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Thylakoid Membranes and Pancreatic Lipase/Colipase

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Thylakoid Membranes and Pancreatic Lipase/Colipase

Thylakoid membranes retard digestion of fat and suppress appetite

Sinan Cem Emek

Doctoral Thesis
Department of Biochemistry and Structural Biology
2010

LUND UNIVERSITY

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To my family

Dokumentblad

Abbreviations

apo A-IV	Apolipoprotein A-IV
ATP	Adenosine triphosphate
BMI	Body mass index
CCK	Cholecystokinin
Chl	Chlorophyll
CL	Colipase
CNS	Central nervous system
DGDG	Digalactosyldiacylglycerol
EM	Electron microscopy
GI	Gastrointestinal
GLP-1	Glucagon-like-peptide-1
HF	High-fat diet
HFT	High-fat diet enriched with thylakoids
IES	Inter-envelope space
IO	Inner-envelope
Kd	Dissociation constant
LF	Low-fat diet
LHCII	Light harvesting complex II
LHCI	Light harvesting complex I
L	Lipase
MGDG	Monogalactosyldiacylglycerol
OE	Outer envelope
Oxm	Oxyntomodulin
PG	Phosphatidylglycerol
PL	Phospholipids
PM	Plasma membrane
PP	Pancreatic polypeptide
PSI	Photosystem I
PSII	Photosystem II
PTL	Pancreatic triglyceride lipase
RC	Reaction center
Rubisco	Ribulose-1,5-bisphosphate carboxylase oxygenase
SQDG	Sulfoquinovosyldiacylglycerol
TG	Triacylglycerols

List of Publications

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals. The papers are appended in the end of the thesis.

Paper I
Albertsson PÅ, Köhnke R, ***Emek SC***, Mei J, Rehfeld JF, Åkerlund HE, Erlanson-Albertsson C. *Chloroplast membranes retard fat digestion and induce satiety: effect of biological membranes on pancreatic lipase/co-lipase* Biochem J. 2007 Feb 1; 401(3):727-33. Erratum in: Biochem J. 2007 Nov 1; 407(3):471.

Paper II
Emek SC, Szilagyi A, Åkerlund HE, Albertsson PÅ, Köhnke R, Erlanson-Albertsson C. *A large scale method for preparation of plant thylakoids for use in body weight regulation*. Prep Biochem Biotechnol. 2010; 40(1):13-27.

Paper III
Emek SC, Åkerlund HE, Clausen M, Erlanson-Albertsson C, Albertsson PA. *Pigments protect the light harvesting proteins of chloroplast thylakoid membranes against digestion by gastro-intestinal proteases.* Submitted to Food Hydrocolloids 2010.

Paper IV
Rayner M, Ljusberg H, ***Emek SC,*** Sellman E, Erlanson-Albertsson C, Albertsson PÅ. *Chloroplast thylakoid membrane stabilized emulsions.* Journal of the Science of Food and Agriculture 2010 (accepted).

Paper V
Emek SC, Åkerlund HE, Erlanson-Albertsson C and Albertsson PÅ. *The pancreatic lipase/colipase complex binds strongly to the thylakoid membrane surface*. Manuscript, 2010.

Additional publications

- Köhnke R, Lindbo A, Larsson T, Lindqvist A, Rayner M, Emek SC, Albertsson PÅ, Rehfeld JF, Landin-Olsson M, Erlanson-Albertsson C. *Thylakoids promote release of the satiety hormone cholecystokinin while reducing insulin in healthy humans*.
  Scand J Gastroenterol. 2009; 44(6):712-9.

- Köhnke R, Lindqvist A, Göransson N, Emek SC, Albertsson PÅ, Rehfeld JF, Hultgårdh-Nilsson A, Erlanson-Albertsson C. *Thylakoids suppress appetite by increasing cholecystokinin resulting in lower food intake and body weight in high-fat fed mice*.
  Phytother Res. 2009; 23(12):1778-83.

- Clausén M, Huang S, Emek SC, Sjöholm I &Åkerlund HE. *Post harvest improvement of zeaxanthin content of vegetables*.
  J Food Engin. 2010; 98: 192-197.

List of Contributions

I	I performed the *in vitro* experiments, participated in the data evolution and writing the manuscript.
II	I performed the experiments, wrote the first draft of the manuscript.
III	I performed the experiments, wrote the first draft of manuscript.
IV	I performed partly the experiments, participated in the data evaluation and writing of the manuscript.
V	I performed the experiments, wrote the first draft of the manuscript

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Chapter I

1.1 Obesity

Human obesity is a complex, chronic disease involving environmental (social and cultural), genetic, physiologic, metabolic, behavioral, and psychological components. Obesity is defined as a condition of excess body fat, and it is associated with a large number of debilitating and life-threatening disorders, such as a major increase in cardiovascular, metabolic, and other diseases (Must et al. 1999). The prevalence of obesity is increasing worldwide at an alarming rate and is now a problem for both developed and developing countries. Because the direct measurement of body fat is difficult, the body mass index (BMI), a simple weight/length^2 ratio (kg/m^2), is typically used to classify overweight and obese adults. Obesity is defined as a BMI $\geq$ 30 kg/m^2 (WHO 2000).

Despite the fact that genetic mechanisms strongly influence the body weight regulation, recent studies clearly demonstrate the important role of the environment. Most individuals retain a relatively stable body weight, which is a result of energy intake and energy expenditure being in equilibrium. Weight gain is a result of a positive energy balance, where excess of energy is stored as body fat. This can be the result from increased energy intake, especially from energy-dense food like fat (Westerterp-Plantenga, 2001; Blundell et al. 2010) or decreased energy expenditure, as reduced physical activity (Westerterp, 2001). Excessive fat or carbohydrate intake and inactivity (long term watching TV/DVD and using computer, motorized transport etc.) are known to induce weight gain (Martinez 2000). The regulation of body weight depends upon 1) food intake 2) nutrient turnover and thermogenesis, and 3) body fat stores (Martinez et al. 1996), and the genetics involved in all of this (Bell et al. 2005).

Maintaining a stable body weight requires balancing food intake with energy expenditure. Information about recent food intake and digestion, communicated from the gastrointestinal (GI) tract and liver to several areas of the brain, has a strong influence on feeding behavior and overall energy balance (Moran 2000 and Friedman et al. 1999).

The health risks associated with obesity are considerable and this epidemic requires new strategies to reduce the substantial health effects linked to an increased body weight.

1.2 Medical treatment strategies for obesity

Reaching the ideal body weight is recommended but not often realistic, however, there is evidence that modest weight loss, in the order of 5-10% (Williamson et al. 1995 and 2000), is associated with clinically reductions in comorbidities, such as hypertension (Aucott et al. 2005) and diabetes risk (Toumilheto et al. 2001). Delays in treatment may increase the risk of future development of diabetes and its related complications, and of heart diseases.

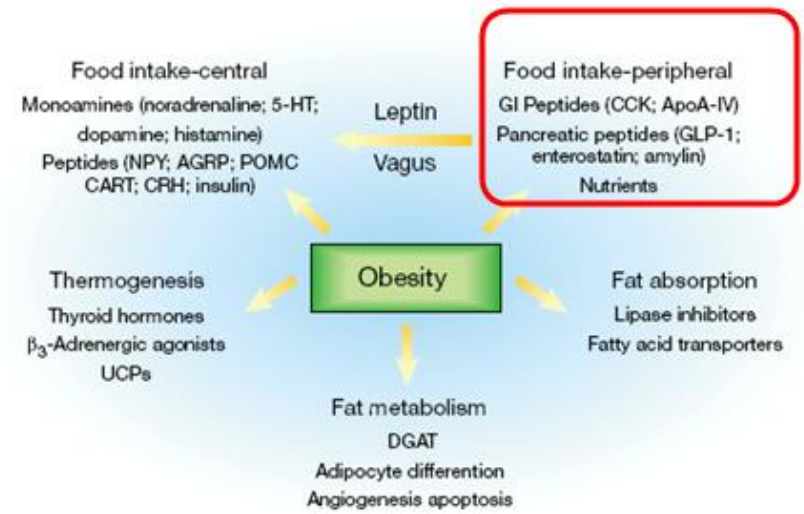


Figure 1. Main strategies for molecules targeted against obesity (Bray and Tartaglia 2000).

Different strategies used in the worldwide to treat obesity, include diet therapy, exercise, behavior modification, pharmacotherapy (figure 1), and surgery. In the pharmacotherapy, the drug should have a significant impact on the body weight ultimately affecting both energy intake and energy expenditure.

1.3 Gut hormones in the regulation of food intake

Hunger signals trigger eating, and satiety signals inhibit appetite for a period subsequently. The gastrointestinal tract is the largest endocrine organ in the body. Many of the hormones that are involved in the regulation of body weight via appetite and satiety are released from the gastrointestinal tract after entering of food. Ingested nutrients, especially long-chain free fatty acids, induce the satiety effect as long as they stay in the intestine (Beglinger and Degen 2004). The satiety effect of gastrointestinal peptides, as potential regulators, has been studied since the early 70´s;

Cholecystokinin (CCK)

Cholecystokinin was one of the first shown to reduce food intake and this satiety effect of CCK occurs to be alike both in animals and humans (Smith 1998). CCK is produced by mucosal endocrine cells (L-cells) in the upper small intestine and released postprandially. CCK exists in several bioactive forms, due to number of amino acids; CCK-8, CCK-22, CCK-33 and CCK-58, with the predominant form CCK-33 (Rehfeld et al. 2001), in human plasma and intestine. CCK is released from gastrointestinal tract, as a local action of digested fat and protein-rich food (Polak et al. 1975). It exerts various functions: prolongation of gastric emptying, appetite suppression, stimulation of gallbladder contraction and stimulation of exocrine pancreatic secretion. The peripheral administration of CCK to both rodents and humans decreases food intake by reducing meal size and duration (Kissileff et al. 1981).

Two CCK receptors, CCK-A and CCK-B, have been characterized (Moran et al. 1982 and Wank et al. 1992). Blockade of CCK-A receptors peripherally and CCK-B receptors in the central nervous system (CNS) increases energy intake (Ballinger et al. 1995; Dourish et al. 1989).

Glucagon-like-peptide-1 (GLP-1)

Food intake can also be reduced, both in animals (Holst 1999) and in humans (Flint et al. 1998), by glucagon and glucagon-like-peptide-1 (GLP-1). GLP-1 is synthesized by intestinal L-cells in two forms: GLP-$1_{1\text{-}37}$ and GLP-$1_{1\text{-}36\ \text{amide}}$. Biologically active fragments, GLP-$1_{7\text{-}37}$ and GLP-$1_{7\text{-}36\ \text{amide}}$, requires further cleavage at the N-terminus (Orskov et al. 1994). GLP-1 is released in response to food intake (Herrman et al. 1995), and produced by processing of the proglucagon gene both in the gut (Wettergren et al. 1994) and in the brain (Williams et al. 2001). Peripheral administration of GLP-1 in humans (Gutzwiller et al. 1999) and in rats (Turton et al. 1996) reduces food intake.

PYY

PYY is a 36-amino acid peptide so called after the tyrosine residues, Y being the single letter abbreviation for the amino acid tyrosine, at both the N and C terminus of the peptide (Tatemoto and Mutt 1980). PYY is synthesized and released into the circulation from L-cells, predominantly located in the distal gastrointestinal tract. Two main forms of PYY have been described; PYY1-36 and PYY3-36. PYY3-36 is a truncated 34-amino acid from N-terminal Tyr-Pro cleavage of PYY1-36 by the enzyme dipeptidyl-peptidase IV (DPPIV) (Mentlein et al. 1993). Levels of circulating PYY3-36 are low during fasting and peak in the second hour after a meal, remaining elevated for up to 6 h. (Ardial et al. 1985). Peripheral administration of PYY was first reported in 1993 to decrease appetite (Okada et al. 1993), and later raported both in rats and humans (Batterham et al. 2002). In the same study with human volunteers, Batterham et al.(2002) reported that infusion

of PYY3-36 to mimic accurately postprandial concentrations reduced food intake by 30%. Further studies confirmed that this anorectic effect of PYY3-36 was preserved in obese human subjects (Batterham et al. 2003).

Pancreatic polypeptide (PP)

PP is a 36-amino acid peptide synthesized and released by the PP cells of the pancreatic islets of Langerhans and to a lesser extent the colon and rectum (Ekblad and Sundler 2002). The circulated levels of PP are low during fasting (Adrian et al. 1977). Physiological effects of PP include inhibition of gastric emptying, gallbladder motility and pancreatic exocrine secretion (Kojima et al. 2007). Peripheral administration of PP to mice and humans reduces food intake (Malaisse-Lagae et al. 1977). Food intake was reduced 21.8% in normal weight human volunteers (Batterham et al. 2003). While peripherally administered PP leads to reduce food intake, central administration of PP stimulates daytime food intake in satiated rats (Clark et al. 1984).

Ghrelin

Ghrelin is a 28 amino acid peptide formed by cleavage from its larger precursor, pre-proghrelin and synthesized in the stomach (Kojima et al. 1999). Ghrelin is currently the only known orexigenic gut hormone. In order to be biologically active, ghrelin undergoes a post-translational modification in which the third amino acid (serine-3) is covalently linked to octanoic acid (Karra and Batterham 2010). Ghrelin has been called the “hunger hormone” because of its stimulatory effect on appetite and food intake. Ghrelin levels are highest in the fasting state, rising sharply before and falling within one hour after a meal (Cummings DE et al. 2002). Peripheral administration of ghrelin stimulates food intake in humans (Wren AM et al. 2001). Central and peripheral administration of ghrelin to rats also increases appetite and food intake (Wren et al. 2000). Additionally, rodents vaccinated against ghrelin with ghrelin immunoconjugates prove to have less weight gain (Zorilla et al. 2006). The gene which encodes ghrelin also encodes another peptide known as obestatin. Both central and peripheral administration of this peptide was shown to reduce food intake, while peripherally administrated obestatin reduced weight gain (Zhang et al. 2005).

Oxyntomodulin (Oxm)

Oxyntomodulin is a 37- amino acid peptide hormone, produced by processing of pre-proglucagon in the gut and brain and released from L-cells in response to food ingestion (Holst 1997). Oxm contains the 29 amino acid structure of glucagon, followed by an octapeptide as a C-terminal extension (Bataille et al. 1981). Several studies have shown that Oxm reduces food intake in rats (Darkin et al. 2001, 2004) and in humans (Cohen et al. 2003). In another study, the pre-prandial administration of Oxm to overweight and obese humans over 4 weeks led to a significant reduction of body weight (Wynne et al. 2005).

Amylin

Amylin is a 37 –amino acid peptide that is synthesized in pancreatic β-cells together with insulin in response to food intake. The anorectic action of amylin appears to be one important factor in amylin´s general role to control nutrients into the circulation. Amylin reduces eating, gastric acid secretion and limits the rate of gastric emptying (Young et al. 1998). Amylin seems to affect satiety via the area postrema/nucleus of the solitary tract (Silvestre et al. 2001). Either peripheral or central administration of amylin reduces food intake (Lutz et al. 1995; Rushing et al. 2002). Alike CCK, amylin shares the typical characteristics of satiating hormones, which are involved in the control of meal size (Geary 2004).

