated with different phospholipid vesicles or with lipoprotein particles as free cholesterol or as cholesterol fatty acids esters (Montet and Gerolami 1978; Staggers and others 1990; Mensink and others 1998). When the cholesterol concentration exceeds the dissolution capacity of these vehicles, cholesterol crystals may be deposited. Solid cholesterol crystals are found in gallstones (Admirand and Small 1968; Lundberg 1985; Small and Shipley 1974).

5.1.2 Plant sterols

Plant sterols are structurally related to cholesterol, and the most common plant sterols, β-sitosterol, campesterol and stigmasterol differ from cholesterol by an additional ethyl or methyl group and/or a double bond in side chain (Figure 30). Saturated plant stanols, such as β-sitostanol, are formed when the double bond is saturated. Although a typical dietary intake of plant sterols is almost equal to a dietary intake of cholesterol only small amounts of plant sterols are commonly detected in human plasma (Jones and others 1997). Serum β-sitosterol and campesterol levels are normally about 1/1000 and 2/1000 of that of cholesterol, respectively (Kuksis, 2001). Increased plasma levels of plant sterols can occur in individuals who suffer from sitosterolemia, which is an extremely rare inherited disorder caused by reduced plant

sterol excretion. The reduction of sterol excretion was caused by the mutations in two adjacent genes that encode ATP-binding cassette (ABC) transporter in patients with sitosterolemia (Berge and others 2000). In sitosterolemic patients, up to 30% of serum and bile sterols are plant sterols (Mietinen 1980). Similar to cholesterol, abnormally high plasma plant sterol concentration has been found to be atherogenic (Glueck and others 1991).

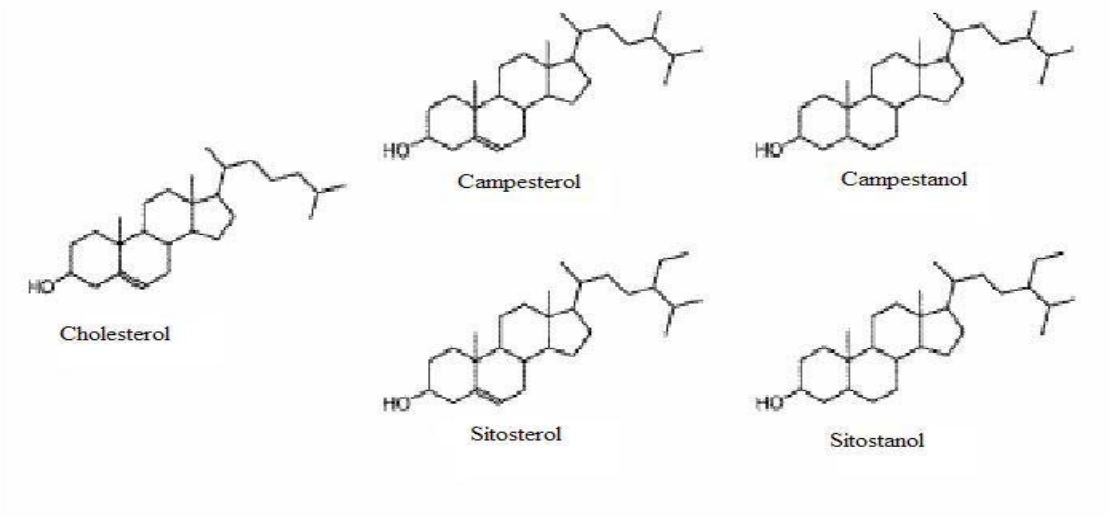

Figure 30- Structure of different sterols.

5.1.3 Cholesterol lowering effect of plant sterols

The recent interest in plant sterols, especially in β-sitosterol and its saturated form β-sitostanol, is due to their cholesterol lowering effect. The cholesterol lowering effect of plant sterols has been known since the 1950's, but poor solubility of these materials and discovery of more effective cholesterol lowering agents limited the interest in them. In the 1990's foods containing plant sterols and stanols as active ingredients came on the European market (Law 2000). The promising cholesterol-lowering results of these products have raised interest in plant sterols and stanols.

Free plant sterols and stanols are crystalline, insoluble in water with limited solubility in edible fats and oils (Jandacek and others 1977; Mattson and others 1977). Thus physico-chemical properties of free plant sterols and stanols limit their applicability in several preparations suitable for oral administration.

5.1.4 Cholesterol metabolism in human body

Cholesterol level in the human body is maintained by balancing cholesterol absorption, endogenous synthesis and excretion. Important regulatory mechanisms involve (1) intestinal cholesterol absorption, (2) hepatic cholesterol synthesis, (3) conversion of cholesterol to bile acids in the liver, (4) biliary secretion of free cholesterol and (5) fecal elimination of steroids of cholesterol origin (Kesäniemi and Miettinen, 1988).

5.1.4.1 Intestinal absorption

The average dietary intake of cholesterol in western countries is 300-500 mg per day (Sehayek and others 1998). In addition to dietary cholesterol, 800-1200 mg cholesterol is excreted daily from the biliary duct into the intestine. A variable proportion of dietary cholesterol is essential (on average 10-15%) esterified with fatty acids, but all biliary cholesterol is non-esterified and incorporated into bile salt-phospholipid micelles (Shen and others 2001). The efficacy of cholesterol absorption (percent cholesterol absorption) in normal healthy individuals is on average 50% of the ingested amount (Bosner and others 1999). Individual subjects have marked differences in cholesterol absorption (ranging from 29% to 80%), but variability in repeated cholesterol absorption studies in the same individual is small (Bosner and others 1993; Bosner and others 1999).

A decrease in the percent cholesterol absorption with increasing dietary cholesterol has been observed in both animal and human studies (Ellegård and Bosaeus, 1994; Sehayek and others 1998; Ostlund and others 1999a). Increased dietary cholesterol intake increases biliary cholesterol concentration, which decreases the absorption of dietary cholesterol. The saturation of cholesterol absorption by either increased dietary cholesterol ingestion or increased excretion of biliary cholesterol into the intestine occurs by saturation of solubilization capacity of the mixed micelles (Sehayek and others 1998).

5.1.4.2 Endogenous synthesis

The main site of cholesterol synthesis is the liver (Stryer 1995; Li and Parish 1998). Appreciable amounts are also synthesised by the intestine. In addition, other tissues are able to synthesize cholesterol, but the primary cholesterol source of tissues other than the liver and intestine is plasma LDL cholesterol particles. The rate-limiting enzyme in the cholesterol biosynthesis is 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoA reductase).

The rate of cholesterol synthesis in the liver is highly dependent on the amount of cholesterol absorbed in the intestine (Kesäniemi and Grundy 1984; Kesäniemi and Miettinen 1988). Inhibition of cholesterol absorption will decrease the quantity of cholesterol returning to the liver with chylomicron remnants. As a result of the reduction of liver cholesterol, a compensatory increase in the synthesis of LDL receptors takes place. Consequently, the uptake of LDL from circulation is enhanced and a decrease in serum LDL and total cholesterol is observed. On the contrary, overloading of the liver cells with cholesterol results in reduction of hepatic cholesterol synthesis regulated by the HMG-CoA reductase enzyme. The activity of hepatic LDL receptors decreases and receptor mediated removal of LDL from plasma lowered which results in increased plasma LDL levels (Kesäniemi and Miettinen 1988). This feedback regulation is mediated by changes in the activity of HMG-CoA reductase. On the other hand, low hepatic cholesterol and bile acid synthesis and/or diminished biliary cholesterol secretion leads to a lower intestinal load of cholesterol and thus enhances the intestinal absorption of cholesterol and vice versa.

