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The effect of heat-treatment of thylakoids on their ability to inhibit *in vitro* lipase/co-lipase activity

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Abstract

Thylakoids has been shown to prolong lipolysis by the inhibition of lipase/co-lipase, which makes thylakoids suitable as a functional food ingredient with satiating properties. The components of thylakoids that provide its function as a lipolysis modulator are primarily photosystems I and II, which are structurally stabilised by chlorophyll. However, chlorophyll is known to be heat sensitive yet the enzymatic inhibiting capacity after heat treatment has not been previously studied. It was hypothesised that the retained function of thylakoids after heat treatment could be correlated to the degree of degradation. Heat treatment at either 60°C, 75°C or 90°C for time interval ranging from 15 sec to 120 min induced a color shift from bright green to olive brown which was attributed to degradation. The ability of heat-treated thylakoids to inhibit lipolysis *in vitro* was also reduced. A correlation between chlorophyll *a* degradation and the enzymatic inhibiting capacity could be established which opens possibilities to use a spectrophotometric method to quantify the ability of thylakoids to inhibit lipase/co-lipase in a more rapid and cost effective way to complement the pH-stat method used today. With the degradation pattern investigated, it is then possible to design a thermal treatment process to ensure a microbiological safe appetite-reducing product and at the same time minimize the loss of functionality.

Keywords

Spinach, photosynthetic membranes, chlorophyll, lipase, co-lipase, thermal processing, heat stability

1. Introduction

1.1 Plant components as functional ingredients in weight management

A functional food ingredient that reduces appetite could be a strategy to counteract the increase in human obesity, which has reached epidemic proportions in Western society[1]. With obesity, occurrence of type 2 diabetes as well as cardiovascular diseases increases, which in turn are closely related to premature death[2]. The Western diet contains a high proportion of fat, which is obesity promoting. A strategy based on regulating the bioavailability of dietary fat could therefore counteract over-consumption. During intestinal lipid digestion, lipase and its cofactor co-lipase are known to be the key enzymes[3]. The use of lipase inhibitors in pharmaceuticals targeting obesity is well established, Orlistat being present at the market today. A known side effect of this drug and similar lipase inhibitors is steatorrhea due to impaired lipid digestion[4]. An alternative to a pharmaceutical approach to lipase inhibition is to use food-based components to prolong lipid digestion and increase satiety, yet without causing negative side effects that reduce patient compliance. Thylakoid membranes isolated from spinach have been shown to inhibit lipase and its cofactor co-lipase without causing steatorrhea.

Palatable food often contains lipids in emulsified form i.e. ice cream, mayonnaise and sauces, and a surfactant is needed to stabilize the lipid droplets present in these products. Thylakoids are efficient emulsifiers[5] and can at the same time decrease the rate of lipolysis. If a functional emulsion-based food with appetite regulative properties shall be created, it is necessary to ensure the microbiological safety and at the same time retain the ability to prolong lipolysis after standard food processing conditions. One such condition is thermal treatment with the purpose of pasteurization and elimination of pathogens[6].

1.2 Structure and function of thylakoid membranes in plants

Thylakoids are the photosynthetic membrane found in the chloroplast of plant cells and is the most abundant biological membrane on earth. The thylakoid membrane is responsible for conversion of solar energy into ATP and NADPH, which are used in the chloroplast for assimilation of carbon dioxide in the production of carbohydrates[7]. Together with their bound pigments, chlorophylls *a* and *b*, luteins, xanthophylls and carotenoids, the membrane proteins constitute up to approximately 70 % of the thylakoid mass. The membrane lipids constitute the majority of the remaining 30 %[8]. Membrane proteins are both extrinsic, i.e. attached to the membrane surface, and intrinsic, i.e. membrane spanning. The main extrinsic membrane complexes are plastocyanin (PC) and ferredoxin (Fd) and the main intrinsic membrane protein complexes are photosystem I (PS I) with light harvesting complex I (LHC I), photosystem II (PS II) with light harvesting complex II (LHC II), cytochrome b_6f and ATP synthase[9].

During photosynthesis reducing power in the form of NADPH is generated by PS I, while PS II transfers the electrons of water to a quinone and simultaneously evolves O_2. Electron flow between the photosystems generates a proton gradient that is used to drive the synthesis of ATP. The X-ray structure of both LHC I and II (associated to the photosystems I and II) has been reported as trimers built up by hydrophobic helices with attached pigments such as chlorophyll *a* and *b*, lutein, neoxanthin and carotenoids[9,10]. It has been shown that chlorophyll *a* separates the helices inside the monomers whereas chlorophyll *b* separates the different monomers from each other (Fig 1). By this arrangement the pigments act to prevent aggregation of the hydrophobic helices inside the LHC I and II and are thereby supporting the structure[11].

1.3 The effect of thylakoid isolates on lipolysis when included in food

In a series of previous studies we have found that thylakoid membranes, the photosynthetic membrane in plant cells, inhibit pancreatic lipase/co-lipase in a dose-dependent way *in vitro,* an effect confirmed both in animal studies on rat[12] and mice[13] as well as in human studies[14,15]. Specifically thylakoids isolated from spinach have been studied due to the high content of thylakoid membranes per plant cell. Thylakoids reduce the lipase/co-lipase activity by up to 80% by primarily adsorbing to the oil-water interface and thereby hindering the enzyme complex to reach its substrate[12]. Since thylakoids are biological membranes composed of proteins and lipids, digestive enzymes such as pepsin and trypsin degrade the thylakoid membranes after approximately four hours *in vitro*[16]. The degraded membranes eventually are detached from the oil-water interface and lipid digestion can continue without steatorrhea[17]. Previous studies both in animal and human models have shown that when thylakoids were included in foods, satiety hormones such as cholecystokinin (CCK), leptin and enterostatin increased while the hunger peptide ghrelin decreased[12-14]. This phenomenon has been attributed to a prolonged lipid digestion, and as long as food is present in the intestine, satiety is promoted[4].