Apolipoprotein A-IV (apo A-IV)

In humans, apolipoprotein A-IV (apo A-IV) is a 46 kDa protein that is synthesized only by the small intestine. In rodents, apo A-IV is a smaller protein (43 kDa), that is synthesized both by the intestine and the liver; the intestine being most important for the contribution of the circulating apo A-IV (Fukagawa et al. 1994; Wu et al. 1979). Intestinal apo A-IV synthesis is stimulated by fat absorption. Apo A-IV has been proposed to physiologically control food intake, as demonstrated by Fujimoto et al. (1992). It has also been shown in both human and rodents that synthesis and secretion of apo A-IV in the small intestine is stimulated by the gastrointestinal hormone peptide YY (Batterham et al. 2002). Administration of apo A-IV either intravenously or directly into the cerebro-ventricular system decreases meal size in rats, whereas central administration of an apo A-IV antiserum increases food intake (Fujimoto et al. 1993).

Enterostatin

Enterostatin was discovered by Erlanson-Albertsson et al. (1988a and b), a digestion-related pentapeptide and closely connected to lipid digestion. The exocrine pancreas is stimulated, with ingestion of fat, to secrete lipase and colipase that are responsible for digestion of fat. Enterostatin is a cleavage product during the formation of colipase from procolipase, catalyzed by trypsin. Enterostatin has been shown to be a potential inhibitor of food intake by Erlanson-Albertsson and Larsson in 1988. Administration of enterostatin reduces food intake when given either systemically (Okada S et al. 1991; Shargill NS et al. 1991) or directly into the brain (Mei J et al. 1992). The interesting effect of enterostatin is that; when rats were allowed a choice of foods to eat, the reduction was specific for fats.

The physiological role of endogenous enterostatin was investigated in a mouse model with deletion of the nucleotides encoding enterostatin from the procolipase by Miller et al. (2009). The authors concluded that mice lacking enterostatin have no significant change in appetite or weight regulation, thus, enterostatin is not certainly requires for satiety or the regulation of fat intake; other pathways may compensate for the loss of enterostatin.

Chapter II

2.1 Digestion of dietary fat and satiety

The main function of the digestive tract is to convert the energy derived from nutrients to make it accessible to the tissues of the body. In the Western world, fat (lipids) cover about 40% of the energy intake in the human diet. Dietary lipids are composed mainly of a large mummer of organic compounds including fatty acids, monoacylglycerols, diacylglycerols, triacylglycerols (TG), phospholipids (PL), sterols, vitamin A and E, carotenoids, and hydrocarbons (Galli et al. 2009).

The major lipid component, about 95%, in the human diet is TG and they carry energy as well as essential fatty acids. TG consists of three fatty acids esterified to a glycerol backbone. TG cannot be absorbed by enterocytes in the intestine; they need to be hydrolyzed before being taking up (Hofmann and Borgström, 1962:1964). A limited digestion (15%) of dietary fat occurs already in the stomach, the major digestion occurring in the small intestine (Carriere et al. 1993).

The dietary lipids are emulsified by bile salts and thereby the surface area of the dietary lipids increases, thus lipases can act more efficiently. This critical emulsification process takes place in duodenum. The digestive process requires a coordinated lingual, gastric, intestinal, biliary and pancreatic function. Usually, the entire fat digestion and absorption lasts for 16-24h if no food is consumed after the initial meal (Bisagaier and Glickman, 1983).

The hydrolysis of TG is fulfilled by a series of enzymes and some cofactors secreted from lingual (gland), stomach, pancreas and intestine (figure 2). Some degree of emulsification of dietary fat in the stomach is required to create a sufficient surface area of substrate to be hydrolyzed by lingual and gastric lipases.

Potential emulsifiers in the acidic media and muscle constriction of the stomach are the forces for emulsification. Most effective emulsification takes place in intestine by bile salts.

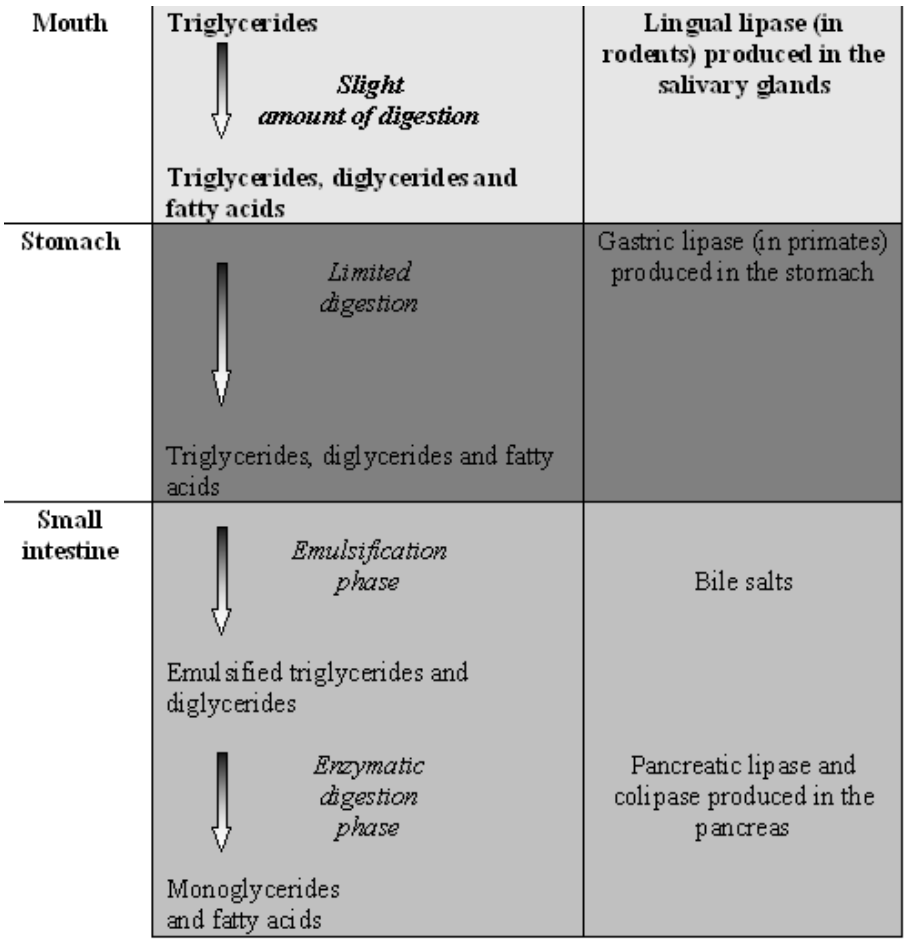


Figure 2. TG digestion pathway in the GI tract.

Following a slight initiation of digestion in the stomach, the main fat digestion occurs in the intestine through the action of intestinal and pancreatic enzymes. In humans, the small intestine is a crucial source of satiety signals. Infusion of nutrients into the small intestine is associated with suppression of food intake (Smith et al. 1984). It has been reported by Welch I et al. (1985) that fat produce satiety and reduce food intake as long they stay in the intestine

The relative efficiency of enzymes that contribute to hydrolysis of fat depends on the species and the age of the individual as well as on the physiological situation. Even though most of the fat digestion occurs in the small intestine by the

pancreatic lipase and colipase the contribution of the preduodenal lipases to TG digestion may be important.

2.2 Preduodenal digestion of fats

The first step of in the hydrolysis of TG takes place in the stomach by gastric or lingual lipase depending on the species. The lingual lipase is released from von Ebner´s glands, the lingual serous glands on the tongue (Fried et al. 1983; Hamosh et al. 1979) and is transferred with the food bolus from mouth into the stomach where its activity is exerted. In humans, digestion of fat begins in the stomach with gastric lipase because humans do not have a lingual lipase (Moreau et al. 1988). Human lipase purified from gastric juice has a molecular weight of about 50 kDa but tends to aggregate and is highly hydrophobic (Hamosh et al. 1990). The gastric lipase is released from the gastric mucosa into the stomach slurry (Hamosh et al. 1990). The relative contribution of these two lipases (lingual and gastric) to the fat digestion depends on the species; rodents in general have high lingual lipase activity and low gastric lipase activity, whereas primates (human) have high gastric lipase activity (DeNigris et al. 1988). The droplet size of fat emulsion also affects lipid digestion; Borel et al. (1994) has shown in a study that gastric lipase activity was higher on the fine mixed emulsion than on the coarse one.

Gastric lipase, in human, is expressed by the chief cells in the stomach. About 10-30% of the dietary fat is hydrolyzed in the stomach (Hamosh et al. 1979). This gastric pre-digestion facilitates intestinal fat digestion resulting in the formation of hydrolysis products that increase the solubilization of the TG, as well as the binding of colipase to lipase (demonstrated by Erlanson-Albertsson and Larsson in 1986). The release of fatty acids also stimulate the release of CCK from the stomach (Go, 1973).

The acidic environment of the stomach serves a required media for gastric lipase, where gastrointestinal lipolysis of dietary fat is initiated in humans (Carriere et al. 1993). In consideration of the gastric juice (pH is about 2, presence of pepsin protease and physiological temperature) the gastric lipase can be observed to be an extremophilic enzyme (Ville et al. 2002).

In contrast to children and adults, in newborns, pancreatic lipase secretion is low and partial breakdown of fats is performed by a lipase, carboxyl ester lipase, present in human milk (Hamosh 1995). When, the baby is weaned onto solid food the major site of fat digestion shifts to the duodenum (Gurr, 1999). Lipase levels, in the small intestine, rise by age and reach at the same level as adults in the first 1-2 years of life (Yang et al. 1998).

2.3 Intestinal digestion of fat

Most digestion of fat occurs in the small intestine. The major enzymes necessary for the chemical changes are not present until fat reaches the small intestine. These specific digestive agents and enzymes come from three different major sources; bile salts from gallbladder, enzymes from pancreas and from small intestine itself. Pancreatic juice, in turn, contains several lipases that have the capacity to hydrolyze TG (table 1).

Lipase	Expression Pattern	Main Site of Action	Substrate Specificity
Gastric	Gastric chief cells	Stomach	• sn-3 positions of glycerides • Also sn-1 and sn-2 positions
Colipase-dependent pancreatic lipase	Pancreatic acinar cells	Small intestine	• sn-1 and sn-3 positions of glycerides
Pancreatic lipase–related protein 1	Pancreatic acinar cells	Small intestine	• None known
Pancreatic lipase–related protein 2	Pancreatic acinar cells Intestinal paneth cells Lymphocytes	Small intestine	• sn-1 and sn-3 positions of glycerides • sn-1 and sn-2 position of galactolipids • sn-1 position of phospholipids
Carboxyl ester lipase	Pancreatic acinar cells Breast milk	Small intestine	• All positions of glycerides • sn-1 and sn-2 positions of galactolipids • sn-1 and sn-2 positions of phospholipids • vitamin esters • cholesterol esters • ceramide
Phospholipase A2	Pancreatic acinar cells	Small intestine	• sn-2 position of phospholipids

Table 1. Classification of human digestive lipases (Whitcomb and Lowe 2007).

When the fat reaches into the duodenum, the first section of the small intestine, it stimulates the secretion of CCK. CCK, in turn, causes the gallbladder to contract and secrete bile into the intestine by way of the common bile duct. Bile is produced first in the liver, in large diluted amounts, and then transferred into gallbladder where the bile is concentrated and stored. Bile salt is an emulsifier and has a very important role for digestion of fats. Because of the detergent function, bile salts break the large fat droplets into smaller particles, greatly enlarging the total surface area available for action of the enzyme. Bile salts also lower the surface tension of the finely dispersed and suspended fat particles.

2.4 Pancreatic duct

The main function of the pancreas is to produce pancreatic juice which consists of the digestive enzymes. The digestive enzymes are delivered into the small intestine, where they hydrolyze ingested nutrients. Table 2 shows the major proteases in human pancreas.

Enzyme	Action	Product(s)
Trypsin	Endopeptidase; cleaves internal bonds at lysine or arginine residues; *activates other pancreatic* proenzymes	Oligopeptides
Cationic trypsinogen (PRSS1)		
Anionic trypsinogen (PRSS2)		
Mesotrypsin (PRSS3)	Endopeptidase – arginine	
Pancreasin (?)		
(Trypsin IV ?)		
Chymotrypsin	Endopeptidase; cleaves bonds at aliphatic amino acid residues	Oligopeptides
Chymotrypsinogen B1		
Chymotrypsin-like protease		
Caldecrin (?)		
Elastase (unclear which form is secreted)	Endopeptidase; cleaves bonds after small amino acid residues – alanine, glycine, serine	Oligopeptides
Elastase 2A, 2B		
Elastase 3A, 3B		
Carboxypeptidase A A1, A2, A3	Exopeptidase; cleaves aromatic amino acids from carboxyl terminal end of protein and peptides	Aromatic amino acids and peptides
Carboxypeptidase B B1, B2	Exopeptidase; cleaves arginine or lysine from carboxyl terminal end of proteins and peptides	Arginine, lysine, and peptides

Table 2. Major proteases in human pancreas (Whitcomb and Lowe 2007)

The pancreatic juice entering the duodenum is a mixture of two types of secretions; an aqueous alkaline-rich and an enzyme-rich secretion. The main function of the alkaline pancreatic secretion with the other alkaline secretions (bile salts and intestinal secretion) is to neutralize the acid chyme arriving from the stomach. This neutralization is important for several reasons; 1) the pancreatic enzymes require a neutral (or slightly alkaline) pH for activity, 2) the intestinal mucosa is protected against damage by excess acid.

Many of the enzymes are secreted as inactive precursors or pro-enzymes (zymogens). Most important of all these digestive enzymes is trypsinogen because it plays a central role in regulating all the other enzymes (Whitcomb et al. 2007). Trypsinogen converts to trypsin by the release of a small peptide, in a reaction catalyzed by enteropeptidase (also called enterikinase); an enzyme present in the epithelial cells of the small intestine. Once a small amount of trypsin has been produced it can catalyze the conversion of more trypsinogen to active trypsin. Trypsin converts chymotrypsinogen, procarboxypeptidase, proelastase, prophospholipase-A and procolipase to their activated forms

2.5 Absorption of dietary fatty acids

The absorption is to transport substrates from the aqueous medium of the intestinal lumen through the barrier of the intestinal mucosal cells to the blood or the lymphatic system. Absorption of fat is generally divided into three components;

absorption into the enterocyte, intracellular processing, and export into the mesenteric lymph.

The lipid-soluble hydrolyzed products of dietary fat are solubilized by bile salts inside mixed micelles, which are composed of bile salt and mixed lipids (fatty acids, monoglycerides, lysophospholipids, and cholesterol). The micelles are small particles; about 1/100 as large in diameter as the emulsion particles (Ratnayake and Galli 2009), and easily diffuse between lumen and microvili of the enterocytes of the intestinal wall.

The micelles reach the membrane bilayer of the enterocytes (intestinal absorptive cells), there, the fatty acids become protonated and leave the mixed micelles to diffuse across the lipid bilayer membrane. Nevertheless, free fatty acids of chain length between C4-C12, which are amphipathic, readily soluble in water, easily cross the gut wall before uptake into the enterocytes (Binder and Reuben 2009).