5.1.5 Proposed cholesterol lowering mechanism of plant sterols

It was assumed that the serum cholesterol lowering effect of plant sterols resulted from the inhibition of cholesterol absorption from the intestinal lumen (Moghadasian and Frohlich, 1999). The typical dietary intake of plant sterols is almost equal to the dietary intake of cholesterol (Ling and Jones 1995). In addition to cholesterol from dietary sources, two thirds of the cholesterol present in the intestinal lumen is endogenous cholesterol from bile (Wilson and Rudel 1994). When effective doses (1-3 g/day) of *β*-sitosterol or plant sterols mixtures are consumed, the ratio of cholesterol to plant sterol entering the intestinal lumen is about 1:1. Besides inhibiting the absorption of cholesterol, plant sterols affect enzymes involved in cholesterol metabolism (Ling and Jones 1995; Moghadasian and Frohlich 1999). The actual mechanism by which the plant sterols and stanols reduce cholesterol absorption has not been confirmed. Several molecular mechanisms of phytosterol action on serum cholesterol level are believed to be of particular importance (Trautwein and others 2003).

5.1.5.1 Mixed crystal formation of *β*-sitosterol and cholesterol

It was observed that cholesterol and *β*-sitosterol formed a mixed crystal from which the two components could not be separated by recrystallisation (Pollak 1953). Davis (1955) observed that cholesterol and *β*-sitosterol formed a new crystal form when 1:1 mixture of the sterols was precipitated from methanol. The solubility of the new crystal in methanol was considerably lower than that of cholesterol but higher than that of *β*-sitosterol. Davis concluded that the conversion of cholesterol in the intestine into a

less dispersible form by the formation of mixed crystal with sitosterol might be the primary mechanism reducing cholesterol absorption. A later in vitro study confirmed that cholesterol and β-sitosterol form a solid solution at 1:1 molar ratio when crystallized from either methanol or ethanol (Karpuj and others 1982).

5.1.5.2 Restriction of the solubility of cholesterol in the intestinal micellar phases

According to the mechanism presented by Mattson and others (1982), absorption of cholesterol is determined by the total sterol concentration (cholesterol plus plant sterols) in the intestinal fat. It is thought that β-sitosterol reduces the solubility of cholesterol in oil presumably by competing for solubility sites (Wright and Presberg 1964). When the sterol solubility in oil is exceeded, solid sterols crystallize and precipitates are not absorbed. The composition of the precipitated sterols in the intestine reflects that of the oil phase. In the lumen of the intestine, sterols are distributed between micellar and oil phases. The surface activity of the free sterols causes them to accumulate at the oil/water interface. At the interface, the transfer of the sterols to the micellar phase and their crystallization are facilitated. Thus, high intake of plant sterol increases the fecal excretion of neutral sterols (Westrate and others 1999).

According to Armstrong and Carey (1987), the binding of β-sitosterol to tri-hydroxy bile salt micelles is energetically favored compared with cholesterol. Both β-sitosterol and β-sitostanol decreased the micellar solubility of cholesterol and reduce the concentration of cholesterol in mixed bile salt micelles, both in vitro and in vivo (Ikeda and Sugano 1983; Ikeda and others 1988; Ikeda and others 1989). In general, the

competition for the micellar solubilization capacity of bile salts may regulate the absorption of different sterols in the intestinal lumen. For example, plant stanols prevent the absorption of plant sterols in addition to cholesterol, resulting in a lowering of the respective serum levels of sterols, but even a twofold increase in serum plant stanol levels is observed (Gylling and others 1999a; Gylling and Miettinen 1999; Hallikainen and others 2000a).

5.1.5.3 Competitive inhibition of cholesterol uptake by intestinal mucosal cells

The early studies suggested that *β*-sitosterol could restrict the absorption of cholesterol by competitive inhibition of the uptake by intestinal mucosal cells (Glover and Green 1957). According to later studies, β-sitosterol does not affect the uptake of cholesterol from micellar solutions or the esterification of cholesterol by the mucosal cells (Ikeda and Sugano 1983; Field and others 1997).

5.1.5.4 Effects on cholesterol synthesis and metabolism

A feedback control of cholesterol synthesis is demonstrated to occur when cholesterol absorption is restricted by plant sterols or stanols (Grundy and others 1969; Gylling and others 1999b; Gylling and Miettinen, 1999). When the intestinal absorption of cholesterol is greatly reduced, a compensatory increase in the cholesterol synthesis is observed. Although intestinal mechanisms are widely accepted as responsible for cholesterol lowering actions of plant sterols, animal studies have suggested that intra-peritoneal and subcutaneous injections of *β*-sitosterol also lower circulating cholesterol

concentrations (Vanstone and others 2001). The mechanism by which β-sitosterol acts parenteral is not known. A potential mechanism is that β-sitosterol reduces cholesterol synthesis by affecting the activity of HMG-CoA reductase in vitro (Field and others 1997). In addition, consumption of added β-sitosterol increased the Lecithin-Cholesterol Acyl Transferase (LCAT) level in blood (Weisweller and others 1984). Conflicting results of the possible effects of plant sterols on enzymes responsible for conversion of cholesterol into bile acids (cholesterol-7α-hydroxylase) have been published (Ling and Jones 1995).

5.1.6 The effect of the dosage of plant sterols

Due to the possible mechanisms by which plant sterols are thought to inhibit cholesterol absorption, a uniform and intimate mixture of plant sterols with cholesterol in the intestine should be important. Thus, the preferred vehicle for plant sterols would be dietary fat, which is also a carrier for dietary cholesterol (Mattson and others 1982). Large doses of β-sitosterol, up to 3 g/day, are needed to achieve effective reduction in serum cholesterol levels (Hallikainen and others 2000b; Hendriks and others 1999). In addition to the large doses, the physical properties of free plant sterols and stanols limit their applicability in food and oral pharmaceuticals. The cholesterol absorption reducing effect of plant sterols seem to be dependent on the physical state of the plant sterols (Mattson and others 1982). The clinical trials that showed the largest effects have used plant sterols and stanols finely dispersed into foods during cooking or esterified with fatty acids and dissolved in vegetable oil (Table 20). Comparison between different

studies does not give reliable information on different dosage forms due to large differences in study conditions. Esterification of plant sterols increases the lipid solubility and thus provides a technically feasible way of introducing plant sterols into edible fats and oils (Wester, 2000). In theory, less soluble crystalline plant sterols enter the micellar phase less effectively than sterols released from fat-soluble sterol esters after their hydrolysis. In the intestine the plant sterol esters are hydrolysed and the resulting free sterols decrease the solubility of cholesterol in oil and micellar phases

Table 20-Examples of different dosage forms of plant sterols and stanols in clinical trials and the observed serum total cholesterol lowering effects[a]

Formulation	**Dose/ Day**	**Effect**
β-sitostanol oil suspention (25%) in capsules	3 g	No significant effect (3 months)
B-sitostanol partially dissolved and suspended in oil in capsules	1.5 g	15% (4 weeks)
Plant stanol suspended in food	1.5 g	15% (30 days)
Plant sterol dispersed (particle size 130 μm) in food	1.25-5.0 g	5% (15 weeks)
Plant stanol esters in margarine	2.6 g	10% (one year)
Plant sterol and stanol esters in margarine	0.8-3 g	3-11% (4 weeks)
Plant stanol esters in margarine	8.6 g	18% (21-28 days)

[a]Source: Christiansen (2002)

(Mattson and others 1977). Only a few comparative studies of free and esterified sterols have been performed. In an animal study with rats, no difference in the cholesterol lowering effects of free sterols and sterol esters was observed (Mattson and others 1977). On the contrary, in a human study where the effect of free β-sitosterol was compared to the effect of esterified β-sitosterol, the free sterols resulted in a statistically significant 9% greater (42% versus 33%) decrease in cholesterol absorption than did the sterol ester form (Mattson and others 1982). In that study, both the free and esterified sterols were dispersed in food. One possible explanation for the lesser effect of the ester form could be incomplete ester hydrolysis in the intestine.

