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New enzymatic approach on the treatment of fructose malabsorption in adults

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List of Abbreviations

AUC	Area under the curve
BBM	Brush border membrane
CAP	Cellulose acetate phthalate
FGD	Functional gut disorder
FM	Fructose malabsorption
HBT	Hydrogen breath test
HFI	Hereditary fructose intolerance
HRPO	Horseradish peroxidase
IGIU	International glucose isomerase units (see 3.1.4)
SCFA	Short chain fatty acid
SGLT-1	Sodium dependent glucose transporter 1
XI	Xylose isomerase
XIU	Xylose isomerase units (see 3.1.3)

1 Introduction and Hypothesis

Fructose malabsorption is a common digestive disorder resulting from incomplete absorption of ingested fructose. Excess fructose is degraded by bacteria in the large bowel into hydrogen, methane, carbon dioxide and short chain fatty acids leading to symptoms like bloating, wind, abdominal pain or diarrhea. Currently the only treatment is a fructose-free diet which can lead to nutritional imbalances.

The aim of this work is to develop a new treatment of fructose malabsorption based on the in vivo elimination of excess fructose in the small intestine. This should prevent the generation of symptoms and allow the affected persons to eat a normal diet. Since the cause for fructose malabsorption is currently unknown no targets exist to increase the intestinal absorptive capacity for fructose. The excess fructose will therefore be eliminated by enzymatic conversion into other, absorbable compounds.

To achieve this goal it is necessary to identify a suitable enzyme, to devise assays for the determination of enzyme activity and presence, to assess the feasibility of an enzymatic supplementation and to develop a purification step for the target enzyme. Then a pharmaceutical dosage form has to be developed that allows administering the enzyme in a highly active and stable form.

A toxicological study will assess the toxicity of the enzymatic supplementation and an observational study is used to determine the required dose for the elimination of symptoms. Furthermore a randomised clinical trial will be conducted to prove the efficacy of the newly developed treatment. The primary outcome of this trial will be the reduction of the exhaled hydrogen after a provocation with fructose by the active treatment when compared to a placebo.

2 Reference to Literature

2.1 Fructose Malabsorption

2.1.1 Carbohydrate Malabsorptions

Carbohydrates are an important energy source of the human diet. The ingested carbohydrates range from simple monosaccharides (glucose, fructose and galactose) to disaccharides (lactose, sucrose) and complex polysaccharides [DROZDOWSKI and THOMSON, 2006]. The various carbohydrates are digested into their monosaccharide components prior to the transport across the brush border membrane (BBM) of the absorptive enterocytes. The terminal digestion into monosaccharides is performed by carbohydrate-specific hydrolases bound to the membrane of the enterocytes. The monosaccharides are then transported into the enterocyte via specific membrane transport systems [SIBLEY, 2004].

Carbohydrate malabsorptions are either caused by a reduction in intestinal surface area digestion, for example villi degradation after intestinal inflammation, an increase in intestinal motility, defects in the digestive process or defects in the transport process [WRIGHT et al, 2003]. Malabsorptions caused by a reduction in intestinal surface area digestion or by an increased intestinal motility are not sugar specific and may present symptoms with a wide variety of carbohydrates. Defects of the digestive process can be hydrolase-specific in which case only certain types of carbohydrates are incompletely absorbed or may, for example, be due to a pancreatic defect which affects the digestion of all carbohydrates. Defects in sugar transporters are always substrate specific. The undigested carbohydrates are fermented by the bacteria in the large bowel which causes common symptoms like diarrhea, abdominal pain and gas.

The most prominent member of the group of malabsorptions caused by defects in the digestive process is lactose malabsorption. The disaccharide lactose consists of a glucose and a galactose molecule linked by a β(1-4) glycosidic bond. Lactase catalyzes the hydrolysis of lactose into the monosaccharides at the brush border membrane [SIBLEY, 2004]. Lactase deficiency occurs mainly in two types: lactase nonpersistence and lactose intolerance. The very rare congenital lactose intolerance is caused by a defect in the gene encoding the lactase enzyme. This condition is already present in infants and can causes life-threatening diarrhea when fed with milk containing lactose. Treatment is simply removal and substitution of lactose from the diet with a commercial lactose-free formula [HEYMAN, 2006].

Lactase enzyme activity is maximal in preweaned mammals and declines markedly during maturation. This normal maturational decline in lactase activity causes the intolerance of most of the world's adult human population to excessive consumption of lactose containing foods. Lactase nonpersistence is therefore the ancestral state. Only after the introduction of milk in the diet of adults with the development of animal husbandry did the mutation causing the persistence of lactase activity spread [PATTERSON, 2008]. Treatment of lactase nonpersistence consists primarily of avoiding lactose-containing foods. An alternative to lactose avoidance is enzymatic supplementation with lactase derived from microbial sources [SIBLEY, 2004].

Monosaccharide malabsorption is caused by a defect in the transport into the enterocytes via specific membrane transport systems. For example glucose-galactose malabsorption is caused by a defect in the SGLT1 transporter. This autosomal recessive congenital defect can cause severe diarrhea in infants when consuming breast milk or standard infant formulas. The treatment consists of a glucose- and galactose-free diet [SIBLEY, 2004].

The most prominent member of the group of malabsorptions caused by defects in the transport process is fructose malabsorption. But, unlike the other carbohydrate malabsorption, the cause for this gastrointestinal disorder is still unknown. This condition will be discussed further in the following chapters.

2.1.2 Fructose in the diet

Fructose is found widely in the diet as a free hexose, as the disaccharid sucrose and in polymerised form as fructan. Only the first two sources can be utilised by the human body whereas fructans are not absorbed and digested by the intestinal microbial flora in the large intestine. Fructose in its many forms has excited interest from its potential role in symptom generation in patients with irritable bowel syndrome (IBS), as a prebiotic, as a contributor to the obesity crisis, in the pathogenesis of non-alcoholic fatty liver disease, in influencing the glycaemic index of foods and in the pathogenesis of dental caries [GIBSON, 2007].

Fructose is considerably sweeter than sucrose and enhances the flavor and physical appeal of many foods and beverages. It is also used in place of sucrose and other carbohydrates to reduce overall carbohydrate content [BEYER et al, 2005]. Some nutritionists regard fructose as a relatively safe form of sugar since it does not require insulin for uptake into cell and does not adversely affect blood-glucose levels. For these reasons fructose is often recommended for people with diabetes and is included in many weight-loss products [GABY, 2005].

Accurate data on the dietary intake of fructose is unattainable but some researchers have made some attempt at estimating it. Examination of the results from the US Department of Agriculture Food Consumption Survey 1977-78 revealed an approximate daily intake of fructose of between 15g and 54g with a mean of 37g in the US [PARK and YETLEY, 1993]. Since the usage of fructose has changed significantly over the last 30 years these results may not represent today's intake. Especially the development of high fructose corn syrup (HFCS), a sweetener based on free fructose, significantly increased the intake of fructose in the US. From 1970 to 1997 annual per capita intake of HFCS in the US increased from 0.23kg to 28.3kg whereas sucrose consumption decreased from 46.3kg to 30.4kg [GABY, 2005]. HFCS only plays a minor role in the European Union and is subject to a production quota. 2010 this quota was set to 270,000t whereas sugar production

amounted to 14.8 million tons [European Commission Agriculture and Rural Development, 2011].

Fructose has been discussed on having adverse effects on human health. It is generally agreed that excessive consumption of refined sugars of any type is undesirable since it primarily consists of empty calories. Additionally fructose as a reducing sugar may form toxic advanced glycation end-products which appear to accelerate the aging process and to play a role in the pathogenesis of diabetes complications and cardiovascular disease. Excessive fructose consumption has also been shown to cause hypertriglyceridemia, hyperuricemia, to induce insulin resitance and to be a main cause of symptoms in some patients with IBS. Finally, there is evidence that it is responsible in part for the increasing prevalence of obesity, type 2 diabetes mellitus, and non-alcoholic fatty liver disease [GABY, 2005]. The impact of this data on dietary recommendations is controversial and especially HFCS in the diet as a source of fructose has been the subject of several studies to assess the impact on health [ANGELOPOULOS et al, 2009; MELANSON et al, 2008]. At the present time, there is insufficient evidence to restrict the use of fructose-containing sweeteners in the food supply. Nevertheless, dietary advice to limit fructose consumption is warranted [MOELLER et al, 2009].

2.1.3 Inborn errors of fructose metabolism

Carbohydrate intolerances are caused by inabilities of the body to metabolize specific carbohydrates. These conditions should be clearly differentiated from malabsorptions and are almost always caused by single enzyme deficiencies. Until now five abnormalities in fructose metabolism have been recognised as caused by genetically determined deficiencies in the metabolism of fructose: deficiencies of fructokinase, aldolase A, aldolase B, fructose-

1,6-diphosphatase and glycerate kinase [HOMMES, 1993]. Of these conditions only aldolase B deficiency occurs with a significant frequency and will be discussed further.

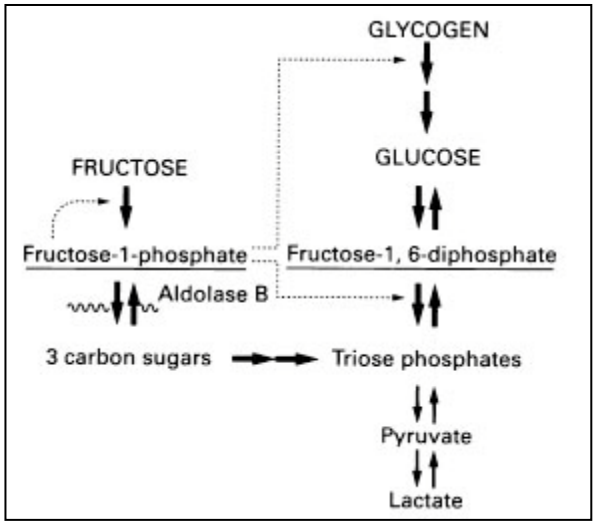

Figure 2.1.3.1 Fructose metabolism in healthy subjects 3-carbon sugars: D-glyceraldehyde 3-phosphate, D-glyceraldehyde, dihydroxyacetone phosphate. Dotted arrows indicate point of secondary metabolic arreast in HFI. Taken from ALI et al, 1998.

Aldolase B is an enzyme of liver, intestine and renal cortex [SANTER et al, 2005] where it catalyzes the reversible cleavage of fructose-1-phosphate. Once exogenouse fructose enters these cells, it is rapidly phosphorylated into fructose-1-phosphate by the action of fructokinase and further cleaved by aldolase B. The generated 3-carbon sugars D-glyceraldehyde and dihydroxyacetone phosphate can be incorporated either into the glycolytic pathway or the gluconeogenic pathway. When aldolase B activity is deficient, fructose-1-phosphate rapidly builds up and depletes intracellular inorganic phosphate and ATP. This further causes an inhibition of fructokinase, glycogenolysis and of the formation of the gluconeogenic intermediates fructose-1-6-bisphosphate and glucose-6-phosphate [ALI et al, 1998].

Aldolase B deficiency or hereditary fructose intolerance HFI (OMIM#229600) is a recessive autosomal genetic defect in which affected homozygotes develop hypoglycemia

and severe abdominal symptoms after ingesting foods containing fructose. Persistent intake of fructose in infants leads to a syndrome of chronic toxicity which can also cause irreversible damage to liver and kidney. Affected patients often develop a self-protective aversion to sweets and fruits and may therefore remain undiagnosed for many years. Treatment of HFI consists of a strict exclusion of fructose from the diet [ALI et al, 1998]. The prevalence of HFI in central Europe is estimated to be 1 in 26,100 [SANTER et al, 2005].

2.1.4 Fructose Malabsorption

The capacity for the human intestine to absorb fructose is limited and varies between individuals. When consuming foods and beverages with fructose as the dominant sugar, the capacity for absorption of fructose can easily be exceeded [BEYER et al, 2005]. Up to 80% of a healthy population test positive for fructose malabsorption when challenged with 50g fructose [GIBSON et al, 2007]. Therefore fructose malabsorption itself is not pathological since the excess sugar does not necessarily cause gastrointestinal symptoms. It can only be considered a disorder if gastrointestinal symptoms are observed in affected patients [GIBSON et al, 2007]. Although absorptive capacity of the intestine is not gender-dependent [GIBSON et al, 2007], an increased prevalence of symptomatic fructose malabsorption has been suggested in the female population [SZILAGYI et al, 2007]

Delivery of free fructose to the lumen of the distal small intestine and large bowel can cause several changes in luminal conditions [GIBSON et al, 2007]:

- *Osmotic load*: Excess luminal fructose exerts an osmotic effect and causes an increase of the liquidity of the luminal content.

- *Substrate for bacterial fermentation*: Fructose is fermented by bacteria of the large bowel into short-chain fatty acids (SCFAs) and the gases hydrogen, carbon dioxide and methane.

- *Gastrointestinal motility*: Products of bacterial fermentation [CHERBUT et al, 1997] and the increased liquidity of the luminal content may shorten the bowel transit time by affecting gut motility and cause diarrhea.

- *Prebiotic effect*: Fructose may promote the growth of bacterial populations and the synthesis of the mucosal biofilm [GIBSON, 2007]. However, excess fructose has been shown to not cause colonic microflora adaption suggesting that this increase in bacterial population is not selective [SZILAGYI et al, 2007].

- *Depression*: Fructose malabsorption has been associated with depression in young women [LEDOCHOWSKI et al, 2000b]. Whether this is a direct result of the malabsorbed fructose, a cause for the symptoms of fructose malabsorption or rather a consequence of the IBS experienced by the patients remains to be investigated.

The most common reported symptoms are abdominal rumbling, abdominal pain, abdominal cramping, diarrhea and flatulence [LEDOCHOWSKI et al, 2000a]. Other reported symptoms include bloating, nausea and constipation [SHEPHERD and GIBSON, 2006]. Some patients claim to suffer from head ache, dizziness, mood swings or fatigue after ingesting a fructose containing meal [PFISTERER M., personal communication].

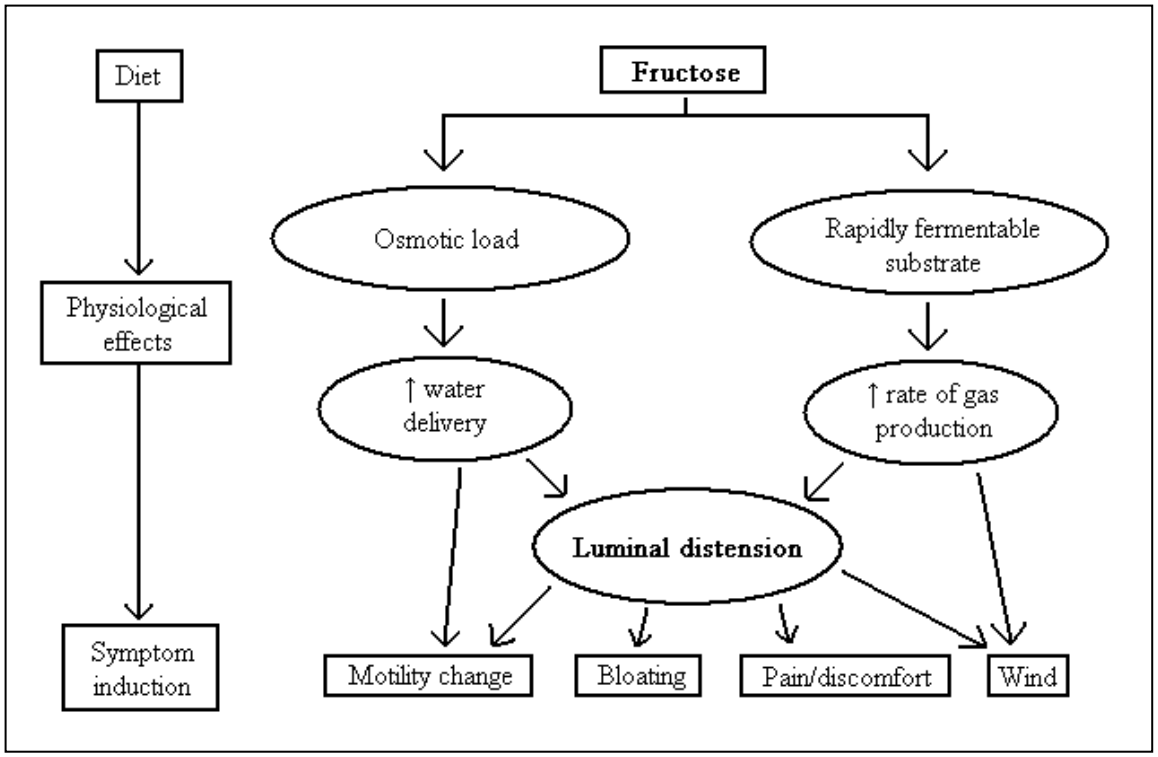

Figure 2.1.4.1 Consequences of malabsorbed fructose Effects of malabsorbed fructose in the distal small intestine and proximal large bowel. Adapted from BARRETT and GIBSON, 2007.

Hydrogen breath tests are now commonly used to diagnose carbohydrate malabsorptions. These tests are based on the fact that the only source for hydrogen gas in humans is the bacterial degradation of carbohydrates in the large bowel [SIMREN and STOTZER, 2006]. For the detection of FM fructose is administered orally and the concentration of end expiratory hydrogen is measured. A rise in hydrogen concentration by 20ppm over baseline within 2 hours is considered as tested positive for fructose malabsorption [LEDOCHOWSKI et al, 2000a]. However, there are several issues of concern with the test [GIBSON, 2007]:

- *Non-hydrogen producers*: Up to 28% of humans utilize hydrogen efficiently in other ways and/or have a predominance of bacteria that do not produce hydrogen [GIBSON, 2007]. These patients will yield a negative hydrogen breath tests even

despite being malabsorbers. These patients can be detected with a lactulose hydrogen breath tests which is usually not done during routine testing.

- *The cut-off value:* While a cut-off value of 20ppm is commonly used, a cut-off of 10ppm has not adversely affected the interpretation [KARCHER et al, 1999]. This problem is further enhanced by the fact that different hydrogen measurement devices may yield different ppm readings.

- *Role of symptoms*: Symptoms during testing do not correlate with the degree of hydrogen production. Furthermore it is not clear whether an absence of symptoms during testing gives any indication as to the role of fructose in the genesis of the patient's gastrointestinal symptoms after meals [GIBSON, 2007]. Some authors have even excluded fructose malabsorption after the lack of symptom induction which is problematic due to the highly artificial nature of the test [RUMESSEN and GUDMAND-HOYER, 1988].

- *Dosage and concentration of fructose*: The higher the used dose and concentration the greater is the chance of a significant rise in breath hydrogen concentration, even in healthy subjects [JONES et al, 2011]. Usually 25 or 50g fructose at a concentration of 10% (w/v) is used for testing. RAO et al [2007] suggest after evaluation of the capacity to absorb fructose to use 25g fructose with a concentration of 10%(w/v) as the dose for testings subjects with suspected fructose malabsorption.