Thylakoids have a complex structure and the mechanism of action on the molecular level is important to elucidate. A series of investigations have previously been undertaken to find specific parts of the thylakoid membrane that could be responsible for the inhibition of lipolysis. First, the thylakoid membranes were delipidated and the inhibiting effect on lipase/co-lipase remained[12]. This indicated that the protein fraction and not the lipid fraction of the thylakoids were mainly responsible for the lipase inhibition. Other plant extracts such as galactolipids, in particular digalactosyldiacylglycerol (DGDG) isolated from spinach have

been reported to inhibit pancreatic lipase[18]. This is in contrast to our previous work, where the inhibitory action was observed in the protein fraction[12].

To further elucidate which specific part of the membrane proteins that contributed to lipase inhibition, the thylakoid membranes were treated by trypsin and the extrinsic proteins were removed, yet the lipase inhibiting effect remained present[12]. When one of the intrinsic protein complexes, an isolate of LHC II, was tested it was demonstrated that it alone had an inhibiting effect[12]. A synthetic polypeptide with the same sequence as one of the hydrophobic α-helices of LHC II was examined and the effect on the enzyme activity was maintained, although not to the same extent as the naturally occurring LHC II with three helices. It was therefore concluded that the hydrophobic α-helical structure played a decisive role in the thylakoid membrane with respect to lipase inhibition, prolonging the lipolysis and in turn enhancing satiety[12]. LHC I was not evaluated alone but since the structure is similar to LHC II (monomers composed by hydrophobic helices) the protein complex likely contributes at least in part to the thylakoids inhibition effect. All biological membranes contain intrinsic membrane proteins that have a conformation including an alpha-helix structure consisting of an amino acid sequence that exposes hydrophobic groups, which are in contact with the lipid portion of the membrane lipid bilayer. Such structures from other biological membranes such as mitochondria from potato tuber and chicken heart and intracytoplasmic membrane fragment from protobacteria have also been shown to inhibit lipase/co-lipase[12]. However thylakoids contain much more of these structures and are therefore of particular interest. Within the thylakoid membrane, photosystem I and II which are the structures harbouring LHC I and II, which together covers almost 70% of the thylakoid surface. Since the thylakoids' ability to prolong lipolysis is a surface-related phenomenon, and the hydrophobic

helices has been shown to play a decisive role, both photosystem I and II are thus believed to be involved in the mechanism of lipase inhibition.

The mechanism behind the enzyme inhibition was first suggested to be a combination of two phenomena: i) thylakoids binding to active sites of the lipase/co-lipase complex and ii) thylakoids interacting with the oil-water interface thereby hindering lipase/co-lipase from digesting the lipid droplets. However, binding studies later showed that only a small fraction of the thylakoids bound directly to lipase/co-lipase[19]. The main mechanism behind thylakoids prolonging satiety was therefore considered to be a surface-related phenomenon with hydrophobic interactions between thylakoids and the oil-water interface. Thylakoids are thereby blocking lipase/co-lipase from their substrate. The thylakoids ability to attach to the oil-water interface was confirmed and quantified by emulsification studies[20]. Lipid droplets have also been shown to protect thylakoids from digestion by proteases[16]. When thylakoids were analysed in an emulsion matrix they retained the ability to inhibit lipase/co-lipase in the gastro-intestinal environment better, compared to non-emulsified thylakoids, i.e when absorbed at an oil-water interface rather than in solution with dissolved lipids such as tributyrine. This may reduce the rate of lipolysis and thereby increase the transit time for the lipids, leading to increased satiety. The modification or structuring of emulsion interfaces to control their rate of digestion had been the topic of several recent reviews[21-23] where the main approaches to increase the GI transit time include increasing droplet size, varying the molecular structure of the lipids and the interfacial composition.

1.4 Functional consequences of heat treatment

It is widely known that when parts of green plants, such as thylakoids, are heat treated, chlorophyll undergoes degradation (Fig 2). This phenomenon has been studied in a wide

variety of vegetables as spinach, mint, coriander, broccoli and peas[24,25]. Chlorophyll degrades to either pheophytin (by heat or acid) or to pheophorbide (by enzymatic breakdown and heat). The degradation of chlorophyll to pheophytin or pheophorbide during processing induce a color shift from intense green to olive brown due to replacement of the central Mg^{2+} ion by two H^{+} ions[26]. The functional parts of the thylakoid membrane are protein complexes stabilised by chlorophyll (i.e. PS I and II, including LHC I and II). Since chlorophyll is heat sensitive, the thermal degradation of chlorophyll may be correlated to the thylakoids function *in vitro.*

1.5 Hypothesis and aims

The question to be addressed in the present study is: How does heat treatment affect the thylakoids ability to prolong lipolysis *in vitro*? The aim of this work was to investigate if thylakoids after heat treatment, still can inhibit intestinal enzymes *in vitro* and if a correlation to chlorophyll degradation could be established.

