Master Thesis

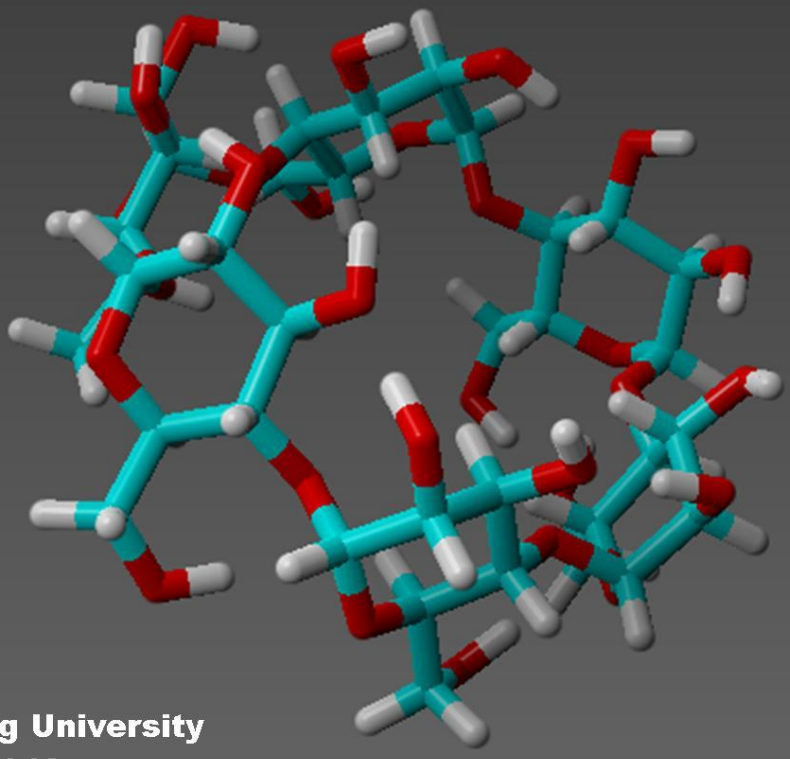
Medicine with Industrial Specialization

α-cyclodextrin

Interferes with

Carbohydrate and Lipid Metabolisms

by Lisbeth Hansen



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Preface

This master thesis was written by Lisbeth Hansen, Translational Medicine, Medicine with Industrial Specialization, Aalborg University, Denmark. The project was compiled from September 1st 2011 to June 1st 2012. The thesis was completed at Aalborg University, Department of Biotechnology, Chemistry and Environmental Engineering, Section of Chemistry.

Kim Lambertsen Larsen, Associate Professor, and Ludmilla Lumholdt Riisager, PhD. Student, were supervisors, both from Aalborg University, Department of Biotechnology, Chemistry and Environmental Engineering, Section of Chemistry.

References are provided as (surname of the first author *et al.*, year of publication) if more than two authors are given. If only two authors wrote the reference both surnames are provided.

Raw and processed data, appendices and a pdf-version of the thesis are available on the enclosed data disc.

I would like to thank the ten volunteers for making themselves available for the blood glucose study. I would also like to thank Stine Hansen and Mark Brenfelt for proofreading.

Table of Contents

Abstract

In the search of an efficient drug with few side effects for the treatment of overweight and obesity, α-cyclodextrin (α-CD) has been suggested as such. Two hypotheses regarding the mechanism behind α-CD's weight loss ability have been put forward; that α-CD affects lipid metabolism and that α-CD affects carbohydrate metabolism. In the present study, both hypotheses were investigated.

The lipid metabolism hypothesis was investigated by lipolysis studies performed to determine if α-CD could reduce lipid degradation. The results, from lipolysis of sunflower oil by porcine pancreatic lipase with different concentrations of α-CD present in the reactions, showed that α-CD was capable of decreasing the enzymatic hydrolysis of triglycerides in a dose dependent manner. It is proposed that the decreased degradation was a result of aggregate formation between α-CD and triglyceride complexes and that this aggregate formation limited lipase's access to the triglycerides. Furthermore, studies of the interaction between α-CD and oil revealed that a white substance easily formed. This resulting product was stable at 37°C, formed under both neutral and acidic conditions and with lipid from a lipid rich food. A prevention of lipid degradation would result in reduced absorption of energy, thus explaining α-CD's weight management property.

The carbohydrate metabolism hypothesis was investigated by degradation studies of starch with α-CD present in various concentrations. Similar to the triglyceride degradation experiments, α-CD inhibited the enzymatic degradation by porcine pancreatic α-amylase in a dose dependent manner. Based on previous research, α-CD is proposed to be a competitive inhibitor of α-amylase when starch is the substrate, where α-CD occupies the active site of the enzyme. Moreover, when smaller oligosaccharides are formed, the inhibition is proposed to be of the noncompetitive type. Furthermore, an *in vivo* crossover study was designed to investigate if this inhibition was evident in blood glucose response after a carbohydrate rich meal. Ten subjects consumed white bread together with water on one day (control) and together with 10 g of α-CD dissolved in water on another day. Comparison of total area under the blood glucose curve between the two conditions revealed that α-CD significantly reduced blood glucose levels ($p = 0.013$). Decreased enzymatic starch degradation probably explains why a reduction in blood glucose response is obtained when α-CD is ingested together with a starch rich meal. This is proposed to account for α-CD's weight loss ability since blood glucose level is better regulated, which is important when weight loss is desired. Furthermore, the prevention of starch degradation could instead lead to fermentation of starch in the colon resulting in a decrease in the calories absorbed from starch.

Results from the present study and from previous research strongly indicates that α-CD affects both lipid and carbohydrate metabolism. It is plausible that both mechanisms work simultaneously explaining the weight management effect of α-CD.

1. Introduction

Obesity represents a major health problem worldwide. In 2008, 1.5 billion adults, 20 years and older, were overweight (body mass index (BMI) > 25 kg/m^2) and of these 500 million were obese (BMI ≥ 30 kg/m^2) globally (WHO, 2011). Individuals may as a result of their overweight or obesity suffer from complications such as dyslipidemia, hypertension, type 2 diabetes, liver diseases, and cardiovascular diseases. Furthermore, the obesity can have great psychological and psychosocial consequences (Pêgo-Fernandes *et al.*, 2011). Several approaches exist in order to induce weight loss, but weight regain remains a great challenge as over 80% of those who lose weight struggle against weight regain (Douketis *et al.*, 2005). As a consequence obesity is considered to be a chronic condition (Burke and Wang, 2011).

1.1. Treatment Options for Obesity and Overweight

The treatment strategy for overweight and obesity can be divided in three parts: lifestyle changes, pharmacotherapy and bariatric surgery (Burke and Wang, 2011).

Lifestyle changes consist of dietary adjustments and regular exercise preferable in cohesion with behavioral therapy. A systematic review from 2005 involving 16 studies with 5698 overweight or obese subjects showed that weight loss from lifestyle changes was < 5 kg after four to seven years. Health benefits in the form of decreased blood pressure and lower risk of diabetes type 2 were measurable in some subjects, but the data were inconsistent (Douketis *et al.*, 2005).

If lifestyle changes are insufficient in inducing weight loss, a few drugs are available as a supplement. The currently available pharmacological treatment in Denmark is either the malabsorptive agent orlistat or the appetite suppressing agent amfepramone (Svendsen *et al.*, 2006). Orlistat can be used as a supplement to dietary adjustments for those with a BMI ≥ 28 kg/m^2 (Dansk-Lægemiddelinformation, 2011a). When taking orlistat it is necessary to follow a low-fat diet in order to prevent significant gastrointestinal side effects. These side effects include loose, oily stool, abdominal pain, and fecal urgency and may happen to some extent even on a low-fat diet (Dansk-Lægemiddelinformation, 2011a; Jain *et al.*, 2011). With regards to amfepramone no clinical studies lasting one year or more exist. Therefore, amfepramone is not generally recommended for treatment of overweight or obesity. It can be considered for patients with a BMI > 30 kg/m^2, or a BMI > 27 kg/m^2 in the presence of comorbidities, if the patient has failed to lose weight after three to six months with lifestyle changes only. Amfepramone exhibits severe side effects such as depression (>10%), psychosis (>10%), and tachycardia (1-10%) (Dansk-Lægemiddelinformation, 2011b). Several other drugs have been approved for treatment of obesity and overweight, but later withdrawn from the market due to a negative benefit/side effect profile (Li and Cheung, 2011).

Lastly, bariatric surgery is a treatment possibility, but it is only applied when all other options have been explored and failed. Furthermore, bariatric operations is reserved for individuals

with a BMI > 50 kg/m^2 or a BMI between 35-50 kg/m^2 in the presence of comorbidities. Various forms of bariatric surgery exist, but two dominate; gastric bypass (65%) and gastric banding (24%). In gastric bypass surgery the small intestine is resected and re-routed to a small gastric pouch and in gastric banding a band is placed around the upper part of the stomach (Burke and Wang, 2011). Figure 1 illustrates the anatomy of the stomach after both procedures. The remaining gastric pouch can contain 20-30 ml as opposed to a normal gastric pouch, which can storage between 0.8 to 1.5 L (Guyton and Hall, 2006).

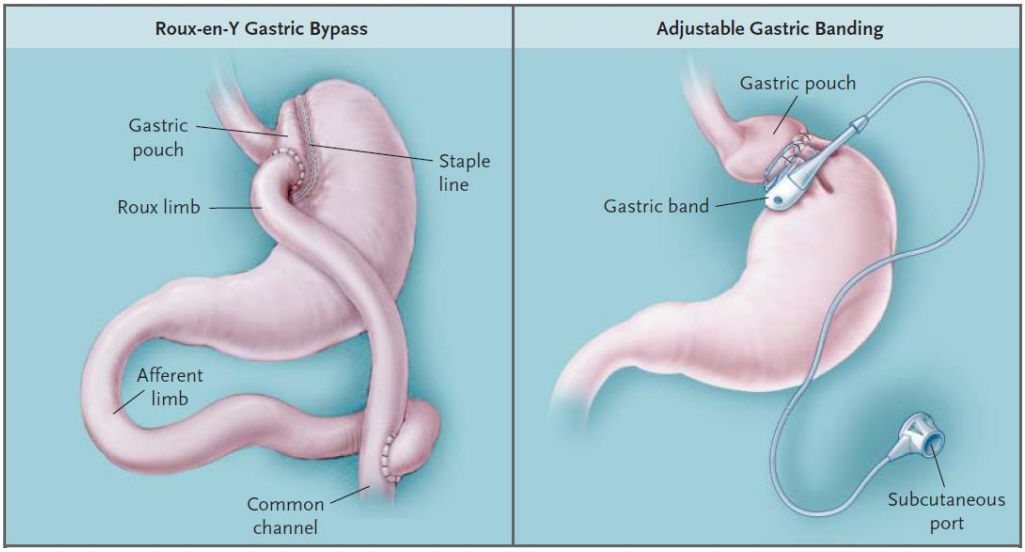

Figure 1. The illustration to the left shows the most common used gastric bypass procedure, the Roux-en-Y gastric bypass. A small proximal gastric pouch is separated from the remaining part of the stomach and connected to a Y-shaped loop of the small bowel. The right panel illustrates a gastric banding procedure. The band, placed near the upper end of the stomach, creates a small pouch and a restricted passage to the remaining part of the stomach. The band can be adjusted by infusion of saline (Steinbrook, 2004).

The systematic review from 2005 investigated the weight loss efficacy of bariatric surgery as well (Douketis *et al.*, 2005). The authors included nine studies with a total of 3622 subjects who underwent surgery. Before surgery all subjects were on a diet and weighed between 110-142 kg. Two years after the surgery, the subjects had lost between 28-76 kg. After three years the weight loss was between 17-73 kg. One study had a follow-up period of eight years and by then the subjects had lost only 20 kg in average (Douketis *et al.*, 2005).

In summary, the three treatment strategies available for overweight and obesity each exhibit some issues. The effects of lifestyle adjustments seem too modest when considering the magnitude of obesity and overweight. Moreover, several drugs have been approved for the treatment of overweight and obesity, but only two are still on the market in Denmark. One of these, amfepramone, can cause so severe side effects that the use of it is very restricted. With orlistat the weight loss is 5-10 kg after two years and there is a risk of mild to moderate gastrointestinal side effects unless a low-fat diet is followed, which can be a challenge for some. Bariatric surgery significantly induces weight loss, but it is a drastic and invasive last resort and is reserved for those

with severe obesity. Last but not least, weight regain remains an issue in all three treatment strategies.

It is necessary to find a treatment option for obesity and overweight that has an acceptable benefit/side effect profile and ideally a treatment option with so few side effects that it can be used for many years in order to overcome the challenge of weight regain.

1.2. α-cyclodextrin as a Novel Weight Loss Agent

One such treatment option might be α-cyclodextrin (α-CD). α-CD is a cyclic oligosaccharide consisting of six glucose units. It is a natural derivate from the degradation of starch but is not further hydrolyzed by human salivary or pancreatic amylases (Marshall and Miwa, 1981). Instead, the α-CD molecules are fermented by bacteria in the colon. Hence, α-CD can be categorized as a soluble fiber and is sold as such in the USA and Japan (WHO, 2006; BioForm, 2011). The term fiber is somewhat misleading as α-CD is not fibrous. Resistant starch is considered to be a subclass of dietary fiber as it possesses the same properties and would be a more appropriate term. Nevertheless, since α-CD is referred to as a fiber by several sources, this term is also used in this thesis.

α-CD is recognized as a safe food ingredient. The World Health Organization (WHO) evaluated in 2006 the safety of α-CD (WHO, 2006). In their report, a study was described investigating the gastrointestinal tolerability of α-CD in 12 healthy volunteers; 25 g of α-CD dissolved in 250 ml of water was given to the participants after an overnight fast. One reported diarrhea and three others experienced abdominal discomfort. These side effects were rated as mild. In the same study, 12 volunteers consumed 10 g of α-CD dissolved in 250 ml of water together with 100 g of white bread after an overnight fast. This dose led to no side effects. WHO describes that mild intestinal discomfort is a well-known occurrence after intake of carbohydrates with low digestibility, especially if ingested in liquid form on an empty stomach. It is caused by an influx of water into the small intestine to achieve isotonicity and by bacterial fermentation later in the more distal part of the intestine. Based on these results, and a calculation of the anticipated use level of α-CD, WHO concluded that there was no safety concern of α-CD. They did however stress that since ingesting a single dose of 25 g of α-CD might cause discomfort, this should be taken into account when choosing the level of usage. Lastly, WHO has granted α-CD an Acceptable Daily Intake of “not specified” meaning that no upper limit of intake is established or deemed necessary (WHO, 2006).

Several studies support that α-CD do in fact possess weight loss capabilities. In one study, a series of experimental setups with rats feeding them different amounts of cyclodextrins (CDs) in different time periods were designed (Suzuki and Sato, 1985). That is, a mixture of *n*-dextrin, α-CD, β-cyclodextrin (β-CD) and γ-cyclodextrin (γ-CD) in ratio of 50, 30, 15, and 5% (w/w), respectively, was used. The growing rats obtained smaller weight gains and body fat deposition when they were fed on diets with higher amounts of CD. Figure 2 illustrates the results over the study period of 110 days. Similar results were achieved when implementing restricted feeding to the rats; rates of weight loss were faster in rats fed a high CD diet than rats fed a diet without CD (control). The rates of weight loss increased proportionally to the amounts of CD in the diet (Suzuki and Sato, 1985). The authors proposed that the suppression of weight gain and the higher rates of weight loss with CDs might be caused mostly by α-CD. They based this assumption on the fact that γ-CD is degraded by human salivary and pancreatic α-amylases to a much higher degree than β-CD and α-CD (Suzuki and Sato, 1985). Furthermore, the authors stated that a six mounts feeding study involving rats and using β-CD in doses of 0.1-1.6 g/kg body weight per day showed no effect on weight gain compared to control. Unfortunately, that study is only available in Japanese (Suzuki and Sato, 1985).

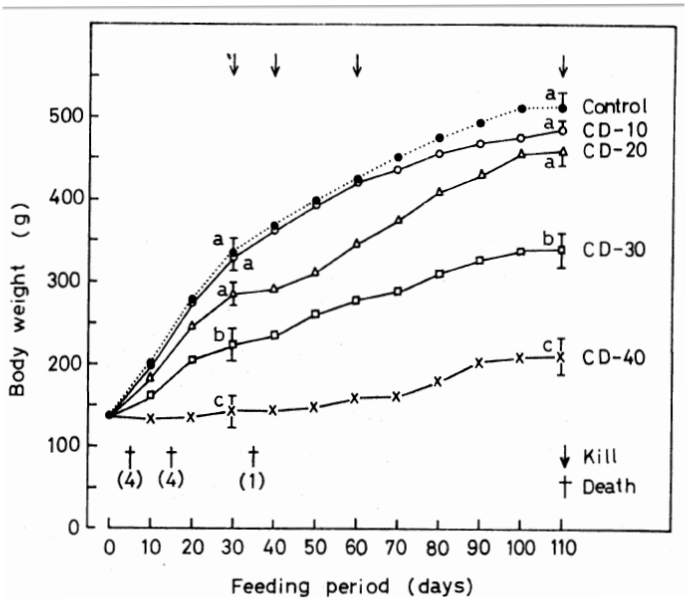

Figure 2 shows the weight changes of rats in an experiment performed by Suzuki and Sato. The rats were fed with a mixture of CDs in 110 days in different amounts (the amount termed CD-40 is the diet with most CD added). Numbers in parentheses are rats of the CD-40 group which died during each 10-day period. Values not followed by the same letter are significantly different from each other ($p<0.05$) (Suzuki and Sato, 1985).