In a preliminary human study, the efficacy of low dose β-sitostanol administered in lecithin micelles showed promising results compared to powdered β-sitostanol as it reduced cholesterol absorption by 37% compare to only 11% when lecithin was not used. (Ostlund and others 1999b). They suggested that naturally occurring phytosterols added with phospholipids might significantly reduce cholesterol absorption. The powdered β-sitostanol administered in capsules was ineffective in that study probably due to slow dissolution of solid stanol in bile. In another study sitostanol suspended in oil at a final concentration of 20% did not show any effect on LDL-cholesterol values during a 3-month trial with 3 g sitostanol / day (Denke 1995).

A preparation of submicron particles of β-sitosterol by precipitation in oil-in-water emulsion has been described (Sjöström and others 1993), but the cholesterol-lowering effect of these sub-micron particles has not been published to our knowledge. In a recent study, the cholesterol lowering effect of a pulverized (< 130 μm) plant sterol

preparation was rather modest in hypercholesterolemic subjects (Tikkanen and others 2001). Recently, a study was conducted on a novel hydrophilic phytostanol (FM-VP4) composed of sitosterol- and campesterol-ascorbyl-phosphate (Figure 31) provided by Forbes Medi-Tech Inc. (Ramaswamy and others 2002). This hydrophilic phytosterol has 15-fold greater cholesterol inhibition activity than free sterol.

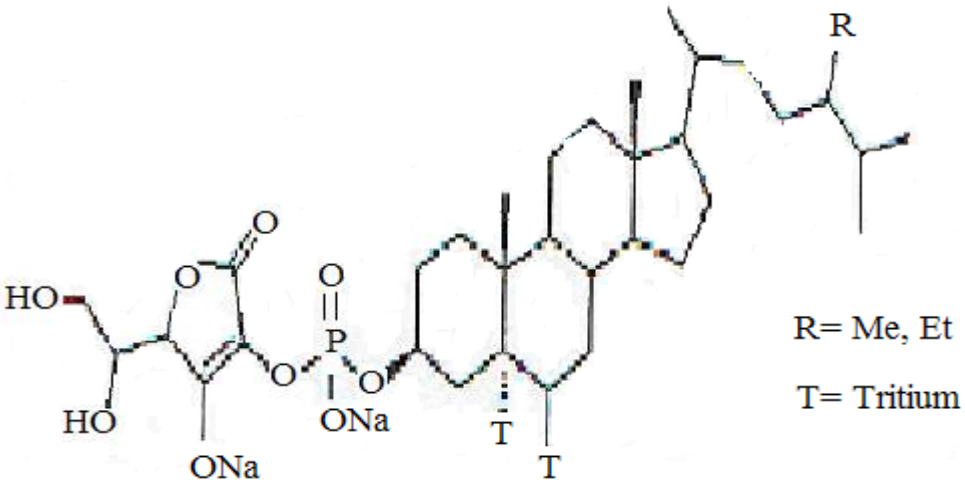

Figure 31-Chemical structure of a hydrophilic phytosterol composed of sitosterol- and campesterol-ascorbyl-phosphate (Ramaswamy and others 2002).

5.1.7 Safety of plant sterols

Harmful clinical or adverse chemical effects have not been observed in studies with human given plant sterols and stanols (Ayesh and others 1999; Plat and others 1999; Plat and Mensink 2000). In theory, the administration of plant sterols and stanols to reduce cholesterol absorption could simultaneously interfere with the absorption of fat-soluble vitamins. In practice, reduced serum carotenoid concentrations, but no reduced vitamin D and retinol concentrations or α-tocopherol/cholesterol proportion have been reported after the administration of plant sterols and stanols (Gylling and others 1999a; Gylling and Miettinen 1999; Hallikainen and others 1999; Hendriks and others 1999; Westrate and Meijer 1998).

Several plant sterols of dietary origin have been found in small quantities in plasma lipoproteins, human bile, and gallstones (Miettinen and others 1986). In healthy subjects, the *β*-sitosterol concentration in human serum is about 1/1000 of serum cholesterol concentration (Kuksis 2001). The consumption of effective doses of *β*-sitosterol (0.8-3 g/day) causes even a twofold increase in the serum *β*-sitosterol concentration (Westrate and Meijer 1998). Plant sterols are not recommended for normocholesterolemic children under 5 as they need large amount of cholesterol for normal development (Berger and others 2004). High serum plant sterol concentrations have been observed in patients suffering from sitosterolemia (Miettinen 1980). Very high plasma concentrations of *β*-sitosterol may have potential cytotoxic effects and may interfere with cellular functions (Moghadasian and Frohlich, 1999). Similar to cholesterol, high plasma plant sterol concentration has been found to be atherogenic

(Glueck and others 1991). According to animal studies, it has been suggested that high plasma concentrations of plant sterols may have adverse effects on reproductive organs (Malini and Vanithakumari 1990, 1991 and 1992; Mellanen and others 1990). In short term studies as well as in a long term study that lasted for 1 year, it has been concluded that plant sterols, within the range that causes desirable reduction of cholesterol, are clinically safe (Berger and others 2004).

CHAPER VI

MATERIALS AND METHODS: SYNTHESIS OF STRUCTURED PHOSPHOLIPIDS WITH PLANT STEROLS*

6.1 Materials

Soybean phosphatidyl choline (PC content not less than 94%) was donated by Lipoid GmBH (Ludwigshafen, Germany). *β*-sitosterol (65% purity) was obtained from Fluka Chemie AG (Buchs, Switzerland).The enzyme Phospholipase D (PLD) from *Streptomyces sp.*(Phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4) was donated by Ashahi Kasei Corporation (Tokyo, Japan). The activity of enzyme was determined by manufacturer as 305 U/mg. Solvents (chloroform and methanol), the matrix (2, 5-dihdroxybenzoic acid, DHB) and trifluoroacetic acid (TFA) were obtained from Sigma Chemical Company (St. Louis, MO).