- *Reproducibility of the test result*: There is limited data on test-retest performance of fructose breath hydrogen tests. Where peak hydrogen concentration of breath tests with lactulose as non-absorbable carbohydrate were reproducible with a deviation of 20% [RUMESSEN et al, 1990], the deviation of peak hydrogen in healthy volunteers after 50g fructose was higher with 36% [TRUSWELL et al, 1988]. The qualitative reproducibility of fructose tests used in routine practice is generally assumed but has received little attention [GIBSON, 2007].

The prevalence of fructose malabsorption cannot be assessed easily because of these problems. Testing whole populations would also include asymptomatic fructose malabsorbers, a condition that is not pathogenic. Several experiments have been done to estimate the prevalence of fructose malabsorption in populations with functional gut disorders (FGDs) [BEYER et al, 2005; BORN et al, 2006; CHOI et al, 2003; FERNANDEZ-BANARES et al, 2006; SHEPHERD et al, 2008]. In these studies patients with unexplained gastrointestinal disorders were examined with results ranging between 55% and 73% fructose malabsorbers. Since approximately 15% of the world population seem to be affected by FGDs [BARRET and GIBSON, 2007] this would set the prevalence of symptomatic fructose malabsorption at approximately 8 to 11%. Usually patients are considered symptomatic fructose malabsorbers only after a successful dietetic intervention. Patients report improvements after a dietetic intervention in approximately 70% of the cases (BORN et al [2006] reported 75% and FERNANDEZ-BANARES [2006] et al reported 67%). This would yield estimates of the prevalence of symptomatic malabsorption of approximately 6 to 8%.

Dietetic interventions are the only used therapeutic measures so far. Usually a diet low on fructose and sorbitol is used to eliminate gastrointestinal symptoms in patients with FM [LEDOCHOWSKI et al, 2000a]. A recent trend has emerged in reducing all poorly absorbed, short-chain carbohydrates in the diet since all fermentable carbohydrates can cause symptoms in sensitive individuals. These carbohydrates are grouped as FODMAPs (Fermentable Oligo-, Di- and Monosaccharides And Polyols) [BARRETT and GIBSON, 2007] and include fructo-oligosaccharides, galacto-oligosaccharides and polyols like sorbitol, xylitol or mannitol [GRABITSKE and SLAVIN, 2009].

2.1.5 Intestinal uptake of fructose

The monosaccharides resulting from the already present monosaccharides in the meal and from the hydrolysis of the carbohydrates by enzymes of the brush-border membrane are transported across the brush-border membrane of the absorptive enterocytes. Whereas glucose and galactose are transported actively across the brush border membrane via the sodium dependent SGLT1 transporter, fructose is only passively transported across the membrane [DROZDOWSKI and THOMSON, 2006].

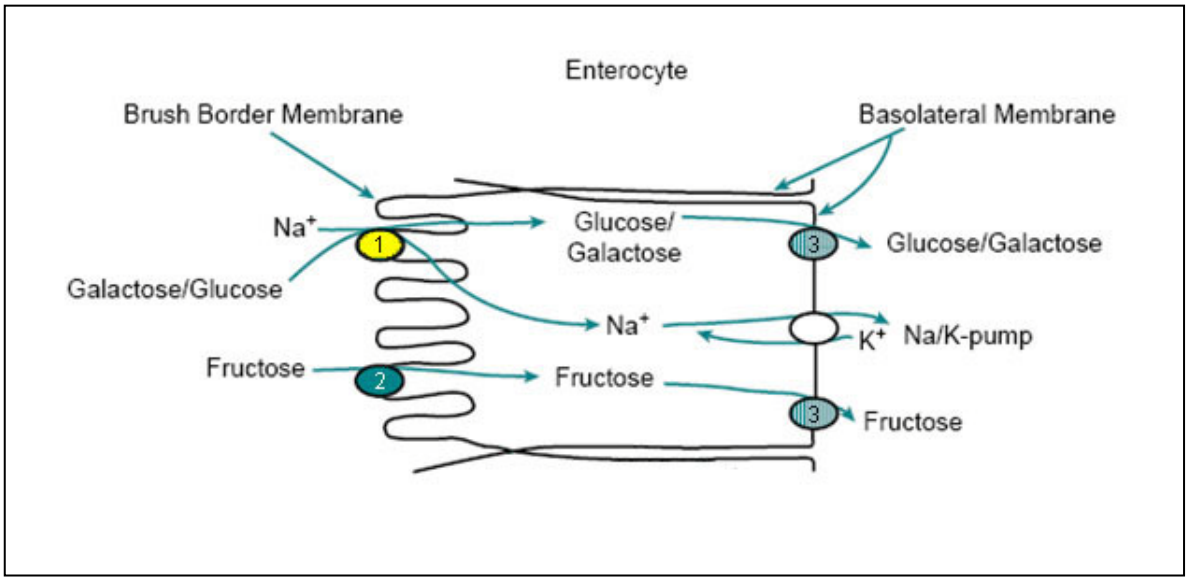

Figure 2.1.5.1 Classical model of the intestinal sugar transport Glucose and galactose are transported actively across the brush border membrane with the Na^+-dependent transporter SGLT-1. Fructose is transported passively into the enterocyte with the GLUT-5 transporter. All three monosaccharides are then transported across the basolateral membrane with the GLUT-2 transporter. The sodium gradient is maintained with a Na^+/K^+-pump.1: SGLT-1; 2: GLUT-5; 3: GLUT-3. Adapted from WRIGHT et al, 2003.

This passive transporter for fructose is GLUT-5, a high affinity transporter exclusively for fructose. In the classical sugar transport model (see 2.1.5.1) GLUT-5 is the only fructose transporter in the BBM whereas GLUT-2, a low affinity passive transporter for glucose,

galactose and fructose, is solely located in the basolateral membrane and transports these sugars out of the enterocyte [DROZDOWSKI and THOMSON, 2006].

Only recently a new model evolved where GLUT-2 is inserted into the BBM when luminal concentration of glucose is increased. In this model the increase of luminal glucose after a meal triggers a response from the enterocyte to increase the absorptive capacity. This increase in absorptive capacity is achieved via integration of the passive GLUT-2 transporter [DROZDOWSKI and THOMSON, 2006]. This might explain the findings of TRUSWELL et al [1988] that glucose increases the absorption of fructose. Their experiments showed no positive hydrogen breath test for fructose when the fructose was combined with glucose or given as sucrose in individuals that malabsorb the same amount of fructose when given alone.

GLUT-5 has been extensively studied because it was long believed to be the main fructose transporter across the BBM. Expression study and localisation of GLUT-5 in humans revealed that GLUT-5 is relatively abundant in the small intestine and localised to the BBM of enterocytes [DAVIDSON et al, 1992]. It is also developmentally regulated and only expressed in adult rats after completion of weaning [DOUARD and FERRARIS, 2008]. Luminal fructose also increases the expression of GLUT-5 [DROZDOWSKI and THOMSON, 2006] which would suggest an increase in absorptive capacity when fructose is added to the diet. This effect has not yet been proven in humans and remains controversial.

No reason has so far been found for the varying absorptive capacity of fructose in different humans. WASSERMAN et al [1996] were unable to find mutations in the GLUT-5 transporter of patients with fructose malabsorption suggesting that the transporter itself is not the reason.

2.1.6 Generation of symptoms in fructose malabsorbers

The mechanism of gastrointestinal symptom generation by malabsorbed carbohydrates is not fully understood [SKOOG et al, 2004]. The primary reasons for symptom generation are quite easy and simple. The fructose is metabolised by bacteria in the large bowel yielding degradation products like short chain fatty acids SCFAs and the gases hydrogen, carbon dioxide and methane. SCFAs in turn cause diarrhea via osmotic pressure and an increase in intestinal motility [MADSEN et al, 2006] while the gases cause symptoms like bloating and flatulence [BEYER, 2005]. These reactions are most likely to occur in every person with fructose malabsorption but do not always cause symptoms. The symptom generation by malabsorbed sugars does not depend on the type of carbohydrate. In patients with more than one carbohydrate malabsorption symptoms occur either after each malabsorption or after none [BORN, 2007]. So the main question is: what affects symptom and pain perception in patients with fructose malabsorption and causes the distinction between symptomatic and asymptomatic malabsorbers?

One influence that is often suggested is the pattern of the colonic flora. BORN et al [1995] found a difference in the colonic bacterial activity of symptomatic and asymptomatic malabsorbers but failed to reproduce their results in a larger study [Born et al, 2000]. A study by VALEUR et al [2009] also found no difference after analyzing the stool of patients with carbohydrate sensitivity when compared to controls although symptom generation seemed to be induced by microbial fermentation. Currently a lot interest is focused on the microbial flora in the intestine. In the past years several attempts to analyze the human microbiome and its diversity have been made [ECKBURG et al, 2005; GILL et al, 2006; TRINGE et al, 2005]. Also a combined effort to understand the microbial components of the human metabolic landscape and how they contribute to normal physiology and predisposition to disease (The Human Microbiome Project) is under way [TURNBAUGH et al, 2007]. With the results of this project and the new techniques it may be feasible to precisely analyze the microbioal flora composition in symptomatic and

asymptomatic malabsorbers. This may yield important insight into symptom generation from carbohydrate malabsorption.

Depression has also been associated with symptomatic fructose malabsorption [LEDOCHOWSKY et al, 2000b; VAREA et al, 2005]. A definite reason for this increased incidence of depression is yet to be found but tryptophan depletion because of a formation of fructose-tryptophan complexes in the intestine has been suggested by VAREA et al [2005]. Tryptophan depletion has also been shown to influence the brain-gut response [KILKENS et al, 2004] and to increase gastrointestinal and anxiety symptoms in IBS patients [SHUFFLEBOTHAM et al, 2006]. There is also evidence of increased tryptophan degradation in IBS patients [CLARE et al, 2009]. Therefore tryptophan levels may have a strong influence on gastrointestinal symptom generation. Still, the increased prevalence of depression among symptomatic fructose malabsorbers remains controversial. The associated depression may, for example, be caused by the reduced life quality because of frequent gastrointestinal symptoms without being involved in fructose malabsorption or symptom generation.

Neuromodulatory interventions on the brain-gut axis have been reported to be efficacious in reducing the gastrointestinal symptoms in patients with IBS [GAMAN and KUO, 2008]. This suggests a possible role of the brain-gut axis in the generation of symptoms in patients with fructose malabsorption. Brain-gut-enteric microbiota interactions have also been suggested to be involved in the regulation of gastrointestinal motility, secretion and pain modulation [RHEE et al, 2009]. The significance of this for symptom generation in carbohydrate malabsorption is yet to be determined but may suggest another pathway for the colonic bacterial activity to influence symptom generation.

2.2 Enzymatic supplementation as a therapeutic measure

2.2.1 Lactose nonpersistence

Exogenous β-galactosidase has been used for some time for the treatment of lactose nonpersistence. It is either used to hydrolyze the lactose *in vitro* yielding for example lactose-free milk or to assist the digestion of lactose in the digestive tract [LISKER et al, 1989]. For these two applications two different β-galactosidase preparations are used. β-galactosidase from Kluyveromyces *lactis* or Kluyveromyces *marxianus* with a pH optimum of 6,5 is used for the *in vitro* applications. Since these β-galactosidase preparations would be inactivated in the stomach β-galactosidase from Aspergillus niger or Aspergillus oryzae with a pH optimum of 3 and 3,6 is used for *in vivo* supplementation.

The efficacy of β-galactosidase supplementation has been studied extensively [LIN et al, 1993; LISKER et al, 1989; ONWULATA et al, 1989; RAMIREZ et al, 1994; SANDERS et al, 1992] usually with a positive result. RAMIREZ et al [1994] compared different commercially available β-galactosidase preparations and found significant differences in the efficacy of these products. Interestingly only one product reduced the AUC of the HBT after lactose provocation significantly. This product was also the only one that did not reduce symptoms while all other preparations reduced one or more symptoms but failed to influence the breath test.

The mode of action of the *in vitro* β-galactosidase use is simply the hydrolysis of lactose in the product prior to consumption. Therefore intestinal β-galactosidase is not needed any more. This may however influence the flavor and physical appeal of the manipulated food. For the *in vivo* supplementation all currently available products aim to hydrolyze the lactose in the acidic environment of the stomach. The supplemented enzyme will cease to work once it reaches the intestine because of the drastic change of the pH of the environment.

2.2.2 Fructose Malabsorption

Until now the only treatment for FM is the elimination of fructose from the diet. This restricts the intake of fruits and vegetables and can lead to malnutrition. Therefore possible addition of fructose to the diet would be preferable.

A treatment of FM in adults would need to enhance the intestinal absorptive capacity for fructose, convert the fructose into an easily absorbed carbohydrate or inhibit the symptom generation. Since the reasons for the reduced absorptive capacity and the symptom generation in affected individuals is not known the only possibility is the conversion of the fructose by enzymatic manipulation before it reaches the large bowel. For this only enzymes that have free, unaltered fructose as a substrate can be used. And since the addition of substances is only possible in a limited amount this reaction must work with fructose alone without another reactant.

Only two enzymes are known to act on D-fructose in its unphosphorylated free form without another reactant. These enzymes are mannose isomerase and xylose isomerase.

Mannose isomerase (E.C. 5.3.1.7) reversibly converts fructose into mannose [PALLERONI and DOUDOROFF, 1956]. The in the intestine generated mannose would then have to be absorbed by the intestine to eliminate symptoms in patients with FM. Mannose seems to be absorbed via two transport systems: one sodium dependent active transporter that is not SGLT-1 and a passive transporter other than GLUT-5 [DURAN et al, 2004]. Since the exact absorption mechanism and the absorptive capacity for mannose are unknown the use of mannose isomerase is not advisable although suitable sources for the enzyme exist [HEY-FERGUSON and ELBEIN, 1970; HIROSE et al, 2001].

Xylose isomerase (EC 5.3.1.5) reversibly converts fructose into glucose which would then be absorbed via the SGLT-1 transporter. This conversion would remove the fructose from

the intestine and eliminate the cause for the symptoms in adults with fructose malabsorption.

2.2.3 Xylose isomerase

Xylose isomerase is expressed by saprophytic bacteria and is important for the degradation of hemicellulose. Originally it catalyzes the reversible isomerisation of xylose to xylulose and thus helps the bacteria to degrade the xylose from hemicellulose [BHOSALE et al, 1996]. It soon aroused interest in the industry for its ability to convert other monosaccharides. Xylose isomerase from several sources is able to catalyze the reversible isomerisation of glucose to fructose. This reaction is needed for the production of high fructose corn syrup HFCS, a sweetener that was developed because of the lack of supply of sucrose in the US after the Cuban revolution 1958 [BHOSALE et al, 1996].

HFCS is made from starch in three steps. First the starch is liquefied by α-amylase and then the resulting oligosaccharides digested to glucose with amyloglucosidase and a debranching enzyme [BHOSALE et al, 1996]. The resulting glucose syrup has two major disadvantages to other sweetners: pure glucose is less sweet than fructose or sucrose and glucose syrup is not liquid at room temperature. Therefore in a third step the glucose in this syrup is partly converted with xylose isomerase to form HFCS, a glucose-fructose syrup that has comparable sweetness to sucrose and is liquid at room temperature.

This industrial usage has made xylose isomerase to be one of the three highest tonnage enzymes and caused extensive studies on its function to convert glucose to fructose. It has been purified and characterised from several sources [CHAUTHAIWALE and RAO, 1994; CHEN et al, 1979a; CHEN et al, 1979b; CHEN and ANDERSON, 1979; SCHELLENBERG et al, 1984; VIOT et al, 1987; YAMANAKA, 1968] including higher plants [PUBOLS et al, 1963]. The reaction mechanism is known in minute detail with

crystal structures of every intermediate step [ASBOTH and NARAY-SZABO, 2000; COLLYER et al, 1990; RANGARAJAN and HARTLEY, 1992].

Xylose isomerase from all known sources has several disadvantages for the industrial usage for the production of HFCS: the extremely low turnover rate of about 2 s^{-1} for glucose and fructose [ASBOTH and NARAY-SZABO, 2000], the metal ion requirement of the active center [VAN BASTELAERE et al, 1991; VAN BASTELAERE et al, 1992] and the pH optimum in the slightly alkaline which is different from the pH optimum of the enzymes α-amylase and amyloglucosidase [BHOSALE et al, 1996]. Several attempts at mutating the active center to change these properties have been made [CHA et al, 1994; JENKINS et al, 1992; LAMBEIR et al, 1992; VAN TILBEURGH et al, 1992] but without much success.

The industrial use and the extensive knowledge of this enzyme made xylose isomerase an optimal candidate for enzyme supplementation for the treatment of FM in adults. The enzyme is commercially available which eliminates the need to establish an expression method and is relatively cheap. Unfortunately the enzyme was characterised and is used at conditions (60°C, 2M glucose) very different from the environment found in the stomach or the small intestine. Therefore a complete biochemical characterisation was needed to assess the feasibility of xylose isomerase supplementation. For this work xylose isomerase from *Streptomyces rubiginosus* was used which was provided by Genencor International.

But unlike the β-galactosidase used for enzyme supplementation in patients with lactose nonpersistence xylose isomerase is inactive at low pH. Therefore it cannot be used to convert the fructose in the stomach. The location of activity for xylose isomerase has to be the small intestine which has a favorable slightly alkaline environment. Therefore a different pharmaceutical formulation has to be found for xylose isomerase to ensure the delivery of the active enzyme into the intestine.

One of the main problems will be the extremely low turnover rate of the enzyme. Industrially the enzyme is used at 60°C which increases the activity approximately by the factor 5 when compared to 37°C. This low activity at 37°C makes it necessary to supplement high amounts of enzyme with the highest activity possible. A way has to be

found to administer these high doses of xylose isomerase that will be accepted by the target group.

3 Materials & Methods

3.1 Biochemical Methods

3.1.1 Fructose determination with adapted Carbazole/H2SO4 assay

The assay is a microtiter-plate adapted version of the fructose test by DISCHE and BOHRENFREUND [1951]. The assay was developed in the course of this work.

Add 50µl of a solution containing 0 – 600µM Fructose and 10µl of a 1.5% (w/v) solution of cystein hydrochloride in H_2O per well into a clear polystyrene flat-bottom 96-well microtiter-plate. The plate is incubated at 60°C for 15'. A 80% (w/w) solution of H_2SO_4 and a 0.12% (w/v) solution of carbazole in ethanol p.a. are mixed with the ratio of 24:1 and incubated at 60°C for 15'. Add 250µl of the H_2SO_4/carbazole solution per well and incubate at 60°C for 10'. The absorbance is determined with a spectrophotometer at a wavelength of 560nm.

Assay principle: The exact chemical reaction for this assay is unknown. The fructose reacts with the cystein under water removal by the concentrated H_2SO_4 and forms a violet compound that is determined spectrophotometrically.

3.1.2 Glucose determination with chemiluminescence assay

The assay is based on a diamine oxidase activity test [MAYER et al, 2007]. A luminometer with 2 injectors able to inject 50 and 100µl per well is needed for the assay. The assay was developed in the course of this work.

