Soluble and insoluble fractions from nopal (*Opuntia ficus*) cladodes improve postprandial glycaemic regulation: a crossover randomized study in healthy volunteers

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Abstract

Background and objectives: Type 2 diabetes has been increasing all over the world. A healthy diet, including particular functional foods is an effective strategy to prevent this form of diabetes. Nopal (*Oputia ficus*) is a cactus plant that, according to traditional medicine, has antidiabetic properties. The objective of this research was to examine the effects of different fractions of nopal cladodes on postprandial glycaemic regulation in healthy volunteers.

Methods: Nopal cladodes flour was separated by centrifugation into two fractions (a water insoluble and a soluble fraction). The fractions were incorporated into two different test breads, i.e. insoluble fraction bread (INB) and soluble fraction bread (SNB), and evaluated with respect to acute glycaemic properties in 17 healthy subjects with normal body mass index, applying a randomized cross-over study design. The effects on glycaemic and insulinaemic responses were compared with the responses after a control product, which composed of a similar bread lacking the nopal fractions (white wheat bread, WWB). The products were characterized with respect to starch and dietary fibre content. In addition, starch hydrolysis index (HI) was determined with an *in-vitro* method in order to predict glycaemic index (pGI), and elucidate possibly underlying mechanisms on postprandial glycaemia.

Results: Results regarding glycaemic regulation show that insoluble nopal fractions had a beneficial effect on postprandial glucose regulation. Consequently, the incremental area under the postprandial blood glucose curve (iAUC) after INB was significantly reduced compared to after intake of the WWB during the time periods 0 – 45 and 0 – 60 minutes ($p < 0.05$). In addition, the iPeak value, i.e. the individual highest incremental peak concentrations, was significantly lowered after INB compared to WWB ($P < 0.05$). Regarding the postprandial insulin secretion, both SNB and INB lowered the postprandial iAUC compared to the WWB during the time period 0 – 45 and 0 – 60 minutes ($P < 0.05$). On the other hand, only the INB significantly reduced the insulin iPeak value compared to WWB ($P < 0.05$). In terms of HI, INB had lower HI and pGI compared to WWB and SNB. Also, INB exhibited highest total, soluble and insoluble dietary fibre contents.

Conclusion: The INB promoted lower postprandial glucose and insulin response, while SNB showed beneficial effects only on postprandial insulin secretion. The beneficial effects of nopal on glycaemic regulation might be to some extent explained by the dietary fibre content, especially the INB. However, with respect to the improved postprandial insulin economy observed after the SNB, additional mechanisms are probably involved.

Keywords: Nopal cladodes, Glycaemic regulation, Randomized cross-over study, Dietary fibre, Hydrolysis index

List of Abbreviations

ADA	-	American diabetes association
ANOVA	-	Analysis of variance
ºC	-	Celsius
DF	-	Dietary fibre
DNS	-	3,5-Dinitrosalicylic acid
DS1	-	Dry substance 1
DS2	-	Dry substance 2
df	-	degree of freedom
FAO	-	Food agriculture organization
g	-	Gram
GI	-	Glycaemic index
GIP	-	Gastric inhibitory polypeptide
GLP1	-	Glucagon-like peptide 1
HI	-	Hydrolysis index
iAUC	-	Incremental area under the curve
INB	-	Insoluble nopal bread
iPeak	-	Incremental peaks
IR	-	Insulin resistance
L	-	Liter
m^2	-	Square meter
Mets	-	Metabolic syndromes
MS	-	Mean sum of square
mg	-	Milligram
nmol	-	Nano mole
ml	-	Milliliter
µl	-	Microliter
pGI	-	Predicted glycaemic index
pH	-	Potential of hydrogen
PYY	-	Peptide tyrosin tyrosin

SNB	-	Soluble nopal bread
T1D	-	Type 1 diabetic
T2D	-	Type 2 diabetic
WHO	-	World health organisation
WWB	-	White wheat bread

Chapter 1

1.1 Introduction

There has been a continuous increase in the prevalence of type 2 diabetes (T2D) in both developing and developed countries. It is a severe disease because it is associated with many of the serious diseases that we face today such as heart disease, cancer, Alzheimer's disease, etc. The diet is considered as one of the most important factors that have impact on the development of T2D. In order to prevent and control the increase of T2D, functional foods will play an important role to reduce the risks.

Nopal (*Opuntia ficus*) is a cactus plant originally from Mexico. Nopal is rich in healthy components, i.e dietary fibre, vitamins, and minerals. In a previous study about the postprandial effect of nopal on glycemia, it was shown that nopal cladodes decreased the glucose level peak after food intake. In the previous study it was observed that supplementation of white wheat flour based breads with 25% nopal flour (dry weight) improved glycaemic properties of the bread, compared to a 100% white wheat flour bread, containing similar amounts of available starch. Therefore, the aim of presently described study was to further investigate underlying mechanisms behind the beneficial health effects. The purpose was to examine the effect of soluble and insoluble fractions in nopal on postprandial blood glucose and insulin levels in healthy volunteers, using a randomized cross-over study design. The test products were consumed at breakfast, with 7 days washout period in-between each test product. It was hypothesized that soluble and insoluble nopal fractions would differentially reduce postprandial blood glucose and insulin levels, thereby contributing to knowledge about a healthy food ingredient with the potential to prevent the metabolic syndrome (MetS) and related diseases.

1.2 Background

1.2.1 Metabolic Syndrome (MetS) & Diabetes Mellitus

The MetS describes a cluster of metabolic abnormalities, e.g. obesity, hypertension, disturbed cholesterol concentrations and glucose intolerance, which drastically increase the risk of T2D and cardiovascular diseases[1]. The definition of MetS has varied over time. However, MetS is associated with abnormalities such as insulin resistance, high blood pressure, obesity, and dyslipidaemia[2]. However, the most recent definition, the risk factors in MetS include increased blood pressure, high level of triglyceride, decreased HDL (high-density lipoprotein), increased triglyceride, fasting glucose level and central obesity; the person with three of these factors can be diagnosed as having MetS[3]. In 2016, World Health Organization (WHO) reported on the global challenge with obesity, stating that more than 1.9 billion adults were overweight, and one – third of these were obese [4], defined as having a body mass index (BMI) above 30 Kg/m^2 [4]. One of the severe illness related to obesity is T2D, as shown by a number of studies[5].

Diabetes mellitus, a non-communicable disease, is a chronic disorder able to interrupt the glucose metabolism. There are two categories of diabetes mellitus, which are type 1 (T1D) and T2D[6]. T1D is caused by the lack of insulin secretion from the islet cells of pancreas, suggested to be caused by environmental or infectious agents. In addition, the main factor in pathophysiology of T1D is considered as autoimmunity[5]. On the other hand, compared to the pathophysiology in T1D, T2D have different underlying factors that cause the illness, such as obesity, inactive lifestyle, poor food consumption/choices as well as urbanization.

T2D results in high blood glucose and insulin concentrations due to peripheral insulin resistance (IR), which eventually may lead to an impairment in insulin production from pancreas[8]. The effects of IR can lead to raise fatty acids in the plasma, subsequently causing lower transportation of glucose into muscle (**Fig. 1**).

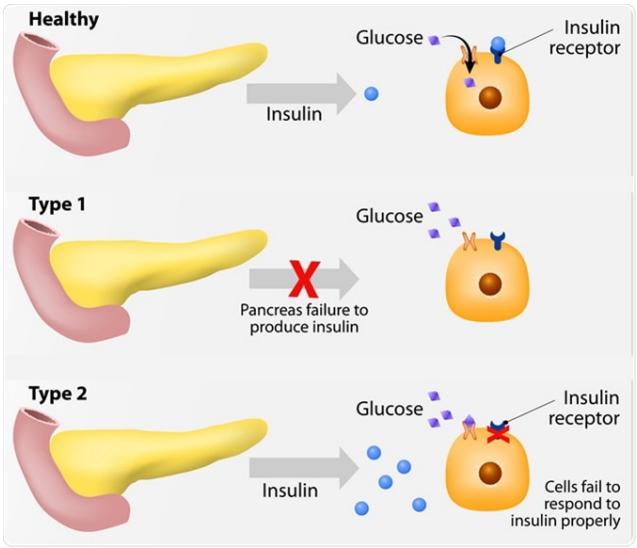


Figure 1: The difference mechanism of diabetes mellitus type 1 and type 2[9]

Moreover, IR also results in elevated fat breakdown and IR in the liver, causing increased blood glucose concentration. However, pancreatic dysfunction must be presented simultaneously with IR in order to develop T2D. In another term, the person who is suffering from T2D possess a background of lack insufficient insulin secretion to compensate for the level of IR, which occur in those who are mostly overweight or obese. T2D patients have initially high insulin concentrations but fail to meet the normality of glycemia level[10]. The pancreas produces insulin, but insulin cannot work effectively to drive glucose into cells because of IR, often as a result of obesity[11]. If hyperglycaemia repeatedly occurs, it can cause inflammation leading to the introduction of disorders and diseases in many organs and the vascular system.

To prevent IR, an adequate dietary pattern is a key factor. High consumption of red meat, saturated fats, refined grain, and low intake of dietary fibre, fruits, and vegetables can increase the risk of IR[12]. Functional foods that can provide good source of nutrients and that are rich in dietary fibre, may help in prevention of T2D. Such foods need further investigations for the purpose to being offered as alternative food choices in the market.

1.2.2 Glycaemic Index (GI) and Hydrolysis Index (HI)

Jenkins and co-workers introduced the Glycaemic index (GI) concept in 1981. GI is referred to as the postprandial Incremental Area Under the Curve (iAUC) of blood glucose concentration after a test meal including 50 g available carbohydrate as a percentage of the response after a reference food including the same amount of available carbohydrates (glucose solution or white wheat bread as a GI 100) taken by the same test person[13]. Therefore, GI differentiates foods rich in carbohydrates by considering how much the blood glucose concentration will rise after intake (**Fig. 2**).

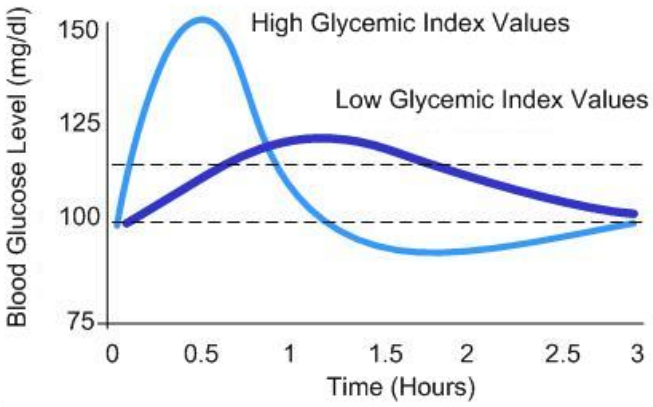


Figure 2: The blood glucose response after a high glycaemic index food and low glycaemic index food, respectively[14]

A high-GI diet is distinguished by high intake of carbohydrate rich foods that are easy to digest and absorb into our body. Consequently, the blood glucose and insulin concentrations will rapidly increase[15]. The high rise in blood glucose concentrations, especially if frequently occurred, can cause inflammation as well as IR and lead to the risk of developing T2D and cardiovascular diseases.

The Food and Agriculture Organization (FAO) of the United Nations and the WHO committees agree to use the GI method for classifying carbohydrate rich foods and use as a guidance for food choices. Moreover, the committees supported the consumption of high-carbohydrate diet (55% of energy) with high proportion of carbohydrate containing foods with a low GI[16]. Studies have shown that low GI foods cause lower insulin responses compared to high GI foods[17].

The method to classify GI is normally assessed through an *in vivo* procedure. However, there are other procedures that can be applied *in vitro*. *In vitro* procedures can predict the GI by mimicking the physiological digestion of carbohydrate containing foods in the human body[18]. One of such *in vitro* procedures is the determination of the Hydrolysis Index (HI). This method will be described in chapter 2 part 6.