Some of the rats which were assigned to the diet denoted CD-40 in figure 2 died during the study. In the CD-40 diet approximately 40% of the food consisted of CD (w/w). The authors described that rats on this diet showed abnormal symptoms such as poor appetite and constipation with gas accumulation in the large intestine (Suzuki and Sato, 1985). The occurrence of these symptoms indicated that the rats' digestive system could not handle this large amount of fibers plausible explaining why some of the rats died.

In 2006 a research group followed up on Suzuki and Sato's results regarding the potential weight loss ability of α-CD. Artiss and colleagues acquired 42 rats and divided them into two different diet groups and further into two subgroups (Artiss *et al.*, 2006). The two different diets were a low-fat (LF) diet containing 4% (w/w) of soybean oil and a high fat (HF) diet with 40% (w/w) soybean oil as the fat source. The subgroups were formed according to the presence or absence of 10% of α-CD to fat content (w/w). That is, 4 g of α-CD was added to one of the LF diets and 40 g of α-CD was added to one of the HF diets. Hence, four groups were formed altogether: HF control, HF diet with α-CD, LF control and LF diet with α-CD. The rats were kept on their respective diet for six weeks and body weight and food intake were monitored continuously. Similar to the results obtained previously (Suzuki and Sato, 1985), this study showed that rats on the HF diet with α-CD gained weight at a significantly slower rate than the rats on the HF control diet. The reduction of body weight in the group of rats which were fed the HF diet with α-CD was 7.4% in comparison to the HF control group ($p<0.05$). The total body fat mass of rats in each group is given in figure 3. Comparison of the total body fat masses revealed that α-CD in the HF diet reduced the mass by 22% compared to HF control diet ($p < 0.05$). It is also worth noticing that the rats in all four groups ate similar amounts of food by weight, but the HF diets were assumable more energy-rich, thus the rats fed with the HF diets seemed to consume more energy than rats in the LF groups. It was therefore proposed that the rats could have lost weight if the amount of energy intake had been the same (Artiss *et al.*, 2006).

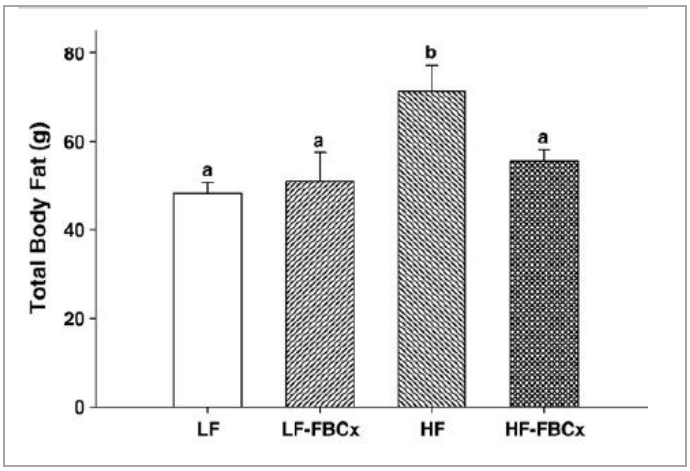

Figure 3. Illustrations of total body fat mass of the four groups of rats at sacrifice (LF: low fat control diet, LF-FBC$_x$: low fat diet with α-CD, HF: high fat control diet, HF-FBC$_x$: high fat diet with α-CD). There is significant difference in body fat mass between the HF diet group when comparing with the other three groups (Artiss *et al.*, 2006).

Two studies with human subjects further support that α-CD exhibits weight loss properties. Grunberger and colleagues performed a three months, double-blind, placebo controlled study with 47 obese patients with type 2 diabetes (Grunberger *et al.*, 2007). The subjects were randomized to take either two 1-gram tablets of α-CD (active group) or placebo per fat containing meal and should not change their eating or exercise habits. The results showed that the placebo group gained on average 1.54 kg (± 0.5 kg (SE)) over 12 weeks, whereas the active group gained 0.27 kg (± 0.8 kg (SE)). This difference was not significant. However, participants in the active treatment group seemed to consume a significantly higher amount of calories, while participants in the placebo group reduced their energy intake. When energy intake was applied as a co-variance, the difference in weight gain became statistically significant ($p <0.05$) (Grunberger *et al.*, 2007). These results are

in agreement with the results mentioned above (Suzuki and Sato, 1985; Artiss *et al.*, 2006). The other study supporting α-CD's weight loss capabilities was performed by Comerford and colleagues and included 28 overweight subjects (Comerford *et al.*, 2010). In this two months, double-blind, crossover study, the subjects were given tablets containing either α-CD (active phase) or placebo for one month and the alternate treatment for the next month. The participants were instructed not to change their eating or exercise habits. The results revealed that the subjects lost weight in both the active phase and the placebo controlled phase, but a significant difference between the two conditions was measurable. Body weight was 0.41 kg (± 0.2 kg (SE)) lower when comparing the active phase with the control phase ($p < 0.05$) (Comerford *et al.*, 2010).

In summary, the results from the four studies – two involving rats (Suzuki and Sato, 1985; Artiss *et al.*, 2006), two involving humans (Grunberger *et al.*, 2007; Comerford *et al.*, 2010) – reveal that α-CD has a weight management effect. The three studies by Artiss, Grunberger, and Comerford were performed by the same research group.

1.3. Hypothetical Mechanisms by which α-cyclodextrin Aids in Weight Management

More than one hypothesis has been stated regarding how α-CD exerts the weight loss ability. One hypothesis about the mechanism responsible for α-CD's weight loss ability is that α-CD prevents or inhibits the intestinal absorption of lipids from the diet. The hypothesis was put forward in the article previously mentioned by Artiss and colleagues, and in order to support the hypothesis an *in vitro* experiment with olive oil and α-CD was performed (Artiss *et al.*, 2006). Nine tubes containing 4 ml of oil and various amounts of dissolved α-CD were mixed for a few seconds. Each tube was labeled with weight percentage of α-CD to oil (for example the tube labeled 10% contained 4 g of oil and 400 mg of α-CD). Figure 4 shows the result of the interaction between oil and α-CD from that experiment.

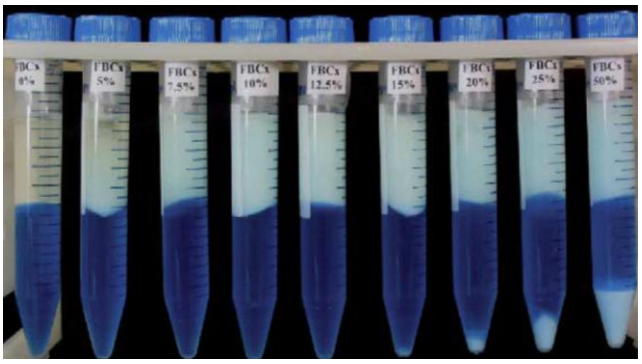

Figure 4. The result obtained by Artiss and colleagues where α-CD dissolved in water dyed blue interacted with olive oil to form a white substance at the top. The tubes were labeled with weight percentage of α-CD to oil (for example the tube labeled 10% contained 4 g of oil and 400 mg of α-CD) (Artiss *et al.*, 2006).

It can be seen that α-CD and oil formed an emulsion-like white substance at the top of the tubes while water (dyed blue) remained at the bottom. With 5% and 7.5% of α-CD some free oil was still detectable. The white precipitate in the tubes with the higher percentages of α-CD was considered to be insoluble α-CD. These results showed that α-CD somehow interacts with oil. An emulsion is defined as a mixture of two or more liquids that are normally immiscible. As the oil was not suspended in the water, actual emulsification did not occur and the product formed between oil and α-CD is named the substance or the product henceforward.

When hypothesizing that an interaction between α-CD's and lipid prevents intestinal absorption of lipid, it is important to take into account that lipid digestion is a dynamic process involving several sequential steps. Firstly, emulsification of the insoluble lipids is necessary before digestion can occur in the gastrointestinal tract. The emulsification is caused by mechanical movements of the stomach followed by micellar reorganization in the small intestine. The micelle formation occurs by means of amphipathic molecules such as phospholipids and bile salts among others (Bauer *et al.*, 2005). The degradation process starts in the stomach where gastric lipase acts at the lipid/water interface. Hereafter, digestion continues in the duodenum by the action of pancreatic lipase. Because the lipases work at the lipid/water interface, proper emulsification is important as is creates a larger area available for the lipases. The degradation of triglyceride, the most common dietary lipid, results in the formation of free fatty acids and monoglycerides. These lipolysis products leave the surface of the lipid droplet and are incorporated into micellar structures consisting of phospholipids or bile salts. The micellar structures then migrate towards the gastrointestinal wall where the lipolysis products diffuse into the enterocytes (Bauer *et al.*, 2005; Guyton and Hall, 2006). In view of the complex nature of lipid digestion it appears that several possible mechanisms for an interaction between α-CD and lipid digestion exists.

Another hypothesis regarding the mechanism behind the weight loss property of α-CD is that α-CD affects carbohydrate metabolism. Two studies with similar setups support this. The first study is an unpublished study report by Diamantis and Bär from 2002, which is mentioned in a WHO report (WHO, 2006). In this crossover study α-CD's ability to reduce the glycemic index of food was investigated. 12 healthy male subjects were recruited to attend two sessions in the morning after an overnight fast. The subjects were randomized to consume 100 g of white bread contained 50 g digestible carbohydrates and 250 ml of water either with or without 10 g of α-CD dissolved in it. Measurements of glucose and insulin levels in the blood were taken before consumption of the meal and at regular intervals for three hours thereafter. The meal consumed without α-CD in the water led to the expected postprandial rise in both glucose and insulin levels. The meal consumed together with α-CD dissolved in water showed a reduced postprandial response regarding both glucose and insulin levels. Calculations of the glycemic and insulinemic index showed a reduction of 57 and 55%, respectively (WHO, 2006). Glycemic index is calculated by dividing the incremental area under the blood glucose curve of a test food by the incremental area of a standard, usually glucose and then multiplied by 100. 50 g of glucose has per definition a glycemic index of 100 and all other foods have a lower glycemic index (Ludwig, 2003).

The other study supporting that α-CD affects carbohydrate metabolism used a similar setup (Buckley *et al.*, 2006). Ten healthy subjects were recruited to attend to four sessions in the morning after an overnight fast. In random order they consumed 64 g of white rice contained 50 g digestible carbohydrates which had been boiled together with 0, 5 or 10 g of α-CD. 250 ml of water was served at each meal. To determine the dose-response relationship between the amount of α-CD and the decrease in blood glucose response, a fourth visit was planned by the authors to allow for the addition of another dose of α-CD. Based on an interim analysis of the responses from the other three visits, 2 g of α-CD was chosen as the subsequent dose. Measurements of glucose and insulin levels in the blood were taken before consumption of the meal and at regular intervals for two hours thereafter. The control meal (0 g of α-CD) led to the expected postprandial rise in both glucose and insulin levels. Calculations of incremental areas under the curve (iAUC) for the blood glucose response revealed a reduction in iAUC for 2, 5 and 10 g doses compared to control. The reductions in iAUC were -1.7% (± 17.2% (SE), not significant), -20.4% (± 15.4% (SE), p = 0.03), and -49.6% (± 9.9% (SE), p = 0.001) for 2, 5, and 10 g of α-CD respectively compared to the control meal. Figure 5 illustrates the blood glucose levels and it can be seen that all three doses of α-CD reduced the glucose concentrations.

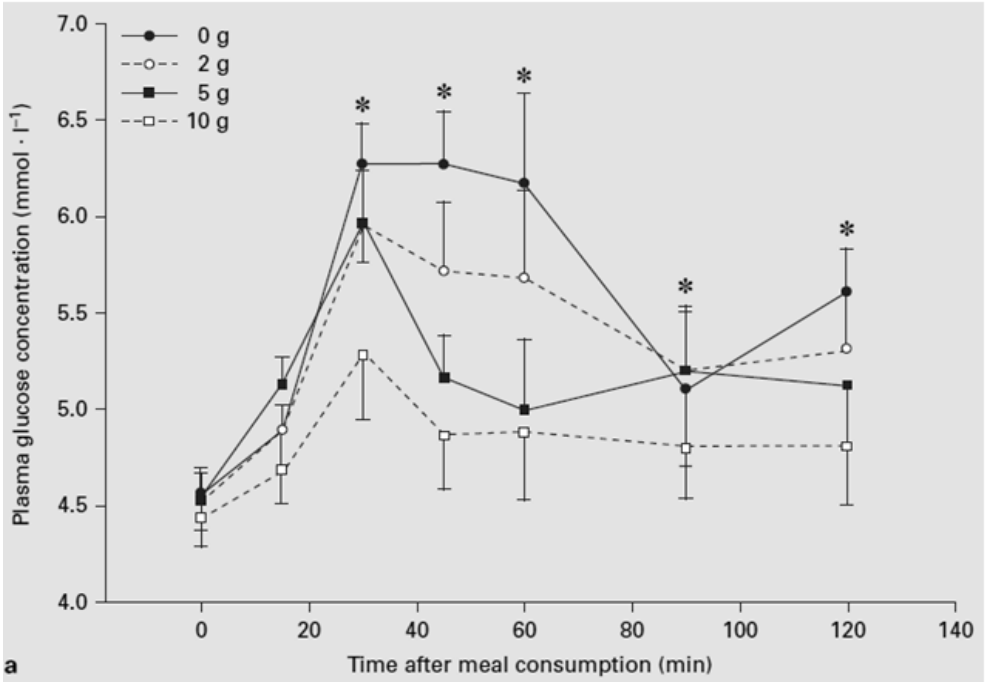

Figure 5. An illustration of the results obtained by Buckley and colleagues. Measurements of plasma glucose levels after consumption of rice boiled with 0, 2, 5, or 10 g of α-CD. The addition of 5 and 10 g of α-CD significantly lowered the iAUC compared to control (Buckley *et al.*, 2006).

In summary, the mechanism behind the weight loss inducing capability of α-CD revolves around two different hypotheses. One states that α-CD affects lipid metabolism in a way that could lead to decreased absorption of lipid from the diet. This could then result in reduced calorie intake

thus explaining why α-CD possesses weight loss abilities. The other hypothesis states that α-CD affects carbohydrate metabolism leading to a reduced glycemic response after a carbohydrate rich meal. The result could be reduced calorie intake and/or a more stable blood glucose level during the day, which have been proven important when weight loss is desired (Ludwig, 2003).

The preceding considerations lead to the following problem statement.

1.4. Problem Statement

Is has been shown that α-CD somehow affects human digestion of nutrients thereby aiding in weight management. However, none of the studies concerning this investigate the actual mechanism behind such weight management. The hypothesis is that the product formed between α-CD and lipid results in decreased lipid degradation. Available literature has not explored if the addition of α-CD to a lipolysis reaction decreases the degradation and this scenario will be investigated in the present study. Furthermore, the mixture of α-CD and lipid is examined by microscopy. The second hypothesis put forward is that α-CD results in a decrease of starch degradation. This scenario is also investigated. Moreover, an *in vivo* study of blood glucose response after a carbohydrate rich meal with the addition of α-CD is performed.

2. Experimental Aspects

2.1. Experiments investigating the Lipid Metabolism Hypothesis

The hypothesis regarding α-CD's effect on lipid metabolism will be investigated through different experimental setups. Firstly, the simple study where oil and α-CD were mixed and formed a white substance will be replicated (Artiss *et al.*, 2006). It will also be examined whether this substance can be formed in hydrochloric acid as stomach content is highly acidic and thereby show if the substance forms under circumstances that are closer to *in vivo* conditions. Likewise, the product will be placed at 37°C to test the stability at a temperature which corresponds to the body temperature of humans. It will also be investigated if the interaction can happen with a lipid rich food to determine whether the substance formation is restricted to occur in a simple, artificial environment or if it is possible to reproduce it in a more complex environment. Furthermore, microscopy of the substance formed between α-CD and oil as well as microscopy of samples from the degradation mixture will be carried out in order to visualize the product between α-CD and lipid.

Preliminary studies of triglyceride degradation will be performed. Since it is possible to perform such degradation using different experimental setups, several approaches will be tested to ensure the most optimal setup for lipolysis. In the actual degradation studies, sunflower oil will be used as substrate for pancreatic porcine lipase (PPL) as it is standard cooking oil consisting of mainly triglycerides. Triglycerides are used as substrate to represent the main source of dietary lipids. Moreover, using porcine lipase instead of human lipase is generally accepted (Zangenberg *et al.*, 2001).

The reactions will be conducted at a pH of 6.5 and at 37°C in order to mimic the environment of the small intestine. Pancreatic lipase is active in a broad pH range between 6 and 10 with an optimum of 8.5 (Bauer *et al.*, 2005). Duodenal pH is around 5.5, and a pH of 6.5 is chosen as a compromise, which has resulted in successful lipolysis previously (Zangenberg *et al.*, 2001). $CaCl_2$ is added to the buffer to ensure that PPL can work properly. Likewise, NaCl is added in order to have the proper ionic strength (150 mM) (Baynes, 1999). NaOH will be added continuously during the degradation process in order to maintain the pH at 6.5. Stirring is conducted during the degradation process to obtain a large lipid-water interface since lipase is only active at the interface (Bauer *et al.*, 2005).