20µl of a solution containing 0 to 20mM glucose is added to each well of a microtiter plate for luminescence assays. 100µl 50mM Tris pH 8.5 with 5mM NaCl and 2.5U/ml glucose oxidase (VWR, CatNr A4669.0001) is added per well with the help of the injector of the luminometer and incubated for 30' at RT. Then prepare a solution containing 50mM Tris pH 8.5 with 5mM NaCl, 3U/ml HRPO (Sigma, CatNr P8375) and a luminescence reagent in a dilution of 1:10 (PSatto, which is not commercially available any more, was used during this work). Add 50µl of this solution per well, wait for 10" and then measure the emitted light over 15". To avoid a drift of the results the time difference between addition of the glucose oxidase and measurement should be identical for all wells.

Assay principle: The glucose oxidase oxidizes the glucose to D-glucono-1,5-lactone and forms H_2O_2. The HRPO degrades the H_2O_2 and excites the luminal in the luminescence reagent which then emits light. This emitted light is measured.

3.1.3 Xylose isomerase activity determination (XI-Units)

One XI Unit (XIU) is defined as the enzyme activity needed to convert one µmol glucose into fructose or one µmol of fructose into glucose per hour in 50mM phosphate buffer pH 7.4 with 5mM MgSO4, 1mM cobalt acetate and 100mM glucose or fructose at 37°C. This assay was usually used to analyze activity of the enzyme. 1 XIU equates to approximately 11 IGIU.

A XI containing solution is diluted or XI powder solubilised to an activity of approximately 30 XIU/ml in 50mM phosphate buffer pH 7.4. 50µl of this solution is pipetted into each well of a microtiter plate. 100µl 75mM phosphate buffer pH 7.4 with 10mM MgSO4 and 2mM cobalt acetate (unless specified otherwise) is added to each well and the plate incubated for 15’ at 37°C. 50µl 400mM glucose solution preheated to 37°C is then added and incubated for 45’ at 37°C. The reaction is stopped by adding 50µl 1M HCl to each well. This reaction mixture is then diluted suitably with distilled H_2O and the fructose concentration measured with the adapted Carbazole/H_2SO_4 assay (see 3.1.1). Negative controls are mandatory to account for all interfering substances in the Carbazole/H_2SO_4 assay.

Other metal ions used in the buffer are $MnSO_4$, $ZnCl_2$, $FeCl_2$, $NiSO_4$ and $CuSO_4$. All these salts were used to replace the cobalt acetate and were used in the same concentration as cobalt acetate.

Assay principle: The xylose isomerase converts the added glucose to fructose which is then measured.

3.1.4 Xylose isomerase activity determination (IGIU-Units)

One International Glucose Isomerase Unit (IGIU) is defined as the enzyme activity needed to convert one µmol glucose into fructose per minute in 100mM maleate buffer pH 6.85 with 20mM MgSO4, 1mM $CoCl_2$ and 2M glucose at 60°C.

A XI containing solution is diluted or XI powder solubilised to an activity of approximately 7.5 IGIU/ml in 100mM maleate buffer pH 6.85 with 20mM $MgSO_4$ and 1mM $CoCl_2$. 1ml 4M Glucose is mixed with 0.4ml 400mM maleate buffer pH 6.85, 64µl 500mM $MgSO_4$, 16µl 100mM $CoCl_2$ and 120µl distilled H_2O in an appropriate reaction tube. The enzyme

solution and the reaction tube are incubated for 15’ at 60°C. Then 400µl of the enzyme solution is added to the reaction tube, vortexed briefly and incubated at 60°C for 60’. The reaction is stopped by adding 500µl 1M HCl. The reaction mixture is then diluted suitably with distilled H_2O and the fructose concentration measured with the adapted Carbazole/H_2SO_4 assay (see 3.1.1). Negative controls are mandatory to account for all interfering substances in the Carbazole/H_2SO_4 assay.

Assay principle: The xylose isomerase converts the added glucose to fructose which is then measured.

3.1.5 Xylose isomerase activity determination for routine measurement

This assay was developed for routine measurements because the normal Carbazole/H_2SO_4 assay is not user friendly and stable enough for routine handling.

A XI containing solution is diluted or XI powder solubilised to an activity of approximately 30 XIU/ml in 50mM phosphate buffer pH 7.4. 50µl of this solution is pipetted into each well of a microtiter plate. 100µl 75mM phosphate buffer pH 7.4 with 10mM MgSO4 and 2mM cobalt acetate is added to each well and the plate incubated for 15’ at 37°C. 50µl 400mM fructose solution preheated to 37°C is then added and incubated for 60’ at 37°C. The reaction is stopped by adding 50µl 50mM EDTA to each well. This reaction mixture is then diluted suitably with distilled H_2O and the glucose concentration measured with the chemiluminescence assay (see 3.1.2).

Assay principle: The xylose isomerase converts the added fructose to glucose which is then measured. The EDTA removes the metal ions from the xylose isomerase and thus inactivates it.

3.1.6 Pellet acid resistance and dissolution testing

100mg pellets are weighed into 5ml reaction tubes. If the acid resistance is to be tested 1.5ml 0.1M HCl are added to the pellets and incubated on a rotamix for 15' at 37°C. Then the HCl solution is removed and 4ml 50mM phosphate buffer pH 7.4 is added. The pellets are incubated with the phosphate buffer on a rotamix for 60' at 37°C. 1ml is then removed and centrifuged for 1' at 6000g. The supernatant is diluted accordingly and used for further testing.

3.1.7 Xylose isomerase sandwich ELISA

WPL:

2.42g Tris (=20mM)

0.88g NaCl (=15mM)

1ml Tween20

Add distilled H_2O to 900ml, adjust pH with 1M HCl to 9.6 and top up to 1L.

The primary purified α-XI antibody is diluted to 3µg/ml in 20mM carbonate buffer pH 9.6 with 0.01%(v/v) thimerosal. 100µl of this solution is pipetted into each well of a high binding optical microtiter plate and incubated over night at 4°C. The plate is then blocked with 20mM carbonate buffer pH 9.6 with 0.01%(v/v) thimerosal, 0.1%(v/v) gelatin from cold water fish and 5%(w/v) saccharose for 1 hour at RT. The microtiter plate is then dried and can be stored dry at 4°C for several months.

Xylose isomerase is diluted in 50mM phosphate buffer pH 7.4 to a concentration of 0 to 25 ng/ml and 100µl pipetted into each well. The plates are incubated over night at 4°C and

then washed 4 times with 300µl WPL per well. The HRPO-coupled secondary α-XI antibody is diluted in StabilZyme HRP Conjugate Stabilizer (SurModics, CatNo SZ02), 100µl of the diluted antibody added to each well and incubated for 3h at 37°C. The plate is then washed 4 times with 300µl WPL per well. Then 100µl TMB are added and the reaction stopped with 1M HCl when enough color developed. The absorbance is determined with a spectrophotometer at a wavelength of 450nm.

Assay principle: The primary α-XI antibody is coated and binds the xylose isomerase. After washing a HRPO-coupled secondary α-XI antibody is added and binds the bound xylose isomerase. The coupled HRPO later converts the TMB to yield a blue product which turns yellow after addition of acid. This product is then determined spectrophotometrically.

3.1.8 Ammonium sulfate precipitation

Gensweet SGI was provided by Genencor International.

300g of Gensweet SGI from Genencor containing XI at 3000-3500 IGIU per g of solution is topped with distilled H_2O to 1L. Then 1L saturated ammonium sulfate solution is added slowly under stirring at RT. After another 20’ of stirring at RT the solution is centrifuged for 40’ at 10,000g and the pellet suspended in 500ml 25mM maleate buffer pH 6.85 with 5mM MgSO4, 1mM CoCl2 and 500mM NaCl. The suspended pellet can then be stored at -20°C for at least 2 years.

3.1.9 Crystallisation

Gensweet SGI was provided by Genencor International.

1kg of Gensweet SGI from Genencor containing XI at 3000-3500 IGIU per g of solution is cooled to 4°C. 654g ammonium sulfate (Molekula CatNo 10565375) and 45.5g magnesium sulfate heptahydrate (Merck CatNo 1.72572.9025) are added to 2.52kg distilled H_2O, stirred until completely dissolved and cooled to 4°C. The ammonium sulfate solution is then added to the Gensweet SGI solution over 3 hours under constant slow stirring at 4°C. Then 8g XI crystals from an earlier crystallisation are added to speed up the crystallisation process. The obtained solution is then left stirring slowly for 3-5 days at 4°C. At this stage a turbidity sensor can be used to control the crystallisation process. This solution is then either centrifuged for 15' at 5,000g to obtain crystal slurry or processed further into powder (see 3.1.10).

3.1.10 Filtration and Lyophilisation

The solution containing the crystals is filtered through a cellulose paper filter grade 1288 (e.g. Sartorius, CatNo Ft-2-206-580580). The filter cake is then frozen at -30°C and lyophilised until completely dry. The temperature during lyophilisation is raised from -30°C to 4°C over 6 hours and remains at 4°C until the end of lyophilisation. Add 126µmol $MgSO_4$ per g dried filter cake to the dried filter cake. The dry filter cake with the $MgSO_4$ is then grinded on a MF10 basic Microfine grinder drive from IKA with a cutting-grinding head and a 0.5mm sieve. The obtained powder is ready for further processing and stable for at least 1 year at 4°C.

3.1.11 Antibody purification

An Äkta purifier FPLC machine from GE Healthcare was used.

A HiTrap NHS-activated column (GE Healthcare, CatNo 17-0716-01) was coupled with xylose isomerase according to manual. The bleed was diluted 1+4 in 100mM borate buffer pH 8.0 and loaded with 0.2ml/min onto the HiTrap XI-coupled column preequilibrated with borate buffer. The column was then washed with borate buffer until the UV reading returned to base levels. The bound antibody was eluted with 100mM citrate buffer pH 3.0 and fractions taken when the UV detector recorded elevated absorbance at 280nm. These fractions were then tested and all fractions with a high titer pooled. If needed the volume of the eluate was reduced with a 3kDa microcon centrifugal filter device from Millipore.

3.1.12 Testing bleeds for specific antibodies

APF:
23.83g Hepes (=100mM)
9.56g Guanidin-Hydrochlorid (=100mM)
0.5g Brij 35
1ml Gelatine Cold Fish
0.1g Thimerosal
Add distilled H_2O to 1000ml

XI powder was solubilised in 20mM carbonate buffer pH 9.6 with 0.01% thimerosal and diluted to a concentration of 2µg/ml. 100µl of this solution was pipetted into each well of a high binding optical microtiter plate and incubated over night at 4°C. The plate is then

blocked with 20mM carbonate buffer pH9.6 with 0.01%(v/v) thimerosal, 0.1%(v/v) gelatin from cold water fish and 5%(w/v) saccharose for 1 hour at RT. The plate is then emptied and dried and can be stored dry at 4°C for at least 6 months.

The bleed containing the desired antibody is diluted appropriately (1:1,000-1:125,000) in APF, 100µl of the diluted antibody added to each well and incubated for 3h at 37°C. The wells are then washed 4 times with 300µl WPL (see 3.1.7) per well. A HRPO-coupled secondary antibody (e.g. α-rabbit) is diluted appropriately in Stabilzyme HRP Conjugate Stabilizer (SurModics, CatNo SZ02), 100µl of this dilution added to each well and incubated for 1h at 37°C. The plate is then washed 4 times with 300µl WPL per well and 100µl TMB is added per well. The reaction is stopped with 50µl 1M HCl when enough color developed. The absorbance is determined with a spectrophotometer at a wavelength of 450nm.

Assay principle: The α-XI antibody in the bleed binds to the coated xylose isomerase. After washing a HRPO-coupled secondary antibody then binds the bound antibody and the coupled HRPO later converts the TMB to yield a blue product which turns yellow after addition of acid. This product is then determined spectrophotometrically.

3.1.13 Determining the protease resistance of xylose isomerase

A pancreatic protease preparation by the International Pharmaceutical Federation FIP Centre for Standards with an acitivity of 1.48U/mg was used.

Approximately 3.3mg/ml pure xylose isomerase is added to a 100mM phosphate buffer pH7.4 with 5mM MgSO4, 1mM cobalt acetate and 20U/ml protease and vortexed. This mixture is then incubated at 37°C and aliquots taken after different times of up to 180’. The enzyme activity is then immediately determined in these aliquots. The incubation of the

enzyme during activity measurement is reduced to 15’ to minimize degradation of the enzyme during measurement.

3.1.14 Total fructose conversion with glucose elimination

In this experiment the by the XI generated glucose is eliminated with glucose oxidase from the reaction. For the reaction buffer 500µl of a solution containing 800mM Hepes pH 7.8, 10mM $MgSO_4$, 2mM cobalt acetate, 660 units catalase (Fluka, CatNr 60640) and 50 units glucose oxidase (VWR, CatNr A4669.0001) is prepared in an appropriate reaction tube. To this buffer 400µl containing 300 XI units xylose isomerase (diluted with distilled H_2O) is added and incubated at 37°C for 15’. To this solution 100µl preheated (37°C) 1M fructose solution is added, vortexed briefly and incubated at 37°C. 100µl aliquots are removed at different time intervals and the reaction in the aliquots stopped by addition of 50µl 1M HCl. The aliquots are then diluted appropriately with distilled H_2O and the fructose concentration measured with the adapted Carbazole/H_2SO_4 assay (see 3.1.1).

Assay principle: The xylose isomerase converts the added fructose to glucose and the remaining fructose is measured. The glucose oxidase oxidizes the glucose to D-glucono-1,5-lactone and forms H_2O_2 which is degraded by the catalase. This prevents the isomerisation reaction from reaching equilibrium.

3.1.15 Size exclusion chromatography

An Äkta purifier FPLC device from GE Healthcare was used.

A column is loaded with approximately 50ml Sephacryl S-200 HR (GE Healthcare, CatNo 17-0584-10) and equilibrated with 100mM citrate buffer pH 2.5 with 0.5M NaCl. 90µl of protein is loaded and the run performed with 100mM citrate buffer pH 2.5 with 0.5M NaCl at 1ml/min. Fractions are taken when the UV detector recorded elevated absorbance at 280nm.

3.1.16 HRPO coupling of antibodies

EZ-Link Plus Activated Peroxidase from Thermo Scientific (CatNo. 31488) was used. The coupling is performed according to manual.

3.1.17 Generation of antibodies in rabbits

The rabbits are immunised intraperitoneal with 250µg of purified xylose isomerase (1mg/ml in phosphate buffer pH 7.4) mixed with 250µl Freund's Adjuvant (Sigma, CatNo F5506). Two weeks later 125µg of purified xylose isomerase mixed 1:1 with Freund's Adjuvant are administered and after two more weeks first bleeds taken and tested. 125µg of purified xylose isomerase mixed 1:1 with Freund's Adjuvant are administered after two more weeks and the blood collected after further two weeks (final bleeds).

The collected blood is incubated over night at 4°C and then centrifuged twice at 10,000g for 5 minutes. The serum is stored with 0.02% sodium azide at 4°C or -20°C.

3.2 Pharmaceutical Methods

3.2.1 Spraydrying

The experiments were done with a Büchi mini spray dryer B-290. Cellulose acetate phthalate (CAP) was used as matrix substance.

30ml of a 10%(w/v) or 6.66%(w/v) solution of CAP was neutralised to a pH of 8.0 with ammonia under constant stirring. To this solution different amounts of crystallised enzyme and helper substances are added and topped to 40ml with H_2O. This solution is then applied to the spray dryer with an inlet temperature of 130°C, an aspiration of -40mbar and air flow of 700-750L/h. The resulting powder is then tested for activity and acid stability.

3.2.2 Pellet production

For the production of pellets an extruder by Caleva (type 20) and a spheroniser by Caleva (type 250) were used. The acid resistant coating was applied with a bottom spray fluid bed coater made by Glatt (type Midi-Glatt).

Ingredient	Weight (%w/w)
Microcrystalline Cellulose	40 – 45%
Hydroxypropyl Cellulose	3 – 4%
Rice Starch	14 - 19%
Croscarmellose	1.5 – 2%
Maltose	0 – 15%
Saccharose	0 – 21%
Polyvinylpyrrolidone (Crospovidone)	4 – 6.5%
Sodium Chloride	0 – 2.5%
Purified XI	5 – 17%

Table 3.2.2.1 Composition of XI containing pellets without acid coating The percentage of each ingredient was adjusted with each experiment.

All substances except for the purified XI were provided by RIEMSER Speciality Production GmbH. The purified XI was produced according to methods 3.1.9 and 3.1.10.

All ingredients are mixed until homogeneity and 12.5ml of a 350mM $MgSO_4$ solution is added slowly per 100g dry pellet mix. Approximately 40ml distilled water per 100g dry pellet mix is added slowly under constant mixing to humidify the pellet mix. The sufficiently humid pellet mass is then applied onto the extruder that has been equipped with a sieve of 1.0mm and the rotation set to 20rpm. The resulting strands are then broken and rounded to pellets in the spheroniser for 150” at 400rpm. The pellets are then dried at 35°C to constant weight. The constant weight was usually reached after 24h.

The dried pellets are then tested for enzyme activity before being processed further. If the pellets pass the quality control the acid resistant coating is applied next. For the acid restistant coating Eudragit L-100 supplemented with triethyl citrate was used.

For the coating solution for 100g dry pellets 40g isopropanol and 40g acetone are mixed and 10g of Eudragit L100 added very slowly under constant stirring. To this solution a mixture of 10g isopronal with 10g acetone and 1g triethyl citrate is added and mixed.

The bottom spray coater is set to an air pressure of 2 bar, the inlet air valve to 20 and product temperature to 50°C. The batch size should be approximately 500g dry pellets. The pellets are loaded into the coater and preheated for 5'. The coating solution is then applied at 17.5g/min. When the coating solution is used up the pellets are dried for another 5' in the coater. The pellets are then dried to constant weight at 35°C in an incubator. The constant weight was usually reached after 24h. The obtained pellets should have a diameter of 0.5 to 2mm and a weight of approximately 0.5 to 2mg.

3.3 Study designs

3.3.1 Subchronic 90 day oral toxicity study

A subchronic 90 day oral toxicity study is performed in rats in cooperation with HAMELN RDS. 32 male and 32 female rats are divided in four groups. One group is the control group and is treated only with phosphate buffer. The other three groups receive 8 $mg.kg^{-1}.d^{-1}$, 40 $mg.kg^{-1}.d^{-1}$ or 80 $mg.kg^{-1}.d^{-1}$ xylose isomerase powder (see 3.1.9 and 3.1.10) dissolved in 250mM phosphate buffer pH 7.3 with 5mM $MgSO_4$ at a concentration of 0.1g/ml. These groups are named "low dose", "mid dose" and "high dose". The low dose of 8 $mg.kg^{-1}.d^{-1}$ represents 100-fold the human daily dose as estimated by the content of the capsules used for the clinical trial when 3 capsules per day are the human daily dose. The medium dose of 40 $mg.kg^{-1}.d^{-1}$ represents 500-fold the human daily dose and the high dose of 80 $mg.kg^{-1}.d^{-1}$ represents 1000-fold the human daily dose.

The reaction of the animals to the applied enzyme and their well being are followed daily and the body weight recorded every two days. Blood for hematological investigations is collected at the start of the study, after 30 days and after 90 days. After 90 days all aminals are sacrificed and autopsied.