However, contrary to the traditionally assumed mechanism, that all of the breakdown products of fat digestion entered enterocytes across the apical membrane by simple diffusion through the lipid bilayer, a protein-dependent diffusion model has been described (Mansbach and Gorelic 2007). The researchers concluded that the hydrolysis products are carried by specific binding proteins to the endoplasmic reticulum, where they are resynthesized to TG either by acylating monoacylglycerol with 2 fatty acids or by dephosphorylating phosphatidic acid and acylating the resultant sn-1,2-diacylglycerol.

Chapter III

3.1 Pancreatic lipase and colipase

As it was mentioned in chapter II, in humans, digestion of fat begins in stomach where gastric lipase hydrolysis about 10-30% of ingested fat. Fat digestion completes in small intestine by other lipases secreted from pancreas (table 1). The majority of dietary fat is digested by colipase dependent pancreatic triglyceride lipase (PTL). Congenital disorder of PTL causes 50-60% of dietary fats are not absorbed (Figarella et al. 1980 and Ghishan et al. 1984). Pancreatic acinar cells secreted PTL enters the pancreatic duct and mixes with biliary lipids and bile salts before entering duodenum.

PTL is water-soluble enzyme acting on insoluble substrates (the dietary TG) at oil-water interfaces. The activity of PTL is much higher against water-insoluble components like triglycerides than water-soluble components (Verger 1997 and Roussel et al. 1998).

PTL can bind alone to the surfaces of lipid emulsions but its activity and adsorption at the water-lipid interface is inhibited in presence of bile salts (Borgström and Erlanson 1973), phospholipids (Larsson and Erlanson-Albertsson 1986) or proteins (Gargouri et al. 1985). A small protein cofactor, colipase restores activity of PTL through binding to the non catalytic C-terminal domain of PTL (Brockman 2000).

Human PTL is a 465 amino acid long protein (Lowe et al. 1989) with a signal peptide, the first 16 amino acids. The purified PTL has an estimated molecular mass of 48 kDa (De Caro A et al. 1977) and cDNA predicted human PTL has a mass of 51,558 kDa, while the 449 amino acids long mature protein is 49,551 Da (Lowe et al. 1989).

The three dimensional structure of human PTL has been reported by Winkler et al. (1990). The single polypeptide chain has two structural domains, an N-terminal domain (residues 1-336) and a C-terminal domain (residues 337-449) (figure 3).

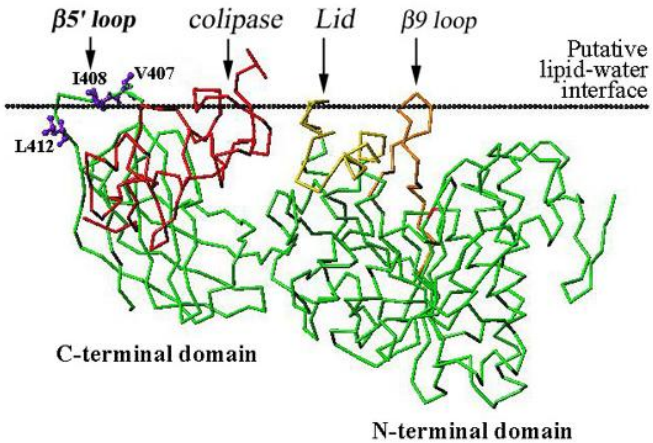


Figure 3. Schematic representation of the orientation of the PTL-colipase complex at a putative lipid interface (Freie et al. 2006)

3.2 The N-terminal domain of PTL

The N-terminal domain belongs to the α/β hydrolase fold, which is a domain structure present in other lipases and esterases (Ollis et al. 1992; Lowe 2002). The N-terminal domain contains the active site, with an analogous catalytic triad (Ser152-Asp176 and His263) which is present in serine proteases (Winkler et al. 1990). The β-strand/Ser/α-helix structural motif including the Gly-X-Ser-X-Gly consensus sequence has only been found in lipases and esterases (Derewenda et al 1991).

3.3 The lid domain of PTL

A surface loop, (Cys237-Cys261) of N-terminal domain forms (by a disulfide bridge) so-called lid or flap (figure 3), which prevents (in the closed conformation) the substrate to reach the active site (Winkler et al. 1990). As demonstrated in figure 4, the lid-domain is stabilized by van der Waals contacts with the β5-loop (residues 76-85) and β9-loop (residues 204-224). These three structural elements (the lid, the β5- and β9 loop) sterically hinder access of substrate to the active site creating an inactive conformation of PTL (Whitcomb and Lowe 2007).

The 3-D structures of the PTL-colipase complex were obtained in the presence and absence of mixed micelles of octylglucoside and phospholipid (van Tilbeurgh et al. 1993 and 1992). The lid domain, in the absence of micelles, remained in the same closed position as observed in earlier studies on the human PTL structure. When, crystals of PTL-colipase complex were allowed to grow in the presence of

mixed phospholipid-bile salt micelles, change in the conformation of the lid occurred (van Tilbeurgh et al. 1993). The lid of the PTL seems to remain closed (inactive) until PTL absorbs to a lipid-water interface. The active conformation of PTL is obtained by a 29 Å hinge movement of the lid domain to open position, which moves this region away from the active site and the β5-loop folds away from the catalytic site (Lowe 2002). As shown in figure 4, the open lid, the colipase fingers and the unchanged β9-loop form a large, continuous hydrophobic plateau, extending over more than 50 Å, which is able to interact strongly with a lipid surface (Chahinian and Carriere 2000).

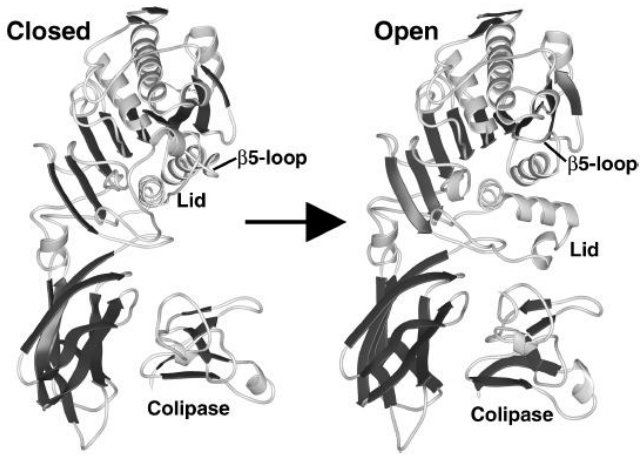


Figure 4. Open (active) and closed (inactive) conformation of the lipase/colipase complex (Lowe ME 2002)

Verger R (1997) has concluded that an oil-water interface triggers a change to the open conformation change of the lid domain in PTL. On the other hand, many other researchers have reported that the open conformation of the lid domain can exit in absence of an oil-water interface. Miled N et al. (2000) has demonstrated that monoclonal antibodies, which only recognize the closed conformation of human PTL, did not bind to the PTL in presence of bile salt and water-miscible organic solvents (dioxane, acetonitrile, tertbutanol, formamide). Thus, the result indicated that bile salts micelles alone can trigger the open conformation of PTL.

3.4 The C-terminal domain (colipase binding domain) of PTL

The C-terminal domain has a *β*-sandwich structure and provides the major binding surface for colipase (van Tilbeurgh et al. 1993 and 1992). C-terminal domain of PTL contains an exposed hydrophobic β5´-loop (residues 405-414), which has a large accessible surface area (877 $Å^2$). This exposed 877 $Å^2$ surface area is

occupied by 488 Å^2 hydrophobic, 105 Å^2 charged and 284 Å^2 semi-polar residues (Chahinian and Carriere 2000).

The β5´-loop is located on the same side as the hydrophobic loops surrounding the active side (the lid and the β9-loop). The β5´-loop, together with open lid, the β9-loop and the tips of the colipase fingers, forms an extension to the large hydrophobic plateau because the bound colipase does not mask the hydrophobic surface of the β5´-loop (Lowe 2002).

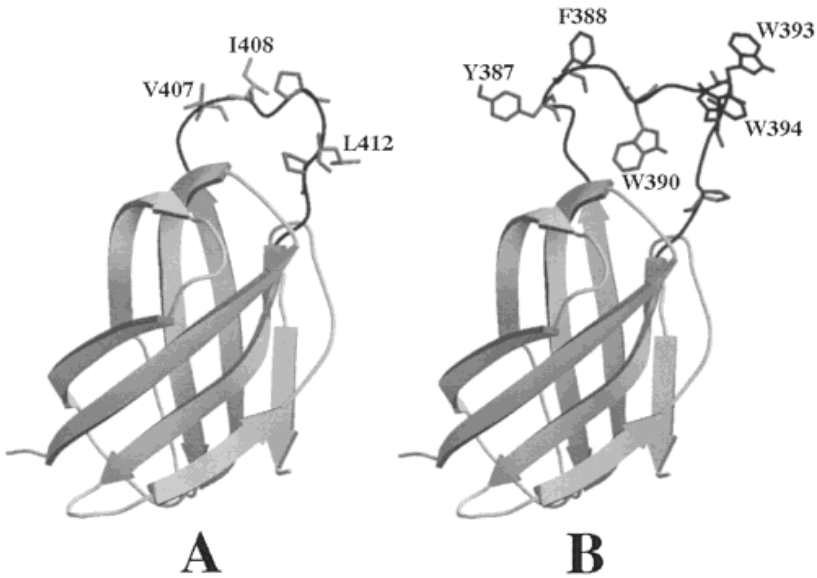


Figure 5. Ribbon models of C-terminal domains of human PTL (A) and human PTL-β5´LPL mutant (B) (Chahinian et al. 2002)

In a study Chahinian et al. (2002) has reported that the human PTL- β5´LPL mutant had decreased affinity for colipase in the presence of bile salts. The investigators determined the contribution to lipid interactions of a hydrophobic loop (β5´) in C-terminal PTL by investigating a human PTL mutant in which β5´-loop hydrophobicity was increased by introducing the homologous lipoprotein lipase (LPL) β5´- loop (figure 5). They conclude that β5´-loop mutation could impair the interaction between PTL and bile salt micelles thereby decreasing the affinity of the human PTL- β5´LPL mutant for colipase.

In a later study, Freie et al. (2006) has characterized the contribution of three residues in the β5´-loop (Val407, Ile408 and Leu412), to the function of human PTL. They changed the hydrophilic ratio to lipophilic ratio of the β5´-loop,

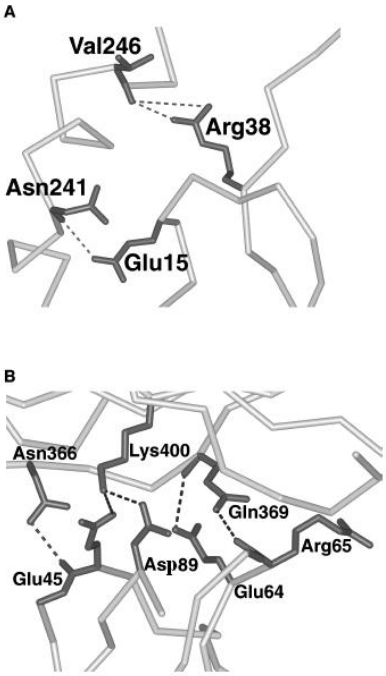
through substituting these three hydrophobic residues (Val407, Ile408 and Leu412) to charged residues, Asp or Lys. The experiments suggest that the β5´-loop, especially the region containing Val407 and Ile408 residues, make major contributions to binding of colipase in micellar concentration of bile salts. The effect of substitution at the points Val407 and Ile 408 was greater than at the point Leu412. Another interesting result was that, both the Asp and Lys substitutions at position 407 reduced the activity of mutant PTL in the presence of bile salts micelles.

3.5 Colipase and interactions with PTL

PTL requires a small protein cofactor, colipase, for the enzyme to be able to bind to the bile salt-covered water-triglyceride interface. Certain duodenal lumen constituents, such as bile salts, phospholipids, dietary lipids, cholesterol esters and dietary carbohydrates inhibit PTL (Erlanson-Albertsson 1992; Borgström and Erlanson-Albertsson 1982), and colipase restores activity of PTL in the presence of inhibitory constituents (Borgström et al. 1979). Colipase binds to PTL in a 1:1 molar ratio to form an active-stable lipase on bile salt covered water-oil surface (van Tilbeurgh et al. 1993).

Colipase is a low molecular mass protein (10 kDa) and has no lipolytic enzymatic activity of its own (Borgström and Erlanson-Albertsson 1984). Colipase is synthesized and secreted, as a procolipase, in pancreas. Procolipase consists of 95 amino acids which is rapidly proteolyzed, presumably by trypsin in the duodenum, to a shorter form (colipase) by cleavage of the Arg5-Gly6 peptide bond (Erlanson and Borgström 1972; Lowe 1990). The N-terminal cleavage product pentapeptide (Val (Ala)-Pro-Asp-Pro-Arg=V(A)PDPR) of procolipase is named enterostatin, as it discussed in chapter I, reduces food intake.

In the open conformation of PTL, the interactions between the lid domain and colipase stabilize the complex. As it shown in figure 6A, hydrogen bounds form between Glu15 of colipase and Asn241 of PTL, and between Arg38 of colipase and Val246 of PTL (Lowe 2002)



The cycteine-rich colipase is a flattened protein and consists of three finger-shaped regions defined by disulfide bridges (Lowe 2002). The size of colipase is 25x30x35 Å in the lipase/colipase complex and mean surface area is about 720 $Å^2$/molecule (Egloff et al. 1995). Two hairpin loops formed by residues 44-46 and 65-87 and Asp89 interact with amino acids in various β-strands of the PTL C-terminal domain (figure 6B). Polar interactions, a salt bridge and hydrogen bounds dominate the interactions (Lowe 2002).

Figure 6. Contacts between colipase and PTL. A: Colipase and the PTL lid domain. The side chains of the interacting residues between are shown in dark gray. The α-carbon trace in the region is shown in light gray. The interacting atoms are connected by dotted lines. Glu15 and Arg38 are colipase residues. Asn241 and Val246 are residues in the PTL lid domain. B: Colipase and the C-terminal domain of PTL. The side chains of the interacting residues are shown in dark gray. The α-carbon trace in the region is shown in light gray. Colipase residues Glu45, Glu64, Arg65, and Asp89 are at the bottom of the figure. Asn366, Gln369, and Lys400 are in the C-terminal domain of PTL (Lowe 2002).

The polar interaction between colipase and C-terminal domain of PTL is likely to play an important role. Strictly conserved residues Lys400 on lipase and Glu45 on colipase, the importance of ion pair, have been studied (Ayvazian et al. 1998). Site directed mutations; E45K and E45N colipase, and K400E and K400N PTL were constituted and investigated. The results showed that preventing the creation of the ion pair between lipase and colipase results in a loss of activity against emulsified substrates; concluded that the ion pair between colipase and C-terminal of PTL plays a critical role to form a lipolytic PTL-colipase complex.

The effect of the colipase residues that interact with the C-terminal domain of PTL were studied through introducing mutations into the PTL binding domains of human colipase (Crandall and Lowe 2001). Two supposed lipase binding domains, Glu45/Asp89 and Glu64/Arg65, form between colipase and lipase. In presence of bile salt micelles, most of the colipase mutations induce a decreased ability to introduce a lipolytic active PTL, above all mutations at Glu64/Arg65 (figure 7). The authors concluded that the mutations decreased the affinity of the colipase mutants for PTL and thereby the formation of PTL-colipase complexes hindered.