1.2.3 Nopal

Nopal (*Opuntia ficus indica*) is a domestic species of the Cactaceae family originally from Mexico. It has been part of the human and animal food for ancient dietary habit restricted to Mexico. Both the nopal fruit and the nopal cladodes (pad; **Fig 3**), as a vegetable in different stages of maturation, are consumed. The benefits from consuming nopal are well known in traditional medicine to treat metabolic diseases such as T2D[19].

Figure 3 Nopal Cladodes[20]

Some studies report that nopal affects the metabolic parameters due to its dietary fibre (DF) content and other bioactive components[21]. Thus, chemical characterizations of nopal have shown that it contains high concentrations of vitamins (especially vitamin C and folic acid) and minerals (calcium). However, the concentrations differ, depending on the maturation state[22,23], and both environmental conditions and maturity stage of nopal must be taken into account regarding concentrations of phytochemical compounds. In the case of dietary fibre, it has been shown that the soluble DF content decreases by about 27% during maturation (growth from 60 g to 200 g of nopal cladodes weight). The insoluble fibre shows the opposite trend with a 31% increase during maturation[24]. Moreover, an important mineral in nopal is calcium, resulting in a low-cost supplier compared to dairy calcium sources. The calcium content in nopal pads increases during the maturation process. It contains 1.56% (dry basis) of calcium at small pad

size (60 g of weight), while large pad size (200 g of weight) contains 3.72% of calcium on dry weight[24]. However, calcium oxalate also occurs in nopal, being located in the external part of cladodes, and increases in crystal size along maturation (from 60 g to 200 g of weight)[24]. The presence of calcium oxalate could affect the calcium bioavailability.

A study in human T2D patients fed a high carbohydrate diet with nopal seed oil resulted in lower postprandial glycaemia and intestinal glucose absorption after consumption[25], and also increased antioxidant activity has been reported in a mice study[26]. In rodents, nopal cladode dried into flour have shown to improve glycaemia, insulinaemia, pancreatic function as well as to inhibit α-glucosidase[27, 28].

1.2.4 Dietary Fibre

DF encompassing a range of plant constituents, such as indigestible polysaccharides and oligosaccharides, associated with health benefits e.g. regarding cardiometabolic health[29,30]. There are various ways to define DF but, generally, DF is classified by considering its solubility in water (soluble and insoluble), viscosity, and fermentability in the gut[31]. According to CODEX Alimentarius Commission 2009, DF is defined as "carbohydrate polymers with ten or more monomeric units, which are not hydrolyzed by the endogenous enzymes in the small intestine of human"[32]. However, the European Commission and, Cereals and Grains Association (formerly American Association of Cereal Chemists) have agreed to include indigestible carbohydrates with DP of 3 – 9[33,34]. Therefore, it can be said that DF are carbohydrate polymers or oligomers that resist digestion in the small intestine and pass through the large intestine, where they are fermented to different degrees depending on the type of DF[35]. This present study, however, focuses both water soluble and insoluble DF. Soluble DF consists of polysaccharides, gums, and pectins, whereas lignin, hemicellulose and cellulose are part of insoluble DF[36]. It was previously believed that only soluble DF were related to beneficial effects, such as decreased serum lipid and cholesterol, while insoluble DF only contributed to faecal bulking. After many years of research and considerations, the physiological effects of the insoluble DF are as well attributed to its degree of viscosity and colonic fermentation. In addition, it can also influence resistance to absorption of particular nutrients in the intestine[37].

1.2.5 Randomized, Crossover Study

Randomized, crossover studies or crossover trials are an important tool for investigating human responses to different exposures, especially in health science. A crossover study is a research design where subjects receive different types of treatments or products with a washout period in-between treatments[38]. All participants receive the same product at difference in random order, which is randomized study design. This procedure allows us to perform the comparison of results between treatments within a participant under the same condition, and thereby, the results come with lower variations due to differences between the participants.

1.3 Objectives

1. To perform literature review for achieving useful information regarding effects of nopal cladodes on blood glucose regulation, which can be applied in the study
2. To determine whether different fractions obtained from nopal cladodes differ in their effect on postprandial glycaemic regulation in healthy volunteers
3. To investigate the predicted glycaemic index of nopal test products in an *in-vitro* experiment
4. To determine the content of soluble and insoluble dietary fibre in the test products

Chapter 2
Material and Methods

2.1 Subjects

To investigate the possible anti-diabetic effect of soluble and insoluble fractions of nopal, a breakfast meal study was performed. The recruitment of this study was advertised in Kemi centrum, Lund University. Twenty-four healthy volunteers were recruited, but only 17 healthy subjects completed the entire test series (see detail below: chapter 3.1)

2.1.1 Inclusion and exclusion criteria

The inclusion criteria for participating in this study were as follows; BMI should be between 18.5-25 kg/m^2, age should be between 20-40 years, and test person should follow the Nordic Nutrition Recommendations[39]. Exclusion criteria were fasting blood glucose higher than 6.1 mmol/L, known diseases that can affect the metabolism such as, diabetes, lactose intolerance, gluten intolerance. Moreover, Smoking, snuffing, vegetarian diet, intake of antibiotics or intake of probiotics were not consistent to participate in this study. These exclusion criteria may affect the results of the study. All test subjects were asked to read and sign a consent, including protocols and information, before participating in this study. They received a small economic incentive to compensate for possible inconvenience and cover possible expenses connected to the study. Test subjects had their own right to leave the study whenever they desired.

2.1.2 Ethical aspects

All experimental research and human trial took place at Kemicentrum, LTH, Lund University and the study was approved by the Swedish Ethical Review Authority in Uppsala (Dnr: 2019-00980). Ethical approval was obtained before starting the study, which included temporary personal data storage and collection of blood samples. The study was registered at ClinicalTrials.gov (NCT04439630).

2.2 Preparation of the Test Products

2.2.1 Separation of Nopal cladodes Fractions

The study required dried nopal cladodes flour (produced by VERALMEX, Sayula, Mexico) which was separated into water soluble and insoluble fractions. Firstly, the separation method was optimized by mixing nopal flour (1 g.) with water (4 g.) at different temperature (4°C, 10°C, 25°C, and 90°C) and then it was stand at room temperature for 10 mins. After that, it was centrifuged at 4200 rpm for 0, 30, 60 and 90 minutes. It was found that the most effective time and temperature for separating nopal fractions were extraction with water at 10 °C and 60 mins centrifugation, as showed in the **Appendix 1**. The water-soluble fractions was captured in the supernatant, meanwhile the insoluble fractions was defined as the pellet after centrifugation.

2.2.2 Test products

Three different breads were prepared for the meal study; one control bread that did not contain any nopal fraction, and two breads with nopal flour fractions; one with the soluble and the other insoluble fractions of nopal flour.

According to a previous study performed at our research department (Caroline Ritke, diploma work 2019: "Stabilizing Effect of *Opuntia Ficus Indica* on Blood Glucose Regulation in Healthy Humans : a Study to Investigate the Possible Prevention of T2D"[40]) it was shown that a white wheat based bread (WWB) supplemented with 25% (dry matter) nopal flour significantly improved postprandial blood glucose regulation. Consequently, the bread recipes developed in this study were based on the previous study with respect to nopal supplementation. However, in this study the total amount of the nopal flour previously investigated was divided in two fractions depending on their solubility in water before the inclusion into separated bread products. To optimize the bread properties regarding the texture and appearance, the recipes were modified with respect to baking settings and/or water content compared to the previous study.

After separating the soluble and insoluble fraction of nopal flour, the soluble fraction of nopal flour was suspended in water, and mixed with white wheat flour, salt, and dry yeast. The ingredients were kneaded and baked in a bread baking machine. While, due to the viscosity, the insoluble fraction was mixed with all ingredients and kneaded by hand before putting the dough into the bread baking machine for baking. The respected recipes for the three breads are presented in **Table 1**.

Table 1. Recipes for the breads that were used in the meal study

Type of Bread	Flour (g)	Nopal flour (g)	Water (g)	Salt (g)	Dry yeast (g)
White Wheat Bread* (WWB)	530	-	360	4.6	4.6
Soluble Nopal Bread (SNB)	530	Soluble fraction from 134 g	360	4.6	4.6
Insoluble Nopal Bread (INB)	396	Insoluble fraction from 134 g	360	4.6	4.6

*WWB is a reference product.

The bread products were baked in a baking machine (SAGA by Heston Blumenthal BBM800BSS) according to the final recipes in table 1. The program used correspond to the standard recipe for a white bread with the basic program setting with light crush and 1.25 kg. (approximately 3 hrs.). After baking, the bread loaves were cooled down, wrapped in a kitchen towel and left to rest for about 2-3 hrs. Thereafter they were cut in serving portions with the basis of 50 g of available starch. The bread portions were then wrapped with aluminum foil and packed into plastic bags with written date, name, and stored in the freezer. The bread portions were thawed overnight in the package before the day of experimental.

2.3 Available starch analysis

In order to determine the size of the test portions, available starch and the total dry matter was determined in each bread.

2.3.1 Dry substance content

Two dry matter calculations were needed to calculate the portion size of the bread, based on fresh weight intended to consume. Dry substance 1 (DS1), represent the dry matter in the fresh bread. Dry substance 2 (DS2) represented dry matter in a sample that had dried outside its package in ambient conditions. For the determination of DS1 two petri dishes (duplicate for each test products) were weighed and recorded, and then approximately 3 – 5 g of fresh bread was crumbled into each petri dish. The samples were left to dry overnight in an oven at 105 °C. After that the samples were put in an exicator for at least one hour to avoid absorption of moisture, at the room atmosphere. The samples were then weighed to measure the DS1 in fresh bread. The starch analysis was performed at ambient temperature on dry ground bread, for which DS2 was required. For this purpose, fresh bread samples were crumbled down and left overnight in a fume hood with air flowed to dry, and become ambient dry. The next day the ambient dry samples were weighted and further dried in 105 ºC as described above. The results of the both dry substances (DS1 and DS2) are available in **Appendix 3**.

2.3.2 Starch Analysis

The test and reference products were analyzed with respect to potentially available starch according to Holm et al.,1986[41]. The principle of the starch analysis is the digestion of starch molecules into glucose by α-amylase and amyloglucosidase and then measurements of the available glucose with glucose peroxidase. The procedure was done in duplicates for each test product. Air-dried bread samples were grounded down into flour. A sample of 0.5 g of each product were collected in a 50 ml beaker and mixed well with 10 ml phosphate buffer. After it was homogenous, 10 ml of phosphate buffer was added. Termamyl, 40 µl, was added while stirred, and the beakers were placed in a boiling water bath for 20 minutes, placed on a magnetic stir plate. The samples were diluted with Millipore water by using a 50 ml volumetric flask. One ml of sample, 1 ml of Millipore water, 1 ml of 0.3 NaAc-buffer and 50 µl amyloglycosidase were pipetted into a set of glass tubes. The tubes were mixed well and incubated in 60°C in a water bath for 30 minutes. The samples were transferred to 100 ml Erlenmeyer flasks and then diluted with Millipore water to the maximum level (100 ml). The Glucose standards were prepared according to the chemical list in **Appendix 5**. One ml sample and 1 ml water were added to test tubes. Four ml Glox-reagent was added to the tubes with glucose standard and samples, respectively, and the tubes were then mixed and incubated for 60 minutes. After that, the tubes were stirred 20 times and centrifuged for 5 minutes. The standard and samples were measured in spectrophotometer with the absorbance at 450 nm[41]. The absorbance data are presented in **Appendix 3**.

The results of dry substances in each bread and the amount of available starch help us to determine the portion size according to 50 g available starch. In **Table 2**, the available starch in each bread is shown along with portion sizes.

Table 2. The available starch for each bread, the final weight for every portion and the percentage of nopal flour per portion

Test products	Available Starch (%dry weight)	Portion weight*(g)	The percentage of nopal flour per portion** (% dry weight)
White Wheat Bread (WWB)	42.70	117.10	0
Soluble Nopal (SNB)	43.86	116.66	19.38
Insoluble Nopal (INB)	31.49	158.78	26.15

*The portions are based on 50 g available starch.
**The percentage is based on the amounts of nopal flour from which the respective fractions are separated.