Pancreatic lipase hydrolyzes triglycerides into free fatty acids, diglycerides and monoglycerides (Bauer *et al.*, 2005). In this study, the degradation process will be investigated by quantifying the amount of converted product as oleic acid equivalents. The amount is quantified using the copper soap method (Pinsirodom and Parkin, 2001). Fatty acids complex with copper(II) acetate to form cupric salts or soaps that absorb light at 715 nm and this can then be quantified by spectrophotometry. The spectrum obtained when using the copper soap reagent with α-CD present

is determined prior to the degradation studies. This is done to test if α-CD affects the spectrophotometric analysis by forming inclusion complexes with the molecules present, which is a well known phenomenon of CDs (Szejtli, 2005). Furthermore, samples of buffer, oil, denatured PPL, and α-CD in concentrations, corresponding to the concentrations in the treated samples, are analyzed with the copper soap method and if necessary data will be adjusted. Lastly, standard curves for each α-CD concentration present in the degradation studies and a standard curve for the control degradation are going to be made to adjust for α-CD's possible effect on the spectrophotometry. All standard curves can be found in appendix I.

2.2. Experiments investigating the Carbohydrate Metabolism Hypothesis

The carbohydrate hypothesis will be investigated by degradation studies of starch with and without different concentrations of α-CD present to see if α-CD affects the enzymatic hydrolysis of starch. The reaction will be conducted at a pH of 6.5 and at 37°C to mimic the environment of the small intestine. The optimum pH for pancreatic α-amylase is between 6.7 and 7.0 (Baynes, 1999). As for the lipid degradation study, a pH of 6.5 is chosen as a compromise between pH optimum and duodenal pH. $CaCl_2$ and NaCl are added to secure the functionality of PPA.

Porcine pancreatic α-amylase (PPA) is chosen over humane pancreatic α-amylase because it is easy to obtain and it has been shown that PPA can degrade starch (Elödi *et al.*, 1972). Potato starch is chosen as substrate to imitate *in vivo* conditions where starch is the predominant carbohydrate in the form of e.g. potatoes, bread, pastas, and rice. The reactions will contain sodium azide to hinder bacterial growth. A concentration of 0.02 w/v% has been proven to be sufficient to hinder bacterial contamination (Kamiya *et al.*, 1993).

α-amylase hydrolyses starch into intermediate oligosaccharides. *In vitro*, α-amylase is able to degrade starch into small amounts of glucose, but mostly into maltose (Kaczmarek and Rosenmund, 1977). Maltose is a reducing sugar, which can be utilized analytically – when a solution of 3,5-dinitrosalicylic acid (DNS) is added to the samples, DNS is reduced to 3-amino-5-nitrosalicylic acid, which absorbs light at 540 nm and this can be measured by spectrophotometry (Miller, 1959). The degradation reaction will be evaluated by quantifying the amount of reducing ends as maltose equivalents by means of a maltose standard curve. The standard curve can be seen in appendix I. Additionally, it is investigated if the absorbance maximum of 540 nm is affected by the presence of α-CD prior to the degradation studies

Samples of buffer, starch, denatured PPA, and α-CD in concentrations corresponding to those in the degradation studies, are going to be analyzed as described above and if necessary data will be adjusted. With concentrations of α-CD that are not soluble, filtration and centrifugation is performed to prevent that any excess starch causes turbidity, which would interfere with the Spectrophotometric measurement. Furthermore, the analysis is going to be verified using an internal standard of known maltose concentration.

Prior to the *in vivo* blood glucose study an approval by the local Research Ethics Committee of Region Nordjylland will be obtained and all experiments are to be conducted at the University. A crossover design is chosen as blood glucose exhibits great interpersonal variability. This issue is solved when subjects function as their own control, which is the case in a crossover design. In order to further reduce the variability, it is attempted to have a group as homogeneous as possible. Therefore, inclusion criteria are ages between 18 and 40 years and a BMI between 18.5 and 24.9 kg/m^2 (normal weight). Likewise, exclusion criteria are diabetes mellitus type 1 and 2, and any form of metabolic disease. Since the participants are going to ingest white bread, gluten allergy is an exclusion criterion as well.

The participants will be fasting 12 hours prior to the experiment and asked to have a similar eating and exercise pattern 24 hours prior to the two test days, respectively. This is done to obtain reasonable similar energy deposits on both days. Moreover, the experimental days are at least one day apart to eliminate any possible carryover effect. The meal at each day consists of 50 g of digestible carbohydrates. This amount is expected to cause a sufficient rise in blood glucose levels. Furthermore, a dose of 10 g of α-CD is chosen in order to see a clear effect and to limit the risk of side effects caused by α-CD.

Randomization of the sequence of the meals (with α-CD or without) will be carried out by flipping a coin by an unbiased person who will also prepare the meals. The person will not have any direct contact with the subjects to ensure double-blinding. Moreover, in order to mask any possible undertaste from α-CD lime juice will be added to the water in both occasions.

Based on previous similar studies and the variance reported, calculation of the number of subjects necessary to obtain a 50% difference in areas under the blood glucose curve is performed using the following equation (Bowers, 2008);

$$\frac{N > (Z_{2\alpha} + Z_{\beta})^2 \cdot S^2}{MIREDIF^2}$$

, N = number of subjects, Z = normally distributed sample size, S = variance, MIREDIF = minimal relevant difference. The alpha level is set to 0.05 and the beta level to 0.20 (Bowers, 2008).

For each of the participants the area under the curve (AUC) will be determined in order to investigate if a difference in blood glucose response exists between the control and active condition. AUC can be calculated in different ways e.g. incremental AUC and total AUC depending on the purpose of the calculation. Total AUC is used in the present study as this is preferable when determining whether a new treatment is able to reduce blood glucose levels (Wolever, 2004).

The area under the curve is divided into triangles and rectangles (figure 6). The area of each triangle and rectangle is then calculated and added up.

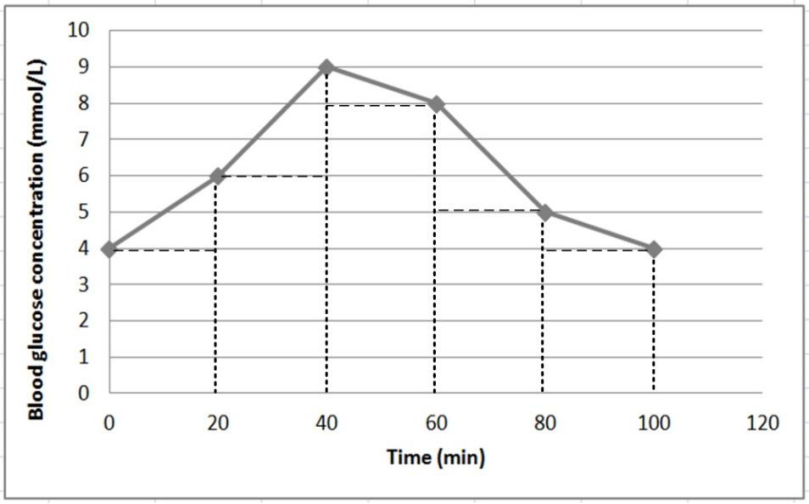

Figure 6 illustrates how the curve is divided into triangles and rectangles in order to calculate the area under the curve.

3. Materials and Methods

Suppliers and lot numbers of all chemicals are listed in appendix II.

3.1. Interaction between α-CD, Sunflower Oil, and a Lipid Rich Food

The study investigating possible product formation was performed as previously described (Artiss *et al.*, 2006) by dissolving various amounts of α-CD in 6 ml purified water followed by addition of 4 mg of vegetable oil. Methylenblue was added for ease of visualization. The content in the tubes was mixed for a few seconds and centrifuged (Sigma, 6-16K) at 2,000 G for 3 minutes. The same procedure was also performed with 1 M HCl instead of water. In addition, the tubes were placed at 37°C for 48 hours.

Furthermore, two times 50 g of deep-fried French fries were blended in a beaker and 400 ml of water was added to each. The mixtures were centrifuged (Sigma, 6-16K) at 10,000 G for 30 minutes at room temperature. The supernatants of each beaker were then transferred to new containers. 7 g of dried α-CD (incubated at 110°C for 12 hours) was added to one container and stirring while the other container functioned as a control. 40 ml of heptane was added to each container and mixed thoroughly. The mixture was then separated into two phases. Quantification of lipids was performed using the copper soap method (Pinsirodom and Parkin, 2001) with a few modifications; 5 ml of the supernatant and 1 ml of copper soap reagent (consisting of 5 w/v% copper(II) acetate in purified water and pH adjusted to 6.0-6.2 with pyridine) was mixed and centrifuged (Sigma, 6-16K) at 1,000 G for 5 minutes at room temperature. Hereafter, 1 ml of the mixture was analyzed by spectrophotometry at 715 nm (Spectronic, 20 Genesys).

3.2. Lipid Degradation

Preliminary studies were performed according to a study that mimicked the fed state in the gastrointestinal tract by first simulating the gastric digestion phase and then the duodenal (Nik *et al.*, 2010); sunflower oil was preincubated in HCl for 20 minutes at 37°C. pH was then adjusted to 6.5 with NaOH. This setup was performed with several different amounts of HCl, oil and PPL. Likewise, $NaHCO_3$ was used to adjust pH instead of NaOH also with different amounts of HCl, oil and PPL.

Other authors use a setup where the gastric phase is left out (Zangenberg *et al.*, 2001). Such a setup was also performed; the lipolysis was conducted in TRIS maleate buffer with a pH of 6.5 without any acidic environment beforehand. Results from the preliminary studies led to the following setup.

The degradation reactions were performed at 37°C with slow rotation in a 2 mM TRIS-maleate buffer prepared from 2 mM maleic acid, 2 mM TRIS-base, 150 mM NaCl, and 4 mM $CaCl_2$. The pH of the buffer was adjusted to 6.5 with 4 M NaOH. Porcine pancreatic lipase (PPL)

activity was approximately 0.10 units (U) in all experiments. U was defined as the concentration of oleic acid equivalents degraded from sunflower oil in three minutes per ml at room temperature. The degradation experiments were performed in duplicates.

3.2.1. Determination of Lipase Activity

PPL was dissolved in buffer and centrifuged (Sigma, 6-16K) at 10,000 G for 15 minutes at room temperature, and filtered through a 0.45 µm filter. Oil was preincubated in buffer for 20 minutes at room temperature while stirring. Dissolved PPL was then added and samples were taken after 30 minutes. The final volume was 5 ml. Samples were analyzed using the copper soap method (Pinsirodom and Parkin, 2001) with a few modifications; tubes containing 5 ml of heptane and 1 ml of copper soap reagent were prepared and 300 µl of sample was added and mixed immediately. The sample tubes were centrifuged (Sigma, 6-16K) at 5,000 G for 5 minutes at room temperature. Hereafter, the supernatant was analyzed by spectrophotometry at 715 nm (Spectronic, 20 Genesys). All analyses were performed in triplicates.

3.2.2. Lipid Degradation by Lipase in the Presence of α-cyclodextrin

Stock solutions of dried α-CD (incubated at 110°C for 12 hours) were prepared with buffer. 20 g of oil and various concentrations of dissolved α-CD were then preincubated in buffer for 20 minutes at 37°C while stirring. A control degradation experiment without any α-CD was also made. PPL solution was freshly prepared each time and added to the mixture. The final volume was 40 ml. Before sampling, stirring was stopped at appropriate time intervals for the mixture to divide in a water phase and a lipid phase. Samples were taken from both phases at time points; 1, 5, 10, 15, 20, 30, 45, 60, 120 minutes, and 3, 4, and 5 hours and analyzed using the copper soap method as described in the previous paragraph. The activity of PPL was stopped when adding the samples to the analytic reagent containing heptane, which denatured the lipase. All analyses were performed in triplicates.

3.3. Optical Microscopy Pictures

Microscopy (Zeiss, Azioskop, Germany) was performed of the product of 4 g of sunflower oil and 400 mg of dried α-CD (incubated at 110°C for 12 hours). The mixture available after degradation of oil with α-CD present in a concentration of 25 mM was also examined under microscope as well as the mixture available from the control degradation study (no α-CD). Furthermore, the substance formation was examined over time without the presence of lipase. That is, 20 g of oil and α-CD in a concentration of 25 mM were mixed and buffer was added to achieve a final volume of 40 ml. Stirring was applied and samples for microscopy were taken at 20 minutes, 5 hours, and 24 hours. All pictures were taken with a moticam 2000 (Motic, Wetzlar, Germany).

3.4. Starch Degradation

The starch degradation reactions were performed at 37°C with slow rotation in a 50 mM TRIS-maleate buffer prepared from 50 mM maleic acid, 50 mM TRIS-base, 30 mM NaCl, and 4 mM $CaCl_2$. The pH of the buffer was adjusted to 6.5 with 4 M NaOH. Porcine pancreatic α-amylase (PPA) activity was 0.012 U in all experiments. U is defined as the concentration of maltose equivalents degraded from starch in three minutes per ml at room temperature. The activity of PPA was stopped by heating to 95°C. The degradation experiments were performed in duplicates.

3.4.1. Determination of α-amylase Activity

PPA was dissolved in buffer and centrifuged (Sigma, 6-16K) at 10,000 G for 15 minutes at room temperature and filtered through a 0.45 µm filter. Activity of PPA was determined by adding 10 mg of soluble starch to 1 ml of PPA solution in a plastic test tube. The tube was then placed in a tumbler with slow rotation in 30 minutes at room temperature. Hereafter, 250 µl of sample was transferred to another test tube and heated to 95°C. The amount of degraded starch was analyzed using the method described by Miller (Miller, 1959) with a few modifications; 750 µl of a solution of DNS consisting of 1 w/v% 3,5-dinitrosalicylic acid, 1 w/v% NaOH, 20 w/v% Rochelle salt, and 0.05 w/v% sodium sulfite were added to the samples. This mixture of sample and DNS solution was heated to 95°C in 15 minutes and then placed on ice for minimum 20 minutes. Hereafter, 200 µl of the mixture was analyzed by spectrophotometry at 540 nm using an ELISA reader (Thermo Scientific, Multiskan FC). The analyses were performed in triplicates.

3.4.2. Starch Degradation by α-amylase in the Presence of α-cyclodextrin

100 mg of soluble starch was heated to 95°C for 20 minutes in buffer with slow rotation, resulting in degradation of starch granules. The mixture was then cooled to 37°C. Sodium azide (0.02 w/v%) and various concentrations of dried α-CD (incubated at 110°C for 12 hours) was added. A control degradation study without any α-CD present was also performed. The freshly prepared PPA solution was added and the time was started immediately. The final volume was 12 ml. Stirring was maintained during sampling and samples were taken at time points; 0, 2.5, 5, 10, 15, 20, 30, 45, 60, 90, 120 minutes and 24 hours. 250 µl of sample was added to a plastic test tube, heated to 95°C and analyzed as when testing the PPA activity. With concentrations of α-CD that was not soluble, filtration with a 0.45 µm filter and centrifugation (Ole Dich, Hermle 2160M) at 10,000 G for 10 minutes was conducted before the spectrophotometric measurements. The analyses were performed in triplicates.

3.5. *In Vivo* Study of Glycemic Response

The study was designed as a double-blind randomized crossover design. At an initial visit, the subjects' height, weight and age were recorded. Participants then attended the laboratory in the morning two times with at least one day apart. The subjects were asked to be fasting 12 hours prior to the experiment and have a similar eating and exercise pattern 24 hours before each test day.

Participants were placed in an armchair for 15 minutes and baseline blood glucose levels were measured. Hereafter, subjects consumed 111 g of white bread containing 50 g digestible carbohydrates, according to the manufacturer, together with 250 ml of water either with or without 10 g of dissolved α-CD. Lime juice was added to the water in both occasions. Additional glucose levels were measured at 5, 10, 20, 30, 45, 60, 90 and 120 minutes after meal consumption. Measurement was performed using a FreeStyleLite glucometer (Abbott Diabetes Care Inc, Alameda, USA). 48 hours after each test day, subjects were asked about possible side effects. Furthermore, calculations of total AUC were performed. Distribution of the difference in total AUC obtained between the two conditions was tested for normality before paired samples T-test was performed using SPSS software (IBM Corporation, US).

4. Results and Discussion

The two hypotheses regarding α-CD's weight loss ability were investigated through a series of experimental studies. Firstly, the results obtained regarding the lipid metabolism hypothesis will be presented and discussed following by presentation and discussion of the results regarding α-CD's effect on carbohydrate metabolism. Moreover, the probability that both of these two hypotheses are true will be addressed. Lastly, the potential for α-CD as a novel drug against overweight and obesity will be assessed.

4.1. Studies regarding α-cyclodextrin's Effect on Lipid Metabolism

4.1.1. The Product Formation of Sunflower Oil and α-cyclodextrin

The hypothesis stating that α-CD might have an effect on lipid metabolism leading to weight loss originated from a previous study (Artiss *et al.*, 2006). In this article, a simple experiment revealed that oil and α-CD formed a white substance when mixed together. In the present study, it was possible to verify the results obtained by Artiss and colleagues. Figure 7 shows two representative tubes where colored water is seen at the bottom and the product between α-CD and oil remains at the top. The tubes were labeled as percentage by weight of α-CD to oil, e.g. the tube labeled 10% contained 400 mg of α-CD and 4 g of oil. The investigations were performed with 1, 2.5, 4, 5, 10, 12.5, 15, 20, and 25% of α-CD to oil. In the tube containing 1% of α-CD some product was formed but free oil was still visible at the top. Before centrifugation no free oil was seen with the remaining percentages of α-CD. After centrifugation free oil was visible at 2.5, 4, and 5%, but not at the higher percentages, which is in accordance with previous results (Artiss *et al.*, 2006). At 25% of a-CD the solubility limit was exceeded, hence excess α-CD was seen at the bottom.