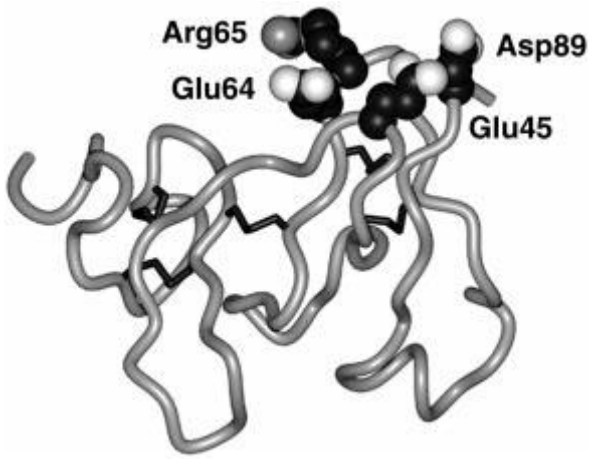


Figure 7. Crystal structure of colipase. The α-carbon backbone and disulfide bonds of colipase are presented as tubes. The side chains of the colipase residues that the crystal structure predicts will form polar interactions with PTL are shown. They form two potential binding sites, Glu64/Arg65 and Glu45/Asp89. The side chain of Ser44 is not shown but sits next to the side chain of Glu45. The coordinates were obtained from the Protein Data Bank, entry 1LPB (Crandall and Lowe 2001).

Only E45A mutation had no effect on the PTL activity, indicating that the ion pair between Glu45 with Lys400 is not essential for complex forming. Similar result was also observed in an earlier study (Jennens and Lowe 1995) that the K400A mutant of PTL had normal activity.

Chapter IV

4.1 Thylakoid membranes

All forms of life require energy for growth and maintenance. Most of the living energy that organisms on earth use originates from the sun. Green plants, algae and certain types of bacteria capture this energy in sunlight and convert it into chemical energy, the processes named photosynthesis. Photosynthesis comes from two Greek words means “light” and “put together”. The major chemical pathway in photosynthesis is the conversion of CO_2 and H_2O to carbohydrates and oxygen. In higher plants and algae photosynthesis takes place in specialized organelles, called chloroplast.

Chloroplasts are highly structured and made up of three types of distinct membrane systems; an outer membrane which is freely permeable to molecules, an inner membrane which contains many transporters and**,** a network system of thylakoid membranes.

Thylakoids are flat, saclike structures located in the stroma and usually arranged in stacks called grana (figure 8). Thylakoids consist of more than hundred different proteins (integral, peripheral and luminal proteins), pigments (i.e. chlorophylls), carotenes (i.e. β-Carotene), xanthophylls (i.e. zeaxanthin), membrane lipids (i.e. galactolipids), plastoquinones, tocopherols and phylloquinones (Juhler et al 1993; DörmZbierzak et al. 2009).

The thylakoid membranes, which are the most abundant biological membranes in nature, are the site of light-harvesting, photosynthetic electron transport, proton translocation, and transduction of an electrochemical gradient in adenosine triphosphate (ATP) synthesis.

Thylakoid membrane proteins, together with their bound pigments, contribute about 70% of whole thylakoid mass and the remaining 30% of mass made by membrane lipids, plastoquinones, tocopherols and phylloquinones.

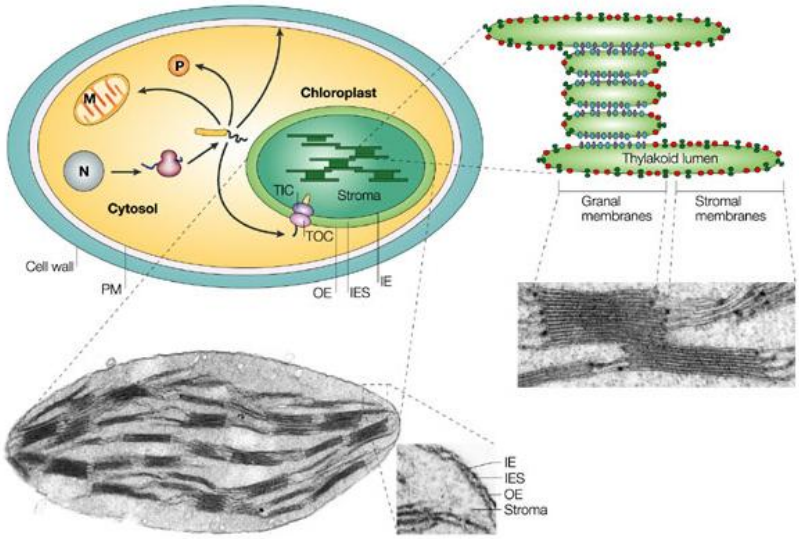


Figure 8. The outer and inner envelope (OE and IE), respectively the inter-envelope space (IES), plasma membrane (PM) the nucleus (N) mitochondria (M), peroxisomes (P) (Soll and Schleiff 2004)

Integral membrane proteins of thylakoids play a crucial role in light-harvesting and light-depended photosynthetic reactions. There are four major thylakoid integral membrane proteins and complexes; 1) Photosystem I, 2) Photosystem II, 3) ATP-synthase, and 4) Cytochrome b_6/f complexes.

Both PSI and ATP synthase cannot be present in the grana membranes because of their bulky stromal exposed parts and also not in the margins because of the large volumes of their membrane-intrinsic parts, thus these complexes can only be located in unstacked thylakoid membranes (stroma lamella), and in the end membranes of the stacks. PSII supercomplexes are located in the stacked grana membranes.

LHCII is mainly located in the grana, but can to some extent be found also in the stroma lamella membrane, where it may bind to PSI. Most models assume a distribution of cytochrome b_6/f between the grana stacks and stroma membranes (Albertsson 2001; Allen and Forsberg 2001). According to a model of thylakoid membrane (figure 9), published by Albertsson (2001), NAD(P)H-PQ is located only the stoma membranes.

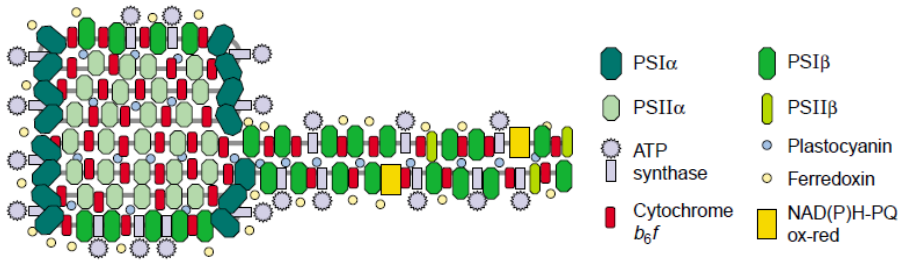


Figure 9. A model of the thylakoid membrane system (Albertsson 2001)

The ATP-synthase complex of green plant chloroplasts, also known as the CF_1CF_0-ATP synthase, belongs to the family of the F-type ATP synthases, similar types of complexes are also found in prokaryotes and mitochondria (Dekker and Boekema 2005). The complex is composed of three specific parts; a hydrophilic headpiece (CF_1), a smaller membrane-bound F_0 moiety and a stalk region. CF_1 headpiece (about 55 kDa) consists of three α-subunits and three β-subunits and located on the stroma side of the thylakoid membrane. Subunit γ (35 kDa) fills most of the central shaft is connected to an 8 kDa subunit III, which forms a fixed ring of 14 subunits (Seelert et al. 2000).

The cytochrome b_6/f complex is a dimeric integral membrane protein complex (about 220 kDa), composed of 8 to 9 polypeptide subunits (Zhang et al. 2001). The 24 kDa cytochrome b_6/f subunit has four transmembrane α-helices and contains heme groups. The subunit IV (17 kDa) has three transmembrane helices, and the Rieske iron-sulfur protein (19 kDa) consisting of an N-terminal single transmembrane α-helix (Dekker and Boekema 2005).

4.2 Photosystem I

Photosystem I (PSI) is a multisubunit protein complex that catalyzes sunlight-driven transmembrane electron transfer and located in the thylakoid membranes. Two major membrane complexes; the core complex (the reaction center-RC), where the bulk of the light capturing and the charge separation reactions occur, the light-harvesting complex I (LHCI), which serves as an additionally antenna system that maximize the light harvesting by collecting solar radiation and transmitting the energy to the core complex (Chitnis 2001). The LHCI complex absorbs photons and transfer the energy over chlorophyll molecules to the reaction center (the core complex) where it eventually reaches the special chlorophyll dimmer, P700, resulting in charge separation reaction and transmembrane electron transfer. The plant PSI complex, which is much larger than cyanobacterial PSI, consist of

currently known 19 protein subunits, about 175 chlorophyll molecules, 2 phylloquinones and 3 Fe_4S_4 clusters (Ben-Shem et al. 2003).

As mentioned above, PSI catalyzes the light-driven electron transfer from the soluble electron carrier plastocyanin, located at the inside (lumen) of the thylakoid membrane, to ferredoxin, at the outside (stroma) of the membrane (Bengis and Nelson 1977). The PSI contains both organic (six chlorophyll and two phylloquinones) and inorganic (three Fe_4S_4 clusters) units. PSI generates probably the most powerful reductant (the most negative redox potential) of any of the natural system studied so far (Brettel and Leibl 2001).

In 1966, the first higher plant photosystem I was isolated and characterized with its chlorophyll content and photochemical activities by Andersson J and Boardman N. However, it took nine years before the first report of a purified plant photosystem I complex and its subunits composition was published (Bengis and Nelson 1975).

The recent x-ray crystal determination at 3.4-Å resolution by merging data from 10 different crystals of plant PSI was published by Amunts A. et al. (2007), however, the peripheral parts of complex, including the LHCI, were poorly resolved.

Plant PSI-LHCI complex forms a monomer in the crystalline state (as well as *in vivo*), and the overall structure shown in figure 10 (Amunts and Nelson 2009). The complex has a maximal length of about 100Å and diameter of about 185Å-150Å. Crystallized PSI-LHCI supercomplex consists of 13 protein subunits (PsaA, PsaB, PsaF, PsaG, PsaH, PsaI, PsaJ, PsaK, PsaL, and Lhca1-4) with their 45 transmembrane helices, that transverse the thylakoid membrane.

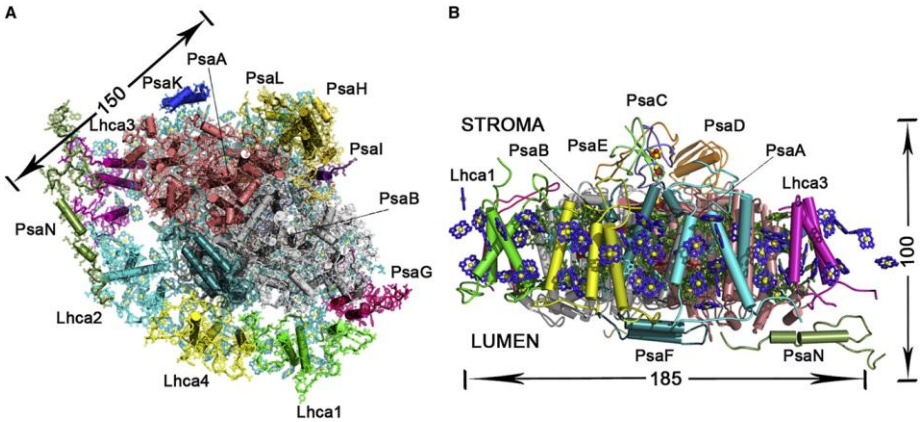


Figure 10. The Overall structure of PSI. Each individual of 17 protein subunits is colored differently. (A) View from the lumen. LHCI is comprised of Lhca1–4 chlorophyll binding proteins, assembled in a half-moon shape onto the RC. (B) View perpendicular to the membrane normal. (Amunts and Nelson 2009).

Subunits PsaC, PsaD and PsaE compose stroma-exposed, while only PsaN composes the luminal subunit of the supercomplex. Total 178 cofactors (168 chlorophylls, 3 Fe_4S_4 clusters, 2 phylloquinones and 5 carotenoids) were identified in the reported structure of plant PSI-LHCI (Amunts et al. 2007). These cofactors contribute about 30% of the total molecular mass (~600kDa) of the PSI-LHCI complex (Amunts and Nelson 2009).

Mullet et al. (1980) was the first researcher to publish evidence for the existence a *Chl a/b* antenna specifically associated to higher plants PSI and in this work, a fraction containing four polypeptides with molecular mass between 20 and 24 kDa of LHCI were identified. In a later study (Lam et al. 1984), LHCI was isolated in two different pigment-protein fractions; one monomeric (Lhca2 and Lhca3), and the other one dimeric (Lhca1 and Lhca4).

The light-harvesting complex I (LHCI) belt, with its associated chlorophylls, is the most distinct addition of the PSI structure by plants and green algae. The belt of LHCI contributes about 160 kDa of about 600 kDa total mass of PSI-LHCI complex. LHCI is made by four subunits (Lha1-Lha4) that are between 20 and 24 kDa polypeptides; belong to the LHC family of the chlorophyll a/b binding proteins.

Plumley and Schmitd (1987) have used an *in vitro* reconstitution of recombinant LHCI, since native LHCI complexes are so hard to purify, to study LHCI, in

presence of chlorophylls and carotenoids. The experiments have showed that Lhca proteins can be grouped in to two pairs with respect to their binding to pigments; the first group comprise of Lhca1 and Lhca3 which have high affinity to *Chl a*, and the other group, Lhca2 and Lhca4 which have a lower *Chl a/b* ratio. Both Lhca1 and Lhca3 bind three carotenoids; mainly lutein and violaxanthin.

4.3 Photosystem II

Photosystem II (PSII) is a protein-pigment complex embedded in the thylakoid membrane of photosynthetic organisms and catalyses the light-driven oxidation of water to molecular oxygen. The complex consists of more than 20 proteins including both integral and extrinsically associated proteins (figure 11). PSII has also a large number of associated cofactors including chlorophylls, manganese atoms, pheophytins, plastoquinones, non-heme iron, calcium, chloride, and heme groups.

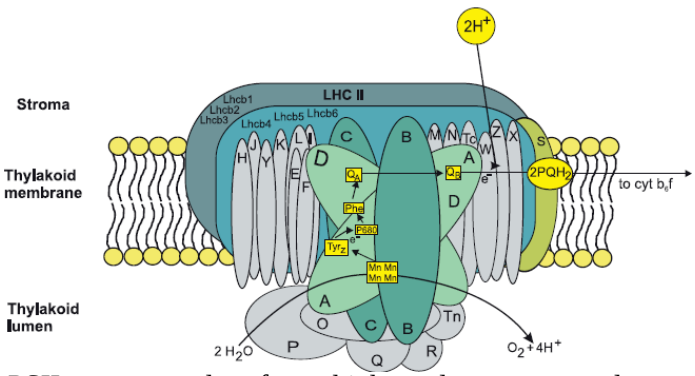


Figure 11. Schematic model of structural organization of PSII within thylakoid membrane. Pigment-binding proteins of PSII are labelled in green. (R. Luciński and G. Jackowski 2006).