3.3.2 Observational study

Pellets from experiment 4 (see 4.3.2) are filled into gelatin capsules size 3 with a filling volume of 205mg (+/- 10mg) pellets per capsule. These capsules are then given to volunteers who were diagnosed with and treated for fructose malabsorption by M.D. Markus Pfisterer (Ordination Nordstraße 28, D-74076 Heilbronn). All participants had a positive hydrogen breath test after a challenge with 25g fructose and responded positively to a fructose-free diet. Diabetes mellitus and pregnancy are reasons for exclusion.

Every participant is instructed to try reintroducing fructose into the diet and to take 1to 3 capsules containing pellets with XI with water 5 minutes prior to each fructose containing meal. They are further instructed to keep a log of the amounts and identities of fructose-containing meals, the experienced symptoms and the amount of capsules taken to each meal. After 10 to 12 days these logs are collected. The participants are then asked to rate the efficacy of the supplementation to reduce fructose-induced gastrointestinal symptoms (“good”, “low”, “none”) and which dosage they would recommend for best results (1, 2 or 3 capsules per fructose containing meal).

3.3.3 Placebo-controlled clinical trial

A placebo-controlled triple-blinded clinical trial is also performed to assess the efficacy of a xylose isomerase supplementation for patients with fructose malabsorption. The study is performed in two centers:

Center 1:

Local study leader: M.D. Peter Komericki

Medical University Graz

Auenbruggerplatz 2/4

A-8036 Graz

Center 2:

Local study leader: M.D. Markus Pfisterer

Ordination M.D. Markus Pfisterer

Nordstraße 28

D-74076 Heilbronn

Participant selection criteria:

Inclusion criteria:

- A positive hydrogen breath test after a fructose challenge.
- Age between 18 and 80 years, mixed gender

- Participant has to be politically mature

Exclusion criteria:

- Pregnancy
- Celiac disease
- Diabetes mellitus
- Small bowel bacterial overgrowth syndrome (SSBOS)
- Gastrointestinal surgery during the last 4 weeks
- Antibiotics therapy during the last 4 weeks
- Hereditary fructose intolerance
- A gastroscopy or a colonoscopy during the last 4 weeks

Study design:

The participants are separated into two groups (A and B) and told that 2 different substances are tested that should enhance the ability of the small intestine to absorb fructose. Two hydrogen breath tests are then performed with a minimal time difference of 4 and a maximal difference of 21 days between the tests. Both tests have to be done at approximately the same time of day.

For these hydrogen breath tests the participants are given 3 capsules size 3 (filled with 205mg +/- 10mg pellets) containing pellets either active xylose isomerase (verum) or heat-inactivated xylose isomerase with otherwise identical chemical properties (placebo) (see 4.3.2 pellets IV). Neither participants nor the local study personnel have any knowledge on the nature of these 2 formulations. The participants take these 3 capsules with still mineral water. 5 minutes after the intake of the capsules 25g (+/- 0.1g) D-fructose (AppliChem,

CatNr. A1299) dissolved in still mineral water is administered orally and the breath analysed for hydrogen with a Gastrolyzer by Bedfont Scientific Ltd after 0', 30', 60', 90', 120', 180' and 240'. The values for breath hydrogen are recorded by the local study personnel (see 3.3.3.3) and not known to the participants to avoid an influence on symptom reporting. The participants are instructed to fill out a symptom report form 24h after the test (see 3.3.3.4).

Hydrogen breath test criteria:

- 12h abstinence from food and drinks before the test with the exception of still water and non-sweetened tee
- Avoidance of leguminous plants and carbohydrate-rich sources in the diet the previous day
- No dental hygiene the day of the hydrogen breath test
- No physical exertion during the test
- No smoking for 12h before and during the test (neither active nor passive)

A case report form is filled out for every participant (see 3.3.3.1 and 3.3.3.2).

1) Participant allocation

Study centre ID: ☐☐

Participant-ID ☐☐☐☐☐☐

Date: ☐☐ . ☐☐ . ☐☐☐☐ (TT . MM . JJJJ)

Participant initials ☐☐☐

Group selection:
Group A ☐
Group B ☐

filled out by (initals) ☐☐☐

2) Participant information

Date of birth ☐☐ . ☐☐ . ☐☐☐☐ (TT . MM . JJJJ)

Gender
male ☐
female ☐

3) Participant informative education

carried out at ☐☐ . ☐☐ . ☐☐☐☐ (TT . MM . JJJJ)

by (initials) ☐☐☐

4) Information on fructose malabsorption

When was the participant diagnosed with FM?

☐☐ years ago unknown ☐

Is the participant free of gastrointestinal symptoms with a fructose-free diet?

Yes ☐ No ☐ unknown ☐

Does the participant sometimes tolerate fructose in the diet?

Yes ☐ No ☐ unknown ☐

Figure 3.3.3.1 Study case report form (points 1 to 4)

5) Hydrogen breath test

First breath test carried out at:

☐☐ . ☐☐ . ☐☐☐☐ (TT . MM . JJJJ) not carried out ☐

Second breath test carried out at:

☐☐ . ☐☐ . ☐☐☐☐ (TT . MM . JJJJ) not carried out ☐

6) Inclusion criteria

Diagnosed fructose malabsorption:

Yes ☐ No ☐

The participant reached his/her 18th birthday:

Yes ☐ No ☐

The participant is politically mature?

Yes ☐ No ☐

7) Exclusion Criteria

Pregancy test (of female participants):

Positive ☐ Negative ☐ not performed ☐

The participant has got diagnosed diabetes mellitus, celiac disease or HFI?

Yes ☐ No ☐

The participant had a gastrointestinal surgery during the last 4 weeks?

Yes ☐ No ☐

The participant had a gastroscopy or a colonoscopy during the last 4 weeks?

Yes ☐ No ☐

The participant took antiobiotics during the last 4 weeks?

Yes ☐ No ☐

Figure 3.3.3.2 Study case report form (points 5 to 7)

1) Participant information

Study center ID

Participant ID

Date of breath test:

TT . MM . JJJJ

Participant initials

filled out by (initals)

2) Hydrogen breath test:

Test number:

Start at :

Fructose batch number:

3) Hydrogen Measurement

Time	Hydrogen value
0' minutes :	ppm H_2
30' minutes :	ppm H_2
60' minutes :	ppm H_2
90' minutes :	ppm H_2
120' minutes :	ppm H_2
180' minutes :	ppm H_2
240' minutes :	ppm H_2

The participant must not know the recorded values!

Figure 3.3.3.3 Study data acquisition form I (for study personnel)

Symptoms reported by the participants include abdominal pain, flatulence, nausea and diarrhea. For the first 3 symptoms the participants have to mark a line to evaluate the severity of the respective symptom. For evaluation the distance between “no symptom” and

the mark is measured and points given in relation to the length of the line with a maximum number of 9 points per symptom. The symptom diarrhea is only recorded quantitatively with 2 symptom points awarded for each diarrheic bowel movement with a maximum of 9 points (see 3.3.3.4).

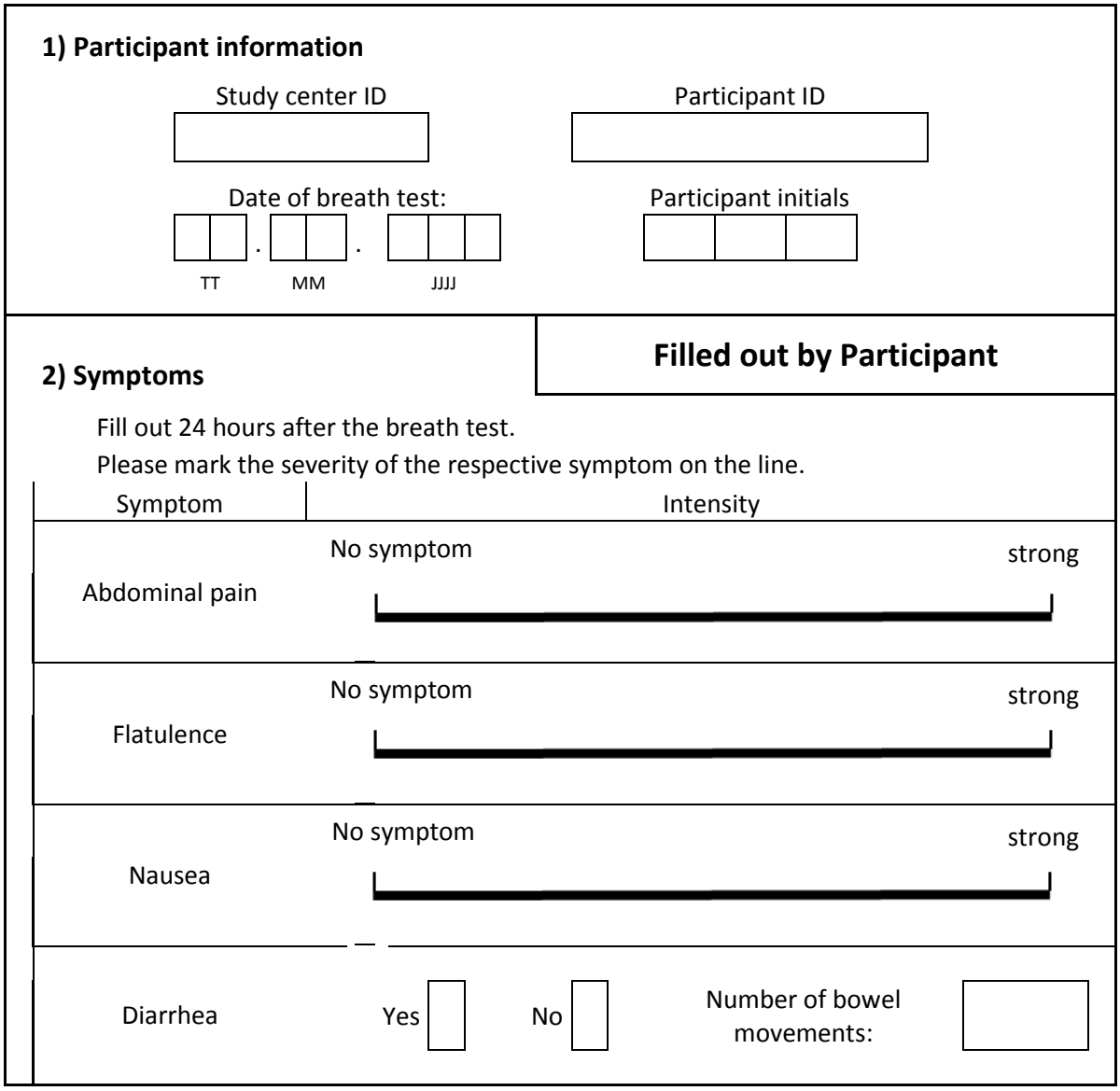

Figure 3.3.3.4 Study data acquisition form II (for participants)

The null hypothesis of the study is that the area under the curve (AUC) of the fructose breath test with the verum (active XI) is not reduced when compared to the AUC of the

breath test with placebo. The rejection of this null hypothesis is the primary outcome of the study. The secondary outcome of the study is the reduction of the fructose-induced gastrointestinal symptoms by verum when compared to placebo.

The study is designed as triple blind. Every participant ID had a specific box with 2x 25g fructose in plastic bottles and 3 capsules of verum and placebo. These capsules are randomly blinded by me and are only known as red and blue to the participants and the local study personnel. The local study personnel in turn separated the participants in two groups, A and B, and decided whether to give the red or the blue capsules for the first breath test. During evaluation it was not know which capsules where used for each test. Only after the calculation of the AUC and the symptom score the code was broken and the hydrogen breath tests assigned to verum or placebo capsules. Therefore participants, local study personnel and the assessors were blinded.

The AUC between two measurements was calculated by multiplying the average of two measurement points x and y with the time difference between these two points. Total AUC was calculated by summing all AUC values.

A one sample t-test was used to calculate the statistical significance of the results. All given p values represent two tailed p values obtained by the one sample t-test.

The study is registered at ClinicalTrials.gov with the identifier NCT00916487 and at EudraCT with the ID EUDRACT2008-005861-80.The study design was examined and approved by the Austrian Bundesamt für Sicherheit im Gesundheitswesen and the ethics committee of the medical university of Graz. Informed written consent was obtained from all subjects before their participation in the study.

4 Results and Discussion

4.1 Characterizing the Enzyme

The first milestone of the project was to establish a proof of concept. Several issues had to be addressed to assess the possibility and feasibility of fructose conversion in the intestine with xylose isomerase.

- Does it survive the acidic conditions in the stomach or are extra measures needed to ensure the activity of the enzyme in the intestine?
- Is the enzyme still active under conditions found usually in the intestine?
- Does it survive the proteases in the intestine long enough to perform the task?
- Is it possible to transform the whole fructose if the produced glucose molecules are consumed?

4.1.1 Influence of the pH on enzyme activity

One of the most important factors was impact of the pH on the activity and the stability of the enzyme.

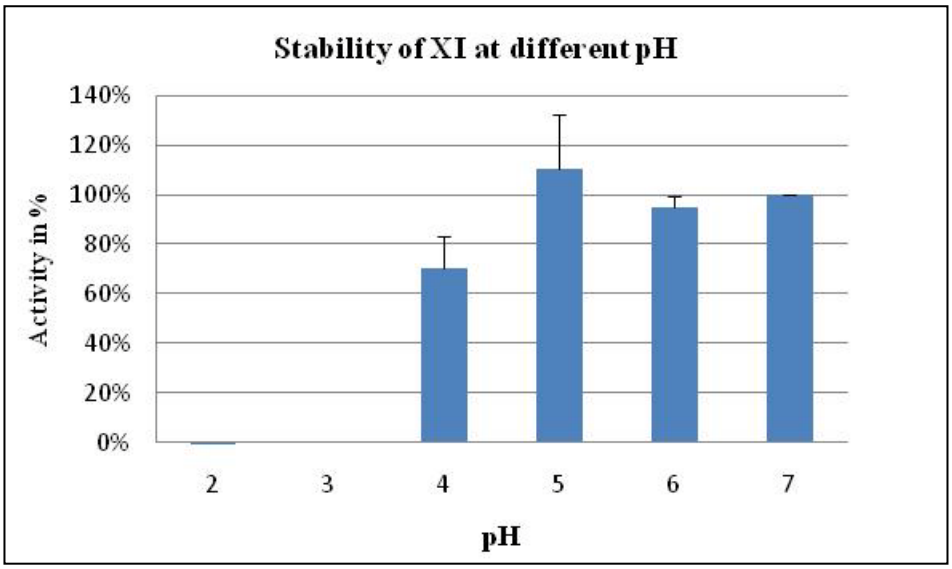

Figure 4.1.1.1 Stability of XI at different pH The enzyme was incubated for 15' at the specified pH and the activity afterwards determined at pH 7.4. No activity could be detected after incubation at pH2 and 3. The activity after incubation at pH 7 was set as 100%.

For this experiment the enzyme was incubated for 15' at RT at the specified pH in different buffers with 100mM concentration of the buffer ion and then the remaining activity determined. For pH 2 and 3 a glycin buffer, for pH 4 and 5 a glutamic acid buffer, for pH 6 a histidine buffer and for pH 7 a Hepes buffer was used. No activity could be detected after the enzyme was incubated at pH 2 or 3 suggesting that the enzyme was not stable in acidic environment. The activity decreased by 30% when incubated 15' at pH4 whereas no decrease could be observed at pH 5 or above. Therefore the enzyme has to be protected from the acidic environment in the stomach to prevent denaturation.

Another experiment was conducted by incubating the enzyme in pH 8 and above. Up to pH 11 no negative effect of 15' incubation in alkaline solutions could be observed (data not shown).

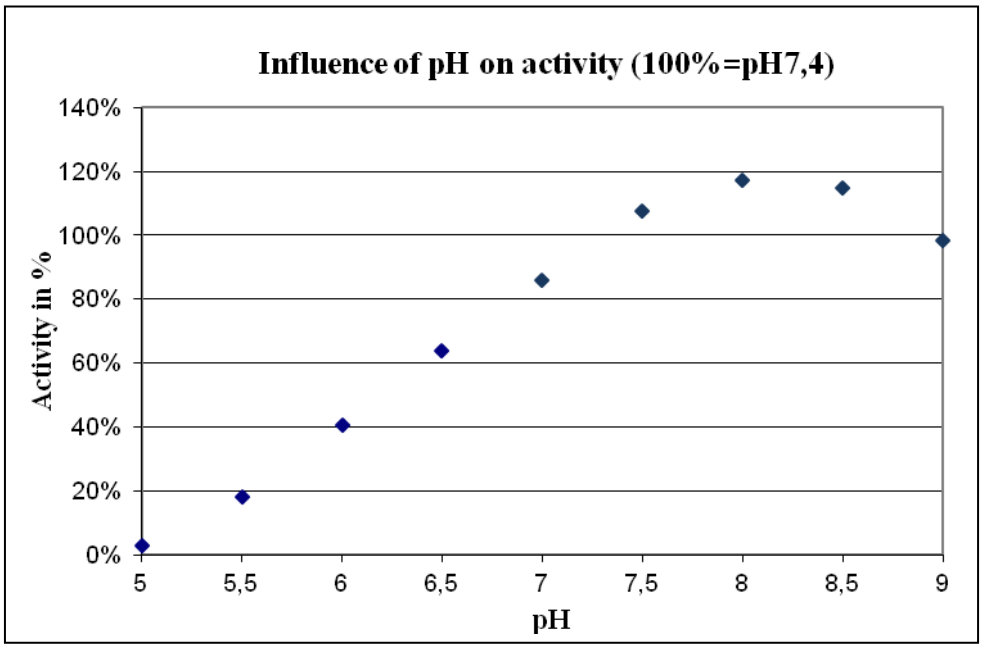

Figure 4.1.1.2 Influence of pH on activity The activity was determined in various buffers at different pH. The activity of the enzyme in the different buffers was normalised to the activity in Hepes buffer (used for pH 7.4). Maximal activity can be observed at pH 8.2.

The activity of XI also greatly depended on the pH and the buffer composition. The influence of the different buffer ions, maleate, Hepes and Tris respectively, had to be determined before the activity at the different pH could be determined. For this the enzyme activity was determined at pH 7.4 with the respective buffers at 100mM supplemented with 10mM Hepes (to provide buffering capacity). This yielded correction factors of 0.78 for the maleate buffer and 0.95 for the Tris buffer. Then the activity of the enzyme was determined at various pH and normalised in regard to the influence of the buffer ions. This yielded the graph of figure 4.1.1.2.

The enzyme exhibited the greatest activity at pH 8.2 and slowly decreased to 3% activity at pH 5. That means that the enzyme will exhibit high activity in the slightly alkaline environment of the small intestine.

4.1.2 Influence of different metal ions

XI requires divalent cations that are bound at the active site of the enzyme for highest activity. Usually Mg^{+2} and Co^{+2} are used for the XI by *Streptomyces rubiginosus* but these ions are only loosely bound and need to be added to the solution. Since the environment in the intestine will not provide these ions they need to be added to the enzyme. Therefore it is important to know:

- Which metal ions can be used?
- How fast does the enzyme lose this ions and the activity when incubated in an ion free solution?