2. Material and method

2.1 Preparation of thylakoid isolates

Spinach leaves (Spinacia oleracea), field grown and harvested under controlled conditions, were a kind gift from Anders Jönsson (Revinge Jordbruks AB, Sweden). The spinach was frozen directly after harvest and used in all experiments. Thylakoids were extracted essentially as described by Emek et al[8]. Briefly, 3 kg of spinach leaves and 3 L of water were homogenised in a blender (Robot Coupe R8, Robot Coupe SA, Bourgogne, France) for 10 min to obtain a smooth green slurry, which was then filtered in room temperature (25°C) through a Monodor polyster filter (20 μm mesh). The filtrate was poured into 750 ml tubes and centrifuged (Beckman Coulter Allegra X-15 R Centrifuge, Fullerton, CA) at 5000 x g,

4°C, 30 min. The supernatant was discarded and the thylakoids in the pellet was collected and re-suspended with fresh water in a glass Potter Elvehjem homogeniser until a homogenous slurry was obtained. This crude preparation obtained, also contains co-precipitation of vesicles from other membranes, i.e., plasma membranes, mitochondria and chloroplast envelopes. This is of no disadvantage with respect to lipase/co-lipase inhibition, since these membranes, like thylakoids, consist of membrane spanning proteins. However, their contribution is only marginal since the thylakoids are the dominating membranes of leaf cells. Dimethyl sulfoxide (DMSO, 99.5%, Sigma, St Louis, MO, USA) was added to a final concentration of 5 vol %, to avoid aggregation during freezing. Thylakoid slurry gave the same enzymatic inhibition with or without 5 vol % DMSO present, and it was concluded that the addition did not influence the results. Dry matter (1.1±0.02%) was determined according to the official method of analysis (AOAC). Analysis was performed in triplicate. The thylakoid slurry was distributed into test tubes, frozen to -18°C and stored frozen until use. Before the thermal degradation experiments were carried out, the isolated samples were thawed in a cold-water bath and stored on ice until use, the samples were therefore never exceeding 1°C until start of the heat treatments.

2.2 Heat treatments

To address the question of how heat treatment affect the thylakoids ability to prolong lipolysis, it is central to control and monitor the times and temperatures correctly. With this as a goal, the experimental set-up was verified and the time required to reach the set temperature (come-up time) was evaluated. Heat treatments were carried out at 60°C and 90°C for time intervals ranging from 15 sec – 120 min and 75°C for time intervals ranging from 15 sec – 4 min. The thylakoid slurry was filled in a bent helical coil of stainless steel (inner diameter = 2 mm) formed as a cylinder (inner diameter = 11 cm, height = 6 cm). A thermostatic oil bath (Julabo HC-8, Julabo, Seelbach, Germany) was used as heating device and the temperature was

measured with ± 0.1 °C accuracy. The come-up time was determined for every sample by inserting a thermocouple (type K, 0.1 mm) into the annular centre of the metal tube, at least 10 cm into the length of the tube. After withdrawal from the oil bath, the samples were immediately cooled in an iced water bath to avoid a lag in cooling. The come-up time for each processing temperature was 20.2 ± 3 sec due to maximized contact surface of the spiral cylinder (Fig 3). This was a considerable shorter come-up time than those reported in other studies of chlorophyll degradation during processing of plant materials which are in the order 3-4 min[24,26]. The resulting heat-treated slurry was distributed to test tubes and stored in -18°C freezer until used for analysis of enzymatic inhibition capacity and determination of chlorophyll. The heat treatment was performed in triplicate at every time-temperature combination. When the temperature and times are controlled, it is possible to quantify the degradation of chlorophyll as well as the heat sensitivity of thylakoids in relationship to their capacity to inhibit lipase/co-lipase *in vitro.*

2.3 Spectrophotometric analysis

2.3.1 Determination of chlorophyll content

Chlorophyll content is proportional to the amount thylakoids and is used as quantifying unit. The chlorophyll content in the non-treated thylakoid slurry was determined by photo spectroscopy according to Porra et al[27]. 30 μl thylakoid slurry was added to 2 ml ice-cold acetone (80 vol %). The samples were vortexed and incubated dark and on ice for 20 min and were thereafter centrifuged (Eppendorf Mini Spin, Eppendorf AG, Hamburg, Germany) at 12 100 x g for 4 min, at 25°C. Absorbance at λ=646.6 nm and λ=663.6 nm was quantified in a spectrophotometer (Varian Cary 50 Bio UV-Vis, Varian Inc., Santa Clara, CA, USA) against a blank of acetone (80 vol %) and the chlorophyll *a*, *b* and total concentration was determined:

$$Chl\ a = 12.25\ \cdot A_{663.6\,nm} - 2.550\ \cdot A_{646.6\,nm} \quad (3)$$

$$Chl\ b = 20.31\ \cdot A_{646.6\,nm} - 4.910\ \cdot A_{663.6\,nm} \quad (4)$$

$$Chl(\ a + b) = 17.76\ \cdot A_{646.6\,nm}\ + 7.340\ \cdot A_{663.6\,nm} \quad (5)$$

The total chlorophyll content in the non-treated thylakoid slurry was 0.767±0.02 mg/ml.

2.3.2 Absorbance spectra

30 µl thylakoid slurry (non-treated or heat-treated) was added to 2 ml ice-cold acetone (80 vol %). The samples were vortexed and incubated dark and on ice for 20 min and were thereafter centrifuged (Eppendorf Mini Spin, Eppendorf AG, Hamburg, Germany) at 12 100 x g for 4 min at 25°C, before spectrophotometric measurements were carried out (Varian Cary 50 Bio UV-Vis, Varian Inc., Santa Clara, CA, USA). Spectra were obtained over the wavelength range 200–1000 nm at intervals of 0.5 nm (1601 data points/spectrum) against a blank of acetone (80 vol %). Samples were analysed in triplicate.