PSII supercomplex from higher plants, green algae and cyanobacteria is an enormously complicated, and in general, can be divided in two major functional domains organized around a P680 molecule; 1) PSII core complex (PSIIcc), existing as a dimer in stacked thylakoids and most probably, as a monomer in unstacked thylakoid membranes (figure 12). PSIIcc is composed of many individual proteins (at last 20), comprising the integral membrane helices, pigments and other cofactors. The oxygen evolving complex (OEC), located on the luminal face of the complex including the extrinsic proteins, catalytic inorganic manganese, calcium and chloride cluster. 2) Six other individual proteins (LHcb1-6) binding an array of numerous pigments (*Chl a*, *Chl b*, lutein, violaxanthin, zeaxanthin and antheraxanthin) in the form of peripheral light harvesting complexes –LCHII, CP29, CP26 and CP24 (Paulsen, 1995).

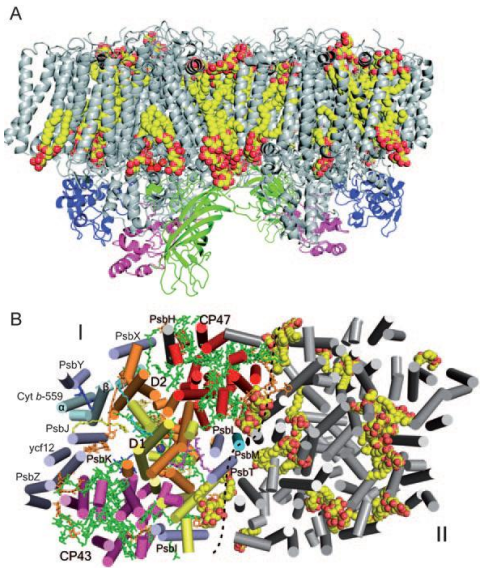


Figure 12. General architecture of the PSIIcc homodimer: A) View of the PSIIcc homodimer along the membrane plane (cytoplasm, top; lumen, bottom). B) View of PSIIcc from the cytoplasmic side (membrane-extrinsic subunits omitted). I. Cofactors are shown in stick mode: *Chl* (green), Car (orange), heme (blue). In monomer II, all protein subunits in gray, lipids and detergents shown as spheres (carbon, yellow; oxygen, red).

The crystal structure of PSII has been solved from two species of thermophilic cyanobacteria (Kamiya and Shen 2003; Ferreira et al. 2004; Loll et al. 2005), and according to the crystal structure, PSII consists of 17 trans-membrane protein subunits and three extrinsic proteins associated with the luminal side. The trans-membrane subunits include D1 (PsbA), D2 (PsbD) and reaction center subunits (*Chl* binding subunits), CP47 (PsbB) and CP43 (PsbC) which are closely associated with the D1 and D2. D1 and D2 proteins of spinach have molecular masses of 38 kDa and 39 kDa respectively. On the other hand, the reaction center subunits of spinach, CP47 and CP43 have molecular masses 56 kDa and 50 kDa respectively (Barber and Nield 2002).

The main trans-membrane subunits in PSII are largely conserved among different photosynthetic organisms, whereas the extrinsic proteins (the OEC proteins of PSII) are significantly different among different species (Enami et al. 2005). There are 13 small subunits with molecular masses below 10 kDa within the PSII reaction-centre core, which are PsbE (large subunit of *Cty b559*), PsbF (small subunit of *Cty b559*), PsbH, PsbI, PsbJ, PsbK, PsbL, PsbM, PsbTc, PsbX, PsbY, PsbZ, and Ycf12 (Kamiya and Shen 2003; Ferreira et al. 2004; Loll et al. 2005). The extrinsic proteins (OEC complex proteins) are 33 kDa PsbO, 23 kDa PsbP and 17 kDa PsbQ in higher plants and green algae, while in cyanobacteria and red algae, PsbP and PsbQ are replaced by the PsbV (15 kDa) and PsbU (11 kDa) respectively (Barber and Nield 2002). According to Barber et al. (1997), there are two further extrinsic proteins associated with OEC; $PsbT_n$ (10 kDa) and PsbR (5 kDa).

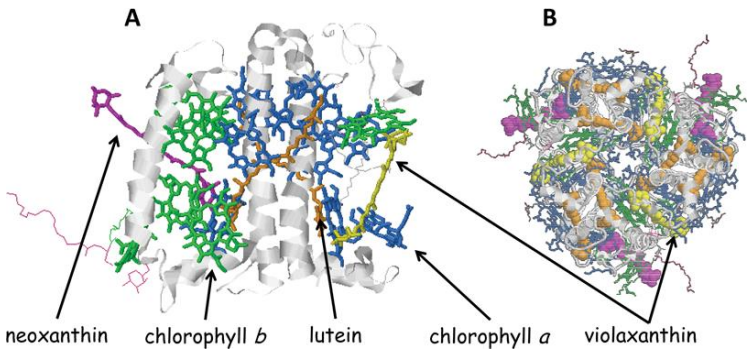
Proteins of PSII supercomplex bind the photosynthetic pigments forming thylakoid membrane-associated pigment-protein complexes; 5 of them, PsbA,

PsbB, PsbC, PsbD and PsbS, which belong to PSIIcc, and the remaining 6 proteins are Lhcb1, Lhcb2, Lhcb3, Lhcb4, Lhcb5 and Lhcb6.

4.4 LHCII

Peripheral light-harvesting complexes of PSII of higher plants and green algae (LHCII does not have a bacterial homologue) are composed of lipids and pigments associated with 6 types of apoproteins, Lhcb1-6. Lhcb1 along with Lhcb2 and Lhcb3 apoproteins together form polypeptide part of light harvesting complex (LCHII) of photosystem II, which is most abundant membrane protein on Earth. Lhcb1 and Lhcb2 together account for 89% of the apoprotein content of LHCII (Jackowski et al. 2001). LHCII is accounting for about 60% of the total chlorophyll content of thylakoid membranes (Lucinski and Jackowski 2006), and roughly 30% of all protein in plant thylakoid membranes (Peter and Thornber 1991). Lhcb4-6, in turn, constitute the polypeptide moiety of minor peripheral light-harvesting complexes; CP29, CP26 and CP24, respectively.

LHCII is nuclear-encoded (Kung et al. 1971), and members of the same protein family are distinguished by en extreme degree of sequence conservation



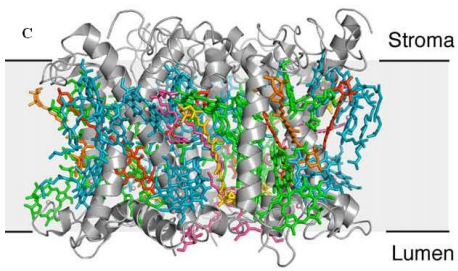


Figure 13. Molecular structure of LHCII with its pigment and lipid content, momomeric (A) and trimeric organization of the complex (B), and from within the membrane (C) (Barros and Kühlbrant 2009).

LHCII exists as a trimer and each monomeric LHCII comprises a polypeptide of about 232 amino acids residues (figure 13), 13-15 *Chl a* and *Chl b* molecules, 3-4 carotenoids and one tightly bound phospholipid (Peter and Thornber 1991; Ruban et al. 1999; Nuβberger et al. 1993). Because of the light-harvesting complexes contain very large amounts of *Chl b*. They have often been

referred to as *Chl a/b* binding proteins (CP43 and CP47 contain only *Chl a* in plants).

Two different lipids, in addition to the polypeptide and pigments, complete the LHCII structure. PG is known to be required for trimer formation (Tremolieres et al. 1981), hydrolysis of PG by phospholipase A2 leads the trimer break up into monomers, emphasize the structural role of PG (Nussberger et al. 1993). It has been shown in the same study that even proteolytic cleavage of the fist amino acid residues brings out the dissociation of the trimer into monomers. This is maybe due to loss of the Tyr44, which makes a polar contact whit the PG head group. The other type of lipid required for the formation is DGDG (PG is present in one copy per monomer, while several molecules of DGDG per monomer is present). DGDG binds more peripherally, whereas PG must bind at the monomer interface (Nussberger et al. 1993).

4.5 Thylakoid membrane lipids

The lipid composition of thylakoid membranes is also highly conserved among oxygenic photosynthetic organisms, and composed of about 50% uncharged monogalactosyldiacylglycerol (MGDG) and about 30% digalactosyldiacylglycerol (DGDG), as well as 5-12% anionic sulfoquinovosyldiacylglycerol (SQDG) and 5-12% phosphatidylglycerol (PG), that means MGDG is the most abundant polar lipid species in nature (Loll et al. 2006; and references therein). An overview of lipid and detergent molecules bound PSII was published in the same study (Loll et al. 2006) shown that DGDG was found almost only at the luminal side of thylakoid membranes, MGDG in both sides, whereas SQDG and PG only at the stromal side of the thylakoid membranes with respect to orientation of head group.

5.1 Thylakoid membranes effect on the lipase/colipase activity

Thylakoid membranes, purified from spinach leaves, inhibit partly the lipolytic activity of pancreatic lipase/colipase *in vitro* (paper I). Other biological membranes, such as mitochondria, plasma membranes and bacterial membranes also inhibited activity of the lipase/colipase (figure 14). Therefore, we concluded that this effect is a general property of biological membranes.

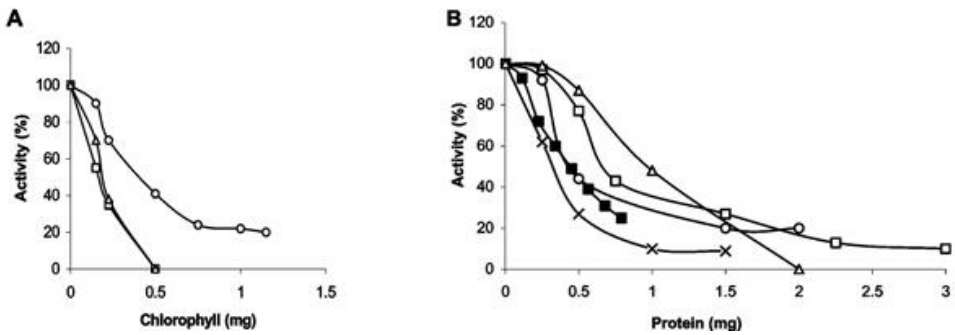


Figure 14. (**A**) Thylakoids isolated from spinach (o), clover (□) and Arabidopsis thaliana (Δ). (**B**) Mitochondria from potato tuber (■), mitochondria from chicken heart (o), plasma membrane from spinach leaf (□), membranes from Synechocystis (×) and chromatophores of Rhodospirillum rubrum (Δ).

Since thylakoid membranes are composed mainly of proteins and lipids, we next separated the proteins from the lipids using methanol/chloroform extraction and investigated the effect of inhibitory potential on lipase/colipase complexes (paper I). The results show that the inhibiting component is the fraction which contains proteins (figure 15A). Furthermore, we treated thylakoids with trypsin to remove most of the extrinsic proteins and exposed loops from the membranes surface, so called "shaving" the membranes surface. The results showed that thylakoids treated with trypsin were still able to inhibit lipolysis (figure 15B), suggesting that the membrane-spanning region of the intrinsic proteins are responsible for the inhibition effect.

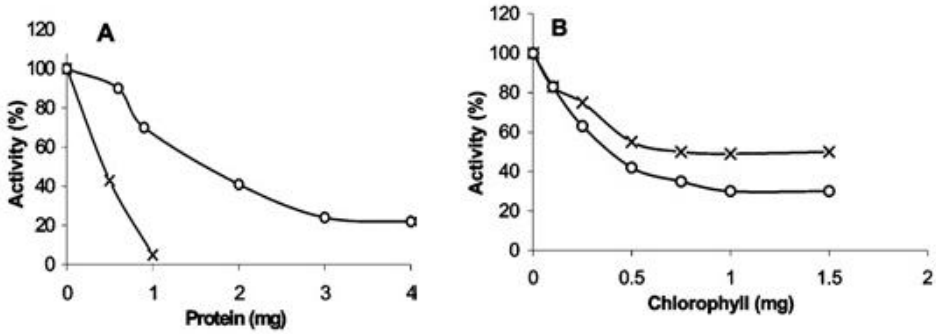


Figure 15. (A) Membrane protein fraction obtained after removing lipids and soluble proteins from thylakoids (×) and untreated thylakoids (o). (B) Thylakoids before (o) and after (×) treatment with trypsin.

LHCII is the most abundant integral-membrane protein in thylakoids (see chapter IV). Therefore we were interested to investigate the effect of the LHCII on the activity of lipase/colipase. As mentioned in chapter IV, LHCII consists of four highly hydrophobic integral-membrane α-helices, and this hydrophobicity, we suggest is responsible for the inhibition (paper I). We found that LHCII alone inhibited the activity of lipase/colipase (figure 16A). Even a synthetic polypeptide with the same sequence as one of the α-helices of LHCII inhibited the lipolysis (figure 16A), but the inhibition effect was not as strong as that with complete LHCII. We also tested other intrinsic membrane proteins, namely transhydrogenase and cytochrome b_6f, which both have several hydrophobic α-helices. Both proteins inhibited the lipolysis. In contrast, water-soluble hydrophilic protein serum albumin did not have any affect (figure 16B), which was another support to effect for the hydrophobicity model.

To elucidate the mechanism behind the inhibitory effect of thylakoid membranes on the activity of lipase/colipase, we studied if thylakoids bind i) directly to the lipase/colipase and/or ii) to the water-oil surface.

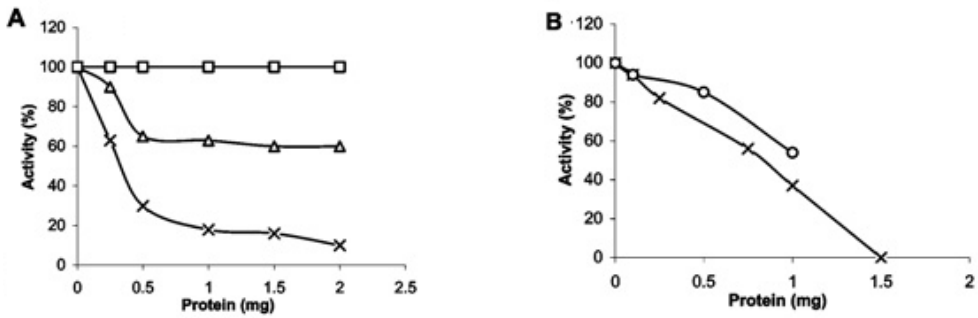


Figure 16. (**A**) LHCII, a membrane protein isolated from spinach thylakoids (×), synthetic polypeptide with the same sequence as one of the membrane-spanning α-helices of LHCII, i.e. VIHCRWAMLGALGCVFPELL (Δ), and BSA (□). (**B**) Transhydrogenase (×) and cytochrome b_6f complex (o).

The affinity of lipase alone and the lipase/colipase complex to the thylakoid membranes were investigated. The results show that the lipase/colipase complex, in absence of bile salts, had strongest affinity to the thylakoids (Paper I), compared to the lipase alone. This suggests that the lipase/colipase complex exposes larger hydrophobic-surface area than the lipase alone.