The first experiments confirm that the metal ions need to be added to the enzyme buffer for full activity. Figure 4.1.2.1 shows that incubation of the enzyme with the appropriate amount of ions (5mM Mg^{2+}, 1mM Co^{2+}) did not increase the activity to the values reached when the ions are added to the buffer in this amount. But the activity still surpassed the activity found when no ions were added directly to the assay. This means that the metal ions are incorporated into the enzyme during an incubation step but are not bound strong enough to sustain the activity of the enzyme in a buffer without ions.

This would be disastrous for the activity in the intestine since it is not possible to reach a concentration of metal ions in the intestinal lumen that would efficiently activate the enzyme.

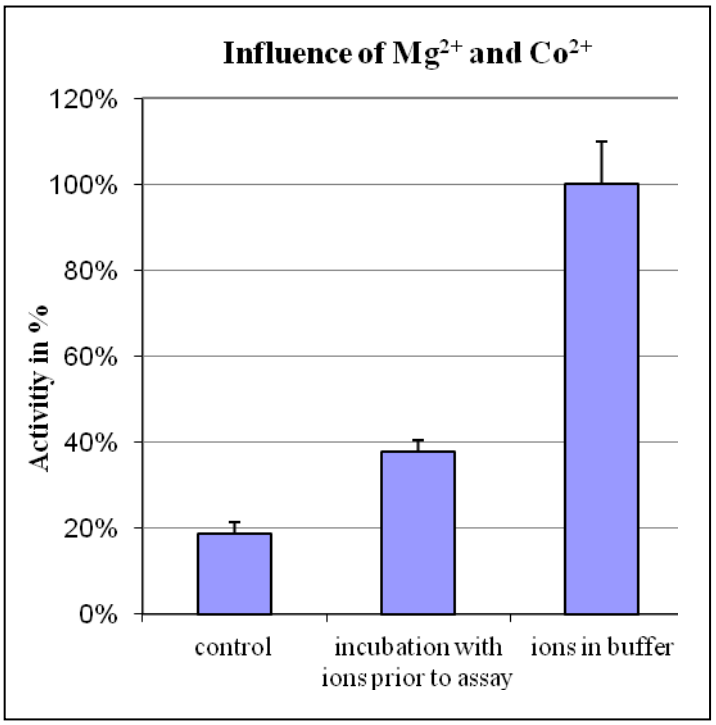

Figure 4.1.2.1 Saturation of the enzyme prior to measurement 5mM Mg^{2+} and 1mM Co^{+2} were added directly to the assay into the buffer, to the enzyme prior to measurement or not at all. When added to the enzyme prior to measurement the final concentration in the assay for the ions was 1:100th of the concentration during incubation. This concentration was also added to the control.

Another goal was to find a suitable substitute for cobalt in the enzyme since high amounts of cobalt can cause a cobalt cardiomyopathy [ALEXANDER, 1972].

For this experiment the 1mM cobalt in the enzyme buffer was substituted with different divalent cations. Figure 4.1.2.2 shows that for complete activation Co^{2+} is needed. Mn^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+} fail to activate the enzyme or even inhibit it under standard analytical conditions (100mM phosphate buffer pH 7.4 with 5mM Mg^{2+} and 100mM glucose at 37°C). These experiments so far confirm the literature that both Mg^{2+} and Co^{2+} are needed and only loosely bound.

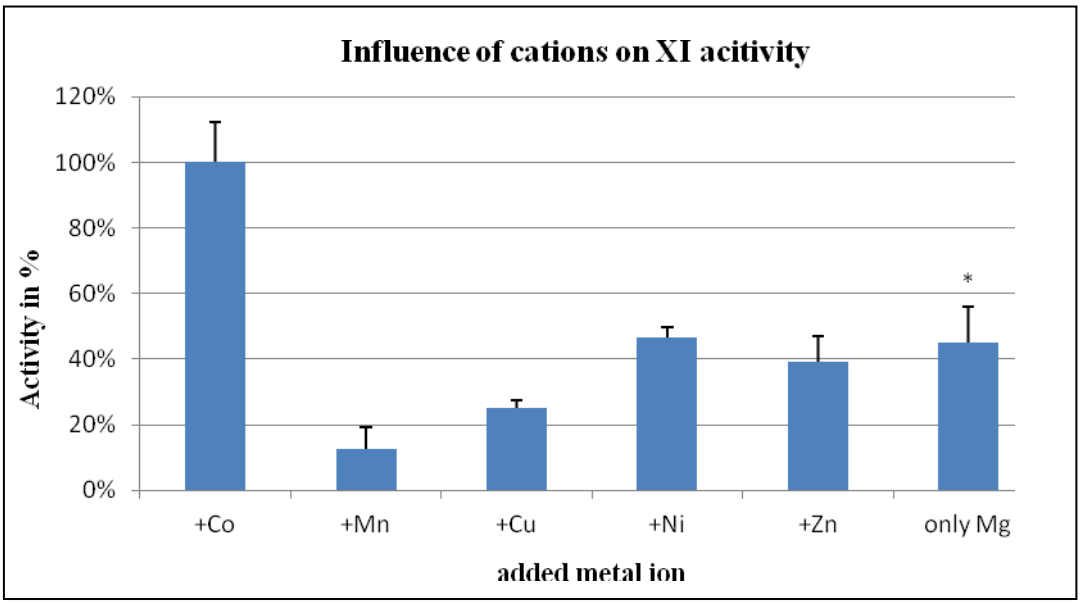

Figure 4.1.2.2 Influence of cations on XI activity 5mM Mg^{2+} and 1mM of the respective metal ion were added to the assay buffer. The activity of enzyme with Mg^{2+} and Co^{2+} in the buffer was set to 100%. *: The experiment with only Mg^{2+} in the buffer was performed separately and included for reference.

An important factor for the enzyme activity that has never been addressed is the used buffer substance. The enzyme exhibits very different levels of activity in different buffers under standard analytical conditions. The buffer that most closely represents conditions in the intestine is the carbonate buffer since the pancreas neutralizes the acid from the stomach by releasing bicarbonate into the duodenum [NOVAK, 2000]. Therefore tests concerning the acitivity of the enzmye in the intestine should be done under these circumstances. For the next experiment the enzyme was crystallised in the presence of Mg^{2+} ions and thus should be saturated with Mg^{2+}. The activity of the enzyme preparation was then determined in a carbonate buffer with or without additional metal ions.

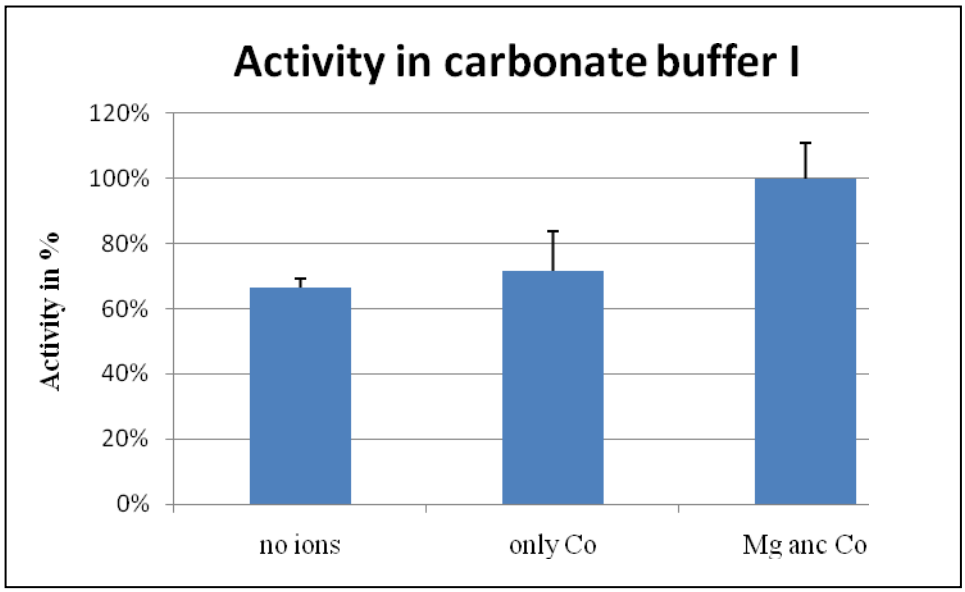

Figure 4.1.2.3 Influence of carbonate buffer on metal ion requirements The activity of purified enzyme that was saturated with Mg^{2+} was measured in 100mM carbonate buffer pH7,4 either without ions in the buffer, with 1mM Co^{2+} or with 5mM Mg^{2+} and 1mM Co^{2+}. The activity in the buffer with Mg^{2+} and Co^{2+} was set to 100%.

As seen in Figure 4.1.2.3 the addition of cobalt increased the activity of the enzyme only slightly by 8%. But when compared to the activity in a buffer where both Mg^{2+} and Co^{2+} were provided the activity was only 66%. Therefore addition of Mg^{2+} further enhanced the activity suggesting that additional Mg^{2+} might be beneficial for the activity of the enzyme in the intestine. After optimisation of the purification process (see 4.2.2) this experiment was repeated with the obtained XI crystals with added $MgSO_4$ (Figure 4.1.2.4). With this optimised purification process the activity of the enzyme in a buffer without metal ions could be increased to 83% when compared to a control with both Mg^{2+} and Co^{2+}. This suggests that the enzyme will have a high activity even in the Mg^{2+} and Co^{2+} free environment of the intestine.

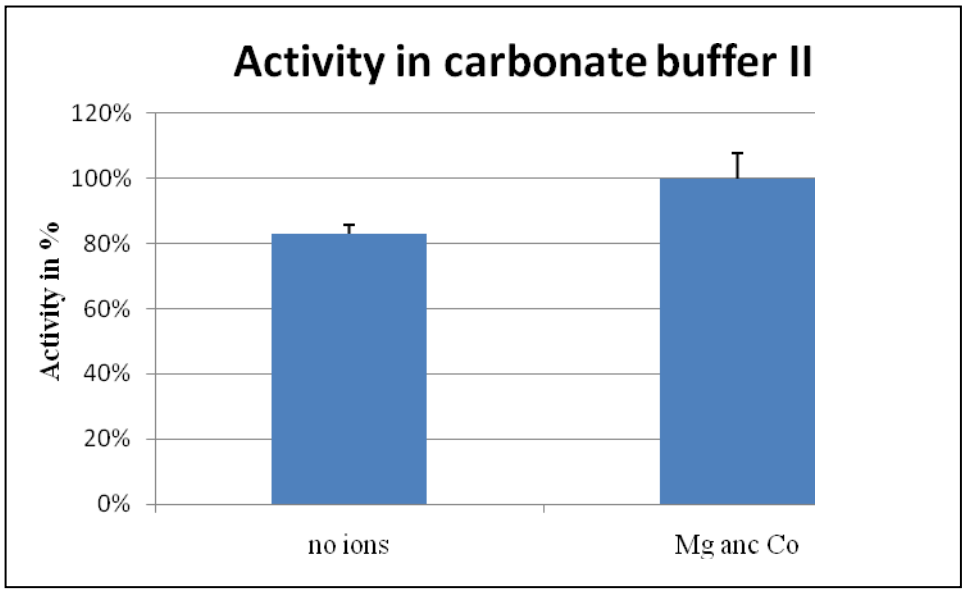

Figure 4.1.2.4 Influence of carbonate buffer on metal ion requirements II The activity of purified enzyme that was saturated and fortified with Mg^{2+} was measured in 100mM carbonate buffer pH 7.4 either without ions in the buffer or with 5mM Mg^{2+} and 1mM Co^{2+}. The activity in the buffer with Mg^{2+} and Co^{2+} was set to 100%.

4.1.3 K_M of xylose isomerase with fructose as substrate

According to the Michaelis-Menten kinetics the affinity of the enzyme for the substrate affects the velocity of the reaction. The affinity determines the Michaelis constant K_M, which specifies the concentration of substrate at which half of the enzyme molecules have a bound substrate molecule. Determining the exact KM for XI is vital to estimate the activity of the enzyme at different concentration of fructose.

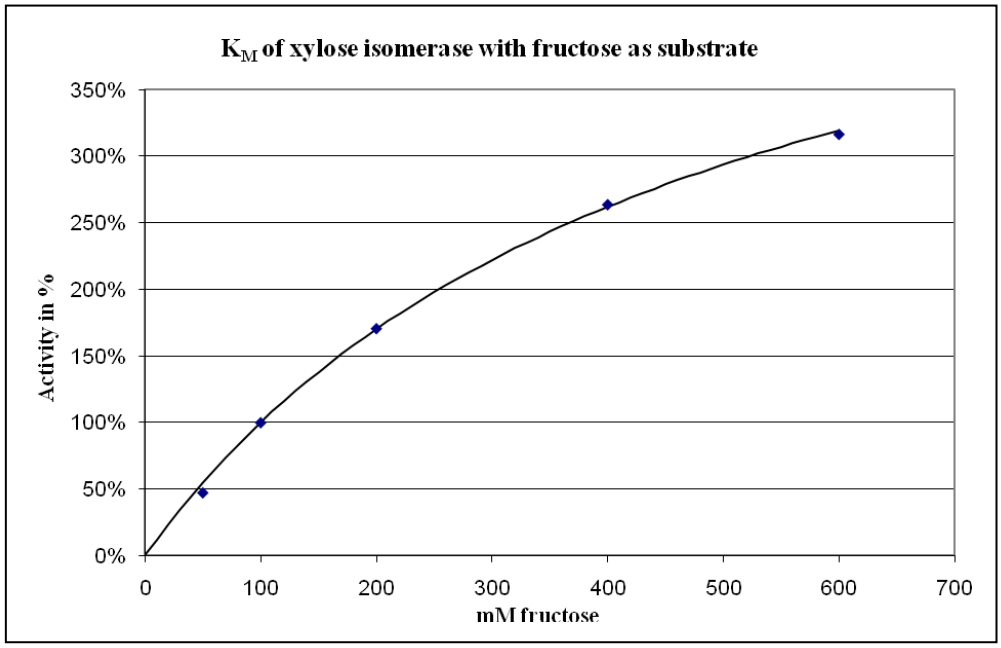

Figure 4.1.3.1 Determination of the KM of XI with fructose as substrate The experiment was done according to 3.1.5 but with different concentration of added fructose. The in the figure indicated concentration represents the concentration of fructose during the activity determination. The glucose concentration was determined according to 3.1.2. The regression is a calculation from the obtained K_M values from this experiment (activity = V_{max}*c/(K_M+c)).

The K_M was determined by measuring the activity with different concentration of fructose and with a Lineweaver-Burk plot. The K_M was calculated to be 470mM fructose with a V_{max} of 570% when compared to 100mM fructose. These values fit the observed values and represent a good estimation of K_M and V_{max} (see 4.1.3.1).

4.1.4 Protease resistance of native xylose isomerase

Xylose isomerase must show resistance to pancreatic proteases in order to be able to work in the intestinal environment. For this experiment a protease standard by the International Pharmaceutical Federation was used.

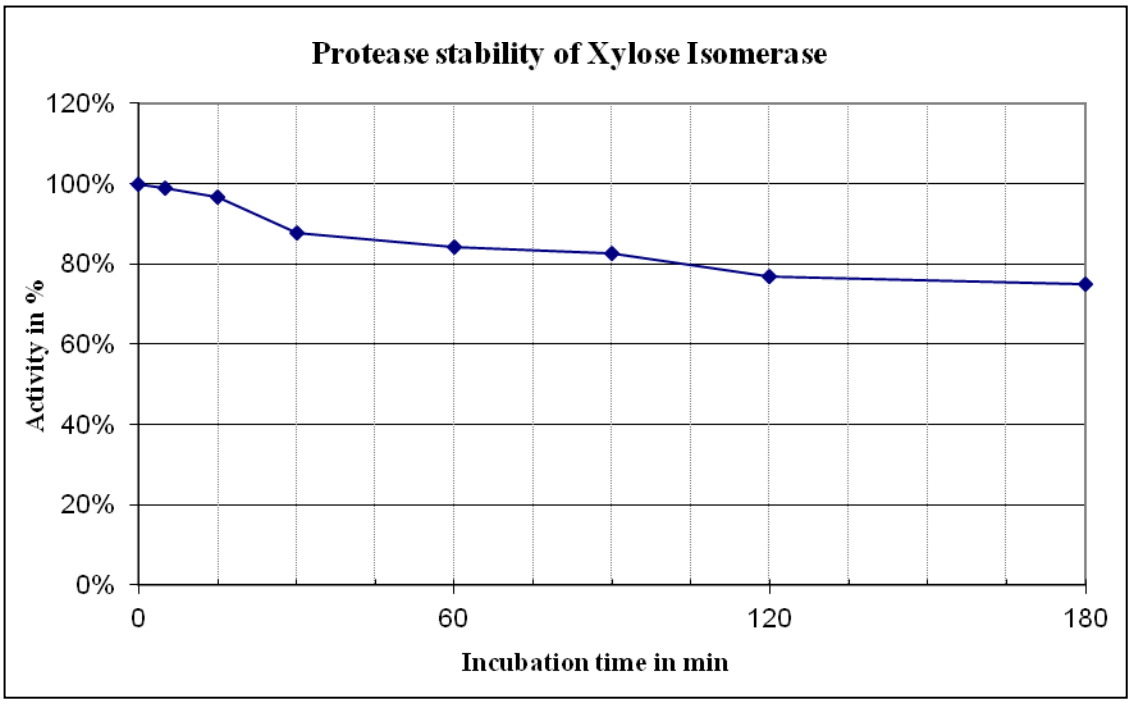

Figure 4.1.4.1 Protease resistance of XI XI was incubated with pancreatic protease and afterwards the activity determined. For conditions see 3.1.13.

Figure 4.1.4.1 shows that the enzyme proved very resistance to proteases. Even after incubation with 20U/ml protease for 180 minutes only 25% of the activity was lost. One unit of protease hydrolyzes casein to produce color equivalent to 1µmol of tyrosine per minute at ph 7.5 at 37°C in a protease activity test. Therefore 20 units should digest the equivalent of 3.6 mg (20µmol) of tyrosine per minute under assay conditions. This means that the 20 units/ml proteases should be able to digest the equivalent of the added enzyme (3.3mg/ml) in roughly one minute. This proved that protease digestion will not significantly reduce enzyme activity in the intestine.

4.1.5 Proof of Concept

The proof of concept consists of an experiment where the generated glucose is eliminated from the reaction so that the enzyme can convert the whole fructose. The degradation of glucose mimics the glucose absorption of the intestine which also causes the glucose to be eliminated from the reaction in the intestine.