6.2 Methods

6.2.1 Phospholipase D (PLD) catalyzed transphosphatidylation reaction

Transphosphatidylation reaction. The transphosphatidylation reaction between phosphatidyl choline (PC) and *β*-sitosterol was carried out according to the reaction scheme 2 (Figure 32). In a typical reaction, 20 mg of phospholipid and 104 mg of *β*-sitosterol (1:10 mol PL/mol sterol) were dissolved in 2 ml of chloroform (HPLC grade)

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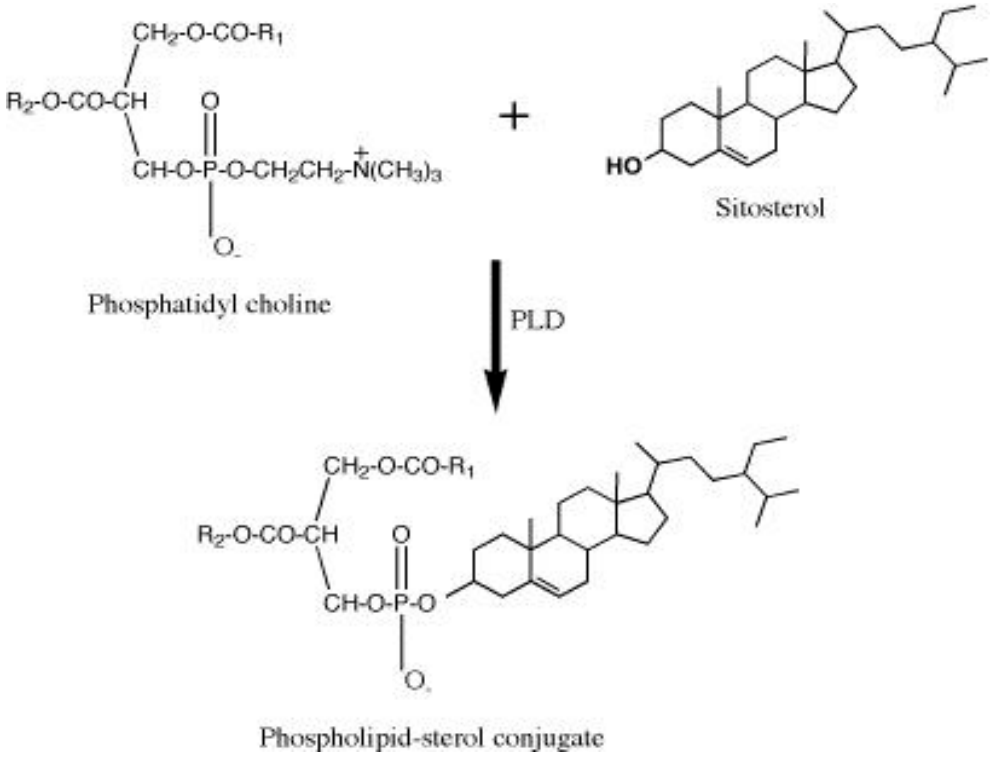

Figure 32- Reaction scheme 2. Phospholipase D (PLD) catalyzed transphosphatidylation reaction between phosphatidylcholine and sterol.

and 100 µl of 0.2M sodium acetate buffer (pH 5.6) containing 40 mM of $CaCl_2$ and 20 units of PLDP (EC 3.1.4.4) and incubated at 40^{o}C with stirring. 50 µl aliquots drawn from the reaction mixture at different time intervals were extracted with 100 µl of chloroform/methanol (3:1, by vol.) after adding 50µl of 0.01 N HCl. The lower organic layer was separated and further extracted with chloroform and analyzed by TLC analysis described above. To confirm the identity of phosphatidyl-sitosterol derivative, a similar reaction was carried out between 1,2-dipalmitoyl-sn-glycero-3-phosphorylcholine

(DPPC, MW 734.1) obtained from Matreya, Inc. (PA, USA) and β-sitosterol (MW 414.7). Reaction aliquots collected in chloroform were analyzed by matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS).

TLC analysis. Aliquots collected in chloroform were applied to TLC plates. The plates were developed with chloroform/methanol/water (65:25:4 by vol.). Eluted compounds were detected by spraying 5% phosphomolybdic acid in ethanol followed by heating.

Time course analysis for the synthesis of PC-sterol. Fatty acid analysis of individual bands (PC, PC-sterol and PLD-hydrolysis product) on TLC plates was used to quantify the reaction products with time. Preparative TLC followed by GC was used for this purpose. About 80 µl of aliquots collected in chloroform was applied on (10cm × 10 cm) TLC plates and developed as described before. Individual bands were scraped from the plates and fatty acid methyl esters were prepared by adding 1.0 ml of 0.25 M sodium methoxide in methanol/diethylether (1:1) as mentioned before. After incubation for 5 min in a water bath shaker at 45^{o}C, 200µl of hexane was added followed by 3 ml of saturated NaCl solution. After vortexing and centrifugation methyl esters extracted in hexane were collected from upper layer and 1 µl of methyl esters with an internal standard (methyl behenate, C22:0 from Nu Chek-Prep, Inc., MN, USA) was injected into GC. The initial column oven temperature was 150^{o}C for 3 min and then raised to 210^{o}C at 6^{o}C/min and held for 15 min. The injector and detector temperatures were 250^{o}C and 300^{o}C respectively. The hydrogen was used as carrier gas. The amount of fatty acids (mol) in each bands were converted into reaction products (mol) after dividing them by

two. The conversion of PC-sterol was calculated as mol% based on their relative amount in total phosphatidyl compounds.

Spectral analysis. To confirm product identities, mass spectra were analyzed with a MALDI-TOF mass spectrometer (Voyager, PerSeptive Biosystems, Framingham, MA, USA) in a linear mode. This system utilizes a pulsed nitrogen laser, emitting at 337 nm. The extraction voltage was set at 20 kV and 100 single laser 'shots' were averaged for each mass spectrum. For analysis, all samples were dissolved in 0.5 M DHB matrix solution in methanol containing 1% trifluroacetic acid. Samples in chloroform (10 μl of aliquots) were premixed with the matrix (10 μl) and the sample/matrix mixtures (1.2 μl) were directly put on the MALDI-TOF sample plate. Samples were crystallized in air without any flow before any spectra were acquired.

CHAPTER VII

RESULTS, DISCUSSIONS, AND SUMMARY: SYNTHESIS OF STRUCTURED PHOSPHOLIPIDS WITH PLANT STEROLS*

7.1 Phospholipase D-catalyzed synthesis of phospholipid-phytosterol conjugates

As a model reaction for monitoring the catalytic activity of PLD, transphosphatidylation reaction between glycerol and phosphatidylcholine was carried out for 8h according to the conditions mentioned in experimental procedures (Figure 33). Synthesis of phosphatidylglycerol (PG) was confirmed by TLC analysis of reaction mixture and standard PG obtained from Sigma (St. Louis, MO). Synthesized PG and standard PG had similar R_f value (Figure 34). The conversion of PC to PG was almost complete as no spot was visible at R_f value similar to standard PC. Enzymatic synthesis of PG from PC by transphosphatidylation reaction catalyzed by PLD from savoy cabbage has been reported by Juneja and others (1987). Several preparative transphosphatidylation reactions between phospholipids and nucleophile alcohols of complex structure have also been reported (Takami and others 1994; Rich and Khmelnitsky 2001). As PLD catalyzes transphosphatidylation of phosphatidylcholine with an acceptor, we proposed that similar reaction can be carried out between phosphatidylcholine and sterol (having a hydroxyl group) to synthesize novel

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phospholipid-sterol conjugates. We first examined the reaction between natural phosphatidylcholine and *β*-sitosterol by PLD (from *Streptomyces sp.*) for 24h. Samples in chloroform collected at different time intervals were put on TLC plates. A new spot having a lower R_f value than that of sitosterol but higher than that of phosphatidylcholine was observed on TLC (Figure 35). For structural analysis, similar reaction was carried out between DPPC and *β*-sitosterol for 72 h according to the similar reaction conditions mentioned in experimental procedures.