Apart from that the lipase/colipase complex has strong affinity to the thylakoids, discussed above; we investigated also another possible mechanism for inhibition; binding membranes or membrane proteins to the triacylglycerol/water interface, thereby covering the substrate surface and hindering the access of the lipase/colipase complex to its substrate (paper I). Thylakoid membranes were mixed in an aqueous/oil two-phase system and electron microscopy of this mixture shows that the thylakoid membranes are firmly bound to the interface of the oil drops (figure 17A). There were no thylakoid membranes found in the surrounding aqueous phase.

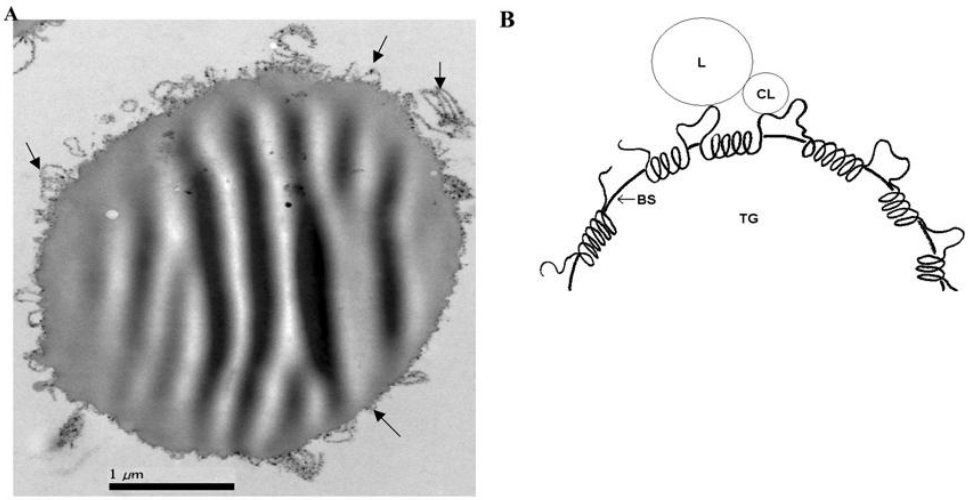


Figure 17. A) Electron microscopy of the thylakoid membranes (arrows) covering the entire interface a lipid droplet in water. B) Schematic model of how an intrinsic membrane protein chain, adsorbed at the surface of a bile salt (BS)-covered triacylglycerol (TG) droplet, sterically hinders the anchoring of the lipase (L)/co-lipase (CL) complex to its triacylglycerol substrate. The hydrophobic helices are embedded in the lipid–bile salt surface layer, and together with the hydrophilic loops and tails they hinder the contact between lipase/co-lipase complex and the lipid (B) (paper I).

We propose that the inhibition of lipolysis by biological membranes/membrane proteins can occur via two types complementary mechanisms;

1) Biological membranes or membrane proteins bind, both by ionic and hydrophobic interactions, to the lipase/colipase complex that block the active site of the enzyme complex and prevent it from coming into contact with the lipid substrate. Binding of lipase/colipase to the thylakoid membranes discussed more detailed in paper V.

2) Binding of membranes or membrane proteins to the lipid/water interface, thereby covering the substrate surface and hindering the lipase/colipase to come in contact with lipid surface (figure 17).

5.2 *In vivo* effect of the thylakoid membranes

After finding that the thylakoid membranes inhibit activity of lipase/colipase *in vitro*, we investigated the *in vivo* effect of thylakoids in added food (paper 1). The

results showed that thylakoid membranes, added to high-fat food, significantly suppressed food intake in rats (figure 18) and thereby reduced body weight gain (table 3). The satiety hormone CCK was raised in the thylakoid group. Surprisingly the activity of lipase/colipase, in the intestine, was higher as well as the expression of pancreatic lipase protein level, in the thylakoid-treated group than the control group (table 3 and figure 19).

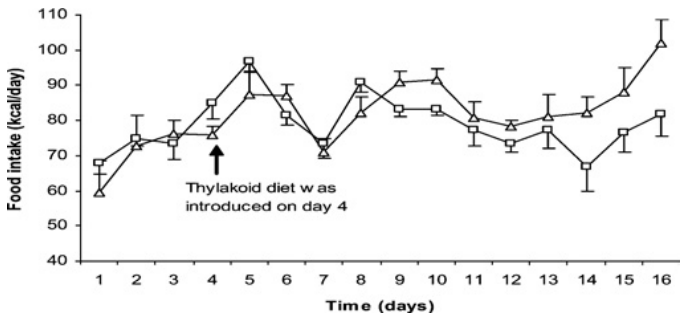


Figure 18. Effect of added thylakoids on Sprague–Dawley rats on a high-fat diet. Food intake with thylakoids (□) and without thylakoids (Δ) (paper I).

The higher level of lipase protein expression in thylakoid-treated animals might be due the prolonged existing of undigested lipids in the intestine; causing increased activity of lipase/colipase hence containing more lipase in the secreted pancreatic juice. The prolonged digestion of fat in the intestine requires more lipase/colipase expression. This, in turn; cause the satiety hormone CCK level to increase in the plasma.

The high level of lipase/colipase activity induced by thylakoid membranes caused the level of enterostatin (see chapter I) to increase, which in turn also promote the satiety.

Another important observation was the absence of steatorrhoea, which is a common side effect by the treatment of lipase inhibitors

	Control diet	Thylakoid
Body weight gain (g)	60.5 ± 3.55	49.9 ± 3.05*
Serum triacylglycerol (mmol/l)	1.02 ± 0.13	0.62 ± 0.04*
Lipase/co-lipase activity (units/mg)	130.5 ± 18.6	222.1 ± 37.5*
Lipase protein expression (arbitrary units/mm^2)	2269 ± 539	5305 ± 809**
Plasma CCK (pmol/l)	0.675 ± 0.08	0.862 ± 0.12*

Table 3. Effect of added thylakoids on rats on high-fat diet (paper I).

The absented steatorrhoea is probably due to the hydrolyzing of thylakoid membranes by gastro-intestinal digestive enzymes since the inhibition of lipase/colipase in the intestine is temporary, eventually lipids are digested in the intestine. Digestion of thylakoid membranes by gastro intestinal duct (gastric and pancreatic juice) and proteases (pepsin and trypsin) were studied in paper III.

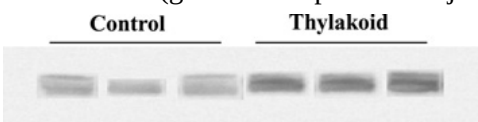


Figure 19. Effect of thylakoids on the lipase expression (paper I).

The effect of thylakoid membranes on the food intake, blood lipid levels and body weight regulation were also examined, using crude thylakoid membranes, isolated by a novel large scale method for preparation of thylakoid membranes (paper II), in long-term studies on mice (paper II), on apolipoprotein (apo) E-deficient mice (Köhnke et al. 2009a) and in a short-term study on healthy humans (Köhnke et al. 2009b).

The long-term feeding experiments in mice clearly demonstrate that thylakoids had a suppressive effect on body fat in the group feeding on thylakoids enriched food (figure 20).

Long-term feeding apolipoprotein (apo) E-deficient mice, a family of mice that is highly sensitive to high-fat diet, with thylakoid membranes enriched high-fat diet resulted in decreased food intake, reduction in body fat gain and amount of body fat (figure 21). The level of satiety hormones CCK, PYY and leptin was increased, whereas free fatty acids, TG, and glucose levels in the serum were decreased in thylakoid groups compare to control groups (Köhnke et al. 2009a).

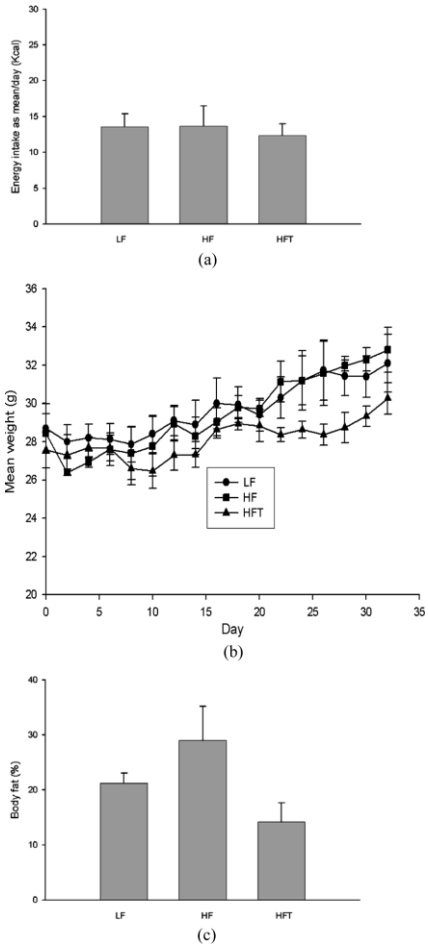


Figure 20. Effect of thylakoids on mean daily food intake (a), body weight over time (b) and body fat (c) in NMRI mice fed either low-fat diet (LF), high-fat diet (HF) or high-fat diet enriched with thylakoids (HFT) during 32 days. There was no significant difference in total energy intake between the three diet groups or in daily food intake over time. The body weight was however significantly reduced in the thylakoid group compared to the other two diets. Measuring body fat in the animals showed a significant reduction ($p<0.05$) in the thylakoid-treated animals compared to high-fat fed animals (paper II).

The effect of thylakoid membranes enriched meal on the healthy humans showed that the level of satiety hormones CCK (figure 22), and leptin increased whereas the hunger hormone ghrelin and glucose in the serum decreased (Köhnke et al. 2009b).

These results suggest that thylakoid membranes promote satiety in humans, as well as in rats and mice

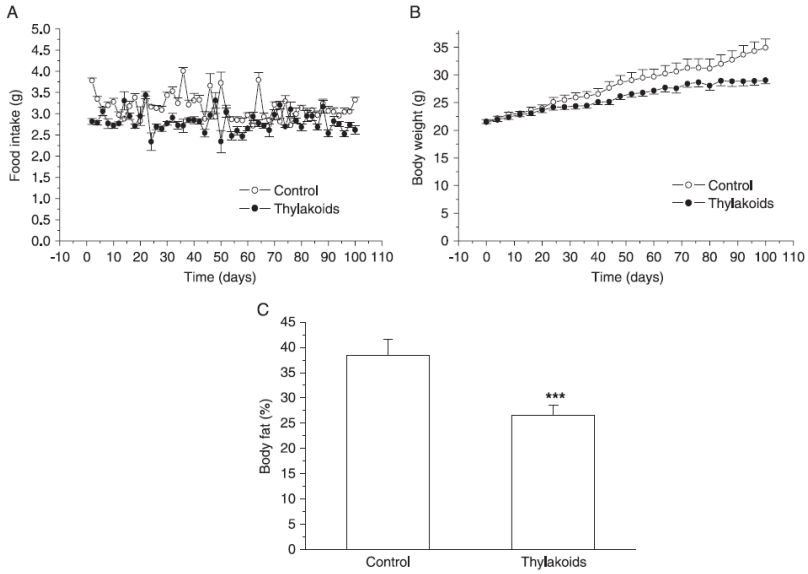


Figure 21. ApoE-deficient mice were offered either a high-fat diet or a high-fat diet with thylakoids for 100 days. The mice offered the thylakoid-enriched diet consumed less food than the control mice [F(1,1372) = 85,197, p < 0.001] (food intake is presented as g/day per mice) (A). The decreased amount of food ingested by the mice offered the thylakoid-enriched diet resulted in a reduced body weight gain compared with the control mice [F(1,700) = 14,34, p < 0.001] (B). The thylakoid diet gave a reduced amount of body fat (C) as assessed using dual energy x-ray analysis (DEXA). *** p < 0.001 (Köhnke et al. 2009a).

5.3 A method for large-scale preparation of thylakoids

To investigate the *in vivo* effect of thylakoids on human, and in industrial-production perspectives for supplement in man for regulation of body weight, larger amounts of thylakoid membranes are needed (paper II). We make use of the isoelectric-point of thylakoids membrane for preparation and the product is characterized with concerning to protein composition (SDS-PAGE and mass-spectroscopy) and the content of carotenoids (HPLC).

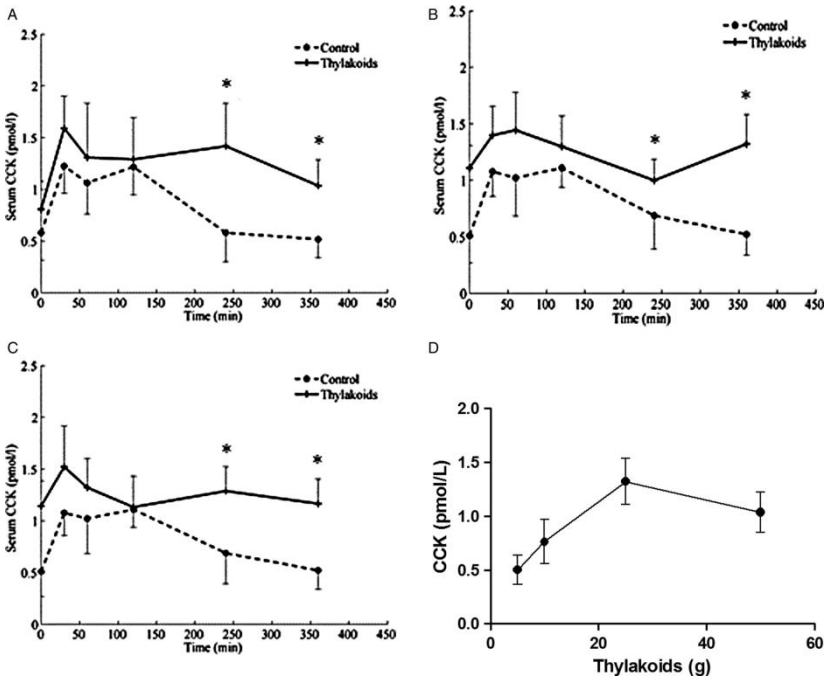


Figure 22. Serum cholecystokinin (CCK) levels in humans after ingestion of a control meal and thylakoid-enriched meals. A. 50 g thylakoidenriched meal; B. 25 g thylakoid-enriched meal; C. 25 g delipidated thylakoid-enriched meal; D. Dose_response curve for the thylakoidenriched meal at time-point 6 h postprandially. Data are given as mean values (9SEM) for 11 subjects (Köhnke et al. 2009b).

Purified thylakoid membranes were precipitated in range of pH 3.5-5.5 and it was observed that, more than 80% of thylakoid membranes were precipitated between pH 4 and 5. Maximum precipitation (above 95%) occurred around pH 4.7 (figure 23), which is the isoelectric-point of the thylakoid membranes (Åkerlund et al. 1989) and was chosen for preparation of crude thylakoids.

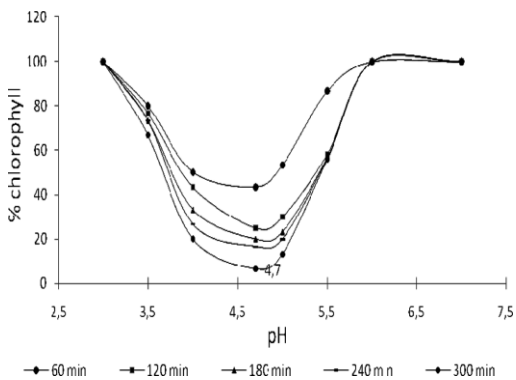


Figure 23. The precipitation curve of thylakoid membranes at different pH.