2.4 Analysis of enzymatic inhibition capacity

2.4.1 Non-treated thylakoid slurry

Lipase/co-lipase activity was determined with pH-stat titration (Autotitrator Titralab TIM 854, Radiometer Analytical, Villeurbanne, France) using 0.1 M NaOH as titrant. The substrate was prepared in a vial by adding 0.5 ml tributyrine to 15 ml buffer [2 mM Tris maleate (pH 7), 0.15 M NaCl, 1 mM $CaCl_2$ and 4 mM NaTDC]. The incubation was performed at 25°C due to

limitations in the equipment. Stirring was maintained with a magnetic stirrer under standardized conditions. Lipase (1 mg/ml; 10 μl) was added followed by co-lipase (1 mg/ml; 10 μl). The activity was recorded for a few minutes to maintain a stable pH at 7, thereafter thylakoid slurry was added and the activity recorded. The mean consumption rate of 0.1 M NaOH (ml/s) during 20 minutes was taken as activity of lipase/co-lipase. Nine measurements of the non-treated thylakoid slurry were performed. The thylakoid slurry was added in different concentrations (given as mg chlorophyll) to establish a dose-response curve. Three measurements were performed at each concentration.

2.4.2 Heat-treated thylakoid slurry

The heat-treated thylakoid slurry was thawed in room temperature, mixed for 10 sec (Polytron PT 1200, Kinematica AG, Lucerne, Switzerland) to eliminate unstable aggregates and was thereafter stored dark and on ice until further analysis.

Determination of the thylakoid's ability to inhibit pancreatic lipase/co-lipase activity was analysed as described above. 260 μl heat-treated slurry was added to the assay, corresponding to 0.2 mg chlorophyll. Six measurements were performed at each temperature-time combination.

2.6 Partial Least Square 1 Regression analysis (PLS 1)

To investigate if the chlorophyll degradation could be correlated to the enzyme inhibiting capacity, a multivariate regression method, partial least square (PLS1) (Unscrambler, version 9.0, Camo Software, Oslo, Norway) was used (Fig 4a and 4b). All wavelengths in the absorbance spectra were used as variables and the enzyme inhibiting effect was used as response. The curve-fitting tool in Matlab (version R2010b, Mathworks Inc., Natick, MA) was used for a correlation analysis between the most predictive wavelengths found and the

enzyme inhibition capacity.

3. Results and discussion

3.1 Thermal degradation of chlorophyll

After heat treatment the thylakoid samples changed color from bright green to olive brown. The color change came gradually and was more pronounced for the higher temperatures and for longer times. A spectral analysis was performed on both non-treated and all the various heat-treated thylakoids to quantify and analyse the chlorophyll content. An absorbance spectra for non-treated thylakoids and thylakoids treated at 90°C for 4 min can be seen in Fig 5.

The non-treated thylakoid slurry displayed a spectrum typical for chlorophyll with two distinct peaks, one in the blue region with a maximum of 436 nm and one peak in the red region with a maximum of 667 nm. The dominating peak in the blue region for non-treated thylakoids is chlorophyll *a* (maximum at 436 nm) and the smaller peak close to the right is chlorophyll *b* (maximum at 465 nm) (Fig 5, dashed line). After heat-treatment the absorbance were both reduced in amplitude and were shifted towards the blue region for the peak around 436 nm, with a new maximum of 413 nm (Fig 5, solid line). During heat-treatment the chlorophyll molecule can be degraded via two different routes (Fig 2). The Mg^{2+} ion in the centre of the chlorophyll can be replaced by two H^{+} ions with a color shift from bright green to olive brown as a visual consequence. Here, chlorophyll undergoes degradation to pheophytin as reported previously[26]. In the second degradation route the chlorophyll are degraded to chlorophyllide by chlorophyllase through removal of the phytol chain. This intermediate molecule is highly unstable and is further degraded to pheophorbide when exposed to acid or heat, by substitution of the Mg^{2+} ion by two H^{+} ions[28,29]. The two

degradation products have structural differences; the phytol chain is still present in pheophytin but is removed in pheophorbide, however they display similar absorbance spectra.

Pheophytin *a* and pheophorbide *a* have both an absorbance maximum at 409 nm to be compared to the absorbance maximum for chlorophyll *a* at 436 nm[30]. The general reduction in amplitude for heat-treated compared to non-treated thylakoids, can thus be explained by the degradation of chlorophyll and the shift towards the blue region can be due to the newly formed degradation products pheophytin and pheophorbide with an absorbance maximum at shorter wavelengths compared to chlorophyll.

3.2 Effect of thermal treatment on the in vitro bio-functionality of thylakoids

To provide a safe and stable food product with appetite supressing properties from plant origin, the microbiological safety of the food must be ensured, thus thermal treatment is commonly used. Quantification of the ability to prolong lipolysis after thermal treatment of thylakoids is valuable knowledge to be able to dose the active ingredient with appetite regulating properties correctly. This is, to the author's knowledge at the time this paper was written, the first time the bio-functionality, i.e. the enzymatic inhibition capacity, of thylakoid membrane after thermal treatment has been studied.

3.2.1 Quantification of initial enzymatic inhibition ability of the non-treated thylakoids

To ensure both a physiologically active starting material and to get a reference level of the thylakoids ability to inhibit lipase/co-lipase *in vitro*, the thylakoid slurry was studied before heat treatment. The relative lipase/co-lipase activity with no thylakoids present was set to 100%. When non-heat treated thylakoids were added in different amounts, the activity was reduced in a dose-dependent way to a final enzymatic activity of 20% (Fig 6). The system

reached a plateau at a concentration of thylakoids equivalent to 0.5 mg chlorophyll. Chlorophyll is proportional to the amount LHC I and II in the thylakoids, suggested to be the primarily active structures in prolonging lipolysis, and is therefore used as the quantifying unit. After addition of thylakoids equivalent to 0.5 mg chlorophyll, no further reduction occurred as demonstrated in previous studies[12,16]. Thus it was confirmed that the thylakoid slurry used was physiologically active.