2.4 Dietary Fibre analysis

In order to determine the dietary fibre, the method originally presented by Asp et al., 1983 was applied[42]. The method simulates the human digestion in the gastrointestinal channel. Carbohydrate, protein, and fat are broken down by enzymes into small fragments. There are three mechanisms in this procedure. Firstly, Termamyl hydrolyzes α bonds of α-linked polysaccharides in starch, resulting in oligosaccharides and small starch fractions, while pepsin break down proteins into peptides[43]. In the last step, peptides, fat and starch fractions are digested by pancreatin, consisting of pancreatic enzymes trypsin, lipase and amylase[44]. In this step the remaining protein is broken-down into amino acids and oligopeptide by enzymes trypsin, the enzyme lipase degraded dietary fat (triglycerides, phospholipids and cholesterol esters) into fatty acids and monoglycerides, and the undigested starch is hydrolyzed into monosaccharides (glucose) by the enzyme amylase[45]. Insoluble fibre components were filtered off with the crucibles filter containing celite previously weighed. From the filtrate, soluble fibre components were precipitated out by ethanol, and filtered with another crucible containing celite. Fibre residues are dried overnight (105 ºC) and weighed. Then the ash content and protein content (see below, the section 2.5 "Protein analysis for dietary fibre determination") in the fibre fractions are determined and subtracted from fibre fractions. Total dietary fibre is the sum of soluble and insoluble fibre.

The process of fibre analysis is as followed. Firstly, sodium phosphate buffer (0.1 M, pH 6.0) was added into the dried samples of the WWB, SNB and INB to adjust the condition that is suitable for Termamyl environment. Then Termamyl was added and incubated in boiling water. Pepsin was incubated at 40 ºC and pH were lowered by adding HCI (0.2 N) to stimulated the conditions in the stomach. For stimulation of small intestinal conditions, incubation with pancreatin was performed by setting the pH to 6.8 at 40 ºC, using NaOH (5 N). In order to stop the incubation, HCI (0.5) was added to adjust pH to 4.5. Ethanol 95%, ethanol 99% and Millipore water were used to wash the insoluble fibre. The crucibles were added with celite. The precipitation of the soluble DF in the filtrate was performed with warm ethanol 95% (60 ºC) for 1 hour. The samples were then filtered, and the fibre washed with ethanol 78%, ethanol 95% and ethanol 99%. The analysis was performed in duplicate for each sample. The fibres were dried overnight in the oven at 105 ºC. Then the crucibles were place in the desiccator for cooling before weighing.

To estimate the final soluble and insoluble dietary fibre, the protein and ash analyses needs to be performed. The amount of protein in the three test products were analyzed using a protein

analyzer (FlashEA® 1112 Elemental Analyzer). The preparation was initiated by packing the day sample in a capsule made of fine thin foil. The cycle for analysis requires a blank, two standards (25 and 50 g of aspartic acid) and verification standard (25 g of aspartic acid) before running the samples into the analyzer. The aim of this procedure is to know the mass that combusted at 900 °C in the presence of oxygen to release H_2O, CO_2 and N_2. The H_2O and CO_2 were removed by passing the gasses over adsorption filters that allow to absorb them. A thermal conductivity detector measured the nitrogen content at the end of the process. The calibration of this method is done by pure material (aspartic acid). The nitrogen content is converted to protein crude content using a conversion factor i.e. 6.25 (Jones Factor). To determine ash content in the DF residues, the crucibles with the residues were put in an oven overnight at 550 ºC. After that crucibles were put in an exicator and thereafter weighted. To determine the DF content, the DF residues were adjusted for the protein and ash content.

2.5 Starch Hydrolysis

HI was determined in order to estimate starch digestibility, which is of importance for the glycaemic properties of the bread. The products were analysed by using chewing as a first step of the experiment [18]. WWB, SNB and INB were chewed by six participants on 3 selected days. The method is designed to mimic physiological steps in the human digestion. The HI of bread products normally correspond well with the products Glycaemic Index (GI). Instead of taking blood from a finger when determining the GI, dialysate samples were taken.

The participants chewed a portion of each product containing 1 g of available starch for 15 times. After that the sample was spitted out into a beaker containing a pepsin solution (2000 FIB-U/g; MERCK, Darmstadt, Germany) mixed with an amylase buffer (0.2 Molar, pH 6.9) and HCI (2 M). Then, subjects rinsed their mouth with buffer during 1 minute and then spit it again into the mixture. Followed by collection of the saliva into the mixture. This procedure aimed to make a fine ground sample well mixed with salivary amylase and added pepsin for simulating physiological conditions of digestion in the mouth and stomach, respectively. HCI (2 molar) were used to adjust the pH to the same as in stomach, which is approximately pH 1.5 - 3. Then the sample solution was incubated at 37 ºC for 30 minutes with stirring every 10 minutes, in order to breakdown proteins.

After the incubation, NaOH (2 M) was added to adjust the pH to 6.9. α amylase was added to digest the starch in the sample solution, and then the sample were put into a dialyse tube, which was supposed to mimic the small intestine of human. The dialyse tubes were immediately put into beakers containing Tugg buffer and along with a triangle magnet. The beakers were placed in a water bath for incubation (37 °C) on a magnetic plate (**Fig 4**). For every 30 minutes until 180 minutes, 1 ml of the dialysate was collected from the beakers and mixed with 1 ml of DNS in a glass tube.

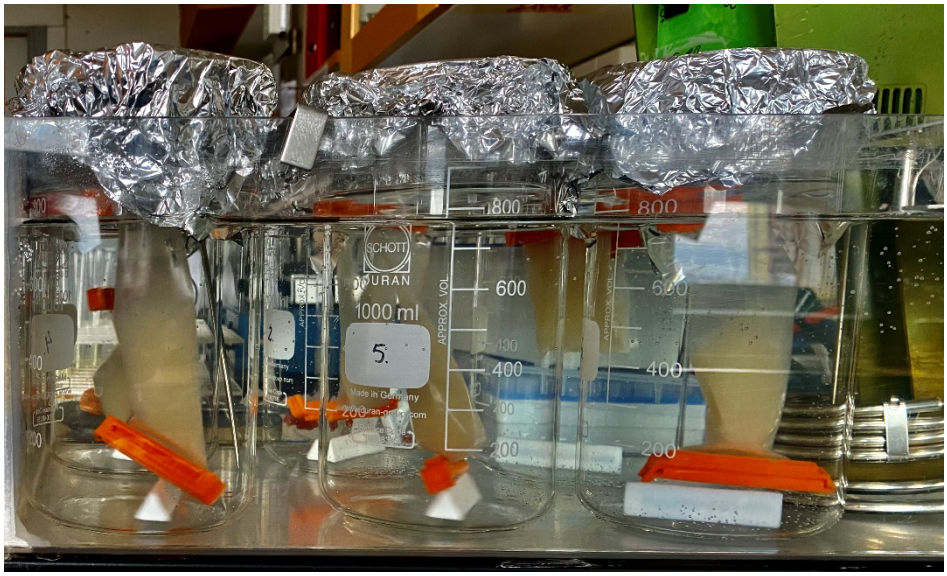


Figure 4: Dialysis tubes incubated in amylase buffer at 37° C on a magnetic stir plate

The samples from the beakers and maltose standards were incubated in boiling water (DNS reacts with reducing sugars). The colour from yellow turns to red. Then 8 ml of Millipore water was added to dilute the samples, which then were measured by using a spectrophotometer at 530 nm.

The sampling was performed in time intervals to mimic the blood sampling intervals conducted in GI studies. Thereafter, result of the starch hydrolysis in the sampling period within 3 hrs were plotted (hydrolysis curve) against time in a similar manner as compared to the determination of GI in order to calculate Area Under the Curve (AUC). The HI was then calculated in a similar approach as used for GI determinations.

HI = (Area Under Curve for the test product/ Area Under Curve for reference product) x 100

In this experiment the reference product was WWB. The HI for the test products was calculated based on the mean of the individual HI value. From the HI values obtained, the GI values of the products can be predicted with an empiric formula. The predicted Glycaemic Index (pGI) was determined using an empirical formula set out by Granfeldt, et. al., 1992[46], pGI = 0.862 HI + 8.198. The HI and pGI value of the test products investigated in this study are presented in **Table 6.**

2.7 Glucose and Insulin Measurements

Fingerprick is the method to collect capillary blood. This procedure has been applied for determination of glucose and insulin levels or even haemoglobin levels[47]. In this study, the purpose of blood sampling was to investigate postprandial levels of blood glucose and serum insulin after intake of the test product. The procedure for capillary sampling followed World Health Organization guidelines for drawing blood[47].

2.7.1 Glucose

A capillary blood sample of participants was taken before having breakfast as a fasting value at every experimental day, and then blood tests were taken after having breakfast at 15, 30, 45, 60, 90, 120, 150 and 180 minutes. The blood samples were collected in a hemo-cue cuvette and analysed by using a blood glucose meter (Hemocue^{+R}). At fasting (time = 0 minute), if blood glucose exceeded 6.1 mmol/L, the subjects were asked to rest, and then the blood glucose concentration was measured again. All data were recorded until the last trial day and then used for next step on statistical analysis.

2.7.2 Insulin Analysis

Insulin was determined by using Insulin Elisa commercial kits. The Insulin ELISA is a two-site enzyme immunoassay utilizing the direct sandwich technique with 2 antibody solutions directed against separate antigenic determinants of the insulin molecule[48]. Serum insulin along with standard and control were prepared and put into the wells on the plates, followed by peroxidase-conjugated anti-insulin antibodies. Anti–insulin antibodies were adhered to insulin in the sample, and the peroxidase-conjugated anti–insulin antibodies were also bond to the insulin. Before adding TMB as a labelled solution for binding the conjugated antibodies, washing procedure is needed to remove unbound antibodies. Stop solution was added to the wells to stop the reaction, and the microplate was read on a spectrophotometer at 450 nm.

After taking a blood sample for glucose measurement, 0.15 ml blood was collected in a test tube and rested between 30 minutes and 1 hour for blood clotting. After that, the test tubes were centrifuged at 4000 rpm. for 10 minutes or until the clear separation between red blood cell and serum was achieved. Serum (clear yellow specimen on the top) was transferred to Eppendorf tubes. These tubes were kept in carton box and stored at -20 °C if not running the experiment immediately.

Before start of the Insulin ELISA procedure, all equipment, standard solution and sample must achieve room temperature. Insulin ELISA was performed by preparing enzyme conjugate 1X solution and wash buffer 1X solution according to Mercodia Insulin ELISA procedure. 25 µL of each calibrators, controls and samples were pipetted into appropriate wells. After that, 100 µL of enzyme conjugate 1X solution was added to each well. The plate was covered with an adhesive plate cover and incubated at room temperature on horizontal orbital microplate shaker set at 800 rpm. Next step was to wash the plate with buffer by using microplate washer processed (set the program: 12 rows and wash 4 times). The aim of wash process is to completely remove the liquid at each step, which is essential to achieve good results. After the last wash, the remaining wash buffer was removed by inverting the plate and blotting it on clean tissue paper. 200 µL of TMB (blue reagent) was added to each well by using 6 channels pipette and incubated for 15 minutes with a new adhesive cover. To stop the reaction, 50 µL of stop solution was added in each well (turn into yellow colour). The plate was shaked for 5 sec to ensure mix well and read immediately by microplate reader at 450 nm.

2.8 Experimental Design

The present experiment applied a randomized, controlled, cross-over study design. The meals were provided as breakfasts on 3 different occasions in random order, with approximately 7 days between each test product. Consequently, by the end of the study, all subject had received the same treatments[49]. The randomization was done by using Microsoft excel.

2.8.1 Before the experimental day

In the evening before the day of experimental, test products were stored overnight at room temperature. The evening before each experimental day, the subjects were instructed to consume an individual standardized meal at 21.00, composed 1 or 2 slices of white bread (Jättefranska, Pågen)) with water, tea or coffee (no milk) depending on their desire. Finally, subjects were asked to avoid alcohol, excessive physical activities and foods rich in DF. After the standardised evening meal, the test persons needed to fast until the morning.