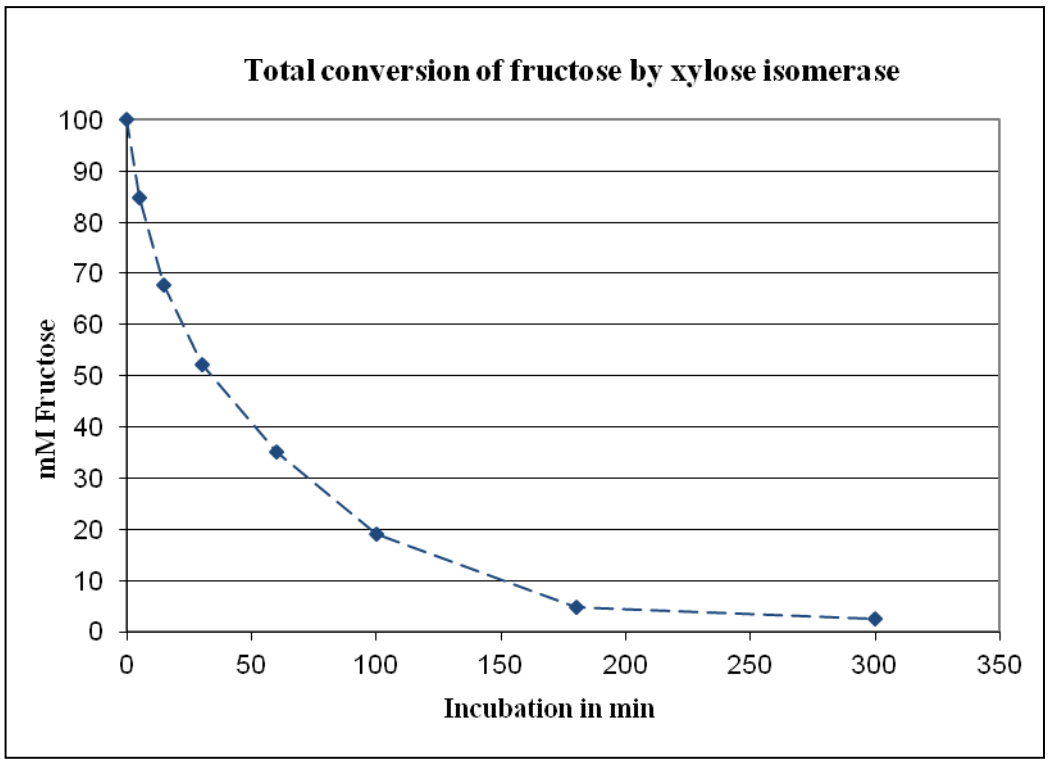

Figure 4.1.5.1 Total conversion of fructose by XI The by the XI generated glucose is eliminated with glucose oxidase. This causes the reaction to proceed until the whole fructose is converted.

This proves that it is principally possible to convert the fructose almost completely in the intestine. Therefore it should be possible to prevent symptom generation from malabsorbed fructose.

4.2 Development of the optimal purification process

The supplied enzyme solution (Gensweet SGI from GENENCOR) already contains the pure enzyme. Unfortunately the solution also contains 30% (w/w) sorbitol which needs to be removed because of its negative impact on enzyme activity. Furthermore sorbitol can cause gastrointestinal symptoms like diarrhea. The low specific acitivity of the enzyme makes it necessary for the preparation to be as pure as possible with the highest possible activity per volume to allow for higher dosage during enzyme supplementation.

First experiments were done with dialysis. Unfortunately this proved not feasible for two reasons:

- The volume increased significantly due to the osmotic pressure of the original enzyme solution.
- The stability of the enzyme in an aqueous solution without additives is low.

Therefore other possibilities had to be assessed.

4.2.1 Precipitation

I tried purifying the enzyme with an ammonium sulfate precipitation step. For this the concentration of ammonium sulfate at which the enzyme precipitates was determined.

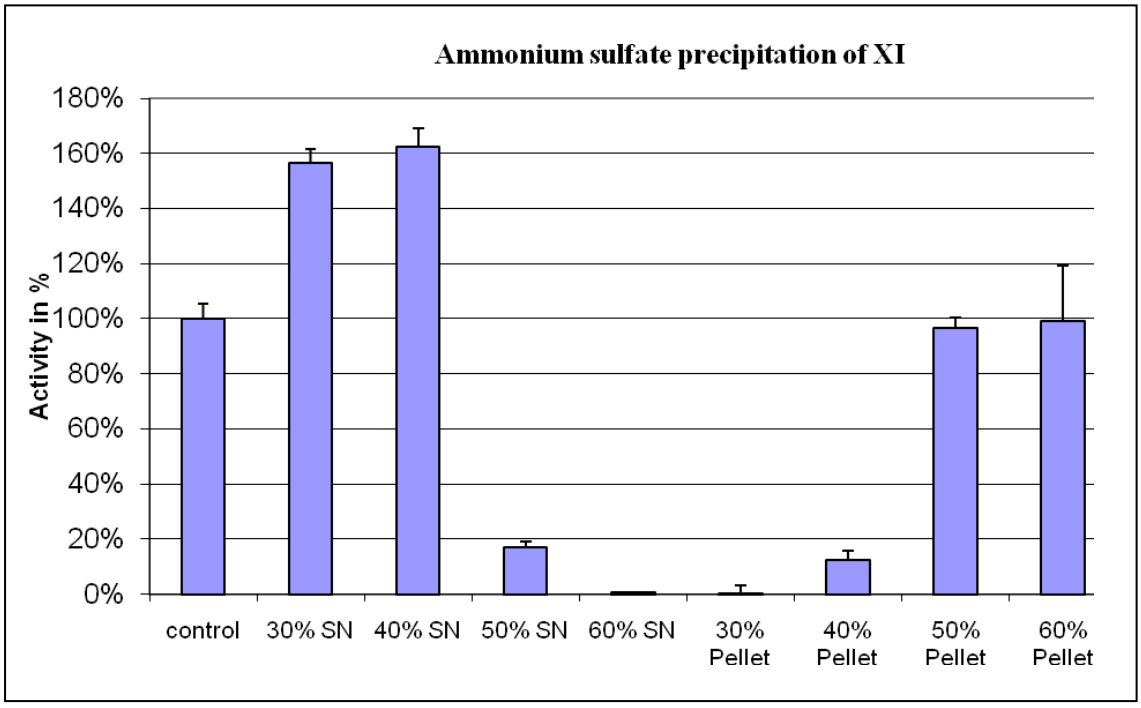

Figure 4.2.1.1 Ammonium sulfate precipitation of XI 5ml enzyme solution (Gensweet SGI from Genencor) was diluted with 15ml distilled H_2O and saturated ammonium sulfate solution added until the indicated percentage was reached (50%: a ratio of enzyme solution to ammonium sulfate solution of 1:1). Then 1ml aliquots were taken and centrifuged 15' at 6,000rpm. The pellet was solubilised in distilled H_2O and the enzyme activity determined in the supernatant and the pellet fractions. The control shows the activity of the enzyme before precipitation. SN: supernatant.

The enzyme precipitated at 50% saturated ammonium sulfate solution and only a small increase in yield is gained by raising the saturated ammonium sulfate solution concentration to 60%. The increased activity (>100%) in the supernatants of 30% and 40% saturated ammonium sulfate shows a positive influence of ammonium sulfate on activity. The enzyme solution needs to be diluted 1:4 with distilled H_2O for a pellet to form. If the precipitation is performed with the pure XI solution the whole solution solidifies and no pellet can be obtained. The reason for this is the high concentration of enzyme and additives in the original solution.

With method 3.1.8 it is possible to obtain an enzyme that is purified from most of the additives of the original solution. A disadvantage of this method is the reduced enzyme concentration in the obtained solution after precipitation. The original enzyme solution (Gensweet SGI) is optimised to for highest solubility of xylose isomerse. Without the additives the solubility of the enzyme is lower.

4.2.2 Crystallisation

The highest possible density of enzyme activity can only be obtained by crystallisation. In crystals the enzyme is tightly packed and therefore the highest activity can be administered for enzyme supplementation.

The main difficulty in crystallizing an enzyme is finding the right buffer. Usually the higher the enzyme concentration the better the crystallisation process will work. In contrast to this a dilution of the Gensweet SGI solution was necessary for crystals to form. This is most likely caused by the additives in the Gensweet SGI which seem to interfere with the crystallisation process.

Ingredient	Experiment I	Experiment II	Experiment III	Experiment IV	Experiment V
Gensweet SGI	400ml	100ml	1L	1L	750ml
Distilled H_2O	600ml	150ml	2L	2,5L	2L
Ammonium sulfate	145.1g (15%)	33.75g (13.5%)	430g (14.5%)	413g(11.8%)	432g (15.7%)
$MgSO_4*7H_2O$	18.7g (1,6%)	3.75g (1.5%)	45g (1.5%)	45g (1.3%)	41,2g (1.5%)
Yield	enzyme precipitated	40%, rest precipitated	enzyme precipitated	10%	75%

Table 4.2.2.1 Crystallisation experiments and their parameters After slow addition of the ammonium sulfate and the $MgSO_4*7H_2O$ under constant stirring the solution was incubated under constant slow stirring at 4°C for 4 days. Crystals were harvested by centrifugation for 15' with 5000g. Precipitated enzyme was discarded. For the yield the activity of the obtained crystals was determined and compared to the original solution used for crystallisation. The percentages in the table represent the w/v concentration of the added salts.

Different experiments to establish the optimal crystallisation conditions can be seen in Table 4.2.2.1. The necessary addition of ammonium sulfate depended on the dilution of the original enzyme solution Gensweet SGI. It was necessary to dilute the original solution more than 1:2.5 for optimal conditions. Now that first crystals were obtained it was possible to add starter crystal to the crystallisation. These starter crystals greatly accelerated and stabilised the crystallisation process.

The used stirrer had a strong impact on the process. If the stirring caused too much disturbances in the solution no crystals formed and the enzyme precipitated. A similar observation could also be made when the crystallisation was performed in a plastic container instead of glass or metal. This suggests that the rough surface of the plastic also had a negative impact on crystallisation.

A turbidity sensor was then used to better observe the crystallisation process. Over a period of 20 different crystallisation experiments the conditions were further optimised by small

changes to the used amounts of distilled H_2O, ammonium sulfate and $MgSO_4$. This then yielded the process described in 3.1.9.

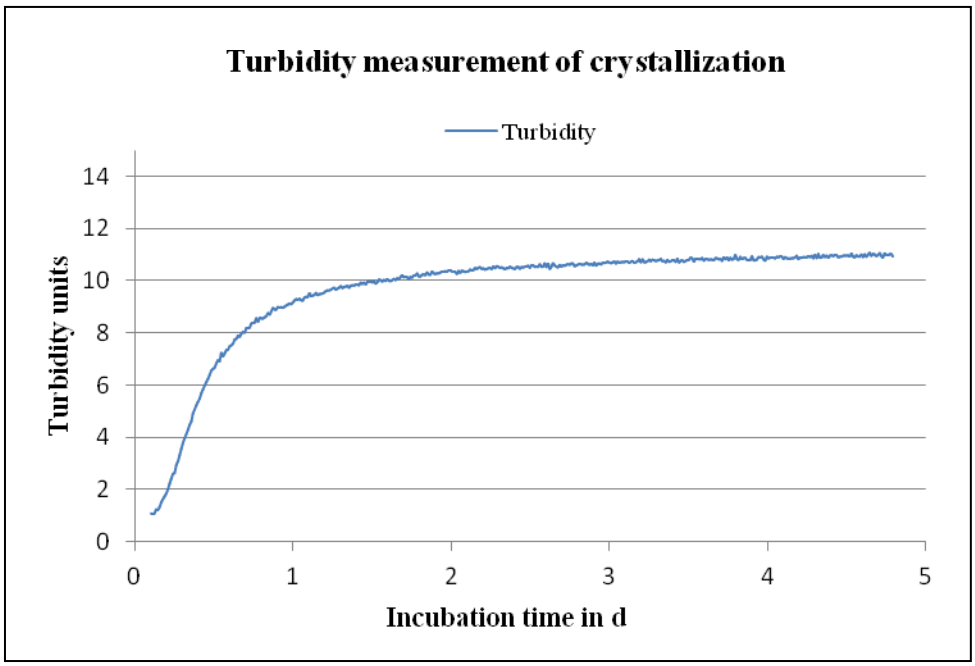

Figure 4.2.2.2 Turbidity measurement of crystallisation This is an example for a turbidity measurement of the crystallisation process. The experiment was conducted as described in 3.1.9 and turbidity measured during the incubation at 4°C. The turbidity units are not an SI unit and have to be calibrated manually on the turbidity sensor. They are therefore arbitrary and cannot be compared to measurements with another turbidity sensor.

Figure 4.2.2.2 shows a turbidity measurement during the incubation of the crystallisation solution at 4°C. During the first 24 hours incubation the turbidity increases strongly. After 36 to 48 hours the increase reduces to a small but constant level suggesting that the crystallisation process is not finished after 5 days. But the small increase in yield does not justify an increase of the duration of the incubation.

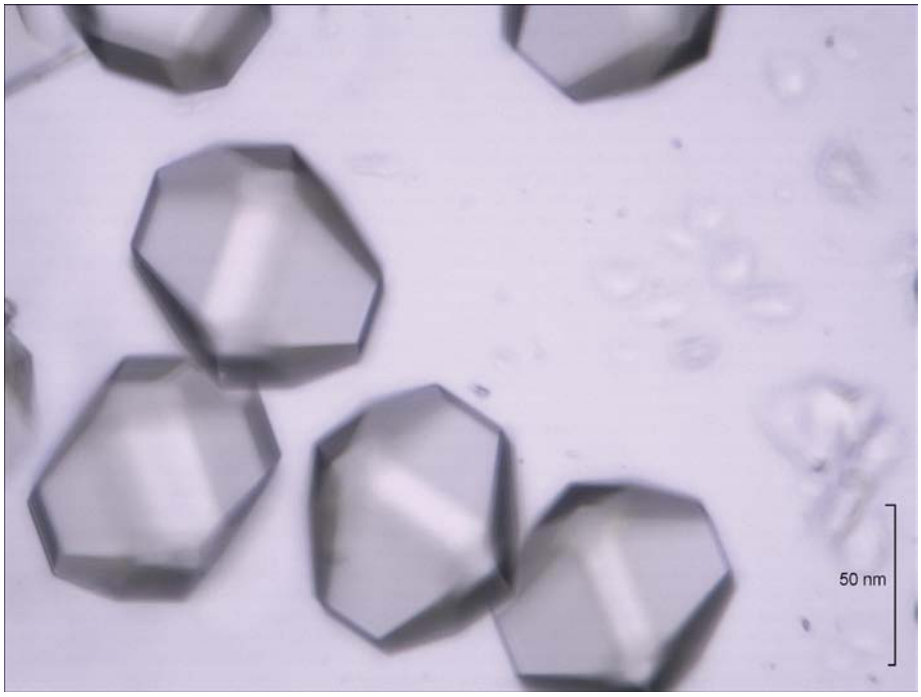

Figure 4.2.2.3 XI crystals I The crystals were obtained as described in method 3.1.9. The diameter of the crystals is approximately 50nm.

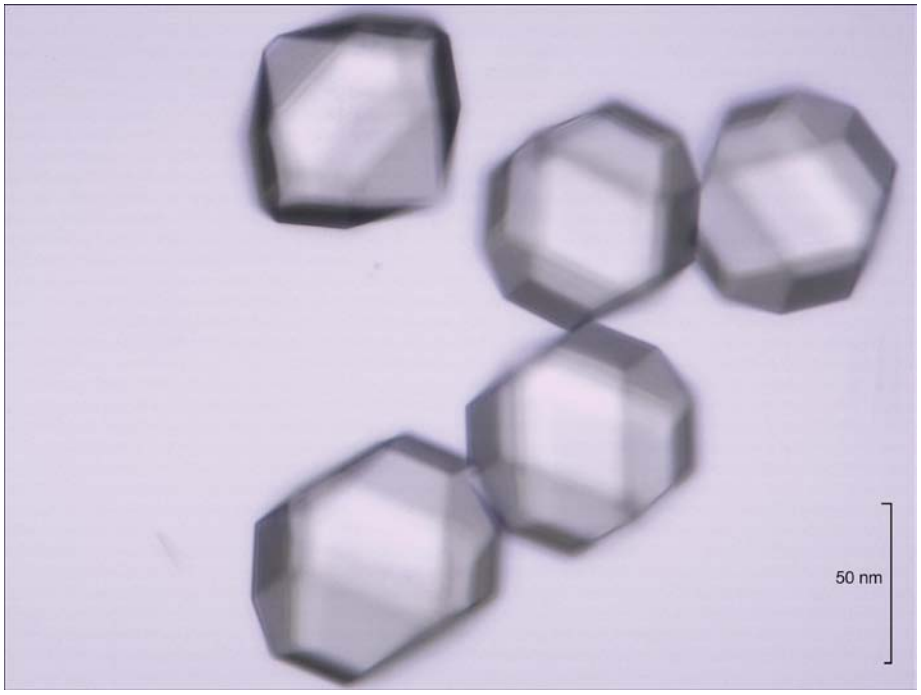

Figure 4.2.2.4 XI crystals II The crystals were obtained as described in method 3.1.9. The diameter of the crystals is approximately 50nm.

Figure 4.2.2.3 and 4.2.2.4 show XI crystals obtained by the described process. The usual crystal size was 50nm, varying between 30 and 70nm from batch to batch.

The crystal slurry that is so far obtained from this process cannot be easily used in further processes because of its physical properties. Furthermore the crystal slurry is not stable enough to be stored for longer periods. Therefore a process had to be found to prepare the crystals for further processing and storage.

First a filtration process had to be developed. Centrifugation cannot be used to separate the crystals from the solution because the resulting pellet is compressed too much for further processing. Therefore filtration with cellulose filters was used and a filter grade 1288 (Sartorius, CatNr Ft-2-206-580580) was found to be especially useful with fast filtration and high yield. This filtration was performed without applied pressure. If the filtration process was accelerated either with compressed air or suction the filter cake was compressed so that no further processing was possible.

Next this filter cake was lyophilised to obtain dry crystals. This step did not work with compressed filter cakes or pellets from centrifugation and the enzyme preparation remained humid causing problems during further processing. The lyophilisation was performed as described in 3.1.10. To the lyophilised crystals $MgSO_4$ is added to increase activity of the crystals in the Mg-free environment of the intestine. The dried filter cake is then grinded to obtain a fine, highly active XI crystal powder (see 3.1.10).

The yield of this process was approximately 125g dry XI crystals with an activity of 70,000-90,000 XIU/g per kg of Gensweet SGI solution. This represented approximately 170% of the enzyme activity that was used for the crystallisation. This increase in activity can be explained by the removal of the inhibiting sorbitol from the solution which causes the enzyme to be less active in the original solution.

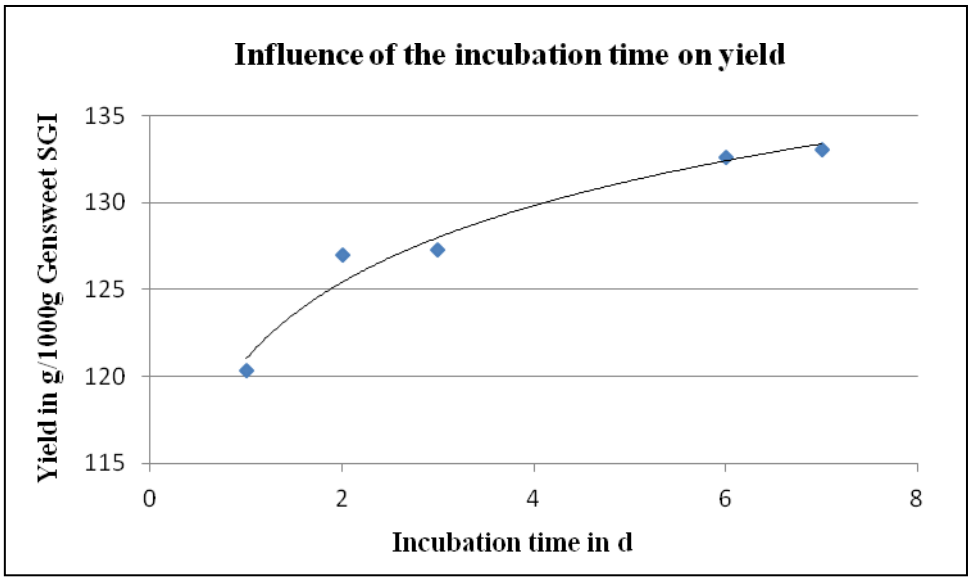

Figure 4.2.2.5 Influence of the incubation time on yield During the incubation at 4°C of the crystallisation process (2.1.10) aliquots were taken and processed according to 2.1.11 to dry powder. This dry enzyme powder was then weighed. For the trend line a logarithmic regression was used.