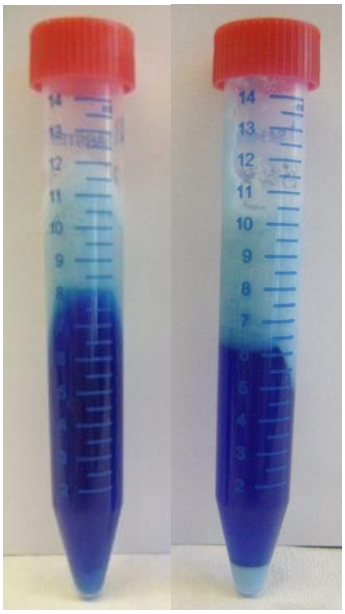

Figure 7 shows the tubes containing 10% (left) and 25% (right) of α-CD to oil. The bottom layer is water with methylen blue and the top layer is the oil/α-CD product. White precipitate is seen at the bottom with 25% of α-CD.

The tubes were placed in an incubator at 37°C for 48 hours in order to see if the substance was stable at a temperature corresponding to the body temperature. No changes of the substance were observed at 48 hours. Likewise, the mixture of oil and α-CD was carried out in HCl to determine if the formation occurred in an acidic environment. With 10% of α-CD to oil in 1M HCl the same result as when using water was obtained; substance was formed and no free oil was visible.

4.1.2. The Product Formation of a Lipid Rich Food and α-cyclodextrin

In continuation of the abovementioned experiments, studies investigating such substance formation with a lipid rich food were also performed. Deep-fried French Fries was chosen as food since it is a common accompaniment to fast food meals in western societies and contain a high amount of fat. Furthermore, deep frying is often done in vegetable oil and using this food is therefore parallel to the degradation studies using sunflower oil. Figure 8 shows the result of blended French fries mixed with α-CD. A white substance is seen at the top in the beaker where α-CD is added, marked as 1, as opposed to the control beaker where free lipid droplets floats at the top, marked as 2. A white precipitate is seen at the picture to the right in the beaker with α-CD and is proposed to be insoluble α-CD. The 7 g of α-CD added was expected to be dissolved in proportion to the solubility of α-CD. The reason the precipitate formation is therefore not known.

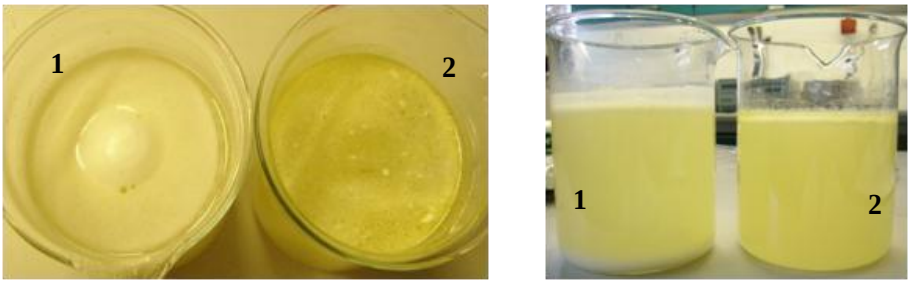

Figure 8 shows the beakers with blended French fries from two different angles. In both pictures, the beaker denoted 1 contains the supernatant from 50 g of blended French fries, where 7 g of α-CD is added. A white substance is seen at the top. The beakers denoted 2 are the control without α-CD. Here, visible lipid droplets float at the top.

Furthermore, spectrophotometric measurements using the copper soap method was performed. That is, the lipid in the beakers was extracted using heptane, and a sample of the heptane phase was analyzed with the copper soap reagent. The concentration of lipid in the control beaker was quantified to be 0.301 mM of oleic acid equivalents, while only 0.015 mM equivalents were quantified in the beaker with α-CD. This indicates that α-CD was able to prevent the extraction of lipid and further indicates that α-CD can interact with lipid from a diverse food matrix. With reference to α-CD's possible weight loss ability, the fact that α-CD somehow affects lipids from fast food as common as French fries is very interesting.

4.1.3. Preliminary Degradation Studies

Artiss and colleagues hypothesized that the product formation between oil and α-CD interferes with lipid digestion in a way that decreases the degradation of lipid. This postulate was investigated in the present study by degradation of triglyceride by PPL. Firstly, the conditions and results from preliminary degradation studies are listed in table 1. Oil was preincubated in HCl before adjusting pH to 6.5 with NaOH. PPL was then added. The purpose was to simulate *in vivo* digestion, where dietary lipid passes through the acidic stomach content, before reaching the intestine. Only low concentrations of oleic acid equivalents were obtained at 30 minutes with this

setup. The result was unaffected by changes in oil amount. Moreover, it was difficult to adjust the pH quickly and accurate with NaOH. The pH was also adjusted with $NaHCO_3$, but with the same results.

Table 1 lists the preliminary triglyceride degradation studies performed. N.A. = not analyzed. [1)]After 20 minutes of preincubation.

Lipolysis performed in	*Amount of oil*	*Amounts of enzyme*	*Oleic acid equivalents after 30 min*	*Oleic acid equivalents after 60 min*	*Oleic acid equivalents after 5 hours*
10 ml HCl, pH adjusted with NaOH[1]	10 g	1 ml from a solution of 15 mg PPL dissolved in 5 ml buffer	0.4 mM	N.A.	N.A.
20 ml HCl, pH adjusted with NaOH[1]	20 g	1 ml from a solution of 15 mg PPL dissolved in 5 ml buffer	0.6 mM	N.A.	N.A.
20 ml HCl, pH adjusted with $NaHCO_3$[1]	20 g	1 ml from a solution of 15 mg PPL dissolved in 5 ml buffer	0.7 mM	N.A.	N.A.
20 ml HCl, pH adjusted with $NaHCO_3$[1]	5 g	1 ml from a solution of 15 mg PPL dissolved in 5 ml buffer	0.9 mM	N.A.	N.A.
10 ml TRIS maleate buffer	5 g	1 ml from a solution of 15 mg PPL dissolved in 5 ml buffer	1.0 mM	N.A.	N.A
20 ml TRIS maleate bufffer	5 g	1 ml from a solution of 30 mg PPL dissolved in 5 ml buffer	4.3 mM	6.2 mM	N.A.
20 ml TRIS maleate buffer	5 g	1 ml from a solution of 200 mg PPL dissolved in 10 ml buffer	4.8 mM	N.A.	N.A.
20 ml TRIS maleate buffer	10 g	1 ml from a solution of 200 mg PPL dissolved in 10 ml buffer	4.4 mM	8.1 mM	N.A.
20 ml TRIS maleate buffer	10 g	1 ml from a solution of 200 mg PPL dissolved in 10 ml buffer	2.3 mM	2.8 mM	3.9 mM
20 ml TRIS maleate buffer	20 g	3 ml from a solution of 200 mg PPL dissolved in 10 ml buffer	4.5 mM	10.0 mM	19.7 mM

When preincubating oil in buffer with a pH of 6.5, better degradation results were obtained and the pH adjustment step was avoided. Consequently, an experimental setup without simulation of the gastric phase of digestion was left out and a simpler setup was chosen for the lipid degradation studies.

4.1.4. α-cyclodextrin Decreases Lipase's Degradation of Triglycerides

Lipid degradation was carried out with equal amount of oil and PPL activity in each degradation study. The degradation occurred with α-CD present in concentrations of 500 µM, and 5, 12, 17, and 25 mM plus a control degradation experiment without the addition of α-CD. The results obtained in the degradation experiments are illustrated in figure 9. The x-axis represents the time and the y-axis the concentration of oleic acid equivalents.

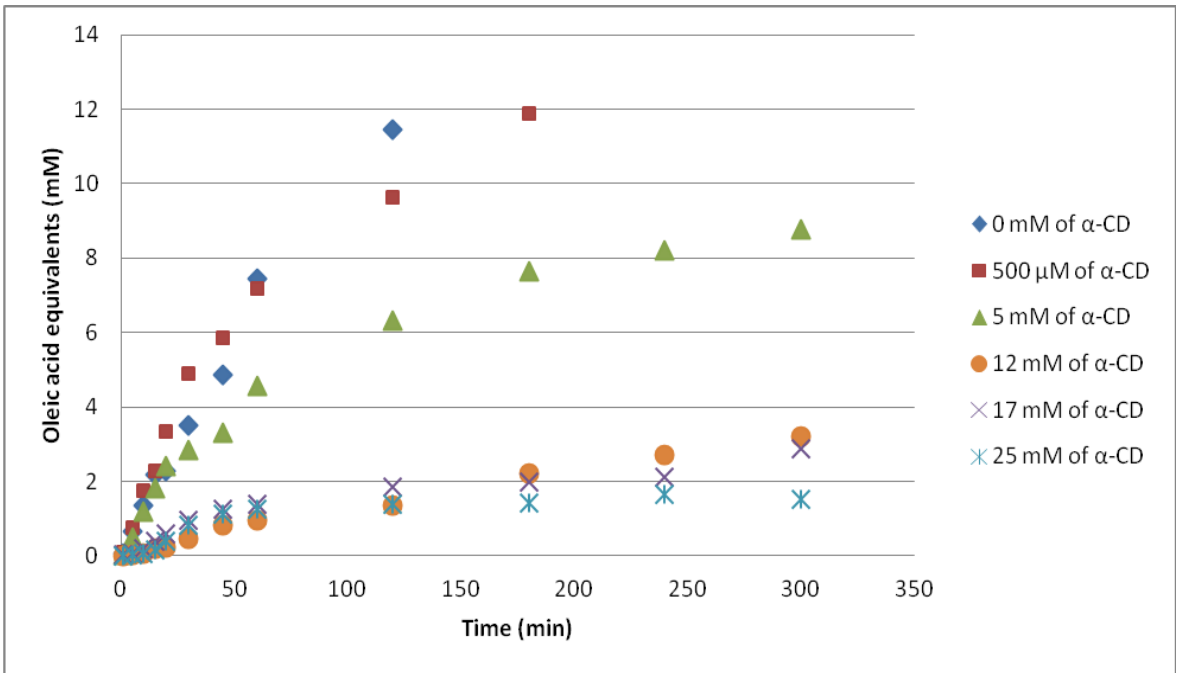

Figure 9 shows the concentrations of oleic acid equivalents quantified over time in the lipid degradation studies. The x-axis represents the time in minutes and the y-axis represents the concentrations. Error bars are applied to each time point (± 2SE).

It is evident that the presence of α-CD decreased the measured concentration of oleic acid equivalents. The difference in concentrations of equivalents in the control samples and samples with α-CD present was calculated in percentages. A concentration as low as 500 µM of α-CD decreased the amount of measured equivalents by 16% at two hours compared to the control. With α-CD in concentrations of 5, 12, 17, and 25 mM, the percentage reductions of measured oleic acid equivalents at two hours were 45, 88, 84, and 88%, respectively. The curves obtained by degradation studies with 12, 17, and 25 mM remained at the same low concentrations until approximately three hours. Hereafter, concentrations of equivalents seems to be even lower in the samples with 25 mM of α-CD than with 12 and 17 mM. This could be a coincidence or could be

caused be inaccurate sampling as the concentrations otherwise seems to lay at the same level with all three α-CD amounts.

The concentration of oleic acid equivalents in the samples from the control degradation reached 11.4 mM at two hours. The concentration was expected to be higher as 20 g of oil was present. It is plausible that the degradation process had not reached the plateau yet and more oil would have been degraded at e.g. 24 hours. Unfortunately, it was not possible to sample for more than two hours due to viscosity of the mixture. The viscosity is proposed to be caused by emulsion formation as a result of the stirring. An analogue to this might be when whipping cream. In future experiments, the stirring speed, the amounts of buffer, and oil, as well as the ratios between the latter two could be varied in order to determine if the problems with viscosity could be delayed. Thereby, sampling from the control degradation experiment could be facilitated for more than two hours.

The degradation studies of triglyceride showed that α-CD reduced the concentration of oleic acid equivalents in a dose-dependent manner. An explanation for this could theoretically be that α-CD formed inclusion complex with the lipolytic products. The ability of α-CD to form complexes with free fatty acids is generally accepted (Szente *et al.*, 1993; Duchêne *et al.*, 2003). If this occurred in the present study, the fatty acids generated by degradation would not be available for the copper acetate to form "soaps". The spectrophotometer quantified the concentration of these soaps. If copper acetate formed complexes with α-CD instead of fatty acids, that would account for the decreased concentrations of equivalents measured. This scenario can be ruled out on the following grounds; standard curves of oleic acid were made with various concentrations of α-CD corresponding to the concentrations used in the degradation experiments. The spectrophotometric measurement was still able to determine fatty acid concentrations. Hence, the copper soap method is valid in quantifying the amount of fatty acid even when α-CD is present. α-CD only affected the measured concentration of oleic acid slightly and this was accounted for by adjusting the data with the standard curve corresponding to each concentration of α-CD. Furthermore, no pronounced shift in the maximum absorbance was seen when α-CD was present in the samples. In summary, the explanation for lower concentrations of oleic acid equivalents when α-CD was present can only be that α-CD was able to reduce the degradation of triglycerides. Thereby, the hypothesis put forward in the problem statement regarding α-CD's ability to decrease lipid degradation is confirmed for the first time. In addition, the decrease occurred in a dose dependent manner as expected.

Figure 10 further illustrates the difference in the rate of triglyceride degradation by showing the initial velocities, that is, the first 20 minutes of each degradation reaction. The concentrations of α-CD in the reactions represent the x-axis. The slopes calculated on the basis of the initial linear part of each degradation curve are represented on the y-axis.

The graph shows that a concentration of 5 mM of α-CD decreased the initial velocity in comparison to control. It also clearly illustrates that 12, 17, and 25 mM of α-CD reduced the velocity to a great extent. The initial velocity of the degradation reaction with 500 µM of α-CD present was faster than in the control degradation, which was the opposite of what was expected. Likewise, the velocity of initial degradation with 12 mM of α-CD was even slower than with concentrations of 17 and 25 mM of α-CD. Despite this, the tendency that α-CD decreased the degradation rate of triglyceride is evident when looking at the initial velocities.

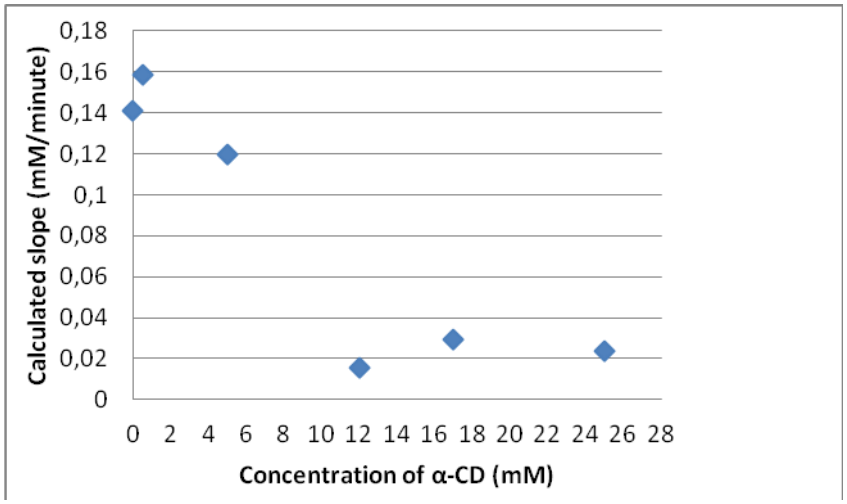

Figure 10 illustrates the initial velocities of the triglyceride degradation studies. The x-axis represents the concentration of α-CD present in each degradation reaction and the y-axis represents the slopes calculated on the basis of the initial linear part of the degradation curves.

An explanation for the irregularities seen in figure 10 might be found in the sampling method. As mentioned, prior to sampling the stirring applied was stopped at appropriate time intervals for the mixture to separate in a water phase and a lipid phase and samples were taken from both. The spectrophotometric measurements showed that few fatty acids were present in the water phase as expected (results are provided in appendix III). This separation of phases was necessary as the risk of inaccurate sampling was too great otherwise. That is, if stirring was maintained during sampling, the lipid would be suspended in the buffer. Then, when samples were taken one could risk to sample mostly water at one time point and mostly lipid at another time point resulting in unusable results. This was attempted to be avoided in the present study by phase division. Still, some water might have been present in the lipid phase and vice versa resulting in inaccurate results. This would account for the irregularities seen in figure 10.

Since the sampling method offered some difficulties, other analytical approached could be considered for future experiments. One such method is pH-stat titration. In this method, the fact that the fatty acid, produced by lipolysis, changes the pH is utilized. That is, the more fatty acid produced the more of a given base e.g. NaOH is added by the pH-stat to maintain the pH. The amounts of NaOH added is then used to calculate the amounts of fatty acid produced (Zangenberg

et al., 2001). pH-stat titration was attempted to be used in the present study as this is the method used with success by others (Zangenberg *et al.*, 2001; Nik *et al.*, 2010). Unfortunately, the equipment for measuring pH in a mixture containing oil was not available.

The reduced concentrations of oleic acid equivalents could be explained by a direct inhibition effect where an inhibitor, in this case α-CD, binds to the enzyme or enzyme-substrate complex causing an inactivation of the catalytic process. However, when considering that α-CD and oil easily formed a stable product, it seems more likely that α-CD interacts with the substrate, in this case triglycerides, in a way that causes decreased lipid degradation.

Two interaction scenarios between α-CD and oil are proposed. The first is that α-CD coats the surface of lipid droplets preventing the access of lipase to the lipid. This complex should occur with one of the fatty acid side chains of triglycerides. A fact that contradicts this scenario is that normally all three side chains are arranged in the same inward direction and the glyceride is the part that is facing the aqueous environment (Guyton and Hall, 2006). Still, it is possible that the interaction of α-CD forces a side chain outwards and that the following proposed inclusion of a side chain stabilizes the lipid droplet (figure 11).