To reduce the risk of thylakoid saturation in the *in vitro* system a probe volume was carefully chosen. This was to avoid a too high concentration of thylakoids in the test vial, which could influence the mass transport of the system investigated. At too high thylakoid concentrations, the particles would constitute a hinder to the lipolysis due to the mere presence (increased viscosity reduces mobility of enzymes), and not due to surface activity or enzyme inhibiting properties, which was the phenomenon of interest in the present study. A probe volume of 260 µl slurry, corresponding to 0.2 mg chlorophyll was selected to avoid the potential saturation problem (Fig 6), yet well above the detection limit of the system. Thus this particular volume was used throughout the study. At the chosen concentration, the slope of the dose-response curve is steep but far from the concentration extremes, which maximize the measurement sensitivity and ability to register changes in enzymatic inhibition ability by thylakoids studied.

3.2.2 Effect of heat treatment on thylakoids ability to inhibit pancreatic lipase/co-lipase

During thermal treatment, the samples changed color but also the formation of aggregates could be detected by visual inspection. This aggregation have previously been found to start around 50°C[31]. Aggregation was more pronounced for the samples treated at higher temperatures and longer times. To examine if thermal treatment and the aggregates formed

affected the function of the thylakoids, the remaining ability to reduce pancreatic lipase activity, was analysed. Thylakoids heat-treated at different temperatures and times were not able to reduce the lipase/co-lipase activity as effective as non-treated thylakoids. The impact of heat treatment on the enzymatic activity were both temperature and time dependent, where thylakoids treated at the highest temperature for the longest time had the lowest ability to inhibit lipolysis.

To better quantify the thylakoids altered ability to inhibit lipase/co-lipase, i.e. ability to reduce enzymatic activity and prolong lipolysis, after thermal treatment compared with before thermal treatment, a new variable was derived. The lipase/co-lipase inhibiting capacity (ϕ) was defined analogous to the dimensionless temperature used in non-stationary heat transfer:

$$\phi_i = \frac{1-\bar{\alpha}_0}{1-\bar{\alpha}_i} \qquad (6)$$

were $\bar{\alpha}_0$ is the average enzyme activity in the presence of non-treated thylakoids and $\bar{\alpha}_i$ is the average enzyme activity for a specific sample of heat-treated thylakoids. Per definition the lipase/co-lipase inhibiting capacity is 1 for non-treated thylakoids.

Heat-treatment of thylakoid slurries at different temperatures and times (60°C, 75°C, 90°C for 15 sec - 4 min) reduced the inhibition capacity of the thylakoids on pancreatic lipase/co-lipase significantly, compared to non-treated thylakoids (Fig 7). After 4 min at 90°C, approximately 20% of the initial inhibition capacity remained. If we allow us to apply these *in vitro* results to a food system, and assume that processing for 4 min at 90°C are needed to ensure a microbial safe food product, up to five times the amount of heat-treated thylakoids have to be ingested to get the same effect on appetite, compared to non-treated thylakoids. In our previous studies,

4-7 g thylakoid powder has showed significant effect on appetite[15]. If heat treatment has to be applied, the dose should be adjusted to 20-35 g to get the same effect.

Heat treatment at 60°C and 75°C caused a stepwise reduction in inhibition capacity, the longer process time the greater reduction. However, heat treatment at 90°C showed a slightly increase in inhibition capacity between 15 sec and 2 min followed by a reduction after further processing. This was unexpected. To further evaluate the influence of time, two extended holding times were tested at the highest (90°C) and the lowest (60°C) processing temperatures. The inhibition capacity after 120 min holding time was unchanged compared to the capacity after 4 min holding time at both tested processing temperatures (Fig 7). It was concluded that the system had reached a plateau at 4 min and that no further reduction occurred independent of holding times.

3.3 Heat-induced changes in structure and function of thylakoids are linked

3.3.1 Theoretical link between heat-induced changes in structure and function

Chlorophyll is an amphiphilic molecule with a hydrophilic porrphyrin and a hydrophobic phytol chain. This allows the chlorophyll molecules to interact with hydrophobic regions inside the thylakoid membrane, for example the hydrophobic helices that constitute the trimers in LHC I and II. Chlorophyll thereby support the helical structure both within the helices (mainly chlorophyll *a*) and between helices (mainly chlorophyll *b*) (Fig 1).

Chlorophyll is sensitive to light, acid, enzymatic degradation and heat. During heat treatment chlorophyll is either degraded to pheophytin by replacement of Mg^{2+} with 2 H^{+}, or pheophorbide by first removal of the phytol chain followed by replacement of Mg^{2+} with 2 H^{+} (Fig 2). Replacement of Mg^{2+} with 2 H^{+} affects both color and polarity of the molecule, due to a decreased hydrophilicity in the pyrrol-part. Removing of the phytol chain also affect the

amphiphilic properties drastically due to removal of the entire hydrophobic part. This together could lead to reduced stability inside the thylakoid membrane, which in turn leads to disruption of the protein-lipid interaction[32].

Both LHC I and II, the intrinsic membrane proteins located in the photosystems I and II inside the thylakoid membrane, includes chlorophyll *a* and *b*. The concentration of *a* is higher compared to *b* (the chlorophyll *a/b* ratio is 4.0 in PS I and 2.2 in PS II[9]) and chlorophyll *a* are more important for the internal structural stability. Heaton *et al*[32] suggest that degradation of chlorophyll destabilize the pigment/proteolipid-complex causing conformational changes thereby promoting association between hydrophobic domains. This in turn leads to aggregation. In accordance, Zhang *et al*[33] reported a complete rupture of the structure above 70°C explained by dissociation of chlorophyll and the hydrophobic helices inside the LHC I and II.