The inhibitory effect on the lipase/colipase activity of crude (precipitated) thylakoids was the same as pure thylakoids (paper II). The co-precipitation of other membranes, i.e. plasma membranes, mitochondria and chloroplast envelop, did not have any negative effect on the inhibition of lipase/colipase, probably since these membranes, like thylakoid membranes, consist of membrane spanning proteins. However, the contribution of these membranes is very limited since thylakoids are dominating membranes of leaf cells (paper II).

The main differences of SDS-PAGE gel electrophoresis of "purified" and "crude" thylakoids were that the two additional bands representing large and small subunits of Rubisco, which were identified with MALDI-TOF analysis (figure 24). Rubisco is the most abundant water-soluble enzyme on earth and located in the chloroplast stroma (Feller et al. 2008). The isoelectric point of Rubisco is 5.5 (Mori et al. 1984), i.e. close to the isoelectric point of thylakoids, which explains the co-precipitation of Rubisco with thylakoid membranes.

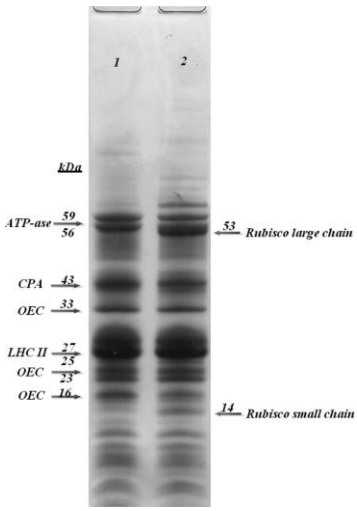


Figure 24. SDS-PAGE of purified (left) and crude thylakoids (right).

Thylakoid membranes contain several pigments, e.g. chlorophyll *a* and chlorophyll *b*, and carotenoids, xanthophylls; violaxanthin, antheraxanthin and zeaxanthin. Zeaxanthin was particularly interesting to analyze (paper II), since it has been suggested to have health promoting properties and protect human eye from age related macular degeneration (Seddon et al. 1997). Thylakoids isolated in the cold at physiological pH showed only small amounts of zeaxanthin, whereas at lower pH isolated thylakoids had more than 5-fold increased amount of zeaxanthin (figure 25).At a combination of low pH and increased temperature even higher values were obtained

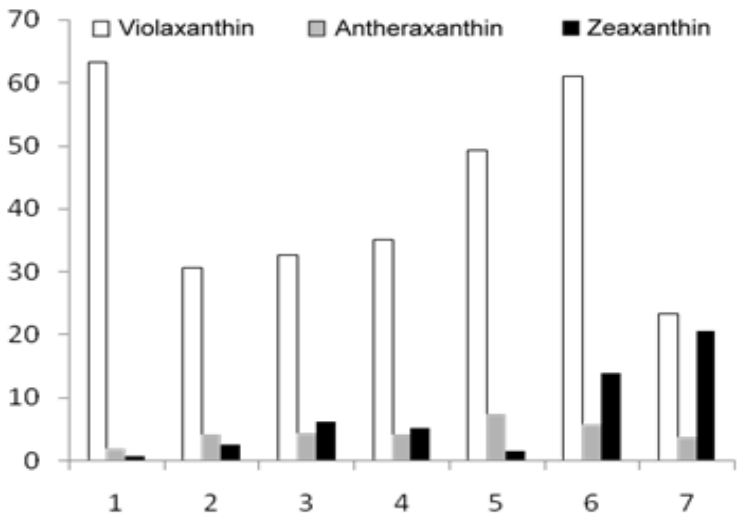


Figure 25. The effect of the pH and temperature on the relative amount of the xanthophyll cycle pigments. 1: Purified thylakoids, pH 7.4, 4^0 C; 2: Purified thylakoids, pH 7.0, room temperature, 3: Crude thylakoids, pH 4.7, 4^0 C; 4: Frozen leaves; 5: Fresh leaves; 6: Crude thylakoids, pH 5.2, 4^0 C; 7: Crude thylakoids, pH 5.2, room temperature. Values are expressed as mmol of pigments per mol of chlorophyll (a+b).

5.4 Digestion of thylakoids

After finding that thylakoid membranes induce satiety when included in food, the question raised how rapidly thylakoid membranes were broken down by digestive enzymes of the gastrointestinal tract. In paper III, we studied the effect of proteases pepsin and trypsin, as well as gastric and pancreatic juice on the thylakoid membranes.

Thylakoid membrane proteins were treated with pepsin and the effect were visualized on the SDS-PAGE (figure 26). Two bands stand out as more resistant against pepsin degradation; one is representing the light harvesting proteins (LHCI and II) around 25 kDa and the other an unidentified band just below 55 kDa. Thylakoid membranes observed in the form of both swollen membrane vesicles and stacked grana-like structures on the EM-picture (figure 26) of the pepsin treated thylakoids in oil-water emulsion (paper III).

The effect of the trypsin treatment on the thylakoid membranes on the SDS-PAGE (figure 27) shows that around 25 kDa LHC I and LHC II proteins, as well as a 55 kDa protein were resistant towards the degradation (paper III). The trypsin treated

thylakoid membranes were again found as slightly swollen membrane-vesicles attached on the oil-water surface on the EM-picture (figure 27).

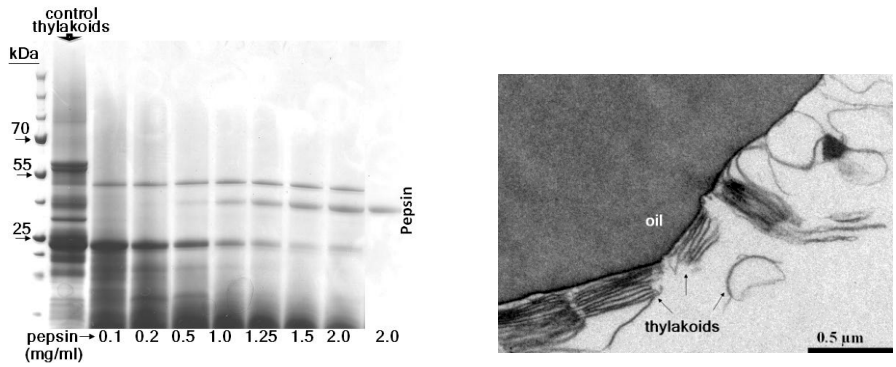


Figure 26. On the left - SDS-PAGE picture of thylakoid membranes treated with pepsin. Thylakoid membranes (1 mg/ml chlorophyll) were treated with varying amount of pepsin at 37° C for 1 h at pH 2.0. EM-picture thylakoid membranes (1mg/ml chlorophyll) treated with pepsin (1mg/ml) at 37° C for 1 h in an oil-water emulsion, pH 2.0. The thylakoid membranes are attached to the oil surface (on the right).

As mentioned in chapter II, pancreatic juice contains several proteases, lipases and other digestive enzymes. The effect of both porcine and human pancreatic juice on the thylakoid membrane was studied also with SDS-PAGE, electron microscopy and mass spectroscopy (paper III). The SDS-PAGE analysis show (figure 28) that after 2 hours treatment, most of the proteins were degraded, except, as treatment by pepsin and trypsin, LHCI, LHCII and the 55 kDa bands. A larger down-shift of LHC was observed compared to the pepsin or trypsin treatments (figure 14 and 15), suggesting that a larger part of LHC protein had been split off (paper III).

Most remaining polypeptide bands after porcine pancreatic juice treatment (the treatment of human pancreatic juice gave same results) on the gel were analyzed with MALDI-TOF mass spectrometry. The analysis showed that the most resistant proteins were the pigment-protein complexes, i.e. PSI, PSII with their respective light harvesting complexes LHCI and LHCII. Both α- and β- subunits of ATP-synthase were also resistant towards the degradation (paper III).

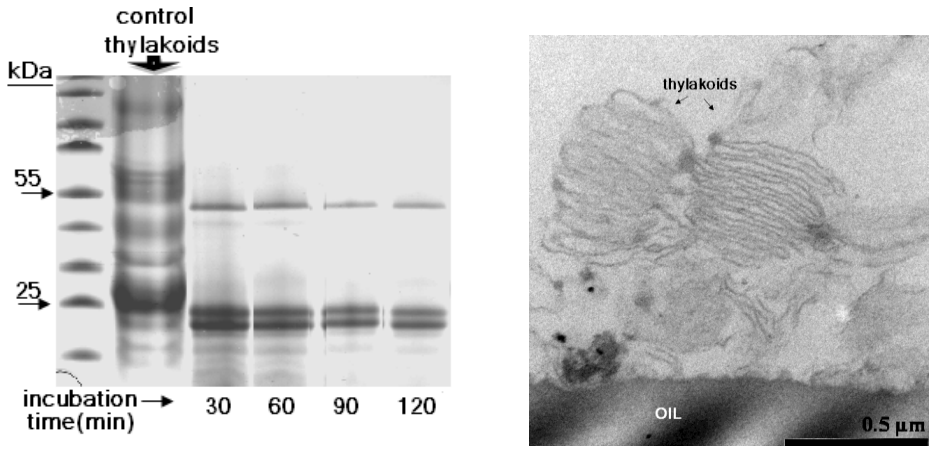


Figure 27. SDS-PAGE picture of thylakoid membranes treated with trypsin. The thylakoid membranes (1mg/ml chlorophyll) were treated with 300 µg trypsin/mg *chl* during different incubation time at 37° C (picture on the left). EM-picture of thylakoid membranes (1mg/ml chlorophyll) treated with trypsin (1mg/ml) at 37° C for 2h in oil-water emulsion with 4 mM NaTDC (picture on the right).

The thylakoid membrane vesicles after treatment of porcine pancreatic juice were more irregular and unfolded on the EM-picture (figure 28) compared to the EM-picture of the pepsin or trypsin treated thylakoids. This is maybe due to the presence of bile-salts and other enzymes.

To simulate the *in vivo* digestion process, thylakoid membranes were treated with human gastric juice followed by human pancreatic juice treatment (paper III). The results, as seen in figure 29, show that the continuous treatment is somewhat more effective than the single treatment with pancreatic juice alone; however, the bands representing LHCI and LHCII proteins are still present to about the same extent.

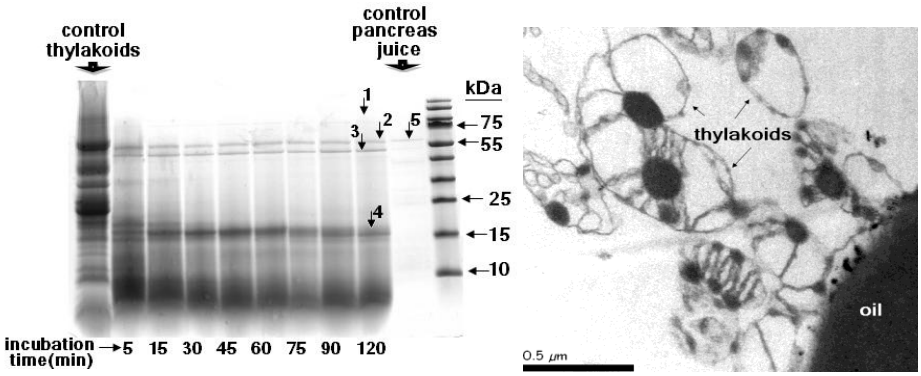


Figure 28. On the left; SDS-PAGE picture of thylakoid membranes treated with porcine pancreatic juice (0.5 mg/ml). Treatment was done at 37° C for different incubation times. Down pointing arrows on the gel picture show proteins identified with MALDI-TOF ms/ms analysis: 1) Photosystem I P700, 2) Pancreatic alpha-amylase, 3) ATP synthase subunit alpha, Photosystem I, P700, 4) LHC Proteins, 5) Pancreatic alpha-amylase. Note the almost complete breakdown of the proteins to the polypeptide size of about 2 kDa. On the right; EM-picture of thylakoid membranes (1mg/ml chlorophyll) treated with pancreatic juice (0.5 mg/ml) at 37 ^{o}C for 2h in an oil-water emulsion with 4 mM NaTDC. The thylakoid membranes attached to the oil surface are unfolded and swollen. The dark bodies represent plastoglobules.

After observation that the pigment containing proteins (LHCI and II) were remarkably resistant toward the breakdown of gastrointestinal enzymes, we treated spinach plasma membranes, which are non-pigment containing membranes, with pancreatic juice (paper III). The digestion of plasma membranes proteins was very rapid and almost all proteins were digested already after 5 minutes treatment (figure 30).

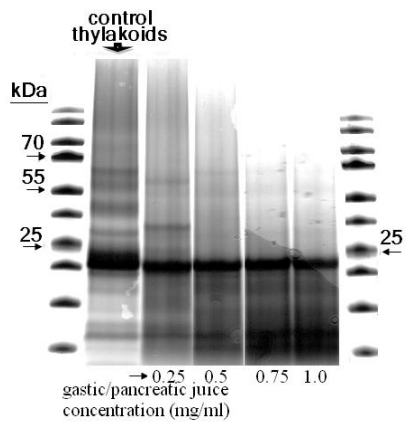


Figure 29. SDS-PAGE of thylakoids treated first with human gastric juice (0,25-1.0 mg/ml) 1h, 37° C, pH 2.0, then human pancreatic juice (0,25-1.0 mg/ml) 2h, 37° C, pH 7.0

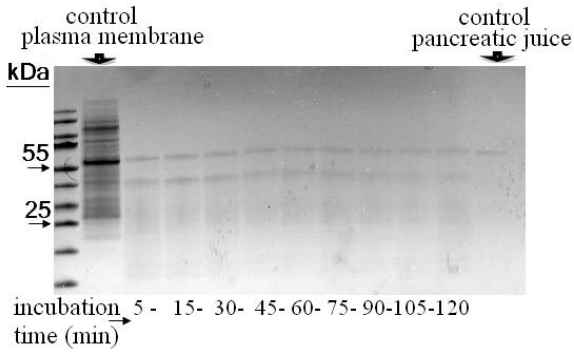


Figure 30. SDS-PAGE of porcine pancreatic juice (0.5 mg/ml) treated spinach plasma membranes (5 mg/ml protein). Membranes were treated during different incubation times at 37^0 C.

Additional support to the effect of pigment to protect the proteins towards the breakdown was obtained in the studies with the delipidated thylakoid membranes (paper III). Delipidated thylakoid membranes (ethanol: chloroform extraction) were treated with pepsin, trypsin and pancreatic juice (figure 31).

Almost all delipidated thylakoid proteins, including photosystems and their light harvesting complexes, except for a polypeptide band about 50 kDa (identified as the alpha chain of ATP synthase) were degraded after the treatment of pepsin (figure 31A). This result is similar to the effect of pepsin on the intact thylakoids, suggests that the lipid and/or pigments protect the pigment containing proteins complexes, i.e. PSI and II reaction centers and LHCI and II, towards degradation of pepsin (paper III).

The effects of the trypsin and pancreatic juice treatment (figure 31B and C) were almost the same and resulted in an almost complete degradation of delipidated thylakoid proteins. We conclude, again, that the more effective degradation of delipidated thylakoids to be due to the absence of the protecting effect from pigments and/or membranes lipids (paper III).