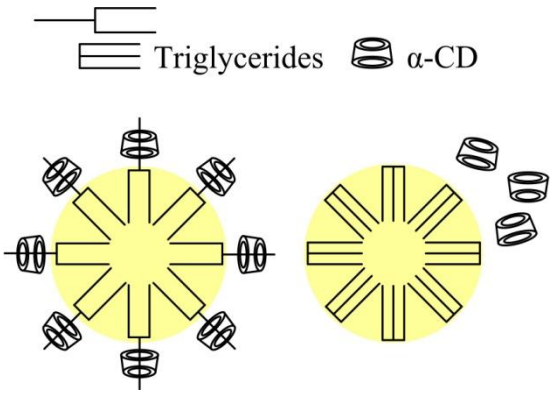

Figure 11. The yellow lipid droplet to the left illustrates how a fatty acid side chain theoretically is orientated outwards and α-CD molecules complexes with these side chains. The lipid droplet to the right shows that triglyceride normally arranges all three side chains toward the middle. Thereby, α-CD might not be able to bind to a side chain.

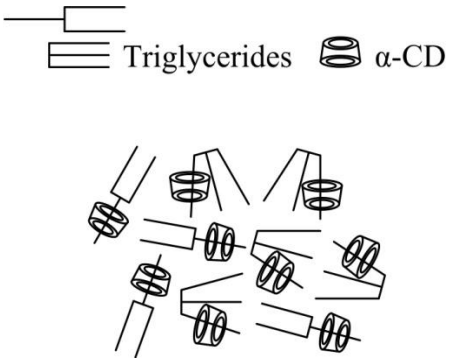

Figure 12 illustrates the possible scenario where α-CD forms inclusion complexes with triglyceride molecules followed by aggregation of these complexes.

Another scenario is that α-CD complexes with a fatty acid side chain of single triglyceride molecules. An article from 1996 indicates that complex formation between CD and triglyceride is possible (Kolossváry and Kolossváry, 1996). In this article, computer modeling was carried out to see if triolein and β-CD could form a complex. The modeling showed that a fatty acid chain could be included in the β-CD cavity, but it slipped out again, suggesting that such a complex is unstable (Kolossváry and Kolossváry, 1996). It is possible that computer modeling would reveal complex formation between α-CD and a given triglyceride to be more stable than when β-CD is used, due to the difference in number of glucose units and thus cavity size. Furthermore, it is proposed that

inclusion complexes of α-CD and triglyceride forms aggregates, which could also account for the stable substance formed between α-CD and oil (Figure 12).

In summary, the results obtained in the lipolysis studies showed for the first time that α-CD has the ability to decrease the degradation of triglycerides by PPL. The mechanism behind is proposed to be formation of α-CD/triglyceride aggregates and/or coating of lipid droplets by α-CD. Such aggregate formations or lipid coating could hinder lipase's access to lipid surface, thereby explaining the reduction in lipid degradation obtained in the present study.

4.1.5. Microscopy of the Product between α-cyclodextrin and Oil

In order to gain insight into the nature of the substance between oil and α-CD, microscopy was performed. All microscopy pictures shown in the following were magnified with a factor of 10. A picture of the sunflower oil prior to any modification is shown in figure 13A as a control. A few small droplets are seen, which plausibly could be impurities in the oil. Otherwise, no irregularities are present indicating a homogenous mixture of lipids as expected.

Figure 13B shows a sample of the mixture from the control degradation of sunflower oil after five hours (no α-CD). Droplets of various sizes are seen, appearing circular and similar in shape. This is in agreement with the expected appearance of lipids suspended in buffer. Hence, the droplets are probably lipid droplets consisting of lipolytic product and non-converted triglyceride.Figure 13C shows the mixture available from the degradation with 25 mM of α-CD present after five hours. Here, a dark appearing phase, marked as (1) in the figure, is seen in between circular formations denoted (2). As the only difference between the two degradation experiments is the addition of α-CD, it seems plausible that substance (1) is the product formed between oil and α-CD. The substance seen is relatively small and variable in size, which is in coherence with the stated hypothesis about the nature of the interaction between α-CD and oil. That is, complex formation between α-CD and triglyceride molecules, which then aggregates, would probably be small and variable in size. The substance could also include small amounts of fatty acids and monoglycerides formed during degradation. The matter denoted (2) in the sample with α-CD looks like lipid droplets. This is unexpected as no free oil was evident with the naked eye. These droplets might be coated with α-CD molecules as illustrated in figure 11.

Figure 13D shows the product formed in the simple study mentioned previously, where 400 mg of α-CD and 4 g of oil where mixed. Here, only the dark appearing phase (1), which is also seen in figure 13C, is present. This further implies that substance (1) must be the product formed between α-CD and oil. No lipid droplets are seen indicating that 400 mg of α-CD was enough to bind all of triglyceride. In that connection it should be mentioned that only 250 mg of α-CD was added in the degradation study (figure 13C). It is possible that 250 mg of α-CD was not enough to form aggregate complexes with all the triglycerides, while 400 mg was (similar amounts of oil and buffer were added in both).

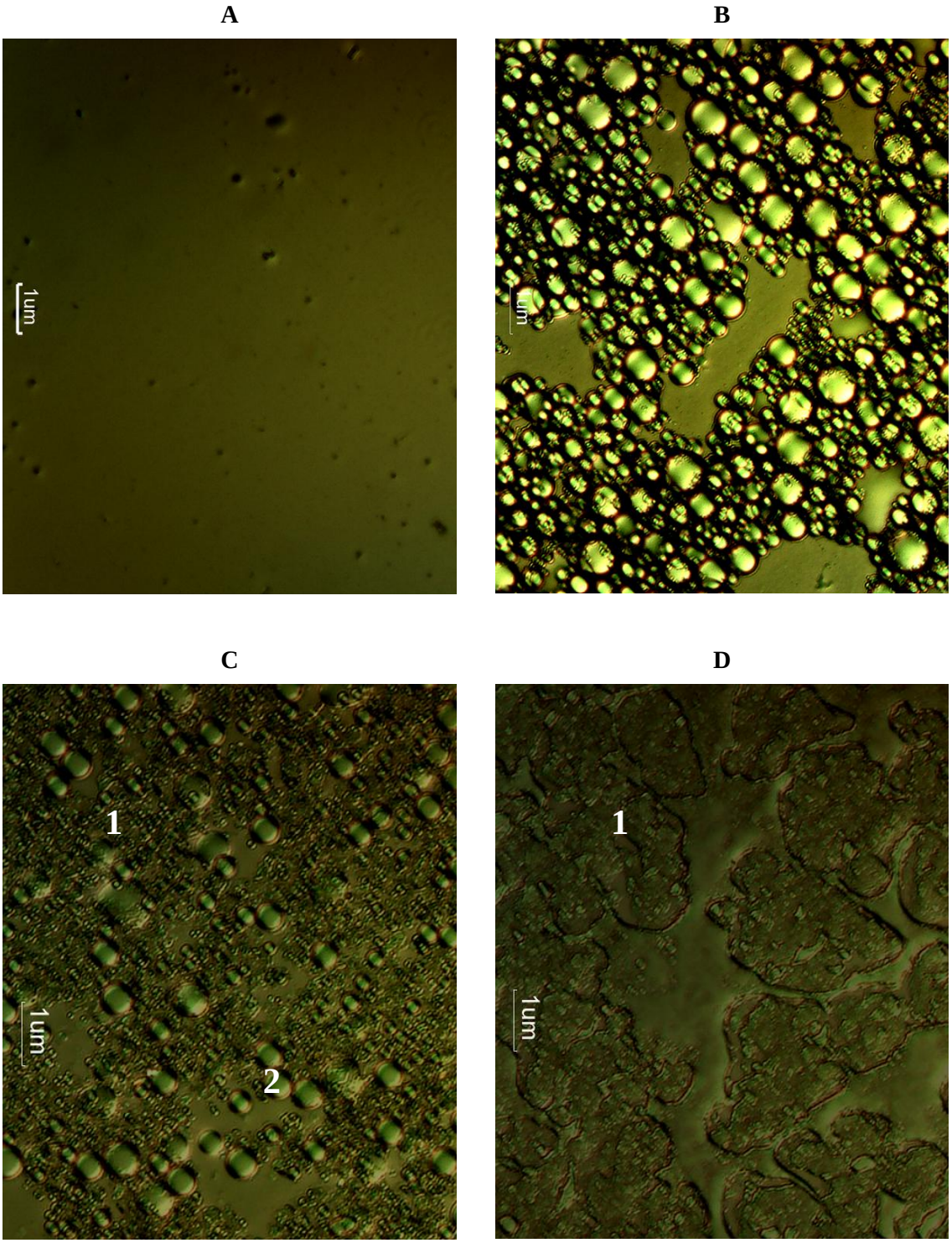

Figure 13. Picture A shows a microscopy picture of the sunflower oil (Magnified 10 times). Picture B shows a sample from the control degradation of oil after five hours (no α-CD). C is a picture of a sample from the degradation of oil with α-CD present in a concentration of 25 mM after five hours. Picture D shows the substance formed between oil with α-CD just after mixing and centrifugation. All pictures are magnified 10 times and scale bars are applied.

The product formation without degradation was examined over time by microscopy as well. This was done to determine if more oil was included in the substance the longer time α-CD and oil had to interact. Oil and buffer was mixed in ratios equal to those used in the degradation studies. 25 mM of dissolved α-CD was added and stirring was applied immediately. After 20 minutes with constant stirring, a white substance formed, which can be seen in figure 14.

Figure 14 shows the white substance formed at 20 minutes between oil and α-CD in buffer.

A microscopy picture of the product at 20 minutes is seen in figure 15A. A dark appearing phase is present marked as (1) in the figure. This substance is expected to be the product formation, which is also seen in figure 13. Furthermore, both small and large formations are seen, marked as (2). It seems likely that these formations are oil not yet included in the substance all though 20 minutes was expected to be sufficient time for this to occur. The picture is taken where the product was seen, but is not representative of the ratio between the product and free oil; significantly more of the oil was present and less of the product when examining the sample in general by microscopy.

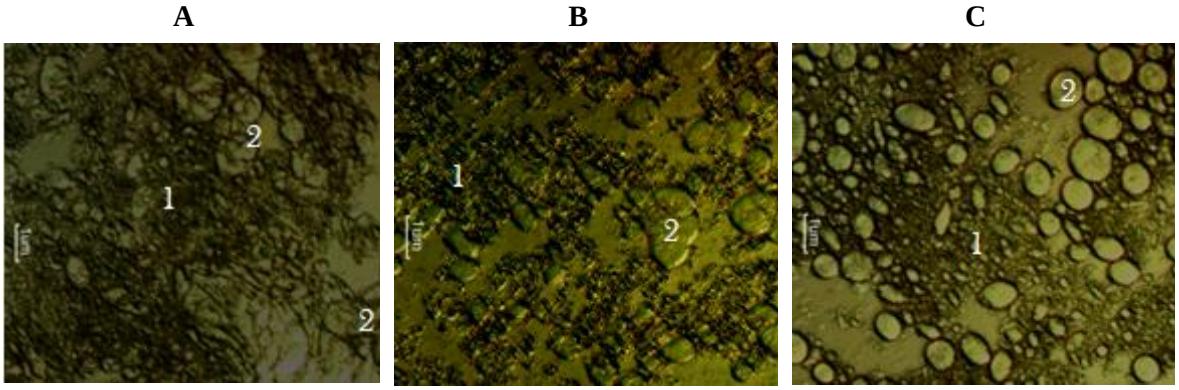

Figure 15 shows the product formed of α-CD and oil in buffer over time. A = at 20 minutes. B = at five hours. C = 24 hours. All three pictures are taken with a magnification of 10. Scale bars are applied.

Figure 15B shows a sample of the mixture after five hours of stirring. The mixture is still not uniform but more of substance (1) is formed in comparison to the amount formed at time point 20 minutes. The oil (2) is present in various sizes and shapes but less than at 20 minutes. α-CD molecules might coat these oil droplets. Lastly, figure 15C shows the mixture after 24 hours of stirring. The ratio of product (1) to oil (2) was the same as at five hours. It seems that at a point between 20 minutes and five hours the amount of α-CD had interacted with the amount of triglyceride that is possible. In figure 13 and figure 15 the same ratio between the amounts of oil and α-CD was used, further indicating that this amount of α-CD was not sufficient to include all of the triglycerides in the product formed.

4.1.6. Possible Mechanisms Affecting Lipid Metabolism

As mentioned, the product formed of lipid and α-CD in the *in vitro* study could account for the decreased lipid degradation. *In vivo*, such a scenario would lead to less absorption of lipids accounting for α-CD's ability to induce weight loss. Several studies indicate that decreased lipid degradation occurs *in vivo* as well; besides reporting that α-CD was able to reduce weight gain in rats in the study by Artiss and colleagues, it also revealed that rats which were fed the HF diet with α-CD had a 30% reduction in plasma triglyceride levels compared to the control condition ($p < 0.05$). Likewise, plasma cholesterol levels were reduced by 9% compared to the control condition (not significant). It has been reported previously that α-CD lowers plasma cholesterol levels in mice (Wagner *et al.*, 2008), rats (Kaewprasert *et al.*, 2001), and humans (Grunberger *et al.*, 2007; Comerford *et al.*, 2010). Lastly, the percentage fecal lipid content was increased by 20% in rats fed on the HF diet with α-CD in comparison to the rats on the HF control diet ($p < 0.01$) (Artiss *et al.*, 2006). This indicates that some lipid absorption was prevented in the group administered α-CD and that this lipid instead was excreted in the feces.

Other mechanisms than the hypothesized could account for such effects as digestion is a complicated process involving factors not included in the *in vitro* study. One such mechanism could be that α-CD is able to prevent the natural emulsification of lipid. Hence, only large lipid droplets would be available for degradation. This reduces the water/lipid interface where lipase is active, and the result might be decreased lipid degradation (figure 16A).

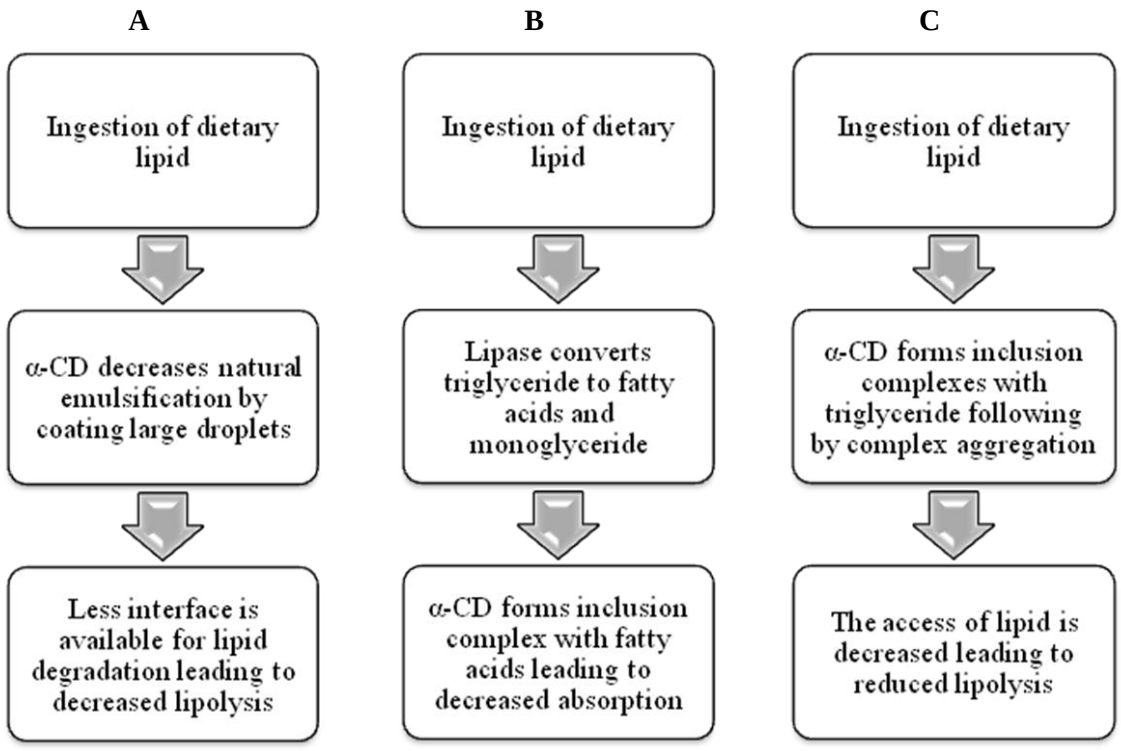

Figure 16 shows an overview of three hypothetical *in vivo* scenarios that could explain the weight loss property of α-CD. Each scenario is explained further in the text.

Another possible scenario is that α-CD binds the free fatty acids and the monoglycerides formed when lipase cleaves triglycerides. A fatty acid/α-CD complex is perhaps not able to diffuse through the gastrointestinal wall thereby preventing absorption of dietary lipids (figure 16B).

Lastly, the scenario of α-CD and triglyceride forming inclusion complexes aggregates, as mentioned in the preceding paragraph, is depicted in figure 16C. Scenario A seems unlikely, also under *in vivo* conditions, due to the structure of a lipid droplet, where no side chains is turned outward. Accordingly, it is questionable if α-CD molecules can coat a lipid droplet. Consequently, scenario B and C is more plausible. For future perspectives, a diet study with for example rats could be performed where α-CD was administered to a diet high in lipid. An increased excretion of fat would then be expected and this fat should be examined to conclude if it consisted mainly of triglycerides or fatty acids. This would give a stronger indication regarding whether scenario B or C occur or if perhaps both acts simultaneously.