The yield of dry powder increased to 133g per kg Gensweet SGI when the crystallisation solution was incubation for 7 days. But after 24 hours of incubation already 90% (120g/kg) of the crystals had formed when compared to 7 days. Longer incubation can therefore increase the yield of this process but 3-5 days are recommended.

4.3 Pharmaceutical formulation

The pharmaceutical formulation has to guarantee a fast delivery of the enzyme in an active form. The enzyme is rapidly denaturated by a pH of 3 or below and therefore has to be protected from the environment in the stomach.

Microparticles with a diameter of less than 2 to 3 mm can rapidly pass the pylorus regardless of filling level of the stomach [RAHMAN and ALI, 2008]. If the enzyme is supplemented as a microparticle the retention time in the stomach is reduced. This will result in a decreased denaturation of the enzyme due to acidic conditions in the stomach. Therefore only acid-resistant microparticle dosage forms were considered as suitable for xylose isomerase supplementation.

4.3.1 Spray drying

Spray drying is a process used in pharmacy to obtain solid dosage forms based on microparticles. The solution containing non-volatile particles is sprayed through a nozzle into the drying chamber with hot air. This hot air causes volatile substance of the used solution to quickly vaporize leaving only small particles comprised of the non-volatile substances.

The obtained powder must be acid-resistant. Therefore cellulose acetate phthalate (CAP) was used as a matrix substance because it is only soluble in aqueous solutions with a pH of 5.5 or higher. CAP is used as an enteric coating in pharmaceutical formulations and can be used in spray drying processes.

Normally spray drying cannot be easily used with enzymes because of the high temperatures associated with this technique. Xylose isomerase has a high temperature resistance and can therefore endure the harsh conditions during spray drying. Still, so far no acid-resistant pharmaceutical formulation of an enzyme has been done with spray drying.

Ingredients	SD I	SD II	SD III
CAP	3 g	2 g	2 g
$MgSO_4$	0.09 g	0.29 g	0.29 g
Enzyme	0.65 g	0.5 g	0.2 g
total amount	3.74 g	2.79 g	2.49 g
Activity determination			
Activtiy in XIU/g	2565	2249	1008
Reference activity in XIU/g	3404	2621	1175
Yield	**75.4%**	**85.8%**	**85.8%**
Acitivty after 5' 1M HCl in XIU/g	3.8	11.9	31.4
Acid resistance	**0.1%**	**0.5%**	**3.1%**

Table 4.3.1.1 Spraydrying experiments For these experiments 40ml of solution were used for spray drying (see 3.2.1). 3g CAP represent 10% (w/v) solution whereas 2g CAP represent 6.66% (w/v) solution. $MgSO_4$ and purified enzyme was added in the indicated amounts. After spray drying the activity of the obtained powder was measured and compared to the activity before spray drying. For acid resistance 30mg powder was mixed with 100µl 0.10M HCl and incubated for 5' at 37°C. Then 100µl of 0.10M NaOH was added to neutralize the solution and to solubilize the CAP and the enzyme. This solution was then used for activity determination (see 3.1.3).

The activity yield of experiment I was 75% (see table 4.3.1.1). Addition of Mg^{2+} further enhanced the stability of the enzyme and increased the activity yield to 85%. By pharmacological standards the CAP would have passed the acid resistance test because it did not dissolve in 0.1M HCl. Unfortunately this did not mean that it protected the enzyme from the acid. After 5' incubation with 0.1M HCl more than 99% of the activity was lost in experiments I and II. For experiment III the amount of enzyme was reduced to increase the amount of CAP per g dry mass. The idea was that an increased amount of CAP can easier envelop the enzyme to provide better acid resistance. But also in experiment III 97% of the activity was lost after 5' incubation with 0.1M HCl.

CAP can therefore not sufficiently protect the enzyme from denaturation in the stomach.

4.3.2 Pellet production

Micropellets with enteric coating are another possible dosage form for xylose isomerase supplementation. Several different additives need to be used to produce enteric coated micropellets. A matrix substance provides the mechanical properties needed for the production of the micropellets. The efficient release of the enzyme in the small intestine is mediated by disintigrants and swelling agents. In my case I used microcrystalline cellulose as a matrix substance with starch as binder, polyvinylpyrrolidone and croscarmellose as swelling agents and hydroxypropyl cellulose as disintigrant. These substances are commonly used in enteric coated pellets.

For the pellets containing XI also maltose or saccharose is added as a water binding agent that increases the stability of enzymes. Sodium chloride was tested as an additive as a substrate for the sodium dependent SGLT-1transporter to facilitate glucose uptake.

For the enteric coating Eudragit L 100, an anionic copolymer based on methacrylic acid and methyl methacrylate, was used. It is stable in aqueous environments with a pH of below 6 and dissolves rapidly at a pH of above 6. This is due the loss of a proton from the methacrylic acid causing the molecule to gain negative charges. It is commonly used for enteric coating in pharmaceutical formulations.

Ingredient	Pellets I	Pellets II	Pellets III	Pellets IV
Microcrystalline Cellulose	38,4%	46,0%	41,9%	40,9%
Hydroxypropyl Cellulose	3,2%	3,3%	3,1%	3,1%
Rice Starch	15,1%	23,2%	19,0%	17,0%
Croscarmellose	1,6%	1,6%	1,6%	1,6%
Maltose	0,0%	0,0%	12,5%	14,5%
Saccharose	22,4%	0,0%	0,0%	0,0%
Polyvinylpyrrolidone (Crospovidone)	4,5%	6,3%	5,2%	6,2%
Sodium Chloride	0,0%	2,2%	0,0%	0,0%
Purified XI	14,9%	17,4%	16,7%	16,7%

Table 4.3.2.1 Pellet production The composition of pellets of 4 exemplary experiments. The pellets were produced and coated according to 3.2.2. Batch size was in all cases approximately 500g of pellets.

Saccharose is a common additive for enzymatic preparations to increase enzyme stability. For the first experiment saccharose was used as enzyme stabilizer. Pellets were produced according to 3.2.2. Experiment I (see table 4.3.2.1) was used to test the applicability of pellets for xylose isomerase supplementation. The dissolution and acid resistance of these pellets was tested as described in 3.1.6.

90% of the activity in the pellets from experiment I was released after 15’ dissolution when compared with 60’ dissolution. After 5’ incubation with 0.1M HCl 20% activity was lost. After 30’ incubation with 0.1M HCl the activity is reduced to 70% and after 60’ incubation with HCl to 45%. Since the pellets should leave the stomach within 10 minutes all tests with longer incubation were for informational purpose only and have no significance for pellet composition or production. This proves that enteric coated pellets are feasible for xylose isomerase supplementation. All the values were above expectations and the pellets were then optimised.

For experiment II two changes were made: removal of the saccharose and addition of NaCl. Saccharose was removed because it constitutes a fructose source. As mentioned earlier NaCl was supposed to act as a substrate for the sodium dependent SGLT-1transporter to facilitate glucose uptake.

These changes proved to be deleterious. After pellet production before the enteric coating the activity was already lower than the activity of the pellets from experiment I even though more enzyme was used for experiment II. The enteric coating further reduced the activity by 82%. This was caused by a significantly reduced heat resistance and stability of the enzyme in the pellets from experiment II. Therefore the composition of pellets II could not be used.

For pellets III maltose was used as enzyme stabiliser. This sugar is in its properties very similar to saccharose but is not a source of fructose. These enteric coated pellets III released 80% of the activity after 10’ dissolution when compared with 60’ dissolution. The acid resistance of the pellets was also good with only 10% loss after 10’ incubation with 0.1M HCl. Unfortunately the activity yield of pellets III when compared to the activity of the initially used enzyme preparation was only 66%. One third of the enzyme activity was lost during processing suggesting a denaturation during the production steps. This is most likely due to the elevated temperature during the coating and drying steps. It was not possible to change these parameters significantly without using different equipment which was not possible.

This pellet composition was found acceptable for xylose isomerase supplementation. Only two small changes were made for the final composition: the amount of maltose was increased slightly to increase enzyme stability and the amount of polyvinylpyrrolidone was also increased slightly. The resulting composition yields pellets IV which were used for all further studies.

These pellets IV lost 10% activity when incubated 10’ in 0.1M HCl and the dissolution rate was 80% after 10’ when compared with 60’ dissolution. These values were identical to the values obtained from pellets III and therefore acceptable.

The pellets were also analysed with an electron microscope. The diameter of the pellets was between 0.5 and 2mm and the weight between 0.5 and 2mg.

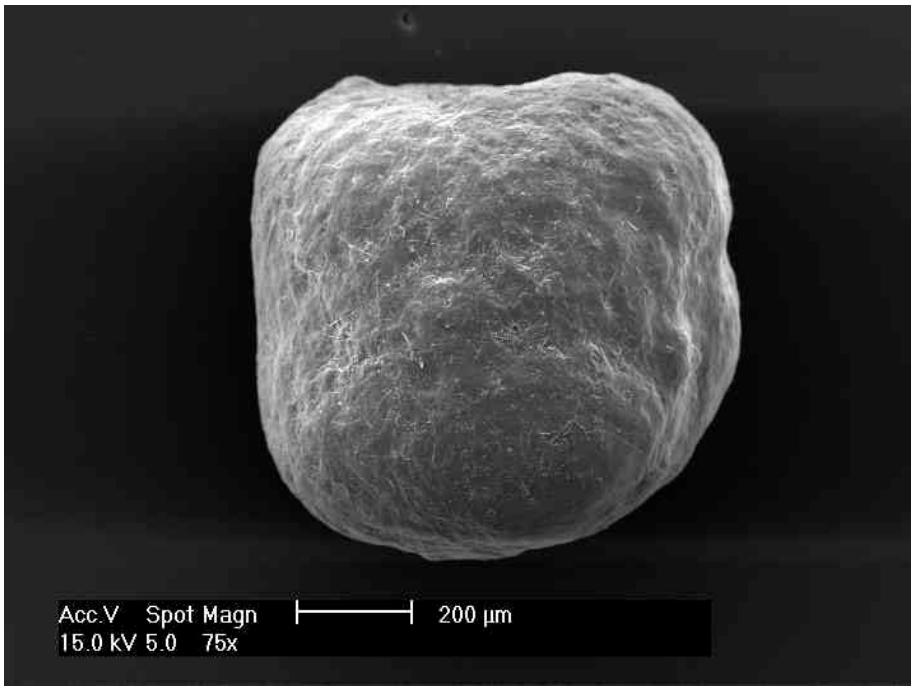

Figure 4.3.2.2 Coated pellet The pellets from experiment IV were analysed with an electron microscope.

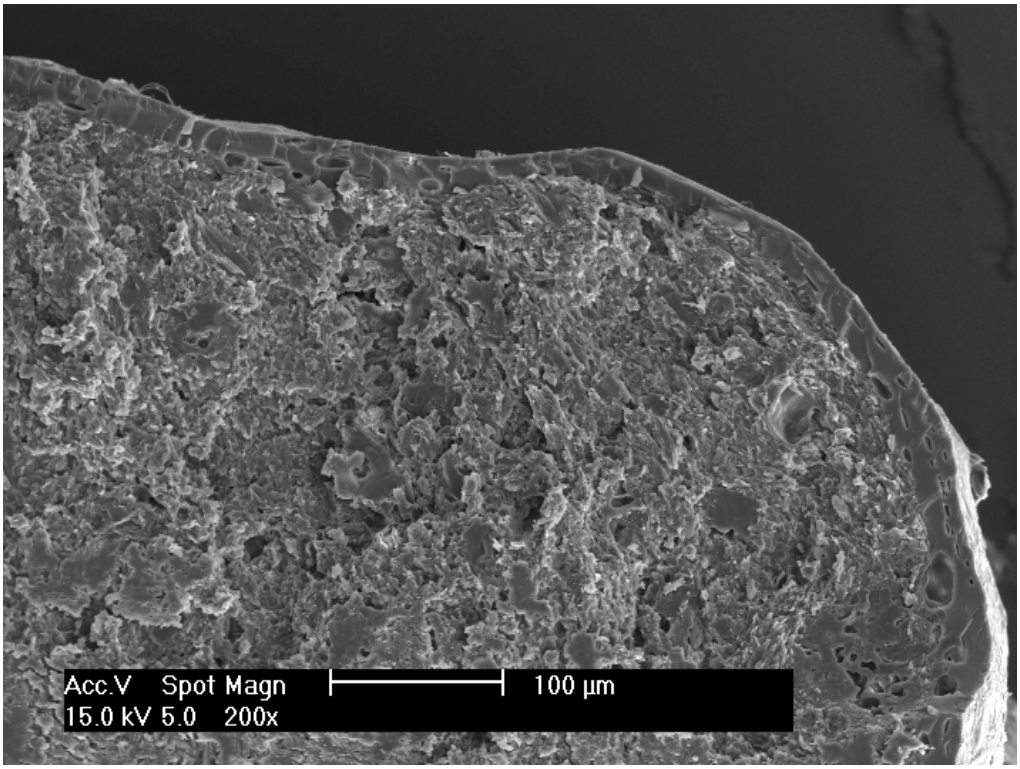

Figure 4.3.2.3 Coated pellet, cross section I This pellet was cut in half to analyze the composition under the electron microscope.

Figure 4.3.2.3 shows a cross section of one pellet under an electron microscope. The slightly lighter mass inside was the pellet mass with the enzyme (for composition see 4.3.2.1). The darker mass on the outside was the enteric coating that consisted of Eudragit L-100. The homogenous thickness (see 4.3.2.4) of the coating and the smooth surface confirmed that the coating process was working as expected. No thinner patches of Eudragit L-100 could be detected where the acid resistance would be compromised. This was confirmed by the very good acid resistance of the produced pellets.

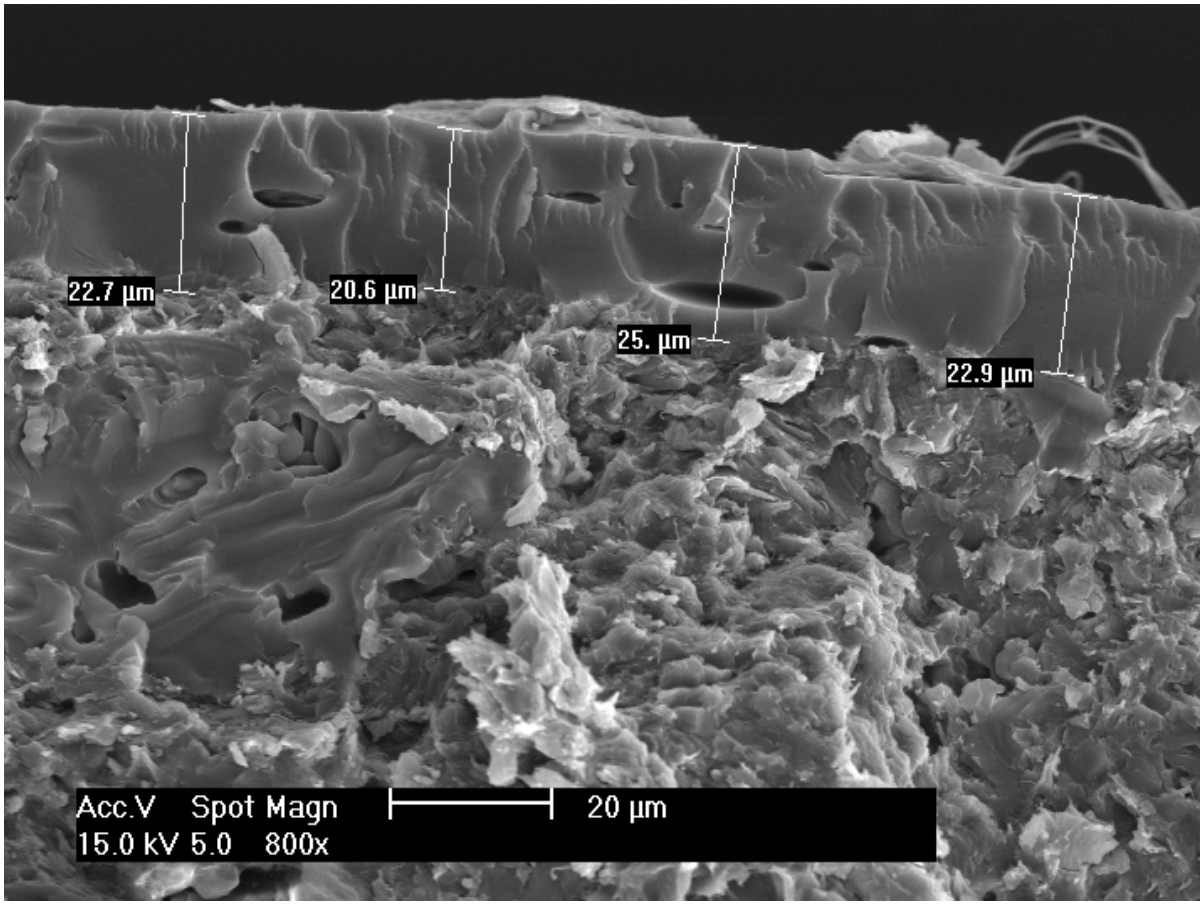

Figure 4.3.2.4 Coated pellet, cross section II This pellet was cut in half to analyze the composition under the electron microscope. The thickness of the enteric coating was between 20 and 25µm

The pellets from experiment IV exhibited an activity of approximately 7,000 XIU per g coated pellets under standard assay conditions (3.1.3). It is not possible to determine the

exact activity of the enzyme in the intestine. It mainly depends on the concentration of fructose in the intestine which varies from meal to meal. For example an apple contains, according to the german Bundeslebensmittelschlüssel, 5.7g free fructose /100g and 85g water /100g. This would give an effective concentration of fructose of 370mM. According to the K_M (see 4.1.3) this concentration of free fructose would give an activity of 17,500 XIU per g pellets. Although no accurate data is available it can be assumed that part of the water will be absorbed quickly in the small intestine which would further increase the concentration of fructose and the activity of the enzyme.

The amount of pellets needed to efficiently convert the fructose of one meal cannot be calculated directly and has to be determined with other means.

4.4 Efficacy and Safety of the final product

4.4.1 Toxicology

A subchronic 90 day oral toxicity study was performed as described in 3.3.1 to evaluate the potential toxicity of xylose isomerase.