2.8.2 Experimental day

If the test persons were thirsty in the morning before the experiment started, they could drink a half glass of water at home if needed. They had to repeat the same amounts of water every experimental day. In the experimental room, test persons arrived at approximately 7.30 am. and were asked to rest at least 15 minutes to avoid a stressful feeling which may affect the experiment. Upon their arrival, the body weight and appetite questionnaires were registered along with fasting breath hydrogen (Gasto$^+$ Gastolyzer). After that, fasting blood samples were collected for glucose and insulin determination. A test product was then served with 250 g of tap water. The subject had to finish the breakfast within 12 minutes. Blood samples were collected for glucose and insulin determinations at fasting and at 15, 30, 45, 60, 90, 120, 150 and 180 minutes after start of breakfast along with registration of appetite.

2.9 Statistical Analysis

All data from blood glucose and serum insulin measurements, and starch hydrolysis were analyzed by using statistical software, Minitab 17 and GraphPad Prism 8.

Before further analysis, a normality test is a prerequisite in order to see if the data is normally distributed. General Linear ANOVA model (Minitab) test was used to investigate if there were any significant differences between the test products regarding effects on test variables. A P-value lower than 0.05 means that there are significant differences. If significant results were detected with the ANOVA analysis, further investigations were preformed using Post hoc test (Dunnett Multiple Comparisons) to see which test products were significantly different compared to the control product.

GraphPad Prism 8 was used for plotting graphs and calculate the incremental area under the curves (iAUC) in different time intervals, which were iAUC 0 – 30, 0 – 45, 0 – 60, 0 – 90, 0 – 120, 0 – 150 and 0 – 180 min. Also the highest incremental value (iPeak) for each individual participant were used to see the overall highest peak concentration for glucose and insulin.

Chapter 3
Results

3.1 Subjects

At the end of the human trial, 3 women and 4 men had dropped out or did not met the criteria to participate (n=3). The dropouts were due to treatment with antibiotics (n=1), difficulties to eat the test product INB (n=2), did not match the criteria (n=3), did not manage to come on the scheduled times (n=1). At the end, the volunteer group consisted of 17 test persons aged between 20 and 32 year (mean ± SD: 24.6 ± 2.9 years) with normal BMI (BMI 21.8 ± 1.6 kg/m^2). After analysing data from insulinaemia, we could only use the data from 15 subjects due to analytical problems.

3.2 Glucose measurements

There was no significant difference between the fasting blood glucose concentration before intake of WWB, SNB and INB. Anova model showed that there were significant differences in glucose responses after intake of test and control products (iAUC, P = 0.014). The *post hoc* test with the Dunnett method showed that the iAUC after the INB was significantly reduced compared to the WWB during the time period 0-45 and 0-60 minutes after intake (P = 0.009 and P = 0.032, respectively). In addition, the iPeak value was significantly lower with INB compared to the WWB (P = 0.015). The results are presented in **Fig. 5 - 6** and in **Table 3**

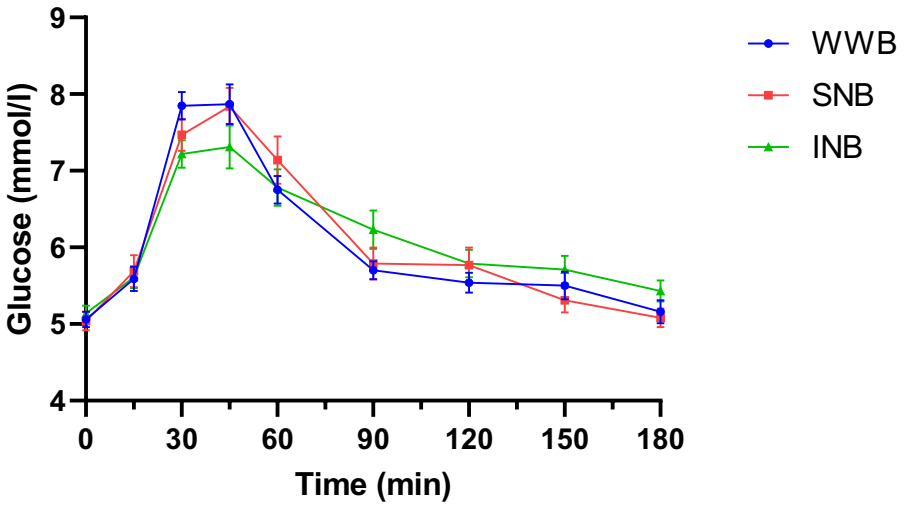


Figure 5: Postprandial blood glucose responses (mmol/l) with the three different bread breakfasts over the period of 0 – 180 minutes. All values are mean ± SEM. n = 15. WWB, White wheat bread. SNB, Soluble nopal bread. INB, Insoluble nopal bread.

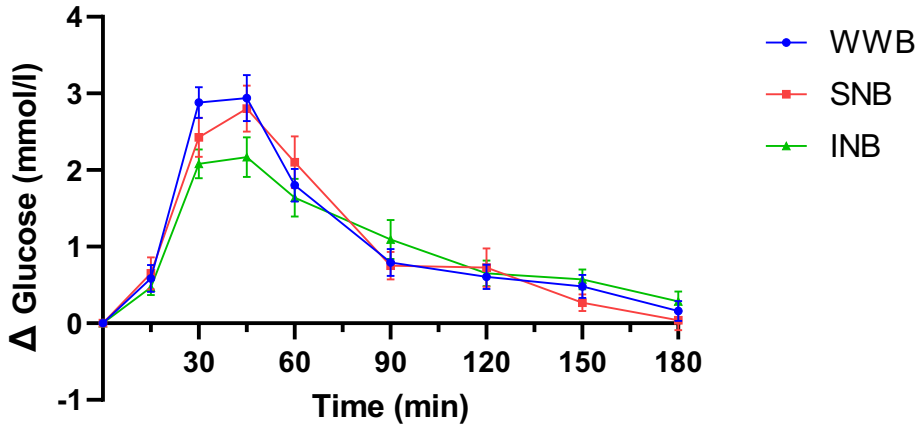


Figure 6: Postprandial blood glucose responses with the three different bread breakfasts over the period of 0 – 180 minutes. All values are mean ± SEM. n = 15. WWB, White wheat bread. SNB, Soluble nopal bread. INB, Insoluble nopal bread.

Table 3. Results regarding blood glucose measurements 180 minutes postprandial to the standardized breakfasts

Test variables, n=15	WWB	SNB	INB	%Δ[a] SNB	%Δ[a] INB
Blood Glucose, Fasting (mmol/L)	5.1±0.1	5.0±0.1	5.1±0.1	-1	2
ΔBlood glucose, iAUC 0 – 45 minutes (mmol•min/L)	72.8±5.3	67.9±6.3	54.5±4.2*	-7	-25
ΔBlood glucose, iAUC 0 – 60 minutes (mmol•min/L)	112.3±10.8	113.4±10.8	92.2±10.0*	1	-18
ΔBlood glucose, iAUC 0 – 120 minutes (mmol•min/L)	161.3±13.1	172.4 ±15.9	151.2±13.6	6	-6
ΔBlood glucose, iPeak (mmol/L)	3.3±0.2	3.3±0.2	2.8±0.1*	0.4	-16

*Different from control bread, P < 0.05 (Post hoc with Dunnett's method). **Δ[a]** The calculated percentage changes between the control bread and the nopal test products. *iAUC*, incremental area under curve. *iPeak*, individual incremental peak values. All values are presented as mean ± SEM. N = 15 Subjects. WWB, White wheat bread. SNB, Soluble nopal bread. INB, Insoluble nopal bread

3.3 Insulin measurements

There was no significant difference in the fasting insulin concentrations between the control product and the test products. Anova model showed that there were significant differences between the products (P = 0.003) with respect to postprandial insulin concentrations (iAUC). The Post hoc test with the Dunnett method showed significant reduced insulin iAUC both after SNB and INB compared to the WWB during the time period 0-45 minutes (P = 0.026 and P = 0.011, respectively) as well as time period 0-60 minutes (P = 0.015 and P = 0.017, respectively). In addition, iPeak values were significantly reduced postprandially after intake

of INB compared to WWB (P = 0.016). The results regarding insulin concentrations are presented in **Fig. 7 – 8**, and in **Table 4.**

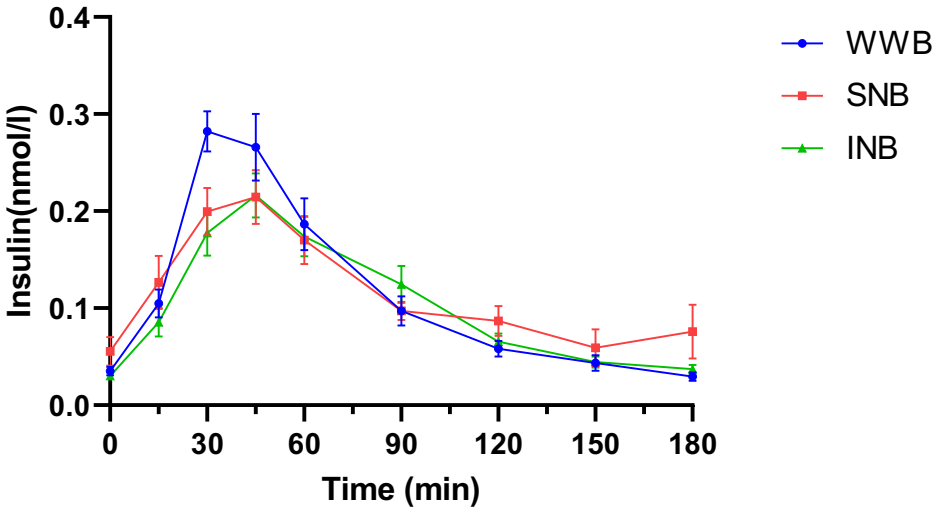


Figure 7: Absolute Insulin responses postprandially with the three different bread breakfasts over the period of 0 – 180 minutes. All values are mean ± SEM. n = 15. WWB, White wheat bread. SNB, Soluble nopal bread. INB, Insoluble nopal bread

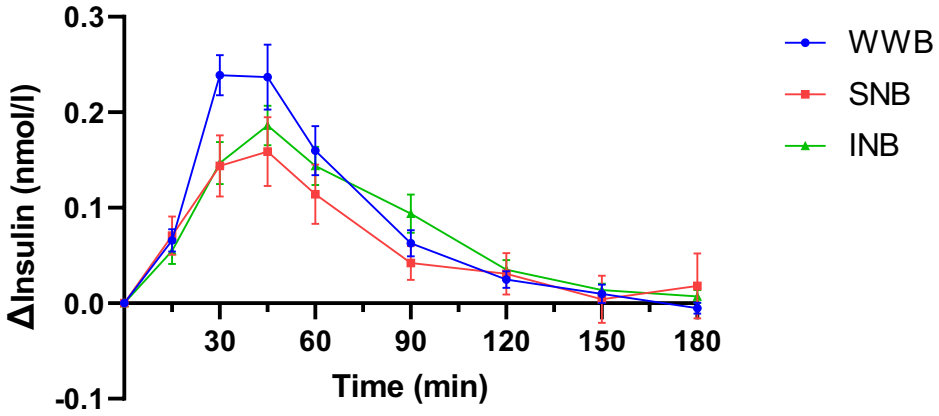


Figure 8: Incremental Insulin responses postprandially with the three different bread breakfasts over the period of 0 – 180 minutes. All values are mean ± SEM. n = 15. WWB, White wheat bread. SNB, Soluble nopal bread. INB, Insoluble nopal bread

Table 4. Results regarding insulin measurement 0 – 180 minutes postprandial to the standardized breakfasts.