The interference of bile salts needs to be considered too. Bile salts aid the emulsification of lipid droplet into much smaller droplets by micelle formation. It is possible that bile salts and α-CD competes for the interaction with triglyceride. Several studies, as mentioned previously, showed that α-CD does affect lipid digestion *in vivo* (Suzuki and Sato, 1985; Kaewprasert *et al.*, 2001; Artiss *et al.*, 2006; Grunberger *et al.*, 2007; Wagner *et al.*, 2008; Comerford *et al.*, 2010). Thus, the scenario where the presence of bile salts completely prevents any effect of α-CD on lipid metabolism seems unlikely. A competitive state between α-CD and bile salts are still possible though. For future experiments, the addition of bile salts in the triglyceride studies should be considered to see if the inhibitory effect of α-CD is unaffected by bile salts or diminished.

The involvement of bile salts in lipid digestion opens for a third new hypothesis for the mechanism behind α-CD's weight loss ability. If α-CD were able to form inclusion complexes with bile salts, then the natural emulsification step and the micellar formation of lipolysis products with bile salts could potentially be inhibited. Following, the lipid degradation would be reduced due to limited access to lipid surface. Moreover, if the lipolytic products formed are not removed from the lipid/water interface by micelle formation, the activity of lipase could be further decreased due to Le Chatelier's principle. A 1:1 complex formation between α-CD and bile salts has been confirmed by capillary electrophoresis and computer modeling although the interactions were weak (Holm *et al.*, 2011). When considering the weakness of this interaction and the several other options stated regarding α-CD's weight loss mechanism, it is proposed that this third hypothesis do not contribute to any significant extent.

4.2. Investigations regarding α-cyclodextrin's Effect on Carbohydrate Metabolism

4.2.1. α-cyclodextrin Decreases α-amylase's Degradation of Potato Starch

The carbohydrate hypothesis was investigated in the present study by performing degradation experiments of potato starch with and without different concentrations of α-CD present to see if α-CD affected the enzymatic hydrolysis of starch. The degradation process was monitored by spectrophotometry and this method was tested to be valid even when α-CD was present, as no pronounced shift in maximum absorbance was observed.

The starch degradation experiments were carried out with equal amounts of starch and equal PPA activities in each degradation. The reactions occurred with α-CD present in concentrations of 50, 100, 300, and 500 mM plus control degradation without the addition of α-CD. The results from the degradation experiments are given graphically in figure 17. The x-axis represents the time and the y-axis the concentration of maltose equivalents.

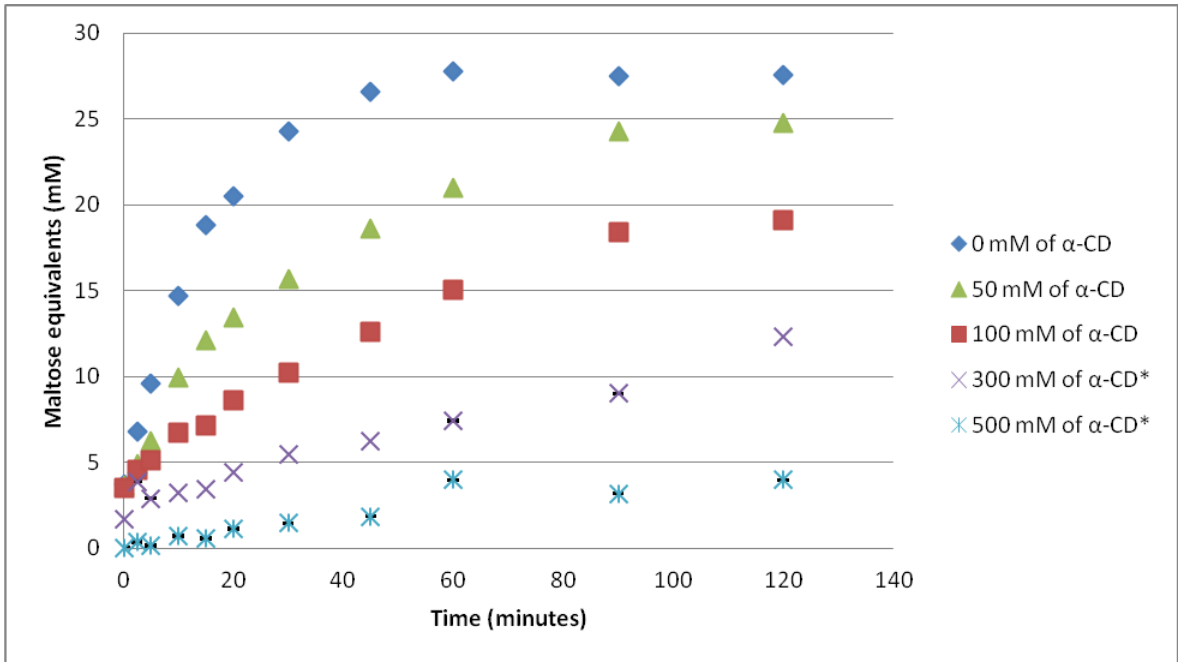

Figure 17 shows the concentrations of maltose equivalents quantified over time in the starch degradation studies. The x-axis represents the time in minutes and the y-axis represents the concentration. Error bars are applied to each time point (± 2SE). In the two concentrations marked with * insoluble α-CD were present.

The graph clearly illustrates that the higher the concentration of α-CD the slower the degradation occurred expressed as lower concentrations of maltose equivalents formed in the same time course. Calculations of the percentage-wise decline in the amounts of maltose equivalents showed a 10, 31, 55, and 86% reduction with 50, 100, 300, and 500 mM of α-CD at two hours, respectively. At the first time point, the concentrations of equivalents in the degradation experiments were not 0 mM. Concentrations of 0 mM was anticipated since the data was adjusted

with the results obtained when analyzing samples of buffer, starch, denatured PPA, and α-CD in concentrations corresponding to those in each degradation study. Hence, it seems that the reaction happened too fast in these degradation studies to take a sample where no product was converted. Moreover, at time point 60 minutes in the degradation with 500 mM of α-CD, the concentration seems to be higher than what could be expected from the concentrations at the remaining time points. This irregularity could be a result of inaccurate sampling or of insoluble α-CD in the sample causing turbidity.

Sampling was also performed at 24 hours and figure 18 shows the degradation results with the addition of this time point. At 24 hours, it is still obvious that α-CD inhibited the degradation in a dose dependent manner as the amounts of equivalents decreased with higher concentrations of α-CD. The results obtained in the starch degradation studies are in agreement with previous research. A study has examined the degradation of amylose and maltopentaose by PPA in the presence of α- and β-CD, respectively. The results revealed that hydrolysis of amylose and maltopentaose was decreased by both α-CD and β-CD (Koukiekolo *et al.*, 2001).

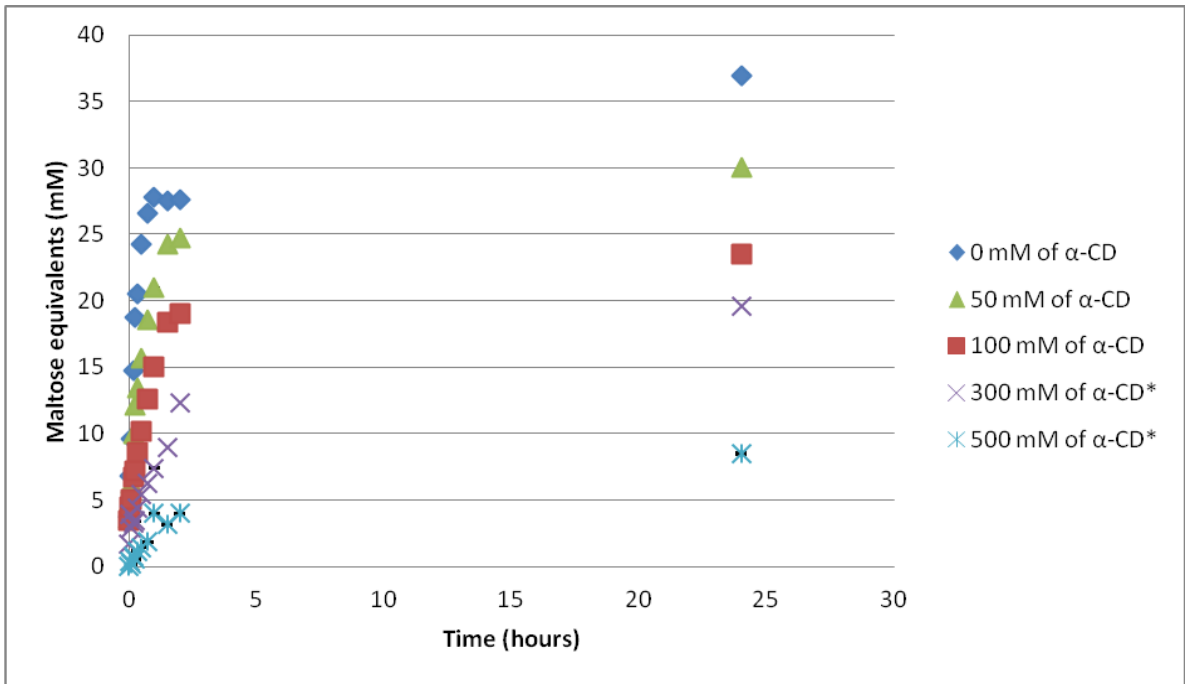

Figure 18 shows the concentrations of maltose equivalents quantified over time in the starch degradation studies with the addition of time point 24 hours. The x-axis represents the time in hours and the y-axis the concentration. Error bars are applied to each time point (± 2SE). In the two concentrations marked with * insoluble α-CD were present.

Figure 19 further illustrates the difference in the rate of starch degradation by showing the initial velocities. The slopes calculated on the basis of the initial linear part of each degradation curve, that is, the first 20 minutes, are represented on the y-axis and the concentrations of α-CD present in the reactions represent the x-axis. It is evident that the initial velocities decreased with

higher concentrations of α-CD as anticipated. Calculation of initial velocities showed a 48, 81, 93, and 96 % reduction in velocities with 50, 100, 300, and 500 mM of α-CD, respectively.

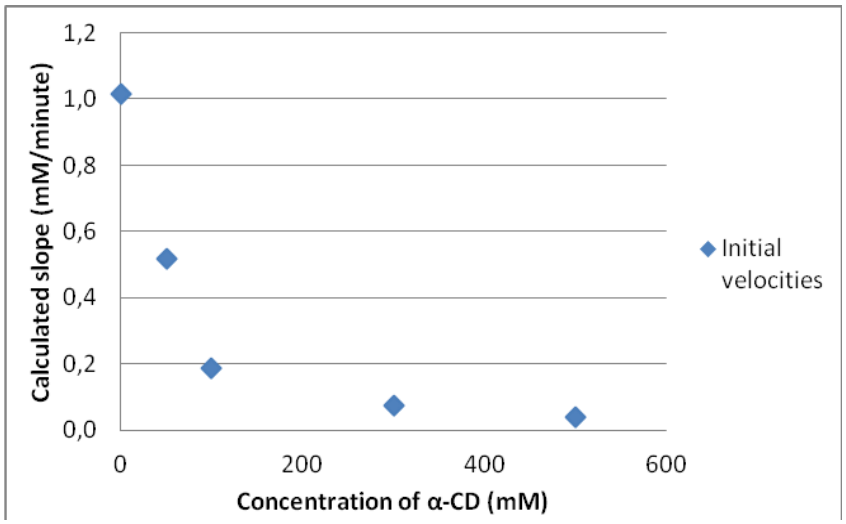

Figure 19 illustrates the initial velocities of the starch degradation studies. The x-axis represents the concentration of α-CD present in each degradation study and the y-axis represents the slopes calculated on the basis of the initial linear part of the degradation curves.

In contrast to the lipid degradation study, no obvious interaction between substrate, in this case starch, and α-CD was seen or have been reported previously. Instead, the reduction in starch degradation could be explained by a direct inhibition effect where α-CD acts as inhibitor of PPA. There are several possible ways for α-CD to inhibit α-amylase as inhibition kinetics of enzymes can be described by more than one model. In competitive inhibition, the enzyme might be bound to the inhibitor or the substrate, but never both at the same time. Most often, the inhibitor resembles the substrate and competes with the active site of the enzyme. In uncompetitive inhibition the inhibitor is first bound to the enzyme when the substrate is bound simultaneously resulting in an enzyme-substrate-inhibitor complex. This complex is inactive. Lastly, noncompetitive inhibition is defined as an inhibition model, where the inhibitor can bind to the enzyme and the enzyme-substrate complex equally well (Fogler, 2008).

Kinetic inhibition studies with α-CD as inhibitor of PPA and amylose and maltopentaose as substrates have been performed in a study previously mentioned (Koukiekolo *et al.*, 2001). The authors analyzed their data by Lineweaver-Burk plots which give a good indication towards which type of inhibition model that are applicable. Interestingly, the results revealed that the type of inhibition seemed to depend on the substrate used. When amylose was hydrolyzed, the inhibition kinetics showed the characteristics for a competitive inhibition (Koukiekolo *et al.*, 2001). In other words, α-CD could plausibly bind to and inactive α-amylase, but if amylose was bound to PPA first, α-CD could probably neither bind at the active site nor at allosteric sites corresponding to the definition of a competitive inhibition. This indicates that when the large amylose molecule is bound

to PPA, it results in steric hindrance of α-CD binding. On the contrary, when maltopentaose was used as substrate, the kinetic data showed the characteristics of a noncompetitive inhibition (Koukiekolo *et al.*, 2001). This indicates that even though maltopentaose was bound to PPA, α-CD could still bind to the enzyme-substrate complex and prevent product formation (Koukiekolo *et al.*, 2001). Since starch consists of amylose and amylopectin, the results from the Koukiekolo study is comparable to the results from the present study. It is therefore proposed that α-CD occupied the active site of PPA resulting in a competitive equilibrium state with starch. Over time, when smaller oligosaccharides were formed such as maltopentaose, the inhibition was plausibly also of the noncompetitive type.

The preceding inhibition scenarios are plausible for the starch degradation experiments where α-CD was solubilized. With the concentrations of 300 and 500 mM of α-CD it is also possible that PPA denatured due to conformational changes caused by extensive complex formation between α-CD and the hydrophobic parts of the amino acids of the enzyme. Hence, the lowered concentrations of maltose equivalents measured in these two degradation studies might therefore be caused by denaturation of PPA in combination with α-CD inhibition. Not all of the PPA could have denatured as the concentration of equivalents reached 20 and 8 mM at 24 hours with 300 and 500 mM of α-CD, respectively. In future experiments, with such high concentrations of α-CD present, the PPA activity at termination of the hydrolysis should be measured in order to rule out this denaturation scenario.

In summary, the degradation studies showed that α-CD was capable of decreasing the enzymatic hydrolysis of starch in a dose dependent manner. Based on previous literature, the proposed mechanism behind this inhibition is that α-CD molecules act as inhibitors when bound to the enzyme and the enzyme-substrate complex.

4.2.2. α-cyclodextrin Reduces Glycemic Response *In Vivo*

The effect of α-CD on starch was further examined in an *in vivo* study. This was done to investigate if the inhibitory effect would be evident on blood glucose levels after incorporating α-CD to a starch containing meal.

Ten healthy subjects completed the *in vivo* study. Subject characteristics are shown in table 2.

The participants consumed 50 g of digestible carbohydrates (control condition) in one occasion. On another day, the same amount of carbohydrates was served together with 10 g of α-CD dissolved in water (active condition). Blood glucose levels were measured

Table 2. Subject characteristics

Age, years	25 ± 0.97
Height, cm	178 ± 0.08
Weight, kg	70 ± 9.65
BMI, kg/m^2	22.0 ± 1.59

BMI = Body mass index. Values are mean ± standard deviations.

before ingestion (T = 0) and continuously over the next two hours. Figure 20 graphically demonstrates the results of mean blood glucose levels for all ten participants at each time point. Time is represented on the x-axis and blood glucose levels on the y-axis. A difference of 0.2 mM in baseline blood glucose levels between the two experimental days was detected. This difference is considered to be coincidental and to be a result of possible difference in amounts of energy consumed prior to the fasting period. At 5 and 10 minutes, the blood glucose levels after consumption of the meal with α-CD were still higher than concentrations measured after ingestion of the control meal, which could be caused be the difference in baseline levels. Hereafter, the meal with α-CD caused a reduction in blood glucose response compared to control. At 45 minutes the difference in concentrations was highest; 6.9 mM for the control condition and 5.7 mM for the active condition. At 60 and 90 minutes the concentration of glucose in the blood was still lower for the active condition than the control. At time points 45 and 60 minutes the difference was significant. At two hours the concentration in the active condition was higher than in the control condition, which was not anticipated (not significant).