Photosynthesis is the most heat sensitive physiological process in plants. When plants are exposed to heat stress, parts of the photosystems, more specifically the light harvesting complexes may aggregate. Aggregation induced by heat stress is suggested to be a part of the biological defence mechanism of the plant. In nature, heat stress most often occurs together with sunlight and when the leaf is exposed to more sunlight than can be utilized, the excess energy must be dissipated. Simultaneously with the aggregation, the carbon dioxide assimilation is reduced. Heat-induced aggregation can therefore be interpreted as a survival strategy to release excess of energy and prevent the leaf from permanent injury. The heat-induced aggregation increases with increased temperature. The phenomenon has been reported both *in vivo* and *in vitro*[34]. The aggregation is reversible *in vivo* if the temperatures do not exceed 35°C, otherwise it may be an irreversible process.

In many food processes the temperature exceeds 35°C, which can lead to an irreversible aggregation[34]. The thylakoid's ability to reduce pancreatic lipase activity, e.g. when used as a functional food ingredient, is explained by interactions between hydrophobic helices in the photosystems I and II and the lipid surface[12]. The thylakoids thereby constitute a barrier to lipase/co-lipase and the enzymes are blocked from the dietary lipids. We suggest that when LHC I and II aggregates due to heat treatment, the available contact surface is decreased, and the interaction between the thylakoids and the lipid droplet is reduced, hence a reduced lipolysis inhibition is possessed by heat-treated thylakoids. But why did heat treatment at 90°C display an unexpected increase in lipase inhibition capacity between 15 sec and 2 min? The enzyme chlorophyllase which degrade chlorophyll to chloropyllide by removal of the phytol chain, are reported to be activated by mild heat treatment (over 40°C)[35] but strongly inactivated by temperatures over 80°C[26,30]. Since the isolation of thylakoids is a crude preparation carried out at low temperatures (4-20°C), a significant fraction of chlorophyllase is likely still present, although not active at the prevailing preparation conditions. We suggest that the chlorophyllase was activated by the lower processing temperatures (60°C and 75°C), which gave two possible degradation routes for chlorophyll: enzymatic or chemical degradation. The highest temperature tested (90°C), rapidly inactivated the chlorophyllase and the number of degradation routes for chlorophyll was then reduced to one single route. The degradation rate was therefore reduced until the combined effect of high temperature and dominate processing time was large enough to dominate. Since degradation of chlorophyll by anyone or combination of these routes, is hypothesised to be responsible for aggregation inside the thylakoid membrane, we suggest that the observed increase in lipase inhibiting capacity between 15 sec and 2 min at 90°C is linked to the competing effects of the heat treatment both causing thermal degradation to the chlorophyll and inactivating chlorophyllase.

3.3.2 Statistical link between heat-induced changes in structure and function

After heat treatment of thylakoid membranes two different phenomena were observed; the absorbance spectra was reduced both in amplitude and shifted towards the blue region compared to non-treated thylakoids, and the lipase/co-lipase inhibiting capacity was reduced. Both effects were more pronounced for higher process temperature and longer times. To evaluate if these phenomena could be linked, a multivariate regression model, PLS 1, was used. It was investigated whether some of the wavelengths in the absorbance spectra, one single or an interval of wavelengths, was a better predictor for the outcome in the pancreatic enzyme assay compared to the others. The PLS 1 was loaded with all spectral wavelengths studied (1601 per sample) as predictor variables and the enzyme inhibition capacity as response variable. Three distinct sets of wavelengths were strongly correlated to the thylakoids lipase inhibition capacity (indicated with circles in Fig 4a). The set of wavelengths displayed in the indicated areas all indicated a narrow interval either around 436 nm, which corresponds perfectly to the maximum absorbance of chlorophyll *a*, or an interval around or close to 409 nm, which corresponds perfectly to the maximum absorbance of the degradation products pheophytin *a* and pheophorbide *a*. The lipase inhibition capacity can thus be closely linked either to chlorophyll *a* or to its degradation products pheophytin *a* and pheophorbide *a*.

To evaluate which variable in the PLS1 analysis above (absorbance at 436 nm or absorbance at 409 nm) that was closest related to lipase inhibition capacity, a correlation analysis was performed. It was concluded that the absorbance at λ=436 nm (maximum for chlorophyll *a* in the blue region) was the closest related variable describing the enzymatic inhibition capacity, ϕ, *in vitro* after heat treatment over the studied interval (R^2 = 0.95). A correlation was established:

$$\phi = 0.08180 \cdot e^{2.344 \cdot A_{436\,nm}} \quad (7)$$

Absorbance at 409 nm displayed a weaker correlation to the lipase inhibiting capacity, which can be due to the two possible degradation products of chlorophyll after exposure to heat: pheophytin or pheophorbide. Thus the remaining amount chlorophyll *a* was strongly correlated to the enzymatic inhibition capacity of the thylakoids after heat treatment. We suggest that chlorophyll *a* appears as an indicator of the structural status within the LHC I and II structure. Previous studies by Heaton and Marangoni[32] have shown that when chlorophyll degrade to pheophytin or pheophorbide the structure changes, which in turn promote aggregation. Aggregation reduces the contact surface to the lipid droplets, which could explain the observed reduction in the enzymatic inhibition capacity *in vitro* of heat-treated thylakoids.

Determination of the thylakoids ability to inhibit lipase/co-lipase by use of the pH-stat titration method has limitations. It is a time consuming, expensive method with a relatively high intra variation coefficient due to the variability of one batch of enzymes to another. It would therefore be an advantage if the titration method could be replaced by a spectrophotometric method, to quantify the thylakoids ability to prolong lipolysis by means of the chlorophyll *a* content in the sample.