Test variables, n=15	WWB	SNB	INB	%Δ[a] SNB	%Δ[a] INB
Insulin, Fasting (mmol/L)	0.04±0.0	0.06±0.0	0.03±0.0	57	-14
Insulin, iAUC 0-45 minutes (nmol/L)	6.4±0.5	4.7±0.7*	4.4±0.6*	-27	-30
Insulin, iAUC 0-60 minutes (nmol/L)	9.3±0.8	6.9±0.9*	6.9±0.7*	-26	-26
Insulin, iAUC 0-120 minutes (nmol/L)	14.1±1.4	10.9±1.5	12.4±1.4	-23	-12
Insulin, iPeak (nmol/L)	0.3±0.0	0.2±0.0	0.2±0.0*	-19	-25

*Different from control bread, $P < 0.05$ (Post hoc with Tukey pairwise method). Δ[a]The calculated percentage changes between the control bread and the Nopal test products. *iAUC*, incremental area under curve. *iPeak*, individual incremental peak values. All values are presented as mean ± SEM. WWB, White wheat bread. SNB, soluble nopal bread. INB, insoluble nopal bread

3.4 Dietary fibre content in WWB, SNB and INB

The results of dietary fibre fractions in WWB, SNB, and INB are presented in Table 5 and Figure 9 (presented as dry matter). The total dietary fibre in WWB is lower than in both SNB and INB. The highest soluble and insoluble dietary fibre were found in the INB.

Table 5. Dietary fibre in the test products (g/100 g of sample in dry matter and g/portion)

Test product	Total DF		Soluble DF		Insoluble DF	
	g/% dm	g/portion	g/%dm	g/portion	g/%dm	g/portion
WWB	1.1 ± 0.1	0.7 ± 0.1	1.1 ± 0.1	0.7 ± 0.1	0.0 ± 0.1	0 .0± 0.1
SNB	4.6 ± 1.2	2.9 ± 0.8	2.9 ± 0.7	1.9 ± 0.5	1.7 ± 0.2	1.1 ± 0.1
INB	10.0± 1.4	7.9 ± 0.7	4.5 ± 1.3	3.6 ± 0.7	5.5 ± 0.2	4.3 ± 0.1

DF, dietary fibre. dm, dry matter. WWB, white wheat bread. SNB, soluble nopal bread. INB, insoluble nopal bread

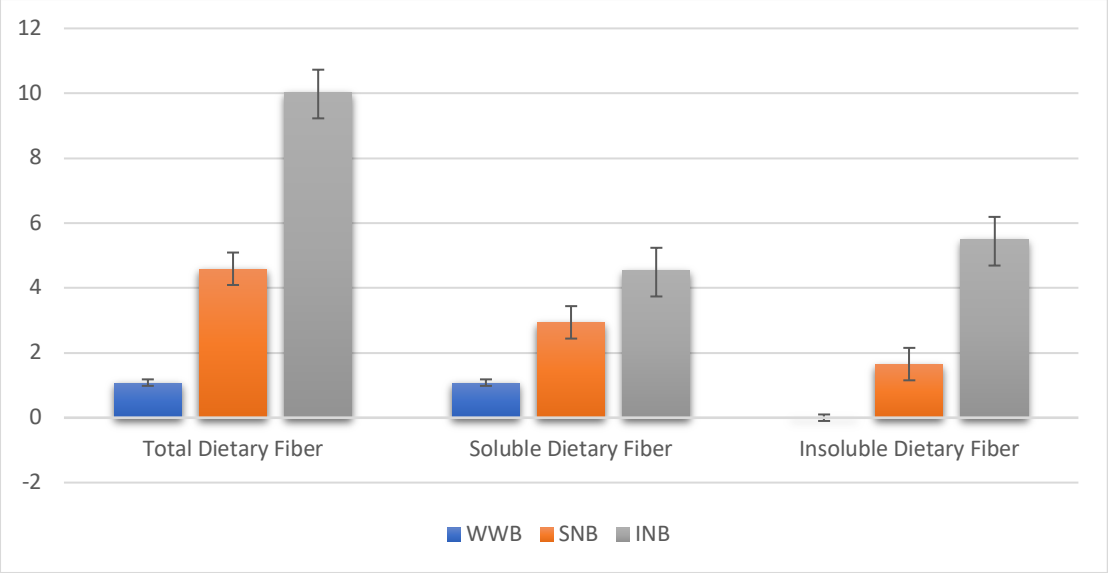


Figure 9 : Total Dietary fibre, Soluble dietary fibre and Insoluble dietary fibre in WWB (white wheat bread), SNB (soluble nopal bread) and INB (insoluble nopal bread)

3.5 Hydrolysis Index and Predicted Glycaemic Index

An *in vitro* starch hydrolysis method was carried out in this study in order to mimic the *in vivo* situation of starch digestion, and to estimate the glycaemic response to tested products. The results of hydrolysis curves used for calculations are displayed in **Fig. 10.** It was found, as expected, that the profile of *in vitro* starch hydrolysis exhibited a gradual increment for all test products as time increased. **Table 6** shows that INB resulted in significantly lower *in vitro* starch hydrolysis and pGI compared to WWB. The HI values of SNB and INB decreased by 13% and 60%, respectively, when compare with WWB. The significant decreases in HI value of the product corresponded to the significant decreases in pGI.

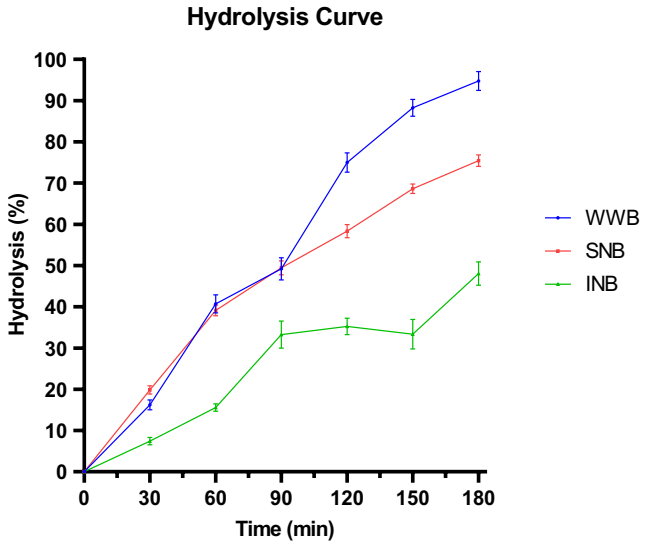


Figure 10: Hydrolysis curve of starch in WWB (white wheat bread), SNB (soluble nopal bread) and INB (insoluble nopal bread)

Table 7. Hydrolysis Index and Predicted Glycaemic Index (% ± SEM)

Products	Hydrolysis index (%)	Predicted Glycaemic Index (%)
WWB	100.0	94.4
SNB	87.1 ± 4.3	83.3 ± 3.7
INB	60.1 ± 3.4*	60.0 ± 2.9

The hydrolysis index was compared with WWB. Values are mean of six subjects chewing and dialysis replicates. * ; Significantly different compared with WWB ($P<0.05$). Predicted glycaemic index = 0.862 HI+8.198[50]. WWB, White wheat bread. SNB, soluble nopal bread. INB, insoluble nopal bread

Chapter 4
Discussion

The purpose of the present study was to examine the glycaemic properties of water soluble and non-soluble fractions in nopal cladodes flour. For this purpose, the fractions were included in separate wheat flour-based breads and compared with respect to their effects on acute postprandial glucose regulation in healthy subjects. The amount of nopal supplementation in the breads was based on a previous dose-response study investigating effects of nopal flour on acute blood glucose regulation[40]. The present study further aimed to investigate the influence of nopal fractions on glucose and insulin responses along with characterization of nopal fractions with respect to dietary fibre content, hydrolysis index and predicted glycaemic index.

4.1 Soluble and insoluble fraction in nopal flour

Separation of nopal fractions is an important issue to consider. The water soluble nopal fraction was dark yellow, with bitter-taste and with slimy texture. This soluble nopal fraction may contain different phytochemicals such as polyphenols and other phenolic compounds that are soluble in water, and the slimy texture may be due to soluble DF. On the other hand, the insoluble nopal fraction was dark green, packed in the bottom of the centrifuge container, and had a sandy texture but less bitter taste compared to soluble nopal fraction. This fraction may consist of insoluble DF and some organic compounds that do not dissolve in water. For instance, nopal cladodes are known to contain tannins[51] and flavonoids[52].

After the separation of nopal flour into water soluble and non-soluble fractions, the presence of soluble DF in INB was still high compared to SNB. This fact, that will be discussed in a later section, might be due to the separation method, which may have been insufficient to separate DF fractions more strictly. In this study, the incubation time before centrifugation was 10 min. For better separation, further studies and optimisation regarding separation techniques should be performed.

4.2 Blood glucose and insulin regulation

Previous studies have revealed that a white wheat bread supplemented with 25% (dry basis) of nopal flour induces a significantly lower postprandial blood glucose response compared to a 100 % white wheat bread. In the presently described study, the INB significantly reduced glucose responses in the early postprandial period compared with the WWB, which is in agreement with the previous study. However, no such effect was observed regarding the SNB. The results thus suggest that it is the insoluble fraction in the nopal flour that is responsible for the beneficial effects previously observed on postprandial glucose regulation. However, it must be noted that the separation method used may be insufficient to draw firm conclusions regarding effects connected to the different fractions, thus, the insoluble fraction studied in the current study included substantial amounts of soluble DF (and perhaps other potentially soluble compounds).

Beneficial effects of soluble DF on postprandial glucose regulation have repeatedly been shown in clinical studies [53,54,55]. However, interestingly, it is insoluble cereal fibre and whole

grain consumption that show the strongest link to lower risk of T2D[51,56,57,58]. It can thus be suggested that there must be mechanisms in addition to the effects of soluble DF on postprandial glucose regulation, that contribute to the health benefits concerning preventive potential of DF against T2D.

Due to the substantial amounts of soluble DF in the INB, the lowering effects on glucose response may be due to mechanisms related to the soluble DF (i.e., pectin) [59, 60]. The separated insoluble fraction showed viscous properties, which also was noticed in the dough preparing steps, which may have influenced the postprandial glucose excursions by delaying the digestion and absorption rate of carbohydrates[55]. The underlying mechanisms may be related to a reduction of starch hydrolysis rate (α-amylase inhibition) due to the increased viscosity[61]. Insoluble DF in INB might also have an effect on insulin secretion, as reported by Isken et al., showing that insoluble DF could resulted in significantly reduced weight and improved insulin sensitivity[53], although this mainly can be attributed to a medium/long term effect of the fibre.

The recommendations for patients with T2D from American Diabetes Association is to maintain blood glucose levels in the normal range, or, at least as close as possible to normal levels[62]. In this study, the glucose concentration peak after intake of INB was significantly improved compared to consumption of WWB, which could be interpreted as an anti-diabetic effect[13].

Interestingly, both the soluble and insoluble nopal fractions resulted in a reduced postprandial insulin secretion. The lowered insulin responses after the SNB ingestion suggest improved insulin sensitivity in healthy humans, thus, lower postprandial insulin secretion was needed to keep the blood glucose at the similar concentrations as compared to after consumption of the WWB. This is in contradiction to the research of *Russell et.al.* reporting that soluble DF has no consistent associations with reduced risk of T2D in prospective cohort studies[63]. The mechanisms behind the increased insulin sensitivity has to be further elucidated. One possible contributing factor may be attributed to an increased release of gut hormones, e.g. GLP-1 and PPY, which have the potential to lower the gastric emptying rate and motility in the gastrointestinal (GI) tract[64]. These effects may improve glucose regulation[61]. Thus, it could be hypothesised that presence of the DF results in delayed digestion and food constituents further down in the GI tract, which may increase the release of the gut hormone from L-cells, which are more frequently located in the lower part of the small intestine (ileum) and large intestine (colon)[65].

Other components in the soluble nopal fraction should be considered, such as phenolic compounds and flavonoids, vitamins, and minerals. The bitter taste in nopal fractions might be tannins. Tannins are polyphenol compounds with high molecular weight and are thought to have several health benefits. For instance, there is a correlation between tannins content in nopal cladodes with their inhibitory activity against α-amylase and α-glucosidases[51], which may be considered a mechanism for the glycemia-regulating properties of nopal. Additionally, the dark yellow colour in soluble nopal fraction might be due to flavonoids. A study conducted by Rodriguez et.al.[52] revealed that flavonoids extract (isorhamnetin) from nopal cladodes stimulated insulin secretion and also improved blood lipid profile in mice. Further investigation regarding these phytochemical compounds of nopal would be needed to evaluate other biological effects of nopal and the mechanism(s) behind these properties.