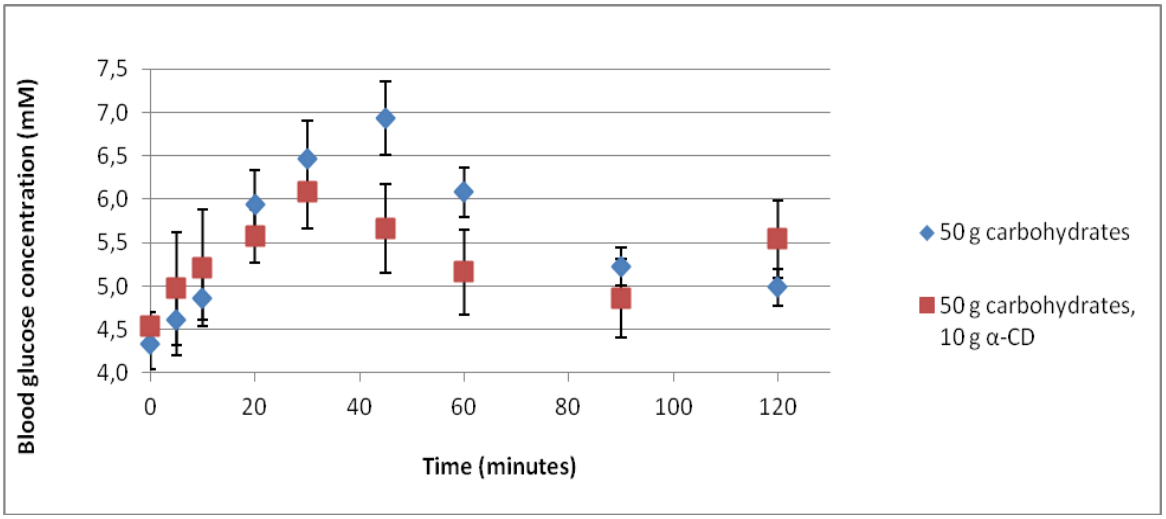

Figure 20 shows the mean blood glucose levels from ten participants after ingestion of 50 g of carbohydrates and 50 g of carbohydrates together with 10 g of α-CD. The x-axis represents the time in minutes and the y-axis represents blood glucose concentrations in mM. Error bars are applied to each point (± 2SE).

Total AUC of blood glucose levels from both experimental conditions was calculated for each subject. Calculation of total AUC was chosen since this is the most optimal method when comparing the effect of two different treatments on blood glucose response (Wolever, 2004). Figure 21 illustrates the difference in total AUC between the control condition and the active condition for each subject. The results revealed that α-CD reduced total AUC in eight out of ten subjects as expected. On the contrary, subject G had equal AUCs at both conditions, and subject A had higher AUC on the active day than on the control day.

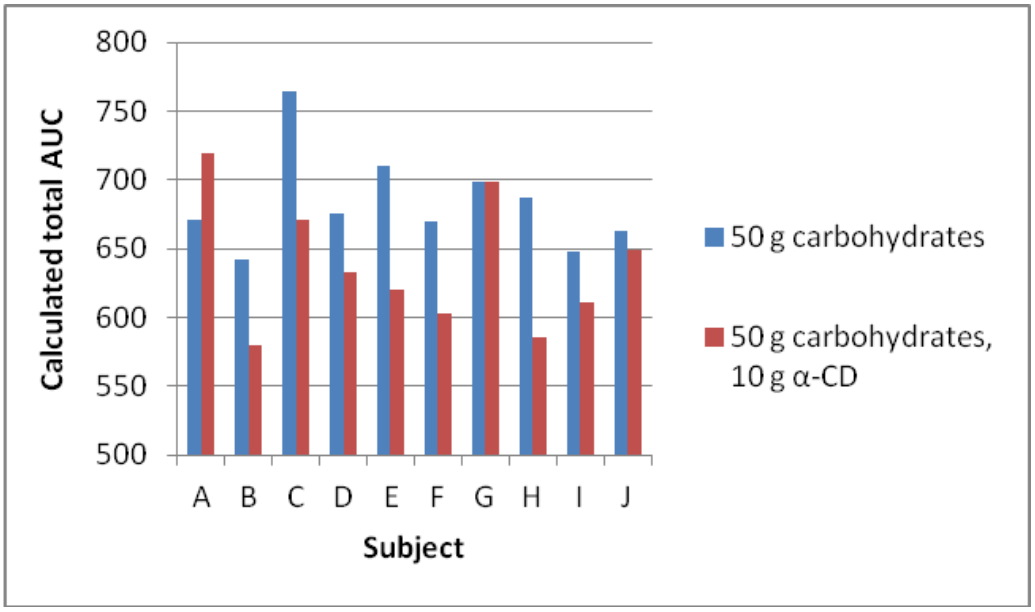

Figure 21 shows the calculated area under the curve (AUC) on the y-axis for each subject (x-axis).

Statistical comparison of total AUC was performed using the paired sample t-test. It revealed that AUCs from the active day were significantly reduced when compared to AUCs obtained at the control day with a p-value of 0.013. The ability of α-CD to reduce glycemic response has been reported previously (Buckley *et al.*, 2006; WHO, 2006). In the study mentioned in the WHO report, 10 g of α-CD was able to reduce the glycemic index by 57%. The subjects consumed 50 g of digestible carbohydrate from white bread and α-CD was served dissolved in water as in the present study (WHO, 2006). A reduction in the range of 57% was not reached here despite the same experimental conditions, but calculations of glycemic index and calculations of total AUC are not directly comparable. Furthermore, the study is unpublished. No information regarding the reason for this is available, but it is possible that the quality of the study was considered to be inadequate for publication.

Still, a greater reduction was expected in the present study based on the previous result. Similarly, the study performed by Buckley and colleagues reported a reduction of approximately 2, 20 and 50% with 2, 5, and 10 g doses of α-CD, respectively, in iAUCs. However, the meal and preparation hereof was different from the present study. The source of digestible starch came from rice instead of wheat flour. Moreover, the rice was soaked overnight in water containing dissolved

α-CD and then the rice was cooked. It is possible that the timing of when α-CD is added to a meal is important for efficient reduction of blood glucose response.

The method for measuring glucose levels might also account for the difference obtained in the magnitude of reduction. Buckley and colleagues took blood samples via a catheter inserted in a forearm vein and in the present study a glucometer was used at the fingertips. The difference in location of measurement and the difference in sampling method might explain the difference in the results. Furthermore, a coincidental difference in baseline blood glucose levels was observed; in general the baseline level was higher at the active day than at the control day. If the difference had not been present, it is likely that the tendency of α-CD to reduce the blood glucose response had appeared clearer.

4.2.3. Side Effects of α-cyclodextrin in the *In Vivo* Study

The participants were asked about possible gastrointestinal discomfort or other side effects 48 after each experimental day. Two subjects experienced stomach cramps and nausea starting two hours after ingestion of the meal with α-CD and stopping after 5-6 hours. Two others reported stomach ache starting two hours after ingestion of the meal with α-CD and lasting for the remainder of the day, while one participant reported a stomach ache of short duration. All five participants categorized the discomfort as mild. The other five participants experienced no side effects.

10 g of α-CD was the selected dose used in the *in vivo* study. This dose was chosen based on previous research reporting that no side effects occurred with 10 g and that the blood glucose lowering effect was evident (Buckley *et al.*, 2006; WHO, 2006). Surprisingly, half of the volunteers in the present study experienced intestinal discomfort even though the same dose of α-CD was used. Gastrointestinal side effects have been reported earlier in a tolerability study using a much higher dose. In that study, 25 g of α-CD was dissolved in water and served as a beverage after an overnight fast. 4 out of 12 subjects experienced some side effects (WHO, 2006). It seems that the high prevalence of intestinal discomfort in the present study might be due to chance, as none of the volunteers reported side effects in the other two studies using 10 g of α-CD (Buckley *et al.*, 2006; WHO, 2006) and as only one third of the subjects from the tolerability study experienced side effects even though the dose was much higher.

The existence of impurities in the used α-CD is also a theoretical possibility. α-CD was of food-grade quality, hence the intended use is ingestion. A technical data sheet can be found in the enclosed data disc. A new container of α-CD was acquired just prior to the experiment and used only for this *in vivo* study. Combined with the fact that the meal preparations were handled cautiously and hygienically, it is considered highly unlikely that the observed side effects is caused by anything but the fiber rich meal since carbohydrates with low digestibility is known to cause intestinal discomfort (WHO, 2006).

Further tolerability studies of ingestion of α-CD in humans are called for to limit the risk of side effects. In such studies, the dose of α-CD should be varied and also the way of ingestion. That is, α-CD could be dissolved in water and served as a beverage and α-CD could be incorporated into

a meal e.g. by baking bread containing α-CD. The latter should be performed due to the fact that especially liquid forms of fibers are known to cause intestinal discomfort (WHO, 2006). Another variable could be that the participants should ingest α-CD on an empty versus a full stomach as an empty stomach seems to worsen the intestinal side effects. Results from such studies would give valuable information regarding the optimal dose and the best ingestion form of α-CD, which would aid in how to reduce the risk of side effects.

4.2.4. α-cyclodextrin Interferes with Starch Metabolism

α-CD can as mentioned be categorized as a soluble fiber. Soluble fibers are defined as indigestible components that are fermented in the colon by bacterial action, whereas insoluble fibers passes through the digestive system metabolic inert (Guyton and Hall, 2006). With reference to how soluble fibers in general exhibit their effect on digestion, α-CD's effect is considered to be multifactorial. Firstly, α-CD as a fiber would increase food volume without increasing caloric content. That is, the fermentation of α-CD leads to production of fatty acids which are absorbed, but the calorie amount is approximately 70% less than if α-CD was degraded to glucose molecules. The increase in food volume would lead to a feeling of satiety. Moreover, soluble fibers are known to attract water forming a viscous gel, which slows the emptying of the stomach and intestinal transit. This gel formation shields carbohydrates from the enzymatic hydrolysis and delays absorption of glucose. The overall result is a more slowly increasing blood glucose level (Guyton and Hall, 2006).

α-CD may also attract water and form this gel, but α-CDs are a small molecules, whereas dietary fibers are non-starch polysaccharides. The reduction by α-CD on blood glucose levels is instead thought to be the mentioned direct inhibitory effect on α-amylase. Thereby, α-CD reduces enzymatic starch degradation and instead the starch passes on to the colon, where it is most likely fermented like α-CD. Plausibly, less energy is available when starch is fermented into fatty acid compared to if it were degraded into glucose molecules. The result would be a decreased absorption of calories. Furthermore, a better regulation of blood glucose levels would be obtained. Normally, starch caused a fast increase in glycemic response. The fast increase can result in a subsequent dive in glucose levels below the baseline level. Such a dive stimulates the appetite leading to food intake, which again results in an increase in blood glucose. These blood glucose fluctuations often lead to overeating. Accordingly, a stable blood glucose level regulates feelings of hunger and satiety better and has been proven to have great importance when weight loss is desired (Ludwig, 2003).

In summary, the interference of carbohydrate metabolism by α-CD plausibly results in a decrease in the calories absorbed from starch and a regulation of blood glucose levels. One or both of these mechanisms could account for α-CD's weight loss ability.

As mentioned previously, relatively high concentrations of α-CD were necessary in the *in vitro* experiments to achieve decreased degradation rates. Regarding the potential of α-CD as a weight loss agent, it seems worrying if large amounts of α-CD can decrease the enzymatic

degradation of only small amounts of starch. In the present study, the amount of starch was 100 mg whereas the amount of α-CD was in the range of 0.6-6 g in the *in vitro* experiments. In this connection, it is important to remember that *in vitro* results are not directly translational to *in vivo* conditions. This is evident when looking at the amounts used in the *in vivo* study; 10 g of α-CD was able to reduce the total AUC after ingestion of 50 g of digestible carbohydrates. It could be interesting to investigate if changing the experimental conditions in the degradation studies would interfere with the concentration of α-CD needed to achieve an inhibitory effect. Such conditions could be e.g. substrate concentration, enzyme and buffer amounts, pH value and temperature. Koukiekolo and colleagues found that the addition of α-CD in a range of 1-14 mM in their *in vitro* study was sufficient to obtain the inhibitory effect they aimed for (Koukiekolo *et al.*, 2001). The experimental conditions in the study by Koukiekolo varied from the present study with regards to the abovementioned factors. This indicates that such factors do affect how great α-CD's inhibitory effect is in vitro. Accordingly, even though the concentration of α-CD had to be high in the degradation studies, this does not imply that patients have to ingest unreasonable amounts of α-CD to obtain an effect on carbohydrate metabolism.

4.3. Is the Effect of One of the Hypothesis Superior to the Other?

Results from the present study and from previous research indicate that both hypothesized mechanisms can account for α-CD's weight loss ability. Furthermore, α-CD per se may account for the weight management seen if the volume of α-CD substitutes the volume of something more calorie-rich. In this connection, Artiss and colleagues stated in their article about the rat feeding study that:

"Based upon the amount of weight gained and the amount of fat consumed by the HF-FBCx– and LF-fed rats, we are able to calculate that 1 g of FBCx prevented the absorption of the equivalent of 9 g of dietary fat" (Artiss et al., 2006).

FBCx is short for Fat Binding Complexer and is the sales name invented by the Artiss group (BioForm, 2011). The prevention of absorption of 9 g of fat per 1 g of α-CD seems to be a substantial amount. Furthermore, such direct calculations are not possible as other *in vivo* factors might have come into play for example an interference of carbohydrate metabolism or the soluble fiber effect. Thus, the statement that 1 g of α-CD prevents the absorption of 9 g of fat seems questionable.

Additionally, the HF diet is compensated for the lack of 40 g of α-CD by increasing the amount of starch correspondingly from 15.530 g to 55.530 g (Artiss *et al.*, 2006). A table provided in the article showing the diet compositions is given in table 3. Such compensation seems unnecessary or overestimated since α-CD is not degraded to glucose but fermented resulting in less available energy/calories. Hence, the calculations of energy available in the diets containing α-CD might be inaccurate. Following, the calculations of how much energy the rats consumed might also be inaccurate. The authors state that the rats ate similar amounts of food by weight and as the two HF diets were more energy-rich the rats in two HF groups consumed more energy de facto. The rats on the HF diet increased their weight faster than rats on the HF/α-CD

diet leading the authors to include that α-CD prevented a weight gain (Artiss *et al.*, 2006). This may not be the case. As stated the HF diet with α-CD might have contained less energy than estimated explaining why the rats gained weight at a slower rate.

A similar scenario might be true in the other diet study using rats (Suzuki and Sato, 1985). In this study, the rats gain weight at slower rates with increasing amounts of CD. It is possible that α-CD's interference of lipid and carbohydrate metabolism accounted for the slower increase in weight gain. However, the amount of starch was replaced with a correspondingly amount of CD (w/w) (Suzuki and Sato, 1985). Therefore, the rats might have gained weight slower because the energy/calories available in the diets were less. The result could also be caused by a combination of both mechanisms.

Table 3 is the diet composition of the four diets used in the rat feeding study (Artiss *et al.*, 2006). Components are given in g/kg.

	LF	LF-FBCx	HF	HF-
Casein	140	140	210	21
Cornstarch	410.692	406.692	55.530	1
Dextrinized cornstarch	135	135	10	1
Maltose dextrin	175	175	175	17
Cellulose	50	50	75	7
FBCx	–	4	–	4
Mineral mix	35	35	52.5	5
Vitamin mix	10	10	15	1
Vitamin E acetate (500 IU/g)	0.00	0.00	0.39	
Soybean oil	40	40	400	40
TBHQ	0.008	0.008	0.08	
L-Cystine	1.8	1.8	2.7	
Choline bitartrate	2.5	2.5	3.8	
Energy (kJ/g)	16.57	15.31	23.85	2
% Energy from fat	9.5	9.5	66.5	6

The HF diets where counterbalanced for energy amount by decreasing the starch content resulting in a HF/low carbohydrate diet in the Artiss study. The opposite was true for the LF diets resulting in a LF/high carbohydrate diet. As the amount of α-CD was added as percentage in proportion to fat content, 40 g/kg was added to the HF/low carbohydrate diet, whereas only 4 g/kg was added to the LF/high carbohydrate diet (Artiss *et al.*, 2006). It is proposed that rats on the LF/high carbohydrate diet could have had a prevention of weight gain too, if equal amounts of α-CD had been present in all diets. That is, α-CD might have caused a decrease in the calorie amount absorbed from starch and might have caused a better regulated blood glucose level if a sufficient amount of α-CD had been added.

Regarding the magnitude of the effect on lipid metabolism versus the effect on carbohydrate metabolism, it is possible that the magnitude of one effect is stronger than the other. For example,

all the α-CD ingested could theoretically bind with triglycerides before the ingested food reached the intestines. Then no α-CD molecules would be free to bind with pancreatic α-amylase. Accordingly, only the decreased lipid degradation would account for α-CD's weight loss ability de facto. On the contrary, α-CD could have a higher binding affinity for α-amylase than for triglycerides resulting in the opposite scenario. Lastly, if α-amylase and triglyceride were to compete for α-CD with similar affinities, the magnitude of both mechanisms could be reduced. To achieve an indication of which scenario that might be true, further degradation studies could be performed. That is, the degradation of oil and the degradation of starch could be carried out as in this study, but in the same beaker. Comparison of the amounts of maltose equivalents and the amounts of oleic acid equivalents converted should then be made with controls. The controls should consist of the two separate degradation experiments performed under exactly the same conditions. If the amount of degradation product from only one of the degradation studies is higher in comparison to control, this would indicate that the inhibitory effect of α-CD inclines toward the other degradation. Although such results are not directly translational to *in vivo* conditions, they would provide information regarding the possibility that one mechanism is superior to the other.

4.4. The Potential for α-cyclodextrin as a Novel Drug against Overweight and Obesity

The lack of an effective treatment option with tolerable side effects for overweight and obesity has led to the suggestion that α-CD could be used as such. Based on the results from the present study and the results from previous research it has been proven that α-CD in fact affects both lipid and carbohydrate metabolisms in a way that could help in weight management. Moreover, α-CD per se could aid in weight management by the properties of a soluble fiber. How great the effect of weight management is and thereby the potential for α-CD as a novel drug against overweight is open to discussion.