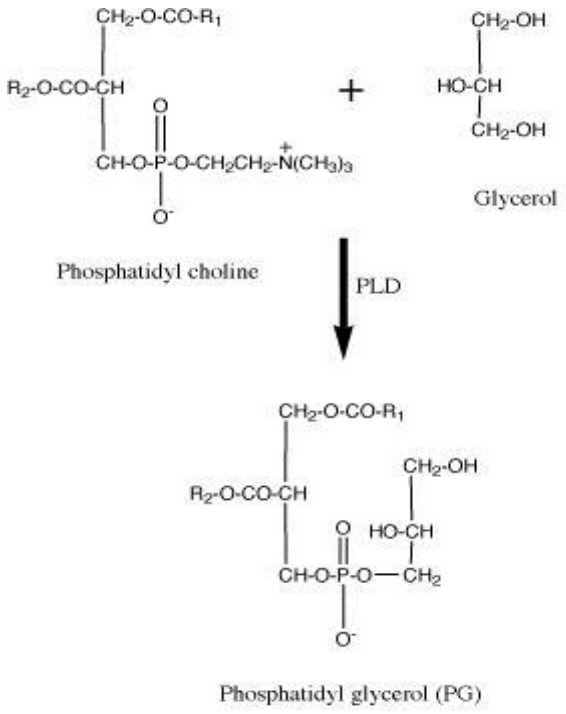

Figure 33- PLD-catalyzed transphospatidylation of PC into PG.

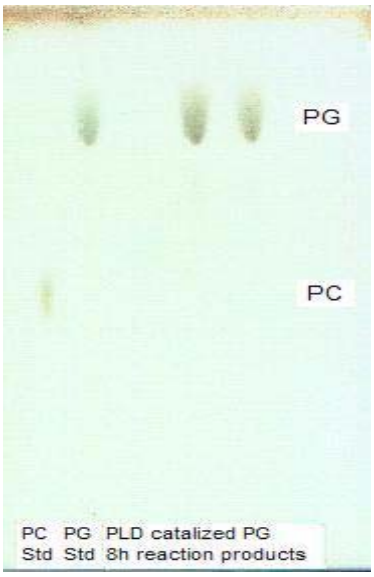

Figure 34- TLC analysis of PLD-catalyzed transphosphatidylation of PC into PG.

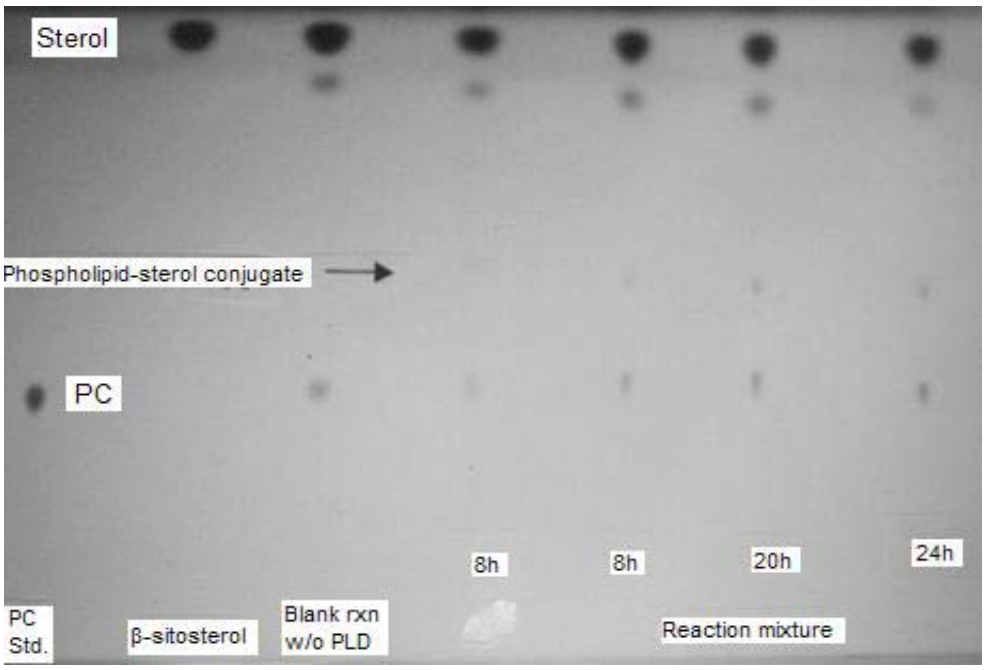

Figure 35- TLC analysis of PLD-catalyzed transphosphatidylation of PC into PC-sitosterol conjugate. Comparing with standard PC and *β*-sitosterol in all reaction products with time, the new unknown spots have R_f value higher than that of PC but lower than that of *β*-sitosterol.

The reaction products extracted in chloroform were isolated by silica gel (Wacogel C-200) column chromatography followed by TLC analysis (Figure 36). Fractions collected with chloroform were identified as sitosterol whereas factions collected with chloroform/methanol (24:1 by vol.) had the same R_f value as unknown spots observed on TLC analysis of reaction mixture (Figure 32). Fractions collected with chloroform/methanol (85/15 by vol.) were identified as PC. Mass spectral analyses of all fractions collected were carried out to confirm the structure of DPPC and *β*-sitosterol and identify the structure of phospholipid-sterol conjugate synthesized from DPPC and *β*-sitosterol. Mass spectral analysis of fractions collected with chloroform/methanol (24/1 by vol.) confirmed the structure of DPPC-sterol conjugate. The MALDI-TOF mass spectrum of this fraction showed a [M-C_2H_6] ion at *m/z* 1013, a [M-C_3H_8] ion at *m/z* 999, a [M-C_4H_{12}] ion at *m/z* 984, and a [M-C_7H_{17}] ion at *m/z* 942 because of the side chain fragmentation of sitosterol of DPPC-sterol conjugate (Figure 37). The time course analysis of the reaction mixture (Figure 38) indicated that the synthesis of PC-sterol was maximum at 12h and then gradually decreased with time. In parallel to transphosphatidylation reaction, hydrolysis of PC and PC-sterol was observed and increased with time after 12h. The amount of PC decreased with time and the rate was much slower after 24 h.