By this close relationship between absorbance and enzymatic inhibition capacity established it is possible to model the thylakoids inhibition capacity *in vitro* from the absorbance at λ=436 nm. This indicates a heat induced conformational change in the thylakoid membrane that both affect the retention of chlorophyll *a* in the LHC I and II structure and the ability of LHC I and

II to maintain their enzymatic inhibition capacity, ϕ. A reduction of chlorophyll *a* hence is correlated to a reduced inhibition capacity of the thylakoids.

Heat treatment, i.e. a combination of a set time and temperature, induce degradation of chlorophyll *a*. The degradation affects the structure as well as the color of the sample which can be determined by absorbance at λ=436 nm which in turn are correlated to the enzymatic inhibition capacity, ϕ, of the thylakoids.

4. Conclusions

Heat treatment reduced the thylakoids ability to inhibit lipase/co-lipase *in vitro* and the effect was both time and temperature dependent. The reduction in lipase inhibiting capacity was hypothesised to be caused by aggregation of LHC I and II, driven by the degradation of chlorophyll. We also have gained a better insight into the structure and function of the chlorophyll-stabilised protein complexes, and how the structure affects the thylakoids ability to inhibit lipase/co-lipase. A statistical link between the enzymatic inhibiting capacity *in vitro* and chlorophyll *a* degradation could be established. This close correlation between chlorophyll *a* degradation and the thylakoids ability to inhibit lipase open possibilities to use a spectrophotometric method to quantify the enzymatic inhibiting capacity of the thylakoids in a more rapid and cost effective way as a complement to the *in vitro* enzyme assay used today, the pH-stat titration method being necessary to provide a reference level of the initial thylakoid capacity before any heat treatment. The observed aggregation in heat-treated samples caused by the formation of inverted micelles may also reduce the available thylakoid surface and thereby reduce lipase-colipase inhibition capacity. This aggregation may also be correlated to the chlorophyll light adsorption and requires further studies. With the degradation quantified, it is possible to design a thermal treatment process to ensure a

microbiological safe appetite-reducing product and at the same time minimize the loss of functionality.

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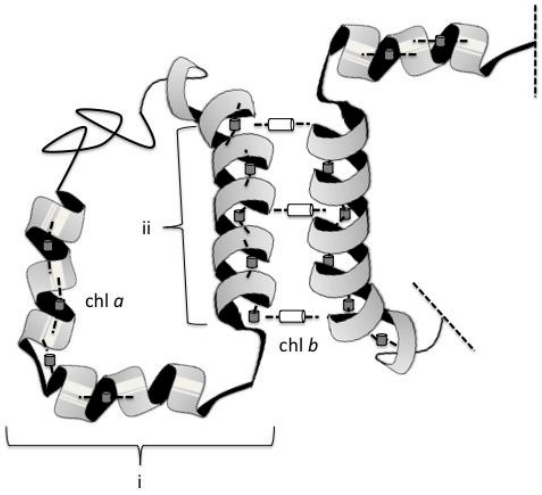

Figure 1. Schematic picture of parts of the light harvesting complex. To the left, a momomer (i) composed of three helices (one of the helices indicated by ii) internally stabilised by chlorophyll *a* (grey). To the right, chlorophyll *b* (white) separates the momomers from each other. Since the alpha-helix structure with hydrophobic side groups pointing out is the proposed mechanism for lipase/co-lipase inhibition, then chlorophyll *a's* role in stabilising this structure is important in retaining its conformation and thus function as a lipase/co-lipase inhibitor.

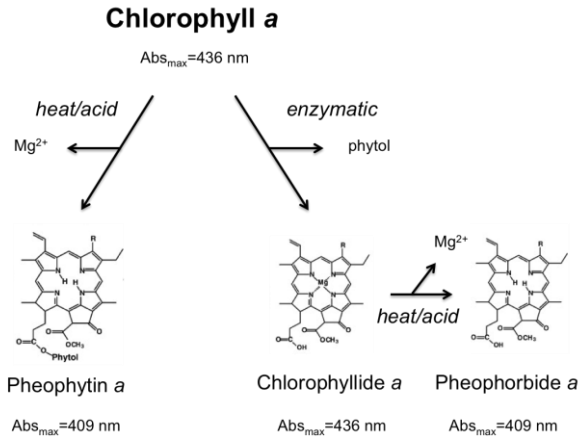

Figure 2. Chlorophyll *a* and its degradation products after heat treatment and/or enzymatic treatment.

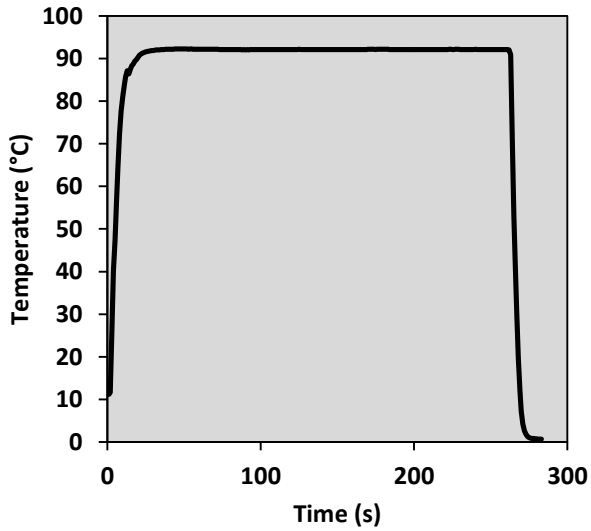

Figure 3. Temperature profile for thylakoid slurry during heat treatment at 90°C for 4 min.