Only one randomized, placebo-controlled crossover study in humans has shown that α-CD can induce weight loss (Comerford *et al.*, 2010). The weight loss was 0.4 kg (± 0.2 (SE)) during one month. The other study investigating a possible weight loss effect in humans is the three months study previously mentioned (Grunberger *et al.*, 2007). In this study, the subjects were asked not to change their eating or exercise habits. Despite this, the group administered α-CD tablets consumed significantly more energy by the end of the study compared to their energy intake prior to the study. This led the authors to state that if the subject consuming α-CD had not increased their energy intake, they could have lost weight. Calculations of energy intake were not described (Grunberger *et al.*, 2007). Therefore, it is not known if α-CD was included in these calculations leading to an inaccurate estimate of energy intake. Hence, the likelihood of the statement that subjects could have lost weight is difficult to assess.

Furthermore, if an increase in energy intake did occur it raised an important issue. Such an increase in energy intake could be explained by the fact that α-CD interferes with lipid and carbohydrate metabolisms in a way that reduces and/or delays the absorption of nutrients. The

control of food intake is a complex neural process regulated by sensations of hunger, appetite and satiety. These feelings are influenced by different physiological signals such as sensory information about stomach filling, chemical signals from nutrients in the blood, signals from gastrointestinal hormones et cetera. These signals stimulate specific feeding centers of the brain (Guyton and Hall, 2006). Although α-CD as a fiber could lead to a feeling of fullness which signals satiety, the decrease in absorption of lipid and/or carbohydrate could result in a prevention of the signal from nutrients in the blood. This signal inhibits the hunger or appetite center of the brain. Hence, increasing the energy intake correspondingly when α-CD is ingested could then be necessary for the subjects to obtain satiety. Thus, it seems necessary to establish if energy intake is increased as a response to a decreased satiety signal caused by fewer nutrients in the blood. If this scenario is true, then the usability of α-CD in the fight against overweight and obesity is questionable since subjects when would not obtain an actual weight loss.

The study by Comerford and colleagues contradicts this as the subjects were able to maintain an equal energy intake regardless if the prescribed tablets contained α-CD or placebo (Comerford *et al.*, 2010). In this connection, it is worth noticing that the subject inclusion criteria varied. In the Grunberger study, obese diabetics were recruited while Comerford and colleagues recruited overweight non-diabetics. In future weigh loss studies, it could be necessary to divide participants in subgroups distinguishing between overweight and obesity as well as if the participants have diabetes. This must be done to ensure that the possible benefit of α-CD is not overlooked.

5. Conclusion

This master thesis investigated two hypotheses regarding the mechanism behind α-CD's weight gain preventing or weight loss inducing properties. The lipid hypothesis states that α-CD interferes with dietary lipid in a way that reduces lipid degradation. Such a scenario would reduce the absorption of lipid from the diet, accounting for α-CD's weight management ability. The possibility that α-CD decreases the degradation of lipid was confirmed for the first time by an *in vitro* lipolysis study. It is proposed that the decreased degradation was a result of complex aggregate formation between α-CD and triglycerides and that this aggregate formation limited lipase's access to the triglycerides. Besides this scenario, other *in vivo* scenarios could also account for a weight loss effect of α-CD.

The other hypothesis states that α-CD affects carbohydrate metabolism. Results from the degradation with potato starch as substrate revealed that α-CD did inhibit the hydrolysis reaction. The reduction is proposed to occur as a result of direct inhibition of α-amylase, where α-CD occupied the active site of the enzyme. Furthermore, when smaller oligosaccharides were converted, the inhibition could be of the noncompetitive type with reference to the research results previously described. The decreased enzymatic starch degradation probably explains why a reduction in blood glucose response is obtained when α-CD is ingested together with a starch rich meal. The result is proposed to be a decrease in the calories absorbed from starch and a regulation of blood glucose levels, which would account for α-CD's weight loss ability.

In conclusion, it seems plausible that both hypotheses account for the weight management property of α-CD. Studies determining the magnitude of the possible weight loss induced by α-CD and investigating the issue of increased energy intake are called for. Furthermore, if the optimal dosing, way of ingestion, and timing of ingestion in correlation with meals of α-CD is established, it is likely that the prevalence of intestinal discomfort is negligible. Therefore, under the prerequisite that future studies can establish that weight loss do occur, α-CD is considered to have great potential as a safe and comfortable treatment option against overweight and obesity.

6. References

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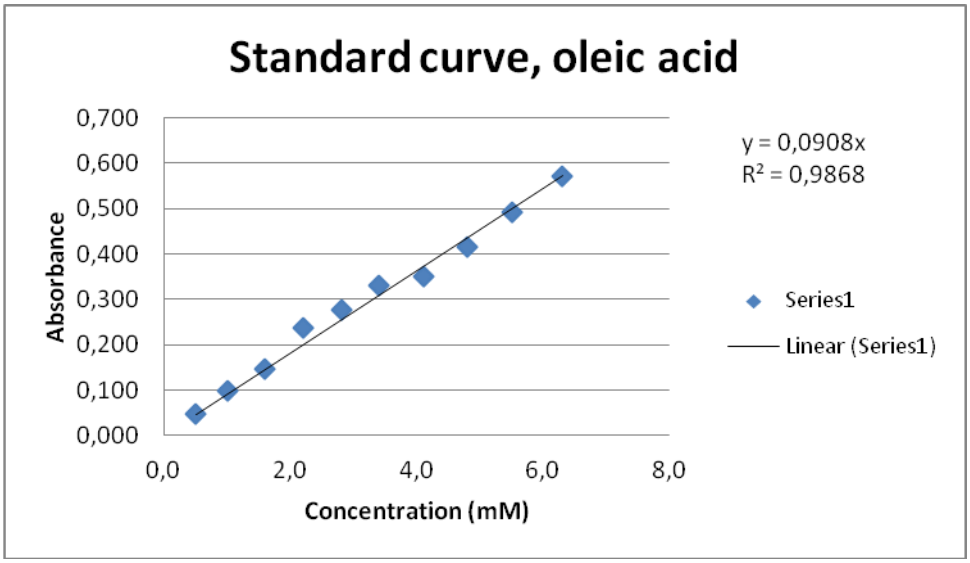
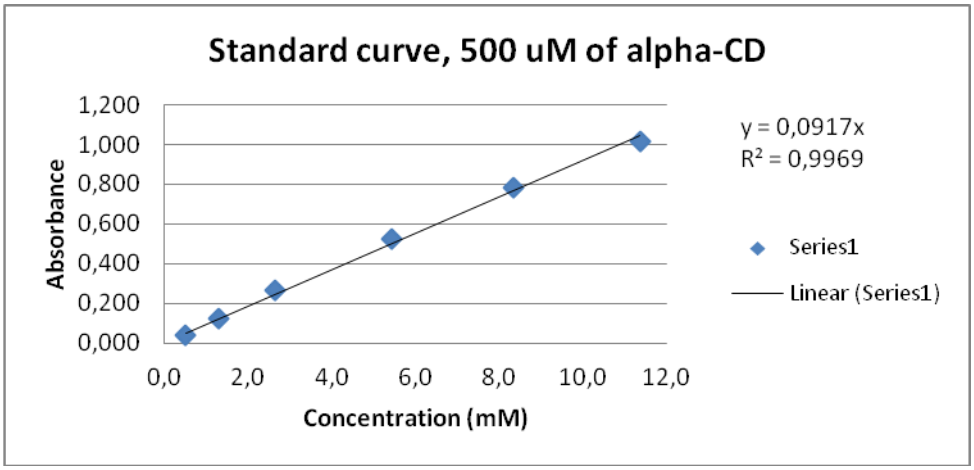
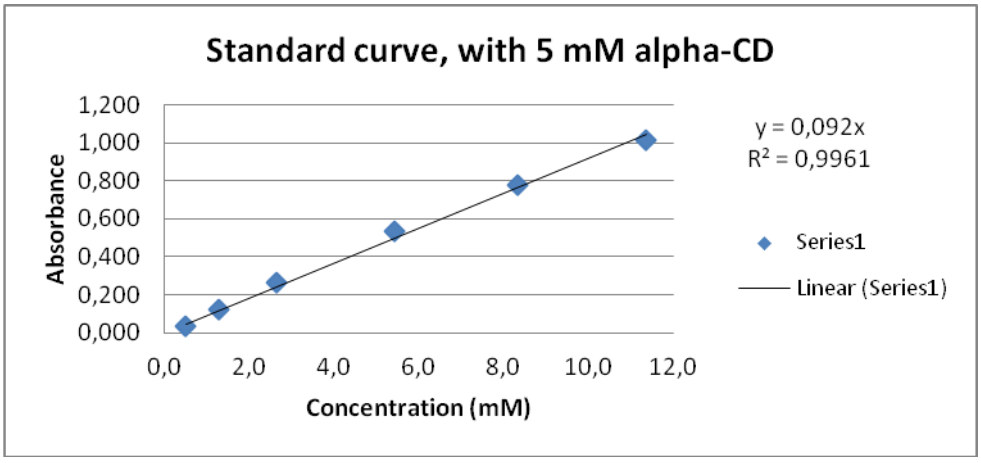
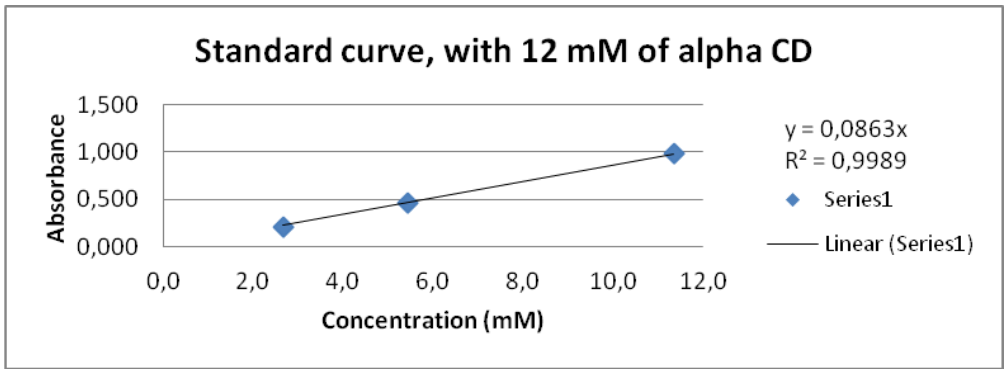
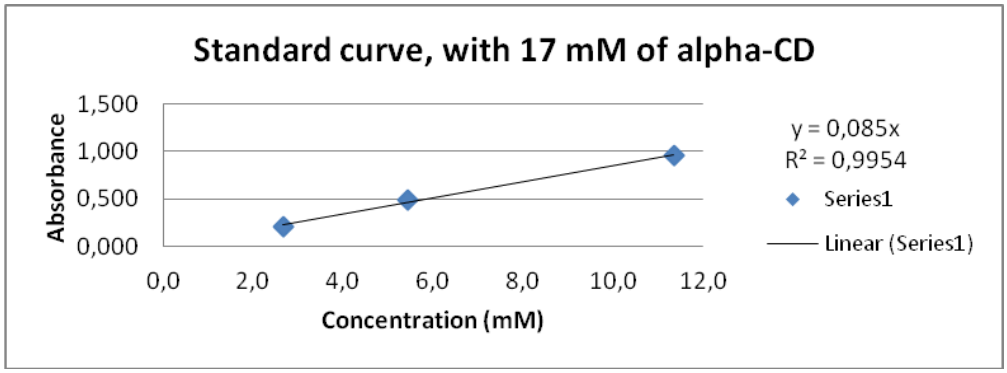
Appendix I

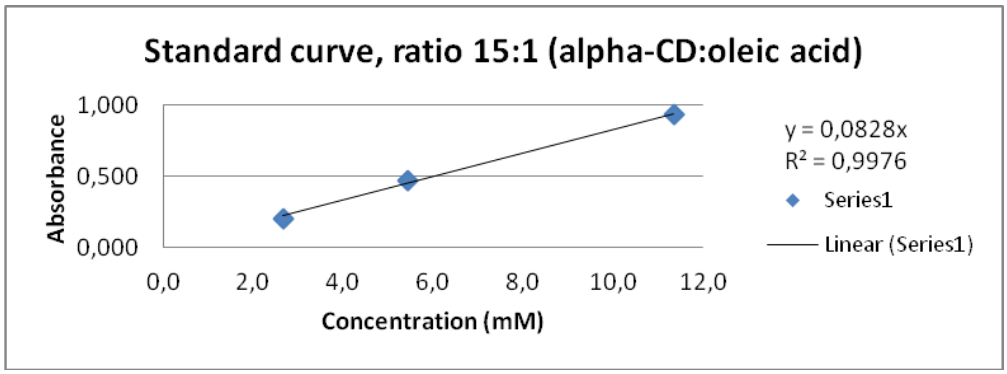

Standard curve, oleic acid
Absorbance
0,700
0,600
0,500
0,400
0,300
0,200
0,100
0,000
0,0
2,0
4,0
6,0
8,0
Concentration (mM)
y = 0,0908x
R² = 0,9868
Series1
Linear (Series1)

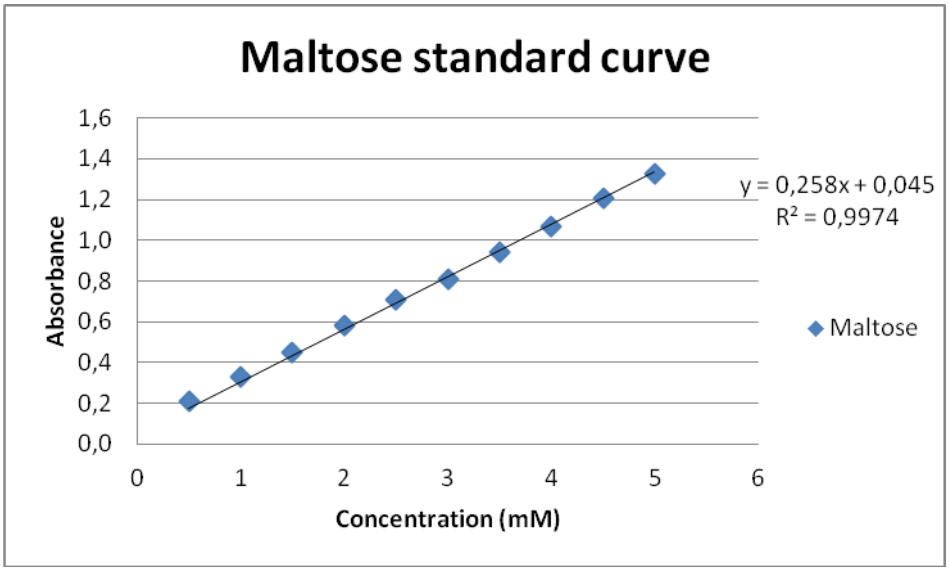

Standard curve, 500 uM of alpha-CD
Absorbance
1,200
1,000
0,800
0,600
0,400
0,200
0,000
0,0
2,0
4,0
6,0
8,0
10,0
12,0
Concentration (mM)
y = 0,0917x
R² = 0,9969
Series1
Linear (Series1)


Standard curve, with 5 mM alpha-CD
Absorbance
1,200
1,000
0,800
0,600
0,400
0,200
0,000
0,0
2,0
4,0
6,0
8,0
10,0
12,0
Concentration (mM)
y = 0,092x
R² = 0,9961
Series1
Linear (Series1)


Standard curve, with 12 mM of alpha CD
Absorbance
1,500
1,000
0,500
0,000
0,0
2,0
4,0
6,0
8,0
10,0
12,0
Concentration (mM)
y = 0,0863x
R² = 0,9989
Series1
Linear (Series1)


Standard curve, with 17 mM of alpha-CD
Absorbance
1,500
1,000
0,500
0,000
0,0
2,0
4,0
6,0
8,0
10,0
12,0
Concentration (mM)
y = 0,085x
R² = 0,9954
Series1
Linear (Series1)


Standard curve, ratio 15:1 (alpha-CD:oleic acid)
Absorbance
1,000
0,500
0,000
0,0
2,0
4,0
6,0
8,0
10,0
12,0
Concentration (mM)
y = 0,0828x
R² = 0,9976
Series1
Linear (Series1)


Maltose standard curve
Absorbance
1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
0
1
2
3
4
5
6
Concentration (mM)
y = 0,258x + 0,045
R² = 0,9974
Maltose

Appendix II

Reagents

All compounds are of analytical grade unless otherwise stated.

From Sigma-Aldrich, MO, USA:
3,5-dinitrosalicylic acid 98%, Lot: 08728MG
Heptane, Lot: SZBB139BV
Maleic acid, Batch: 124K5424
Maltose, Lot: 052K0174
Porcine pancreatic lipase, type II, Lot: 090M1393V
Porcine pancreatic α-amylase, type VI-B, Batch: 098K0730
Sodium azide, Lot: 098K0052
Tris[hydroxymethyl]aminomethane (TRIS base), Lot: 74H5706

From Merck, Darmstadt, Germany:
Calcium chloride dehydrate, Lot: TA853682 605
Copper(II) acetate, Lot: 6331528
Sodium sulfite, Batch: K28344357 102
Soluble starch, Batch: F1170752 234

From Fakta, Denmark:
Denice mineral water, Aqua d'or mineral A/S, Brande, Denmark
Limejuice from lime fruits
Sunflower oil, Nordic Food Partners A/S, Thailand
White bread, Lantmännen Schulstad A/S, Hvidovre, Denmark

Others:
Deep-fried French fries, Food grade, Bella Italia, Aalborg
Pyridine from Fisher Chemical, MA, USA, Lot: 1148951
Rochelle salt from VWR prolabo, PA, USA, Batch: 11A110011
Sodium chloride from VWR prolabo, PA, USA, Batch: 10I210009
Sodium hydroxide from J.T Baker, Holland, Lot: 1022801011
α-CD from Wacker Chemie, München, Germany, Standard grade, Lot: 60014946
α-CD from Wacker Chemie, München, Germany, Food grade, Lot: 70810010

Appendix III