4.3 Hydrolysis Index and Predicted Glycaemic Index

The hydrolysis index values recorded in this study were 87 (pGI 83) and 60 (pGI 60) for SNB and INB, respectively (WWB HI and pGI = 100). The GI concept can be used as a guideline for healthy food choices. Low GI diets can reduce postprandial glucose by retarding digestion and absorption, as well as moderate the insulin responses in our body. Important factors affecting GI of food products are texture and particle size, which strongly influence the digestion and absorption rate[66]. INB contains viscous DF and higher contents of total DF compared to WWB and SNB, which could affect the digestion system in the small intestine or in the dialysis tube model. This is in accordance with the results showing lower postprandial glycaemia, but it was also reflected in the HI and pGI of the INB. These properties can give a beneficial influence to reduce the risk of T2D.

4.4 Dietary Fibre

As discussed above, DF plays an important role in blood glucose and insulin responses. From the result of dietary fibre contents, the INB contains the highest total DF and also highest content of both soluble DF and insoluble DF. The soluble fibre in nopal consists of mainly mucilage and pectin[67], while insoluble fraction contains cellulose and insoluble hemicelluloses[68]. It can be suggested that the high proportion of dietary fibre in the INB is the main determinant for the beneficial effects seen on both glucose and insulin postprandial concentrations. High viscosity of DF, as seen e.g. with glucomannan, relate to anti–diabetic properties[69]. The dietary fibre that enhances the viscosity of the product, it could result in beneficial effects on physiology by extending the retention time of the product in the intestinal tract. The hydration properties of soluble fractions results in swelling gel-like features in the intestine, and restrain or decrease the absorption rate of some nutrients, like glucose[70]. On the other hand, the effects on insoluble DF also play an important role in reducing risk for T2D. There are cohort studies on the consumption of insoluble cereal fibres showing reduced IR, and lower risk of T2D[29,71]. Therefore, the consumption of soluble nopal fraction in combination with insoluble fraction could impart a beneficial effect in reducing the risk of T2D. From this point of view, consumption of whole nopal cladodes should thus be recommended.

Another important aspect is that the contents of soluble DF and insoluble DF in nopal cladodes vary with different maturity stages. From small (young) to large (mature) cladodes, the soluble DF in nopal decreases and the insoluble DF increases overtime. Moreover, total DF also decreases with the age of cladodes[72]. It can be implied that to get the most beneficial anti-diabetic properties, the nopal cladodes should be collected in small or medium size (12 to 20 days or 40 g to 74 g[72]).

Chapter 5
Conclusion

In this study, the postprandial glycaemic regulation of nopal fractions was evaluated in healthy volunteers. INB decreases postprandial glucose and insulin responses, while SNB promoted beneficial effects on postprandial insulin secretion. Thus, the results emanating from this study make it plausible to conclude that soluble and insoluble nopal fractions have the ability to improve postprandial glucose regulation in healthy humans. The current situation regarding the prevalence of T2D has led to a search of functional foods that could be used as tools in the prevention of this non-communicable disease. However, most clinical studies with nopal have been performed in diabetics, and there are not enough scientific studies regarding the ability of nopal fractions to control blood glucose regulation in healthy humans, i.e. possess preventive potential. The results emanating from this study support development of nopal products as functional foods with the purpose to contribute to the prevention of T2D.

The presently described study dealt with effects on glycaemic regulation in humans, focusing on water soluble and insoluble contents of nopal flour. However, in the future research, it is recommended to further investigate DF composition and other components in nopal that could contribute to beneficial effects on cardiometabolic variables in healthy human. In addition, longer-term well controlled studies are needed to investigate the effect of nopal fractions and components. These studies should include determinations of gut hormones and aspects connecting to colonic fermentation.

References

1. Grundy Scott M., Brewer H. Bryan, Cleeman James I., Smith Sidney C., Lenfant Claude. Definition of Metabolic Syndrome. *Circulation*. 2004;109(3):433-438. doi:10.1161/01.CIR.0000111245.75752.C6
2. Kassi E, Pervanidou P, Kaltsas G, Chrousos G. Metabolic syndrome: definitions and controversies. *BMC Med*. 2011;9(1):48. doi:10.1186/1741-7015-9-48
3. Alberti KGMM, Eckel RH, Grundy SM, et al. Harmonizing the Metabolic Syndrome: A Joint Interim Statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and International Association for the Study of Obesity. *Circulation*. 2009;120(16):1640-1645. doi:10.1161/CIRCULATIONAHA.109.192644
4. Obesity and overweight. Accessed May 21, 2020. https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight
5. Martina, Adisasmita AC. Association between Physical Activity and Obesity with Diabetes Mellitus in Indonesia. *Int J Caring Sci*. 2019;12(3):1703-1709.
6. Mahler RJ, Adler ML. Type 2 Diabetes Mellitus: Update on Diagnosis, Pathophysiology, and Treatment. *J Clin Endocrinol Metab*. 1999;84(4):1165-1171. doi:10.1210/jcem.84.4.5612
7. Mathieu C, Badenhoop K. Vitamin D and type 1 diabetes mellitus: state of the art. *Trends Endocrinol Metab*. 2005;16(6):261-266. doi:10.1016/j.tem.2005.06.004
8. Kasuga M. Insulin resistance and pancreatic β cell failure. *J Clin Invest*. 2006;116(7):1756-1760. doi:10.1172/JCI29189
9. Diabetes Mellitus Subtypes. News-Medical.net. Published June 27, 2019. Accessed May 21, 2020. https://www.news-medical.net/health/Diabetes-Mellitus-Subtypes.aspx
10. Roder ME, Porte D Jr, Schwartz RS, Kahn SE. Disproportionately elevated proinsulin levels reflect the degree of impaired B cell secretory capacity in patients with noninsulin-dependent diabetes mellitus. *J Clin Endocrinol Metab*. 1998;83(2):604-608.
11. Czech MP. Insulin action and resistance in obesity and type 2 diabetes. *Nat Med*. 2017;23(7):804-814. doi:10.1038/nm.4350
12. Esmaillzadeh A, Kimiagar M, Mehrabi Y, Azadbakht L, Hu FB, Willett WC. Dietary patterns, insulin resistance, and prevalence of the metabolic syndrome in women. *Am J Clin Nutr*. 2007;85(3):910-918. doi:10.1093/ajcn/85.3.910
13. Wolever TMS. *The Glycaemic Index: A Physiological Classification of Dietary Carbohydrate*. CABI; 2006.
14. Geesha. Difference Between Glycaemic Index and Glycaemic Load. Pediaa.Com. Published April 24, 2016. Accessed May 28, 2020. https://pediaa.com/difference-between-glycaemic-index-and-glycaemic-load/
15. Burris J, Shikany JM, Rietkerk W, Woolf K. A Low Glycemic Index and Glycemic Load Diet Decreases Insulin-like Growth Factor-1 among Adults with Moderate and Severe Acne: A Short-Duration, 2-Week Randomized Controlled Trial. *J Acad Nutr Diet*. 2018;118(10):1874-1885. doi:10.1016/j.jand.2018.02.009
16. Foster-Powell K, Holt SHA, Brand-Miller JC. International table of glycemic index and glycemic load values: 2002. *Am J Clin Nutr*. 2002;76(1):5-56.
17. Kaviani M, Chilibeck PD, Gall S, Jochim J, Zello GA. The Effects of Low- and High-Glycemic Index Sport Nutrition Bars on Metabolism and Performance in Recreational Soccer Players. *Nutrients*. 2020;12(4):982. doi:10.3390/nu12040982

18. Akerberg AK, Liljeberg HG, Granfeldt YE, Drews AW, Bj-orck IM. An in vitro method, based on chewing, to predict resistant starch content in foods allows parallel determination of potentially available starch and dietary fiber. *J Nutr*. 1998;128(3):651-660.
19. Hegwood DA. Human Health Discoveries with Opuntia sp. (Prickly Pear). *HortScience*. 1990;25(12):1515-1516. doi:10.21273/HORTSCI.25.12.1515
20. Teague N. What Do Nopales Taste Like? Get The Facts. Cuisinevault. Published March 19, 2020. Accessed September 1, 2020. https://www.cuisinevault.com/what-do-nopales-taste-like/
21. Butterweck V, Semlin L, Feistel B, Pischel I, Bauer K, Verspohl EJ. Comparative evaluation of two different Opuntia ficus-indica extracts for blood sugar lowering effects in rats. *Phytother Res*. 2011;25(3):370-375. doi:10.1002/ptr.3271
22. Stintzing FC, Carle R. Cactus stems (Opuntia spp.): A review on their chemistry, technology, and uses. *Mol Nutr Food Res*. 2005;49(2):175-194. doi:10.1002/mnfr.200400071
23. Ortiz-Escobar TB, Valverde-González ME, Paredes-López O. Determination of the folate content in cladodes of nopal (Opuntia ficus indica) by microbiological assay utilizing Lactobacillus casei (ATCC 7469) and enzyme-linked immunosorbent assay. *J Agric Food Chem*. 2010;58(10):6472-6475. doi:10.1021/jf100503v
24. Rodriguez-Garcia ME, de Lira C, Hernández-Becerra E, et al. Physicochemical Characterization of Nopal Pads (Opuntia ficus indica) and Dry Vacuum Nopal Powders as a Function of the Maturation. *Plant Foods Hum Nutr*. 2007;62(3):107-112. doi:10.1007/s11130-007-0049-5
25. Lopez-Romero P, Pichardo-Ontiveros E, Avila-Nava A, et al. The effect of nopal (Opuntia ficus indica) on postprandial blood glucose, incretins, and antioxidant activity in Mexican patients with type 2 diabetes after consumption of two different composition breakfasts. *J Acad Nutr Diet*. 2014;114(11):1811-1818. doi:10.1016/j.jand.2014.06.352
26. Berraaouan A, Abderrahim Z, Hassane M, Abdelkhaleq L, Mohammed A, Mohamed B. Evaluation of protective effect of cactus pear seed oil (Opuntia ficus-indica L. MILL.) against alloxan-induced diabetes in mice. *Asian Pac J Trop Med*. 2015;8(7):532-537. doi:10.1016/j.apjtm.2015.06.013
27. Leem K-H, Kim M-G, Hahm Y-T, Kim HK. Hypoglycemic Effect of Opuntia ficus-indica var. saboten Is Due to Enhanced Peripheral Glucose Uptake through Activation of AMPK/p38 MAPK Pathway. *Nutrients*. 2016;8(12):800. doi:10.3390/nu8120800
28. Hwang SH, Kang I-J, Lim SS. Antidiabetic Effect of Fresh Nopal (Opuntia ficus-indica) in Low-Dose Streptozotocin-Induced Diabetic Rats Fed a High-Fat Diet. Evidence-Based Complementary and Alternative Medicine. doi:https://doi.org/10.1155/2017/4380721
29. InterAct Consortium. Dietary fibre and incidence of type 2 diabetes in eight European countries: the EPIC-InterAct Study and a meta-analysis of prospective studies. *Diabetologia*. 2015;58(7):1394-1408. doi:10.1007/s00125-015-3585-9
30. Threapleton DE, Greenwood DC, Evans CEL, et al. Dietary fibre intake and risk of cardiovascular disease: systematic review and meta-analysis. *BMJ*. 2013;347. doi:10.1136/bmj.f6879
31. Kaczmarczyk MM, Miller MJ, Freund GG. The health benefits of dietary fiber: Beyond the usual suspects of type 2 diabetes mellitus, cardiovascular disease and colon cancer. *Metabolism*. 2012;61(8):1058-1066. doi:10.1016/j.metabol.2012.01.017
32. Alimentarius C. Guidelines on nutrition labelling CAC/GL 2-1985 as last amended 2010. *Jt FAOWHO Food Stand Programme Secr Codex Aliment Comm FAO Rome*. Published online 2010.