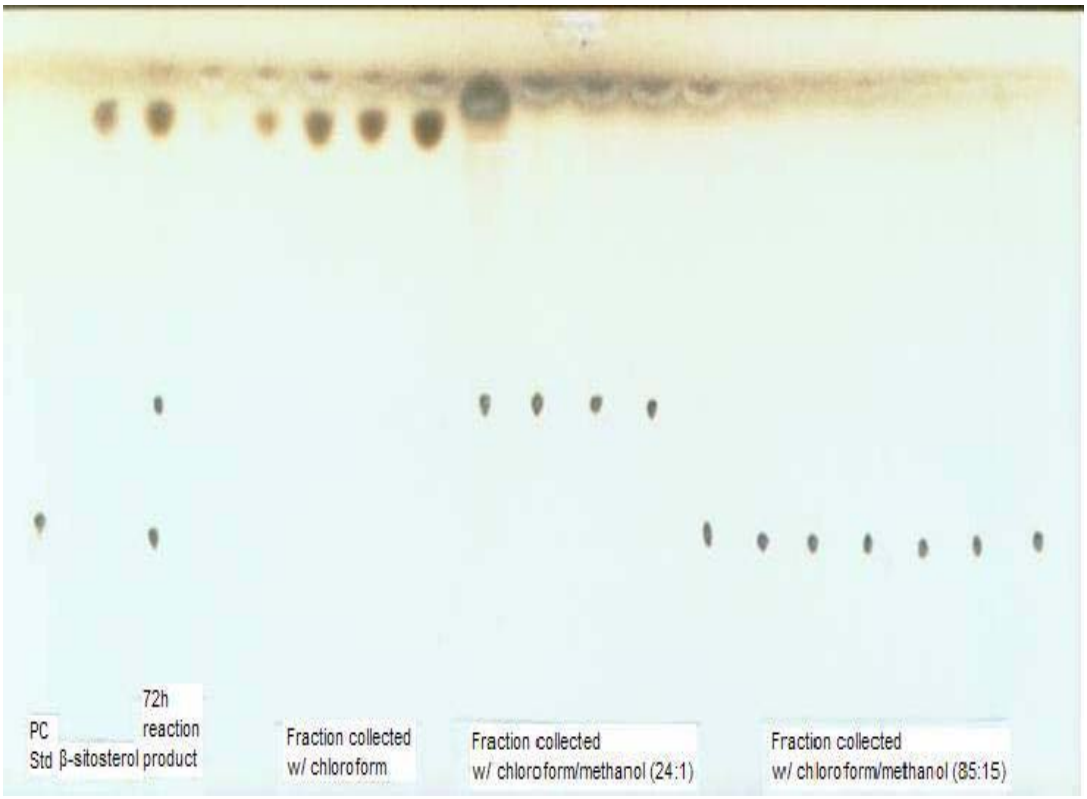

Figure 36- TLC analysis of fractions collected from reaction products of PLD-catalyzed transphosphatidylation of DPPC and β-sitosterol eluted from a silica gel column with gradient solvent. DPPC, 1,2-dipalmitoyl-sn-glycero-3-phosphorylcholine.

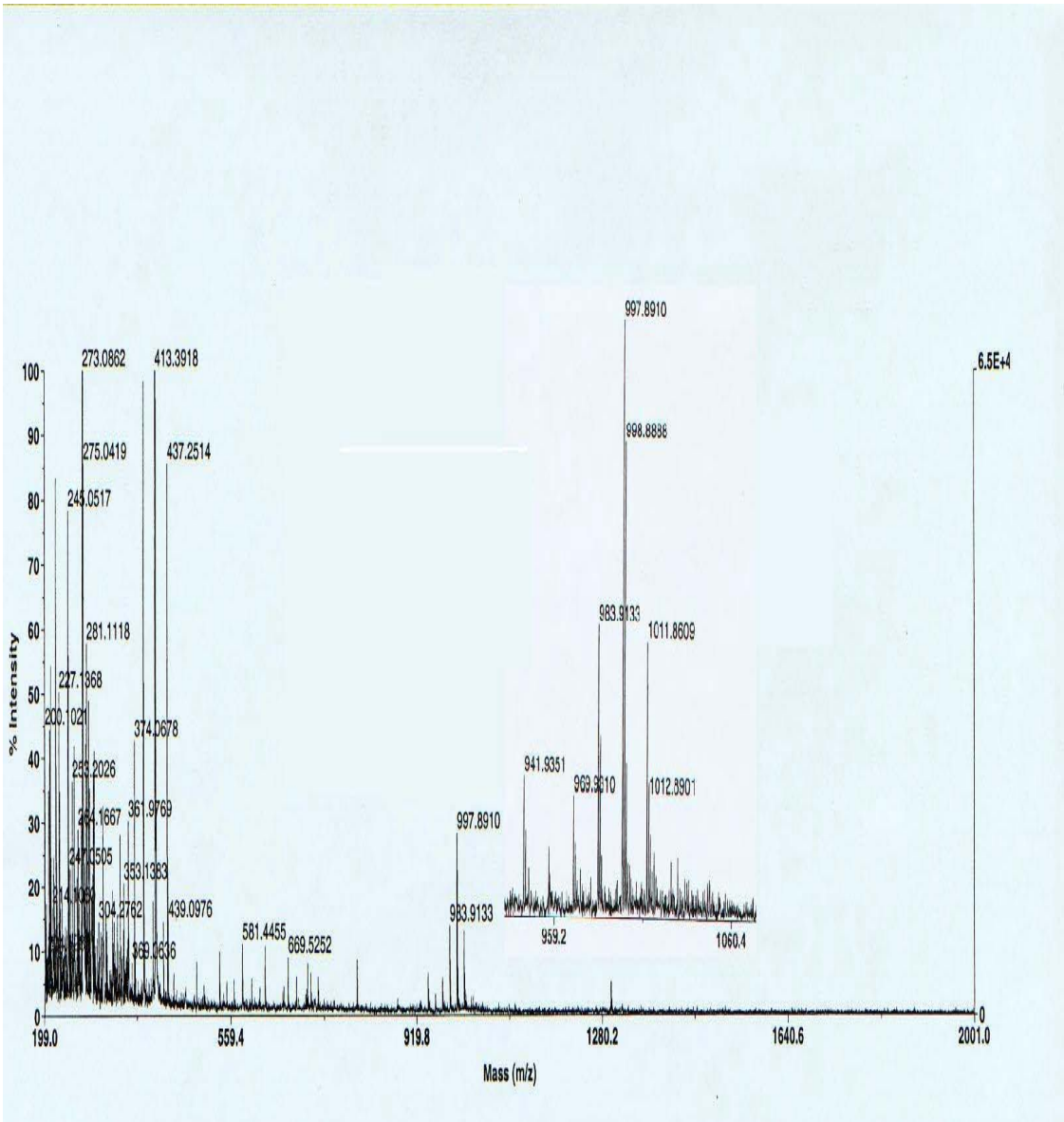

Figure 37- MALDI-TOF mass spectrum of dipalmityl PC-β-sitosterol.