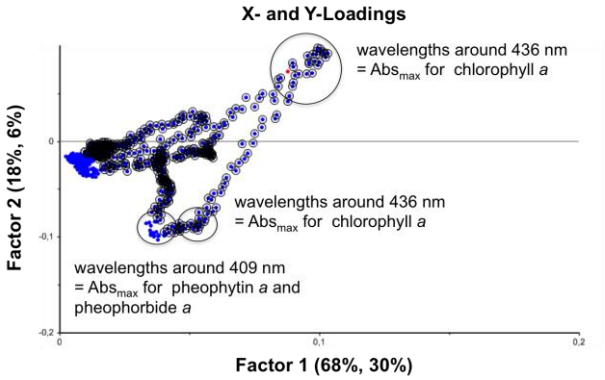

Figure 4a. Partial Least Square analysis (PLS1) based on absorbance at all wavelengths from 800 nm to 350 nm. Loadingplot where wavelengths (loading variables) are displayed in blue and the lipase inhibition effect are displayed in red (response variable). The two first factors are explaining 86% of the variance. Significant data indicated with circles. Sets of wavelengths strongly correlated to lipase inhibiting effect indicated with large circles.

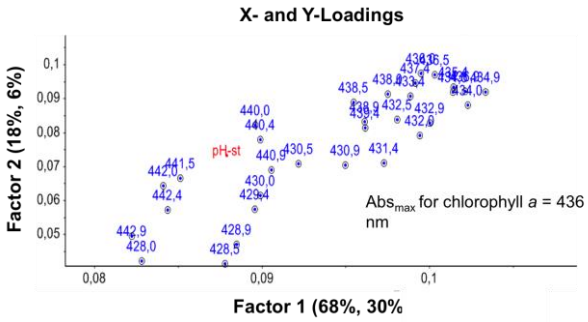

Figure 4b. Magnification of indicated area to the upper right in the loading plot, see Fig 4a. 93 % of the variation explained by the first two factors. Absorbance at wavelengths between 434-438 nm is most correctly describing the response variable (lipase inhibition effect, indicated in red in fig).

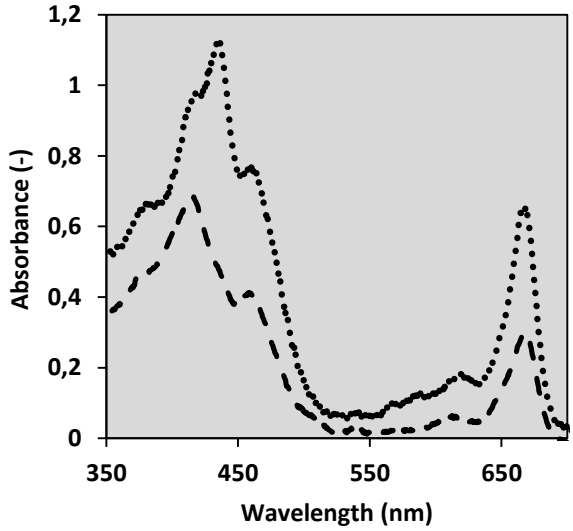

Figure 5 Absorbance spectra for non-treated thylakoids (dashed line) and thylakoids heat-treated at 90°C for 4 min (solid line).

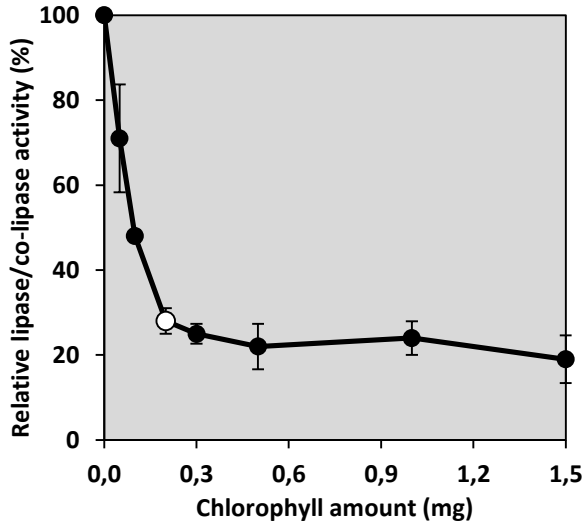

Figure 6. Inhibition of pancreatic lipase/co-lipase activity by non-treated thylakoids. Volume thylakoid slurry corresponding to 0.2 mg chlorophyll was selected as probe volume through out the study to avoid a saturated system (indicated by ◇).

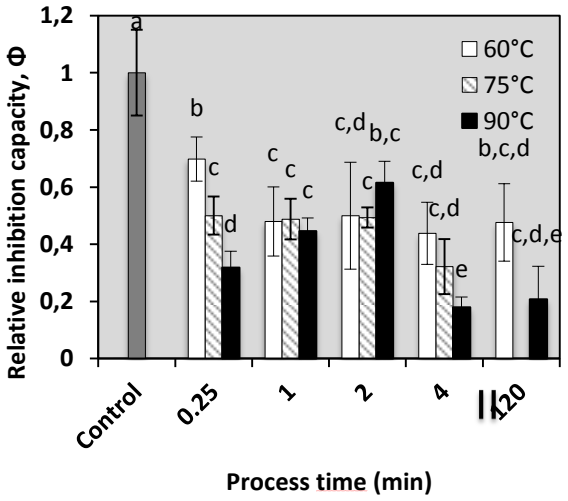

Figure 7. Lipase/co-lipase inhibition capacity by thylakoids heat-treated at different temperatures and holding-times. Inhibition capacity, ϕ, was calculated as (1 – lipase/co-lipase activity (non-treated thylakoids)) /(1- lipase-co-lipase activity (sample)). 1 is equal to the inhibition capacity of non-treated thylakoids and is referred to as control. Columns with different letters are significant different based on t-test on 95% confidence interval.