The capacity to inhibit the activity of the lipase/colipase system *in vitro* by thylakoid membranes treated with pepsin, trypsin and pancreatic juice and the effect of the oil emulsion on the degradation of thylakoid membranes were also studied (paper III). Pepsin treatment of the thylakoid membranes reduced their capacity to inhibit the lipase/colipase complexes from 80% to about 50% (figure 32A).

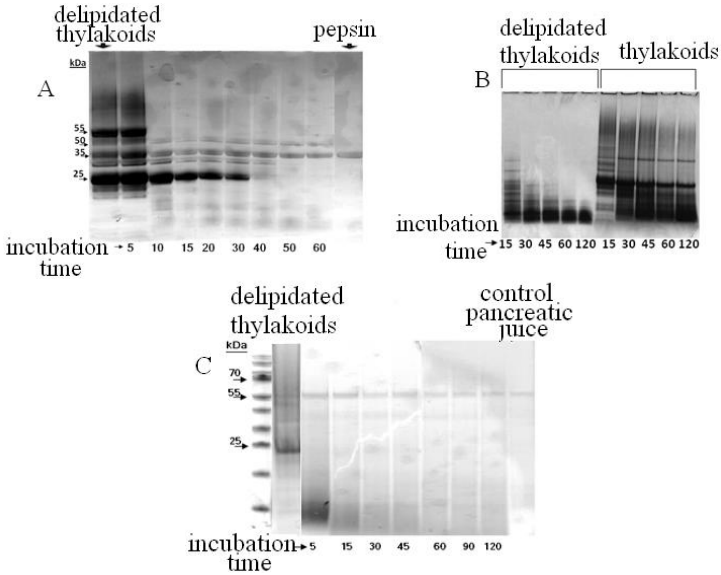


Figure 31. SDS-PAGE pictures of delipidated thylakoids treated with A) pepsin (1mg/ml),B) trypsin (0.3 mg/ml) and C) pancreatic juice (0.5 mg/ml)

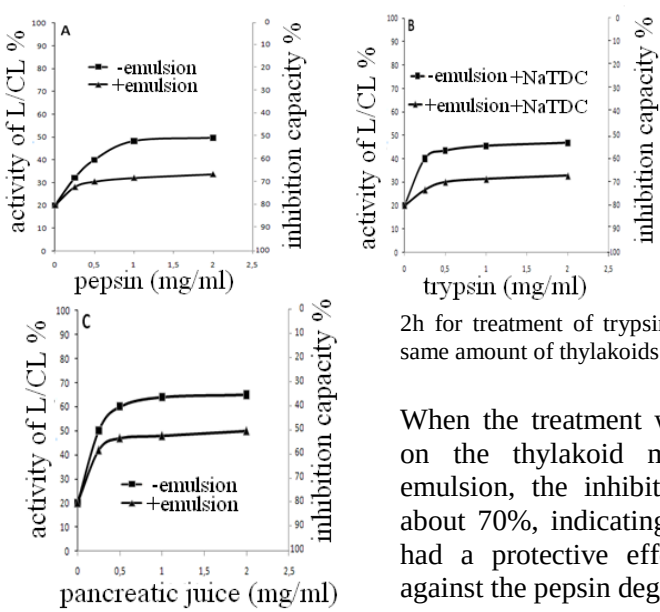


Figure 32. Effect of pepsin, trypsin and pancreatic juice treatment of thylakoid membranes (with and without emulsions) on the activity of lipase/colipase. The treatments were done at 37° C. The incubation times were 1h for treatment of pepsin and 2h for treatment of trypsin and pancreatic juice. The same amount of thylakoids was used as in Fig. 8.

When the treatment with pepsin were done on the thylakoid membranes in an oil emulsion, the inhibition capacity was still about 70%, indicating that the oil emulsion had a protective effect on the thylakoids against the pepsin degradation (paper III).

Similar effects were obtained from the treatment of both trypsin (figure 32B) and pancreatic juice (figure 32C).

The dominating polypeptide of thylakoid membranes is LHCII, which has four main membrane embedded alpha helices (figure 33). Most likely, the proteases acted first on the N-terminal, external polypeptide chain on the stromal side if the thylakoid membrane vesicles.

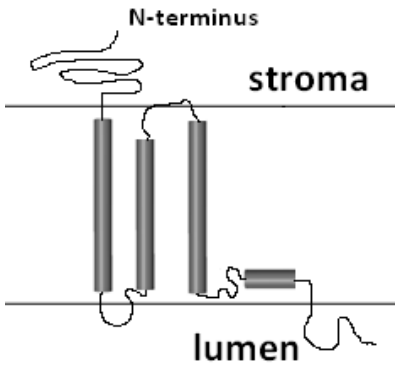


Figure 33. Schematic representation of LHC II monomer (Lhcb1) embedded in the thylakoid membrane. The stroma side is on the outside and lumen side on the inside of the thylakoid membrane vesicles. Only the N-terminus external loop (54 amino acids) is easily available for proteolysis.

This stromal side polypeptide chain is 54 amino acids long with seven theoretical pepsin cleaved sites and the degradation of this fragment can explain the very slight reduction in the molecular weight of the 25 kDa bands. This "shaving" of membranes occurred only on the outside (stromal side) of the membranes since the inside (luminal side) was not available for the protease attack (paper III).

We conclude in paper III that the pigments and lipids around the membrane spanning helices provide a barrier against the proteases to act on their substrate and thereby retard the digestion of thylakoids. The free fatty acids, products after hydrolysis of fat in stomach and intestine (chapter II), and amphiphilic bile salts are incorporated into the thylakoid membranes, thus, may also contribute to the protection of thylakoids towards proteases.

The capacity to inhibit the activity of the lipase/colipase of thylakoid membranes *in vitro* was reduced about 50% in the case of pepsin or trypsin treatments, and about 40% in the case of treatment with pancreatic juice. We suggest that this could either be due to the "shaving" of some hydrophobic groups on the surface of the treated thylakoids causing a reduced ability for the thylakoids to adsorb onto lipid droplets and/or to less adsorption of lipase/colipase onto the thylakoids. The folding of thylakoid membranes might be modified such that the exposed surface area is reduced (paper III).

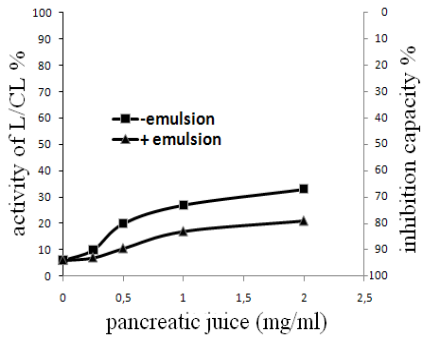


Figure 34. The effect of delipidated thylakoid membranes treated with pancreatic juice with and without emulsions on the activity of the L/CL.

The effect of the pancreatic juice treatment of the delipidated thylakoids to inhibit the lipase/colipase activity is shown in figure 34. Small protein fragments, about 2 kDa representing probably the membrane spanning hydrophobic helices, are still efficient in inhibiting the activity of lipase/colipase.

This result is consistent with earlier results (paper I) demonstrating that a synthetic polypeptide (one of the LHCII membrane spanning alpha helix) is able to inhibit lipase/colipase.

5.5 Thylakoid membranes as an emulsifier

The presence of the oil in the form of emulsion protects the thylakoid membranes towards the degradation by the proteases and the inhibition capacity is less reduced. We explained this effect in the following way that when thylakoids are absorbed into the lipid droplets part of the thylakoid membrane surface will be less available to pepsin, trypsin and other pancreatic enzymes; hence digestion will be slowed down (paper III).

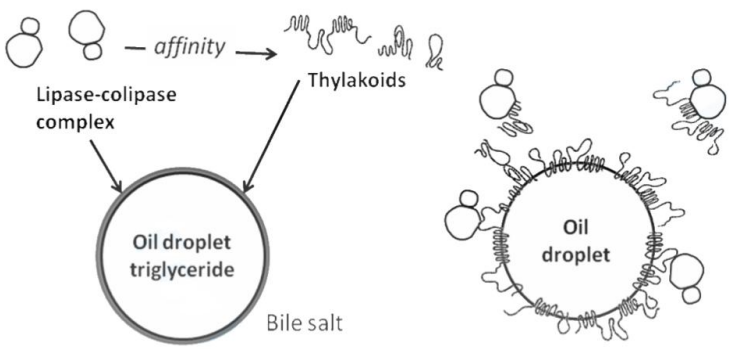


Figure 35. Trilogy mechanism for thylakoids; lipase/colipase complex can absorb both to the bile-salt covered surface of triglyceride oil droplet as well as to the hydrophobic region of the thylakoids.

The two suggested mechanisms for the inhibition of the lipase/colipase activity are that; 1) adsorption of the lipase/colipase complex onto the thylakoid membrane surface such that the substrate binding site of the enzyme is hindered at the

thylakoid surface and 2) adsorption of the thylakoid membranes at the surface of the fat droplets thereby sterically hindering the lipase/colipase to come in contact with its substrate. As described in figure 35, these two interactions together with the affinity of the lipase/colipase complex for its substrate the oil leads to the "triology" (paper IV).

To use thylakoid membranes as an emulsifier they are already present in the food matrix before digestion. In an oil-water-thylakoid emulsion, thylakoid membranes protect the oil from the lipase/colipase and at the same time, oil protects thylakoids from the proteases and other digestive enzymes.

The effect of pH on the thylakoid emulsions was studied in paper IV and the results show that most clear aqueous phase in the range 3-5 (figure 36). Since the isoelectric point of thylakoid membranes is 4.7, at pH 3 and 4 thylakoid membranes have a net positive charge. The electrical potential between the oil/water phases is such that the water phase is more positive than the oil phase and adsorption of the thylakoids to the interface will be facilitated (paper IV).

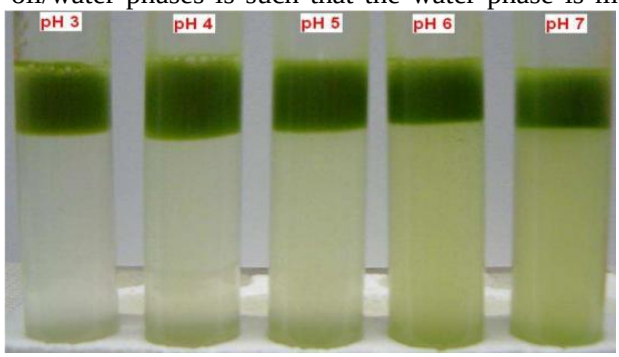


Figure 36. Thylakoid emulsions at different pH

In this study (paper IV), we provide results show that thylakoid membranes stabilized emulsions, thereby, this is the first time a cell organelle has been used as both an emulsifier and as a functional ingredient. Thylakoid membranes provide excellent stability against coalescence, thus, membrane vesicles attaching to the oil surface, creating a particle stabilized emulsion, where both lipids and proteins are part of the active material.

5.6 Strong binding of pancreatic lipase/colipase to thylakoids

The strength and capacity of isolated thylakoid membranes to bind pancreatic lipase/colapase were studied (Paper V). The dissociation constant (Kd) was found to be 1.04×10^{-7} M and the maximum amount of bound lipase/colipase complex to the thylakoid membrane surface was 5.4 nmoles per mg chlorophyll (figure 37). The occupated area of thylakoid membrane surface by lipase/colipase complex was about 5%.

Treatment of the thylakoids with pepsin followed by trypsin or treatment with pancreatic juice showed that the treated thylakoids still had about the same strong binding and about the same capacity for binding (paper V)

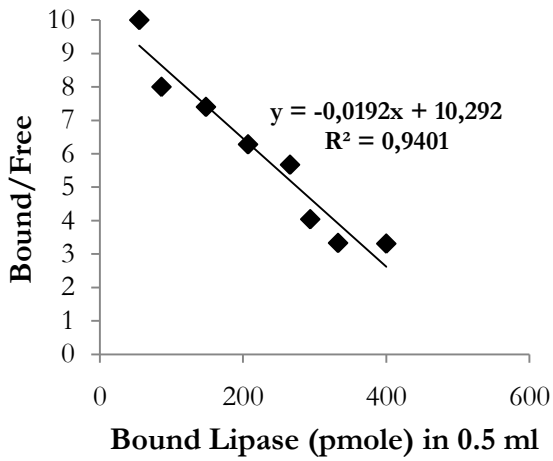


Figure 37. A scartchard plot of lipase/colipase to untreated thylakoid membranes

Delipidated thylakoids showed a more heterogeneous binding strength and a somewhat higher dissociation constant (8.46×10^{-7} M), and less binding capacity, 4,2 nmole/mg chlorophyll, compared to intact thylakoids. Treatment with the proteases or pancreatic juice did not drastically change binding strength or the binding capacity. We concluded that the results show a strong binding for the lipase/colipase complex to the thylakoid membranes; involving both hydrophobic effects and ionic interactions (paper V).

Populärvetenskaplig sammanfattning

Fett som tas in i kroppen bryts huvudsakligen ned i tunntarmen med hjälp av ett enzym, lipas och dess regler-protein colipas. Colipas binder sig till lipas och gör att lipas fastnar på fett dropparna som emulgerats av gallsalter. Både lipas och colipas utsöndras av bukspottkörteln.

I de gröna växternas blad finns kloroplaster som fångar upp ljus och därvid bildar syre och stärkelse genom fotosyntesen. Själva ljusupptagningen och syreproduktionen äger rum i membraner, de så kallade thylakoiderna, som befinner sig inne i kloroplasterna. De består bland annat av proteiner som binder till sig olika pigment som klorofyll och karotenoider som fångar upp ljuset vid fotosyntesen. Thylakoider finns i alla gröna blad och är de biologiska membraner som det finns mest av på jordklotet.

Vi har visat att om vi blandar thylakoider med lipas/colipas och fettdroppar så hämmas nedbrytningen av fettet. Detta beror på att lipas/colipas dels fastnar på thylakoiderna dels att thylakoiderna fastnar på fettdropparna och därmed hindrar lipas/colipas att kpomma i kontakt med fettet som skall brytas ned.

En metod har utvecklats som möjliggör att stora mängder av thylakoider extraheras från gröna blad t.ex.spenat. Tack vare denna metod har vi kunnat göra försök för att se vad som händer när vi tillsätter thylakoider i födan både hos försöksdjur och hos människa.

Eftersom thylakoider fastnar på fettdroppar bildar de lätt emulsioner vilket är en fördel om man vill tillsätta dem i olika livsmedelsprodukter.Thylakoiderna är själva ett födoämne, de innehåller proteiner och fett, och de bryts själva ned vid matspjälkningen, men de bryts ned relativt långsamt tack vare att pigmenten delvis skyddar proteinerna mot matspjälkningen. Thylakoiderna hämmar därför fettnedbrytningen endast temporärt. Så småningom bryts all fett ned och tas upp i tarmen. Nettoresultatet blir att fettspjälkningen endast förlångsammas och detta framkallar i sin tur en ökning av vissa mättnadshormoner såsom cholesystokinin, leptin och enterostatin, och minskning av ett hungerhormon, ghrelin, och detta ger en ökad mättnadskänsla och en dämpad hunger. Det finns därför en möjlighet att använda thylakoider som tillsats i mat för att stärka aptit- och viktkontroll.

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