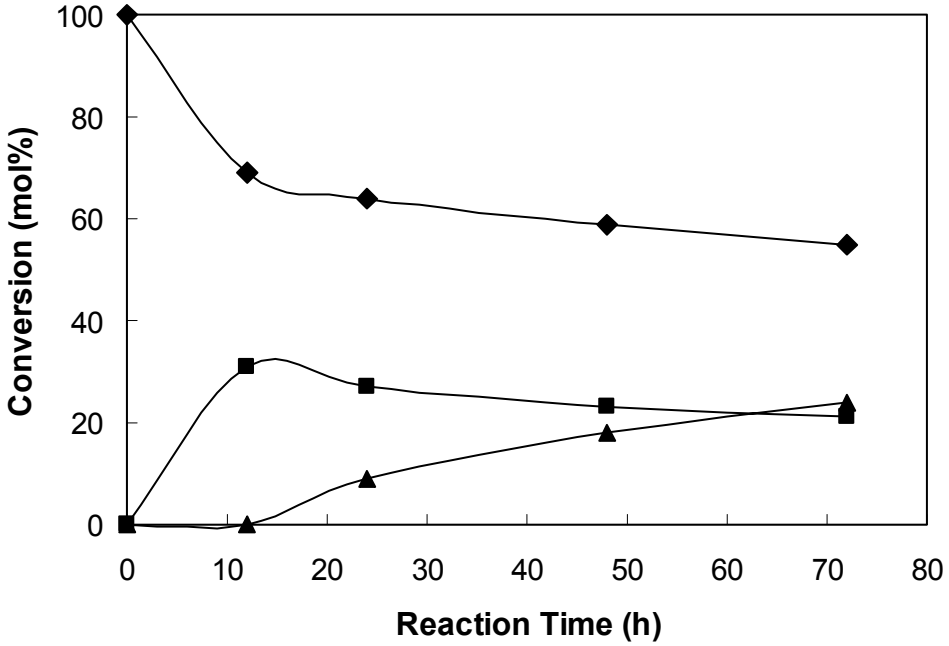

Figure 38- Time course analysis of transphosphatidylation reaction of PC and sterol. ♦, PC; ■, PC-sterol; ▲, hydrolysis products of phosphatidyl compounds.

7.2 Summary

Phosphatidyl-sterol (PC-sterol) conjugates were synthesized by the reaction of PC with sterol by PLD from *Streptomyces sp.* in a biphasic system of an organic solvent with acetate buffer. As it was observed from TLC analysis that the R_f value of phosphatidyl-sterol conjugate was less than that of sitosterol, phosphatidyl-sterol conjugates will have higher polarity compared to sterols. Such types of phospholipid-sterol conjugates have not yet been descried in the literature. There is a demand to improve efficacy of phytosterols and stanols to reduce total serum cholesterol levels by increasing their intestinal bioavailability. As more research need to be focused in this area. In our present study we hypothesize that conjugating phytosterols or stanols directly with phospholipids will increase their cholesterol lowering efficacy even more. Studies to test this hypothesis are required. As commercially available phytosterols, phytostanols and their fatty acid esters form are hydrophobic and limited to oil-based foods such as margarine, more research in line of our present study are needed to have amphiphillic phytosterols with improved food application and absorption aspects.

CHAPTER VIII

CONCLUSIONS

Enzymatic processes were an effective way to produce structured phospholipids. Among the four lipases from *Mucor miechei* (Lipozyme RM IM), *Thermomyces lanuginose* (Lipozyme TL IM), *Candida Antarctica* (Novozym 435), *Rhizopus oryzae* (Lipase F-AP15), and and phospholipase A_2 from *Porcine pancreas* (Lecitase 10 L), only Lipozyme RM IM and Lipozyme TL IM were effective in incorporation of CLA into PLs. The maximum incorporation of CLA achieved was 16% with soy phospholipids in 72 h. The class of phospholipids had a significant effect on the rate of incorporation of CLA compared to source of phospholipids. A three-level, four-factor Central Composite Rotatable Design (CCRD) was developed to predict the rate of incorporation of CLA into phospholipids . The four factors selected were lipase dosage (E_d, wt.% of substrate), substrate ratio (S_r,mol%), reaction time (t_i, h) and reaction temperature (T_e,^oC). The enzyme load and substrate ratio had a greater effect on the rate of incorporation than did reaction time and temperature. The new phosphatidyl derivatives, phosphatidyl-sitosterol, was found to be synthesized by the transfer reaction of phosphatidyl residue from phosphatidylcholine to β-sitosterol by Phospholipase D from *Streptomyces sp*. in biphasic medium. Plant sterols were modified to more polar lipid class by synthesizing phospholipid derivatives of them. When added to a whey protein based oil-in-water emulsion, CLA incorporated structured phospholipids (CLA-PL) had higher heat stability and oxidative stability compare to the controls. Because of phospholipid's uses as emulsifier, wetting agent, dispersing agent, baking stabilizer, and

liposome former, structured phospholipids with CLA and plant sterols would be unique for nutraceuticals and functional food formulations. As it is a challenge to establish the most effective but versatile delivery system of functional food ingredients to increase their bioavailability. Phospholipids with specific fatty acids and/ or polar head group would be efficient dietary fractions to deliver functional components.

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APPENDIX

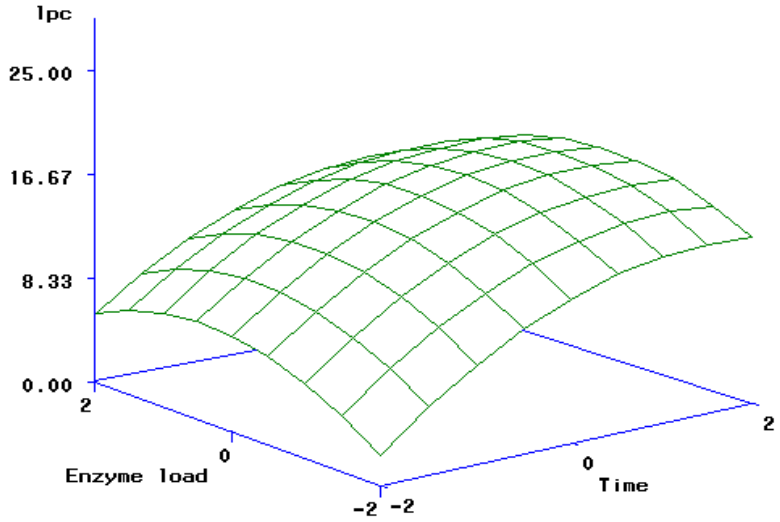

Figure A1- Response surface showing the effect of Enzyme load and time with temperature and substrate ratio held constant at 55°C and 5.0 respectively.

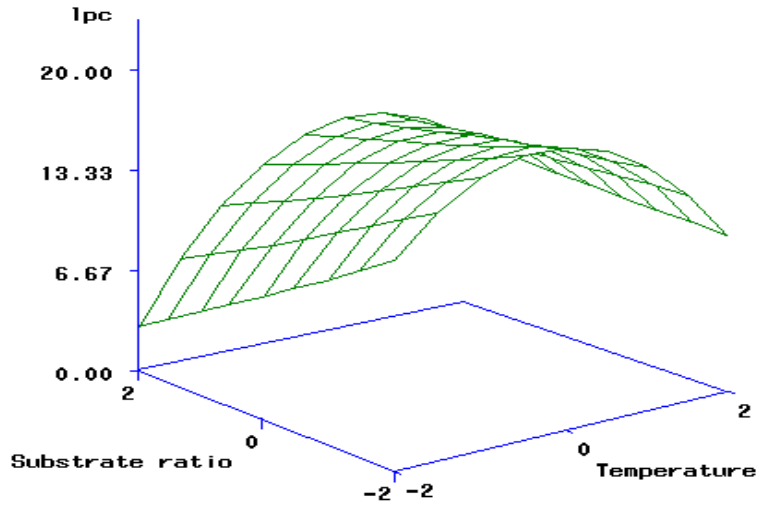

Figure A2- Response surface showing the effect of substrate ratio and temperature with enzyme load and time held constant at 55°C and 5.0 respectively.