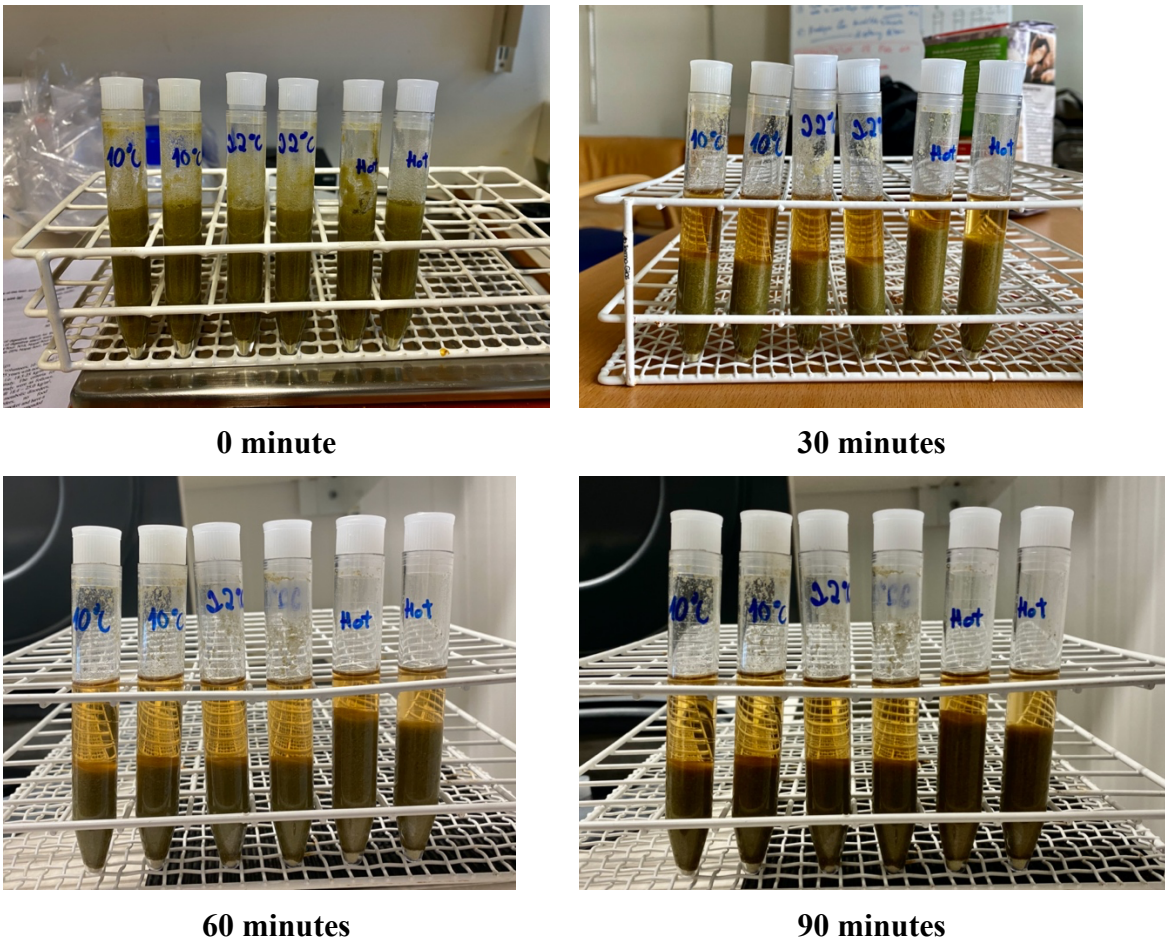
33. Cereals & Grains Association. Accessed June 11, 2020. https://www.cerealsgrains.org/Pages/default.aspx
34. EUR-Lex - 32008L0100 - EN - EUR-Lex. Accessed June 11, 2020. https://eur-lex.europa.eu/eli/dir/2008/100/oj
35. Jones JM. CODEX-aligned dietary fiber definitions help to bridge the 'fiber gap.' *Nutr J*. 2014;13:34. doi:10.1186/1475-2891-13-34
36. Papathanasopoulos A, Camilleri M. Dietary fiber supplements: effects in obesity and metabolic syndrome and relationship to gastrointestinal functions. *Gastroenterology*. 2010;138(1):65-72.e1-2. doi:10.1053/j.gastro.2009.11.045
37. Anderson JW, Baird P, Jr RHD, et al. Health benefits of dietary fiber. *Nutr Rev*. 2009;67(4):188-205. doi:10.1111/j.1753-4887.2009.00189.x
38. Sambandan G, Turcu-Stiolica A. Clinical Trials. In: *Clinical Pharmacy Education, Practice and Research*. Elsevier; 2019:323-344. doi:10.1016/B978-0-12-814276-9.00023-4
39. Livsmedelsverket. Accessed June 30, 2020. https://www.livsmedelsverket.se/bestall-ladda-ner-material/sok-publikationer/artiklar/2016/2016-nr-5-evaluation-of-the-nordic-nutrition-recommendations-2012
40. Ritke C. Stabilizing Effect of Opuntia Ficus Indica on Blood Glucose Regulation in Healthy Humans. Published online May 27, 2019.
41. Holm J, Björck I, Drews A, Asp N-G. A Rapid Method for the Analysis of Starch. *Starch - Stärke*. 1986;38(7):224-226. doi:10.1002/star.19860380704
42. Asp NG, Johansson CG, Hallmer H, Siljestroem M. Rapid enzymic assay of insoluble and soluble dietary fiber. *J Agric Food Chem*. 1983;31(3):476-482. doi:10.1021/jf00117a003
43. Pearson J, Allen A, Venables C. Gastric mucus: Isolation and polymeric structure of the undegraded glycoprotein: Its breakdown by pepsin. *Gastroenterology*. 1980;78(4):709-715. doi:10.1016/0016-5085(80)90672-1
44. DiMagno EP, Go VLW, Summerskill WHJ. Relations between Pancreatic Enzyme Outputs and Malabsorption in Severe Pancreatic Insufficiency. *N Engl J Med*. 1973;288(16):813-815. doi:10.1056/NEJM197304192881603
45. Nagai H, Henrich H, Wünsch P-H, Fischbach W, Mössner J. Role of pancreatic enzymes and their substrates in autodigestion of the pancreas: In vitro studies with isolated rat pancreatic acini. *Gastroenterology*. 1989;96(3):838-847. doi:10.1016/0016-5085(89)90910-4
46. Granfeldt Y, Björck I, Drews A, Tovar J. An in vitro procedure based on chewing to predict metabolic response to starch in cereal and legume products. *Eur J Clin Nutr*. 1992;46(9):649-660.
47. Dhingra N, Safe Injection Global Network, World Health Organization. *WHO Guidelines on Drawing Blood: Best Practices in Phlebotomy*.; 2010. Accessed May 28, 2020. http://www.ncbi.nlm.nih.gov/books/NBK138650/
48. CDC. The Insulin Resistance–Diabetes Connection. Centers for Disease Control and Prevention. Published August 12, 2019. Accessed May 28, 2020. https://www.cdc.gov/diabetes/basics/insulin-resistance.html
49. Shuster JJ. Design and Analysis of Experiments. In: Ambrosius WT, ed. *Topics in Biostatistics*. Methods in Molecular Biology™. Humana Press; 2007:235-259. doi:10.1007/978-1-59745-530-5_12
50. Granfeldt Y. Food factors affecting metabolic responses to cereal products. Published online 1994.
51. Figueroa-Pérez. MG, Pérez-Ramírez IF, Paredes-López O, Mondragón-Jacobo C, Reynoso-Camacho R. Phytochemical Composition and in Vitro Analysis of Nopal (O.

Ficus-Indica) Cladodes at Different Stages of Maturity. *Int J Food Prop*. 2018;21(1):1728-1742. doi:10.1080/10942912.2016.1206126
52. Rodríguez-Rodriguez C, Torres N, Gutierrez-Uribe JA, et al. The effect of isorhamnetin glycosides extracted from Opuntia ficus-indica in a mouse model of diet induced obesity. *Food Funct*. 2015;6(3):805-815. doi:10.1039/C4FO01092B
53. Isken F, Klaus S, Osterhoff M, Pfeiffer AFH, Weickert MO. Effects of long-term soluble vs. insoluble dietary fiber intake on high-fat diet-induced obesity in C57BL/6J mice. *J Nutr Biochem*. 2010;21(4):278-284. doi:10.1016/j.jnutbio.2008.12.012
54. Abutair AS, Naser IA, Hamed AT. Soluble fibers from psyllium improve glycemic response and body weight among diabetes type 2 patients (randomized control trial). *Nutr J*. 2016;15(1):86.
55. de Carvalho CM, de Paula TP, Viana LV, Machado VM, de Almeida JC, Azevedo MJ. Plasma glucose and insulin responses after consumption of breakfasts with different sources of soluble fiber in type 2 diabetes patients: a randomized crossover clinical trial. *Am J Clin Nutr*. 2017;106(5):1238-1245. doi:10.3945/ajcn.117.157263
56. Schulze MB, Schulz M, Heidemann C, Schienkiewitz A, Hoffmann K, Boeing H. Fiber and magnesium intake and incidence of type 2 diabetes: a prospective study and meta-analysis. *Arch Intern Med*. 2007;167(9):956-965. doi:10.1001/archinte.167.9.956
57. Weickert MO, Pfeiffer AFH. Metabolic effects of dietary fiber consumption and prevention of diabetes. *J Nutr*. 2008;138(3):439-442. doi:10.1093/jn/138.3.439
58. Jenkins DJ, Kendall CW, Axelsen M, Augustin LS, Vuksan V. Viscous and nonviscous fibres, nonabsorbable and low glycaemic index carbohydrates, blood lipids and coronary heart disease. *Curr Opin Lipidol*. 2000;11(1):49-56. doi:10.1097/00041433-200002000-00008
59. Galati E m. (1 3), Trovato A(1), D'Aquino A(1), Monforte M t. (1), Tripodo M m. (2). Biological activity of Opuntia ficus indica cladodes II: Effect on experimental hypercholesterolemia in rats. *Pharm Biol*. 2003;41(3):175-179. doi:10.1076/phbi.41.3.175.15087
60. Weickert MO, Pfeiffer AF. Impact of Dietary Fiber Consumption on Insulin Resistance and the Prevention of Type 2 Diabetes. *J Nutr*. 2018;148(1):7-12. doi:10.1093/jn/nxx008
61. Salleh SN, Fairus AAH, Zahary MN, Bhaskar Raj N, Mhd Jalil AM. Unravelling the Effects of Soluble Dietary Fibre Supplementation on Energy Intake and Perceived Satiety in Healthy Adults: Evidence from Systematic Review and Meta-Analysis of Randomised-Controlled Trials. *Foods*. 2019;8(1):15. doi:10.3390/foods8010015
62. Burant C. *Medical Management of Type 2 Diabetes*. American Diabetes Association; 2012.
63. Impact of Diet Composition on Blood Glucose Regulation. *Crit Rev Food Sci Nutr*. 2016;56(4):541-590. doi:10.1080/10408398.2013.792772
64. Drucker DJ. The role of gut hormones in glucose homeostasis. *J Clin Invest*. 2007;117(1):24-32. doi:10.1172/JCI30076
65. Lim GE, Brubaker PL. Glucagon-Like Peptide 1 Secretion by the L-Cell: The View From Within. *Diabetes*. 2006;55(Supplement 2):S70-S77. doi:10.2337/db06-S020
66. Tovar J. Bioavailability of carbohydrates in legumes: digestible and indigestible fractions. - Abstract - Europe PMC. Accessed June 3, 2020. https://europepmc.org/article/med/9137637
67. Goycoolea F, Cardenas A. Pectins from Opuntia spp.: A short review. *J Prof Assoc Cactus Dev*. 2003;5.
68. Monrroy M, García E, Ríos K, García JR. Extraction and Physicochemical Characterization of Mucilage from Opuntia cochenillifera (L.) Miller. *J Chem*. Published online May 10, 2017:1-9. doi:10.1155/2017/4301901