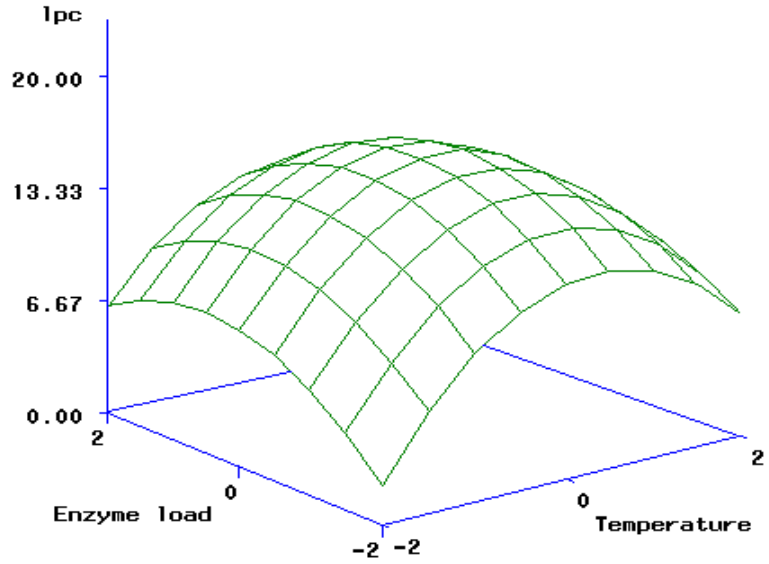

Figure A3- Response surface showing the effect of enzyme load and temperature with substrate ratio and time held constant at 5 and 50 h respectively.

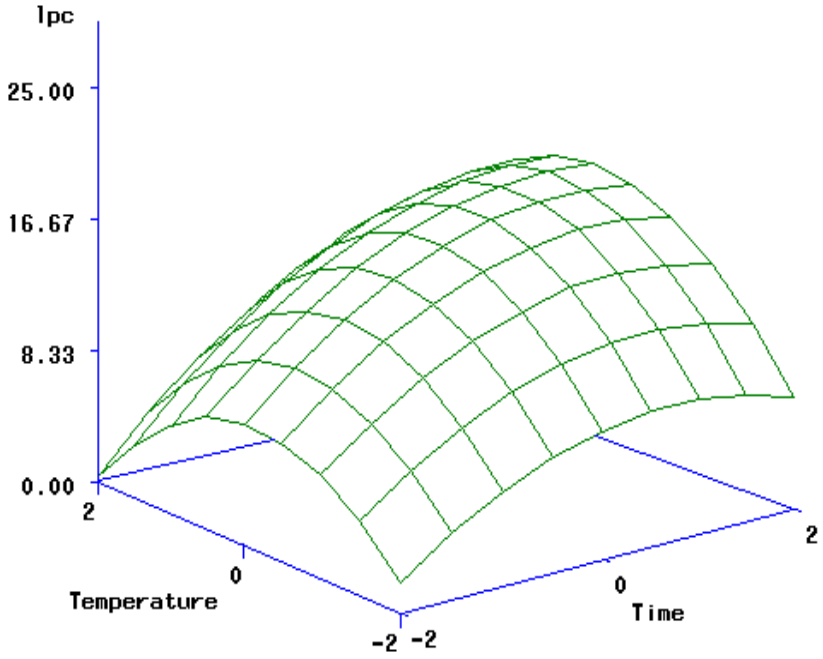

Figure A4- Response surface showing the effect of temperature and time with enzyme load and substrate ratio held constant at 20 (wt.%) and 5 respectively.

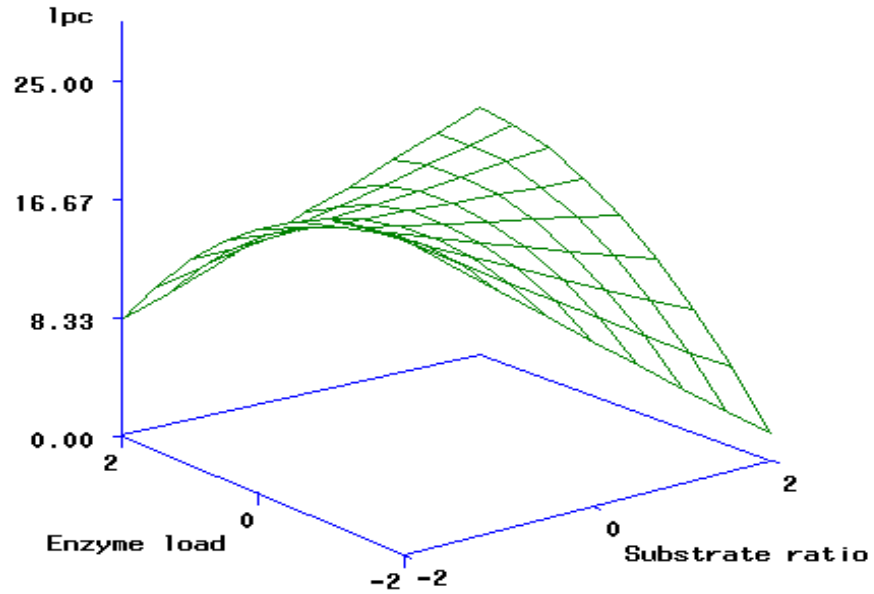

Figure A5- Response surface showing the effect of enzyme load and substrate ratio with time and temperature held constant at 50 h and 55oC respectively.

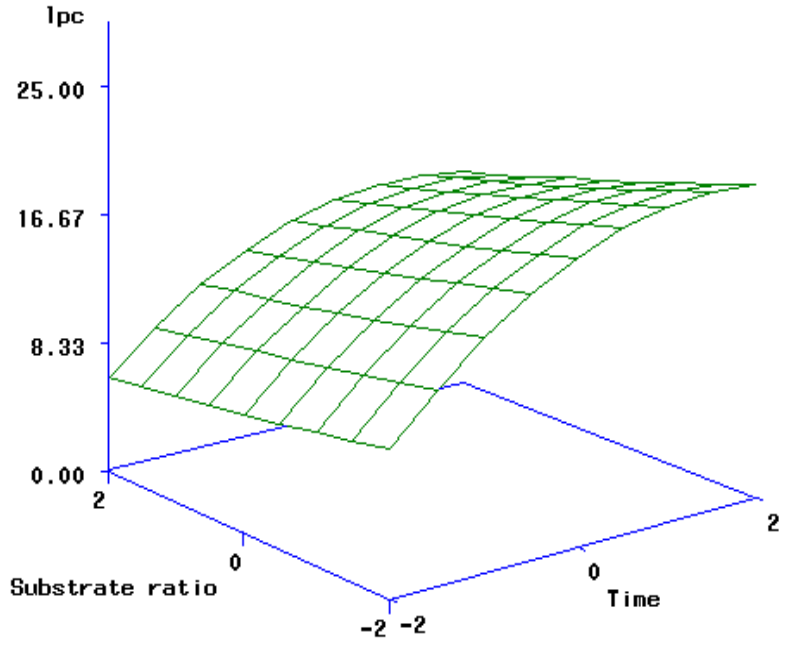

Figure A6- Response surface showing the effect of substrate ratio and time with enzyme load and temperature held constant at 20 (wt.%) and 55^{o}C respectively.

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