69. Palanuvej C, Hokputsa S, Tunsaringkarn T, Ruangrungsi N. In Vitro Glucose Entrapment and Alpha-Glucosidase Inhibition of Mucilaginous Substances from Selected Thai Medicinal Plants. *N Ruangrungsi Sci Pharm*. 2009;77:837-849. doi:10.3797/scipharm.0907-17
70. Eastwood MA. The physiological effect of dietary fiber: an update. *Annu Rev Nutr*. 1992;12:19-35. doi:10.1146/annurev.nu.12.070192.000315
71. Becerra-Tomás N, Díaz-López A, Rosique-Esteban N, et al. Legume consumption is inversely associated with type 2 diabetes incidence in adults: A prospective assessment from the PREDIMED study. *Clin Nutr*. 2018;37(3):906-913. doi:10.1016/j.clnu.2017.03.015
72. Nunez-Lopez MA, Paredes-Lopez O, Reynoso-Camacho R. Functional and Hypoglycemic Properties of Nopal Cladodes (*O. ficus-indica*) at Different Maturity Stages Using in Vitro and in Vivo Tests. *J Agric Food Chem*. 2013;61(46):10981-10986. doi:10.1021/jf403834x

Appendix

Appendix 1 Separation of nopal fractions (Soluble and Insoluble fraction)



0 minute **30 minutes**

60 minutes **90 minutes**

Figure A1 : The separation of soluble and insoluble fractions at 0, 30, 60 and 90 mins in different temperatures (10ºC, 22 ºC, and 90 ºC)

Appendix 2 Weight of test products after baking

Table 6A Weight of the test product after baking and removing crust

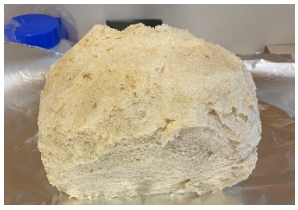
Product	Weight of the product after baking	Weight of the product after removing Crust
WWB		
	798.8 g	546.9 g
SNB		
	806.6 g	512.8 g
INB		
	813.5 g	568.0

Table 8A. The final weight for every portion and the percentage of nopal flour per portion.

Test products	Portion weight*(g)	Weight (g) of nopal fraction per portion
White Wheat Bread (WWB)		
	117.1	0
Soluble Nopal (SNB)		
	116.7	19.4
Insoluble Nopal (INB)		
	158.8	26.2

*The values are rounded to two decimals. The portions are based on 50 g available starch

Appendix 3 Chemical analysis of portion size of test products

Table 7A Determination of Dry substance (DS)

Products	Petri dish	Sample (g)	Petri dish + dry sample	Dry substance 1
WWB 1	1.8731	4.3963	4.1840	0.5256
WWB2	1.8790	4.7064	4.3547	0.5260
SNB1	1.8488	4.3705	4.2496	0.5493
SNB2	1.8652	4.6302	4.4045	0.5484
INB1	1.8746	4.3563	4.0424	0.4976
INB2	1.8583	4.2532	3.9638	0.4950

$$\mathbf{DS1} = \frac{(petri\ dish + dry\ sample\ after\ drying\ in\ the\ oven(g)) - (petri\ dish\ (g))}{Fresh\ sampel\ (g)}$$

Table 8A Determination of Dry Substance 2 (DS2)

Products	Aluminium tin (g)	sample (g)	Aluminium tin + dry sample	DS2
WWB 1	1.8541	1.0275	2.7934	0.9150
WWB 2	1.8699	1.0131	2.7961	0.9142
SNB 1	1.8601	1.0362	2.8111	0.9178
SNB 2	1.8809	1.0092	2.8048	0.9155
INB 1	1.9008	1.0114	2.8273	0.9161
INB 2	1.8887	1.8887	2.8164	0.9167

$$DS2 : \frac{(Aluminum + powder\ sample\ after\ drying\ in\ the\ oven(g)) - (Alumini\ tin\ tin\ (g))}{Powder\ sample\ ambient\ dry(g)}$$

Table 9A Glucose standard concentrations

Standard	D-Glucose (mL)	Water (mL)
0 %	0.0	2.0
25 %	0.5	1.5
50 %	1.0	1.0
100 %	2.0	0.0

Table 10A Raw data for samples in absorbance (450 nm) measurements and glucose

Sample	Well Row	Well code	Absorbance	Average based on Raw data (450)	Linear regression fit in ug (450)
WWB	A	9	0.651	0.6535	74.145
	A	10	0.656	0.6535	74.775
	A	11	0.646	0.65045	73.514
	A	12	0.6549	0.65045	74.636
SNB	B	1	0.6299	0.63635	71.485
	B	2	0.6428	0.63635	73.111
	B	3	0.6418	0.6428	72.985
	B	4	0.6438	0.6428	73.237
INB	C	5	0.5429	0.5323	60.517
	C	6	0.5217	0.5323	57.844
	C	7	0.5183	0.51575	57.416
	C	8	0.5132	0.51575	56.773
Glucose Standard 1 (0 ug) (Blank)	A	1	0.0708	0.07225	1.001
	A	2	0.0737	0.07225	1.367
Glucose Standard 2 (25 ug)	A	3	0.2535	0.2526	24.033
	A	4	0.2517	0.2526	23.806
Glucose Standard 3 (50 ug)	A	5	0.4563	0.45355	49.599
	A	6	0.4508	0.45355	48.906
Glucose Standard 4 (100 ug)	A	7	0.8656	0.8612	101.198
	A	8	0.8568	0.8612	100.089

Table 11A Mean value of digestible starch, DS1 and DS2

Products	Digestible starch (%)	DS 1(%)	DS 2 (%)
WWB	74.27	52.58	91.46
SNB	73.24	54.89	91.66
INB	58.13	49.63	91.64

Table 12A Portion sizes of the test product based on 50 g of digestible starch

Products	Digestible starch (%)	Portion sizes (g)
WWB	42.70	117.09
SNB	43.86	116.66
INB	31.49	158.78

Digestible starch $= \frac{(Mean\ Starch\ for\ Sample)}{Mean\ DS2}\ x\ Mean\ DS1$

Portion size $= \frac{(50.0\ g)}{digestible\ starch}$

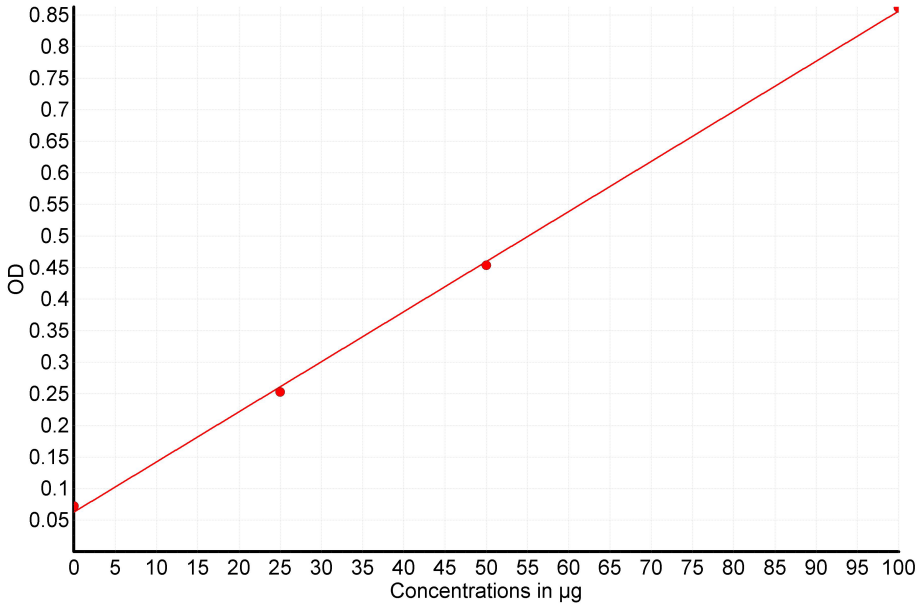


Figure A2: Linear regression of glucose standard measurements. Y = 0.00793X + 0.0289 OD, Optical density

Appendix 4 Starch Hydrolysis

Table 13A iAUC of starch Hydrolysis from 0 – 180 minutes

Person	Product			Hydrolysis Index (HI)	
	WWB	**SNB**	**INB**	**SNB**	**INB**
	As Reference				
x1	103.1	110.3	64.3	107.0	62.3
x2	126.4	96.18	68.7	76.1	54.4
x3	125.5	107.7	60.5	85.8	48.2
x4	135.5	118.4	78.0	87.4	57.6
x5	127.2	107.6	88.8	84.6	69.8
x6	130.3	106.6	89.1	81.8	68.4
Mean	124.7	107.8	74.9	87.1	60.1

HI = (Area Under Curve for the test product/ Area Under Curve for reference product) x 100

Appendix 5 Chemical Solutions

Starch analysis

- 0.30 M NaAc – buffer, pH 4.75: 24.6 NaAc (40.8 NaAc x 3 water) is solved in 900 water. pH is set with acetic acid to 4.75. Solved to a volume of 1000 ml.
- Glox: Frozen Glox is solved in 1000 ml. Tris-buffer.
- 0.50 M Tris-buffer (for Glox-reagens): 61.0 g Tris is solved in 900 ml H_2O. pH is set to 7.0 with 80 – 90 ml. 5M HCI. Solved to a volume of 1000ml.
- Glucose standard: 50 mg D-glucose (anhydrous) is solved in 1000 ml of H_2O
- 4.0 M KOH: 56.1 g dry KOH is solved in 250 ml . H_2O
- 5.0 M HCL: 207 ml concentrated HCI (37%) is solved in H_2O to volume of 500 ml.
- Termamyl 300 L (Novo A/S Köpenhame)
- Amyloglucosidase (3500U/25 ml): 3500U is solved in 25 ml water is frozen in container.
- 0 – 1 M phosphate-buffer, pH 6.0: 12.1 g NaH_2HP0_4 x 2 H_2O is solved in 900 ml H_2O. pH is set to 6.0 and then solved to a volume of 1000 ml.

Fibre analysis

- Aspartic acid
- Termamyl 120 L (300) (Novo A/S, Köpenhame)
- Pepsin (100 mg/ml, 2000 FIP U/g, Merk, Darmstadt, Germany, Art. No. 1.07190.0100)
- Pancreatic enzymes (50 mg/ml, Activity equivalent to 8* USP specifications, sigma, ST. Louis, USA, Art No. P-7545)

- Sodium phosphate buffer (0.1 M, pH 6.0)
- HCI (0.2 N, 0.5 N) to adjust pH
- NaOH (5 N) to adjust pH
- ethanol (78%, 95% and 99%)

Hydrolysis index analysis

- 0.022 M Amylase buffer (tuff – buffer) Dissolve 15.15 g KH_2PO_4, 19.8 g Na_2HPO_4 and 2.0 g NaCl in 4,000 ml. H_2O. Adjust the pH to 6.9 and add water up to 5000 ml. make 10 1/time. Keep in +4 - +8 ºC
- Pepsis – solution (2000 FIB – U/g; MERCK, Darmstadt, Tyskland): Dissolve 5.0 g in 00 ml amylase buffer. Freeze in 7 ml-batches
- α-amylase (SIGMA A – 6255) Make new each analysis time, 30 min before the chewing.
- DNS – Solution: Dissolve 10 g 2 – hydroxy – 3, 5 – dinitrobensoesyra (MERCK 10846) and 300 g K – Na – tartrat – tetrahydrate ($C_4H_4KNaO_6$ x 4 H_2O) in 800 ml water, 16 g NaOH and water up to 1000 ml.
- 2 M NaOH
- 2 M HCI : Dilute 82.3 ml Concentrated HCI to 500 ml.
- Maltose – Standard (1 mg/ml): Dissolve 1,000 g maltos (dry) of 1,050 g maltose (wet) in 1000 ml buffer. Freeze in 10 ml – batches.

Mercodia Insulin ELISA

- Calibrators 1,2,3,4, and 5
- TMB – 3,3´,5´5´ -tetramethylbenzidine
- Stop Solution 0.5 M H2SO4
- Enzyme conjugate 1X solution