There were no xylose isomerase-related deaths of animals during the study, except one unscheduled death. One male animal from the high dose group manifested paralysed hind legs and was killed for ethical reasons. At the post-mortem examination an enlarged spleen was observed.

In some cases smooth stool was observed but did not show relation to the enzyme application. An increased body weight of the males of the medium dose group was registered when compared to control group but considered not significant since it showed

no dose dependency. The food consumption was similar in all groups. No relevant changes in hematological parameters could be observed during the study and the results of the autopsies did not show pathological changes.

The administered enzyme did not cause test article related changes based on the evaluation of survival, clinical signs, body weight, hematological parameters, organs weight and macroscopic pathology. Xylose isomerase can therefore be considered as non-toxic.

4.4.2 Observational study

Ten participants were taken into the observational study and all 10 completed the study. For the study design see 3.3.2.

If possible the fructose content of each meal was estimated with the german Bundeslebensmittelschlüssel BLS. The fructose content of each meal was on average 5.5g with a maximum of 15g and a minimum of 0.2g. All participants recorded between 5 and 14 fructose containing meals. Usually higher fructose content was associated with stronger symptoms in the log.

Of these participants 2 rated the xylose isomerase supplementation to show no efficacy in reducing fructose-induced symptoms. 7 participants rated the efficacy “good” with one participant rating “low”.

All 8 participants that reported a reduction of fructose-induced symptoms recommended the dosage to be 2 capsules per fructose containing meal.

The main goal of the study was to find the dosage needed for the elimination of fructose-induced symptoms in fructose malabsorbers. The unanimity of the participants in recommending 2 capsules per fructose-containing meals is significant. Although six

participants also reported a reduction of symptoms after intake of 1 capsule they still recommended 2 capsules per meal. This suggests that the participants experienced that for meals with high fructose content 1 capsule is insufficient.

Also the high percentage of participant rating the supplementation to be efficient is encouraging. Unfortunately neither the size of the study nor the study design allow for conclusions as to the efficacy of xylose isomerase supplementation for the reduction of fructose-induced symptoms in adults with fructose malabsorption.

4.4.3 Placebo-controlled clinical trial

The placebo controlled clinical trial to assess the efficacy of the xylose isomerase supplementation was conducted with 65 participants, 51 of whom were tested at center 1 (Graz) and 14 at center 2 (Heilbronn). There were no drop-outs and all participants finished the study. For the study design see 3.3.3.

	Verum			Placebo		
Participant ID	AUC	Peak H2	Symptoms	AUC	Peak H2	Symptoms
01	5745	53.0	8.2	2775	29.0	11.8
02	2805	48.0	17.2	210	1.0	0.0
03	7800	63.0	0.0	6555	70.0	0.0
04	4035	39.0	0.0	4560	32.0	0.0
05	2430	14.0	0.0	225	2.0	0.0
06	3765	25.0	0.0	1665	14.0	0.0
07	3135	21.0	0.5	3840	51.0	0.0
08	6270	59.0	1.7	1455	17.0	0.0
09	360	4.0	0.0	2880	36.0	0.0
10	7185	46.0	0.7	11310	67.0	7.2
11	1875	21.0	0.0	2370	27.0	1.6
12	450	4.0	0.2	540	6.0	0.5
13	1605	16.0	0.0	2490	22.0	0.0
14	1770	12.0	0.0	2295	16.0	0.8

Table 4.4.3.1 Measured values for all 65 participants of the clinical trial (cont.)

	Verum			Placebo		
Participant ID	AUC	Peak H2	Symptoms	AUC	Peak H2	Symptoms
15	525	4.0	0.0	1155	9.0	0.0
16	2025	17.0	16.8	2760	26.0	0.7
17	1965	17.0	0.0	3750	26.0	0.6
18	1425	12.0	0.0	1980	18.0	0.0
19	1260	12.0	3.1	2145	27.0	0.7
20	1155	10.0	0.0	1800	14.0	1.2
21	1245	21.0	1.5	2295	22.0	9.5
22	1365	10.0	1.2	3330	23.0	0.8
23	3390	31.0	3.3	990	6.0	0.3
24	3060	22.0	16.0	690	7.0	4.0
25	2850	20.0	2.1	1050	6.0	0.0
26	3960	34.0	1.7	1200	15.0	0.0
27	14145	132.0	0.0	4935	49.0	22.3
28	405	2.0	3.5	2820	23.0	4.0
29	7515	50.0	2.0	28395	232.0	1.3
30	5730	58.0	2.8	4830	53.0	1.0
31	2910	27.0	17.1	4185	40.0	3.0
32	5265	52.0	17.7	615	3.0	1.3
33	9840	89.0	11.3	6930	52.0	0.9
34	10020	62.0	13.2	5730	47.0	6.5
35	5145	30.0	3.8	3720	25.0	2.6
36	12135	90.0	19.1	3075	35.0	12.2
37	495	4.0	3.6	1440	9.0	3.7
38	6450	65.0	6.3	4785	50.0	0.9
39	1800	21.0	8.8	420	3.0	2.9
40	2340	34.0	2.2	2130	20.0	0.0
41	7905	70.0	1.6	3315	22.0	0.0
42	105	2.0	1.2	210	3.0	0.0
43	10845	77.0	2.8	13800	126.0	12.3
44	2520	22.0	0.9	4695	43.0	2.0
45	24630	132.0	2.5	24915	189.0	8.0
46	3465	26.0	1.1	1935	20.0	0.0
47	7635	50.0	0.0	8025	72.0	1.0
48	17910	156.0	31.1	15150	132.0	2.5
49	15015	111.0	6.0	35520	241.0	8.2
50	3090	35.0	7.2	14445	94.0	5.5
51	6330	63.0	4.4	6720	43.0	0.8

Table 4.4.3.1 Measured values for all 65 participants of the clinical trial (cont.)

	Verum	Placebo

Participant ID	AUC	Peak H2	Symptoms	AUC	Peak H2	Symptoms
101	780	5.0	8.3	975	7.0	1.5
103	1095	9.0	0.0	270	2.0	2.5
104	16410	133.0	0.0	31230	193.0	0.0
105	210	1.0	0.0	960	10.0	6.0
106	1890	13.0	0.0	6225	50.0	8.1
107	345	3.0	0.0	3060	36.0	0.0
109	4350	39.0	0.0	2280	21.0	0.0
110	1260	12.0	1.0	705	10.0	0.0
111	3555	23.0	0.0	3150	33.0	0.0
112	3660	27.0	5.0	3225	30.0	2.0
113	3840	31.0	0.0	660	4.0	0.0
114	14085	71.0	7.5	4905	39.0	1.5
115	420	5.0	8.0	885	7.0	23.0
117	1500	10.0	22.0	1200	11.0	0.0

Table 4.4.3.1 Measured values for all 65 participants of the clinical trial The AUC values, the hydrogen peak values and the reported symptom score ("symptoms") are specified for each of the 65 participants for both HBTs. For study design and score calculation see 3.3.3.

The measured values for all 65 participants are given in table 4.4.3.1. The average AUC for the HBT with verum was 4777ppm*min and the average AUC for the placebo test 5058ppm*min. The average hydrogen peak was 38ppm for the verum and 41ppm for the placebo tests. The average difference in AUC between verum and placebo was 281ppm*min with a standard deviation of 5170ppm*min. The one sample t test gave a statistical significance of 0.663 for the difference between the verum and placebo tests making it statistically not significant. The reduction of the hydrogen peak was also not statistically significant (p=0.543).

Noticeable was the high number of participants with a hydrogen peak after placebo of less than 20. Since the placebo should not be able to increase the absorption of fructose this would mean a negative test for fructose malabsorption. The inclusion criteria distinctly stated a positive test for fructose malabsorption which would make these participants not suitable for this study. If these 24 participants are excluded from the study the average values for AUC would change to 6338ppm*min for verum and 7444ppm*min for placebo

raising the difference to 1105ppm*min with a standard deviation of 6259ppm*min (p=0.160). This value was still statistically not significant but represented a reduction of the mean AUC values by 15%. The exclusion of the aforementioned participants changed the average hydrogen peak value to 49.5ppm for verum and 60.2ppm for placebo. This represents a 10.7ppm reduction with a standard deviation of 45.4 yielding p=0.062 which is very close to the p=0.05 for statistical significance.

31 participants (48%) reported abdominal pain during or after the HBT with verum and 18 (28%) of these rated the severity with more than 1 point. Only 22 participants (34%) reported abdominal pain during or after the HBT with placebo and 10 (15%) rated the severity with more than 1 point. Similarly 42 (65%) and 37 (57%) participants reported flatulence during or after the test with verum and with placebo with 27 (42%) and 17 (26%) rating the severity with more than 1 point. The reported incidence of nausea was also lower during or after the placebo test with 20 (31%) for verum versus 15 (23%) for placebo. Of these participants 7 (11%) rated the severity of nausea after verum with more than 1 point while only 2 (3%) did so with placebo. Diarrhea occurred in 15 participants (23%) during or after the test with verum but only in 13 participants (20%) during or after the test with placebo. Therefore all recorded symptoms occurred more frequently and with a higher severity during or after the HBT with verum when compared to the HBT with placebo. The average symptom score for the verum test was 4.9 while the average symptom score for the placebo test was 2.6. The difference of 2.3 symptom score points was statistically significant (p=0.015 with a standard deviation of 7.5).

When the participants with a hydrogen peak value of below 20 for the placebo test (24 participants) were excluded the average symptom score values changed to 4.6 for the verum test and 3.3 for the placebo test. This reduced the statistic significance of the difference between these two values to p=0.151 making it statistically not significant. Furthermore the exclusion of these participants changed the incidence rates of the symptoms for verum and placebo tests. The incidence rates for abdominal pain (34% to 28% verum to placebo, respectively), flatulence (42% to 40%), nausea (20% to 17%) and diarrhea (15% to 17%) were then more similar for both tests.

4.5 Generation of α-XI antibodies and ELISA development

All available assays for the determination of xylose isomerase were based on testing the activity of the enzyme. To test for enzyme presence an ELISA test was developed during the course of this work.

4.5.1 Generation of XI-specific antibodies

Antibodies were raised in two rabbits named R85 and R93. First bleeds of both rabbits showed a good response and both rabbits were sacrificed after the second boost (see 3.1.17). The XI-specific antibodies were purified according to 3.1.11 and tested according to 3.1.12.

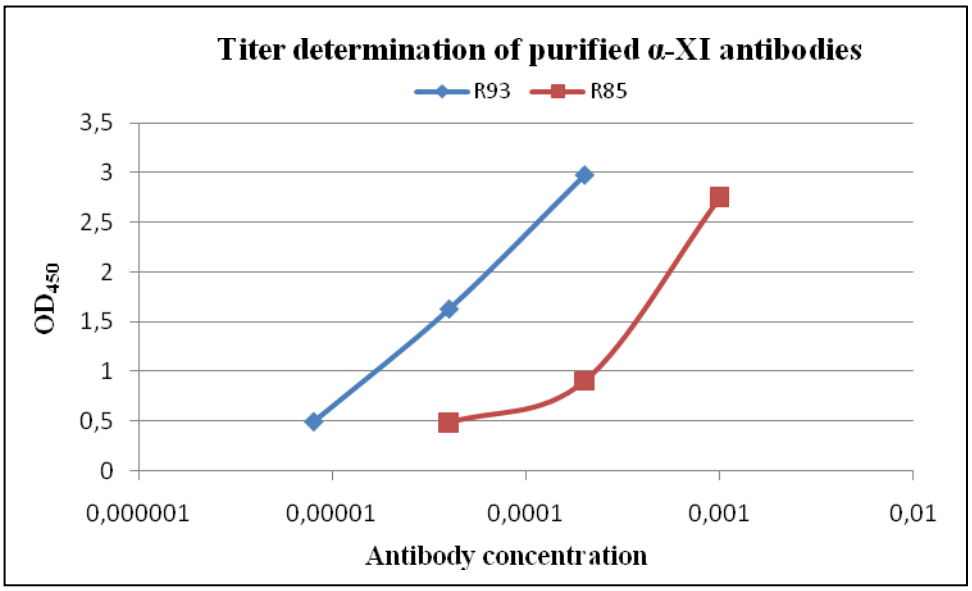

Figure 4.5.1.1 Titer determination of purified α-XI antibodies Final bleeds were purified according to 3.1.11 and the titer tested according to 3.1.12. The antibody concentration is given as dilution factor^{-1}.

Both bleeds showed a good titer with R93 being about 5 times as good.

4.5.2 XI sandwich ELISA development

The purified α-XI antibody from rabbit 85 was then coupled with HRPO (see 3.1.16). An ELISA according to 3.1.7 was performed with antibody from R93 as primary and the HRPO-coupled antibody from R85 as secondary antibody. Unfortunately the background of the ELISA was very high.

The reason for this high background was residual xylose isomerase that was released from the HiTrap NHS-activated XI-coupled column during antibody purification. This was most probably due to a dissociation of the subunits of the homotetrameric xylose isomerase in

the acidic elution buffer. Therefore 40kDa xylose isomerase subunits were present in the purified antibody and caused a strong background in the ELISA.

To eliminate these residual xylose isomerase subunits a size exclusion chromatography was performed as described in 3.1.15 with 900µg purified α-XI antibody from R93. This step proved to not be necessary with the HRPO-coupled antibody from R85.

The ELISA was then repeated with the size-exclusion-purified R93 α-XI antibody as primary antibody and the HRPO-coupled R85 α-XI antibody as secondary antibody.

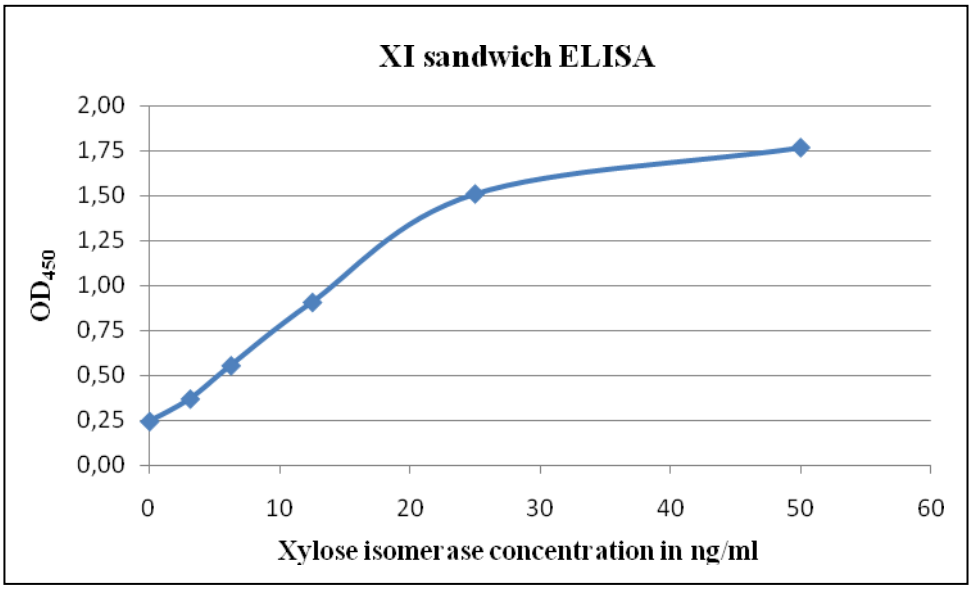

Figure 4.5.2.1 XI sandwich ELISA The ELISA was performed according to 3.1.7 with the size-exclusion-purified R93 α-XI antibody as primary antibody and the HRPO-coupled R85 α-XI antibody as secondary antibody.

The detection limit of xylose isomerase in the ELISA is approximately 3ng/ml with a linear range from 0 to 25ng/ml. The development of a sandwich ELISA for the detection of xylose isomerase was successful.

5 Conclusion

5.1 Evaluation of the feasibility of xylose isomerase supplementation

Xylose isomerase proved to be promising candidate for enzyme supplementation in adults with fructose malabsorption. If purified under the right conditions the enzyme presumably exhibits nearly full (83%) activity in the intestine as can be seen in figure 4.1.2.3. The activity measurement conditions were selected to closely represent the conditions found in the intestine since measurement of the activity in the human intestine was not possible.

A crucial point for xylose isomerase supplementation is the duration of the enzyme activity in the intestine. If the enzyme is quickly deactivated it cannot fulfill its function. The measured high protease resistance of the enzyme (see 4.1.4) suggests that the enzyme will be able to retain its function for prolonged duration in the intestine. Furthermore neither the pH nor the temperature found in the intestine affect the stability of the enzyme. It can therefore be concluded that the enzyme will retain its activity in the intestine and will be able to fulfill its role.

For the pharmaceutical formulation it was necessary to obtain an enzyme with the highest possible specific activity. The developed crystallisation process proved effective because the specific activity could be increased from approximately 5,000 XIU per g of original solution to approximately 80,000 XIU per g of enzyme powder. The high density of the enzyme in the crystals also reduced the needed amount of space, increased the stability and improved handling. It would otherwise not be possible to produce pellets with up to 20% (w/w) enzyme. The physical properties of the free enzyme would prove troublesome during extrusion and spheronisation which would make it necessary to increase the amount of texturiser and binder.

With the developed preparation of XI crystals it seems possible and feasible to supplement xylose isomerase in patients with fructose malabsorption.

5.2 Pharmaceutical dosage form

Only acid-resistant microparcticles have been found to be usable pharmaceutical dosage forms for xylose isomerase supplementation. Xylose isomerase is quickly denatured by a pH of 3 and below and has to be protected from the acidic environment in the stomach. For this the enteric coating is mandatory. Pharmaceutical dosage forms with microparticles have the added advantage to rapidly pass the pylorus which decreases the time in the stomach and in its environment.

Spray drying can be used for the generation of acid resistant microparticles [PALMIERI et al, 2002] but has not been used so far for the production of acid resistant microparticles containing an enzyme. While it was possible to obtain enzymatically active acid resistant microparticles with spray drying the process could not be used for the production of a pharmaceutical dosage form for xylose isomerase supplementation. The reason for this was the failure of the acid resistant compound CAP of these microparticles to protect the enzyme from denaturation in 0.1M HCl.

Therefore enteric coated pellets were developed with xylose isomerase as active ingredient. The developed pellets proved to be highly resistant to acid with only 10% activity loss after 10’ incubation and had a very fast dissolution rate with 80% dissolution after 10’ when compared to 60’dissolution. However, during pellet production approximately one third of the enzyme activity is lost. While it was not possible to reduce this loss in this work it should be possible to optimize this process when batch sizes are increased and other equipment is used.

5.3 Efficacy of xylose isomerase supplementation

After the assurance that the developed product is non-toxic it was necessary to determine the needed dosage. For this an observational study was designed and performed with 10 participants diagnosed with and treated for FM. The results of this study were very encouraging with 70% of the participants rating the efficacy of xylose isomerase supplementation for the treatment of FM as good and 10% rating the efficacy with low. The participant rating the efficacy low volunteered to participate in another study because he/she felt that the low efficacy might have been the result from other, external influences. Two participants reported to notice no reduction in fructose-induced symptoms after xylose isomerase supplementation. The meal log of these two participants did not differ significantly from the log of the others suggesting that it was not due to a difference in food intake.

All participants who felt an effect recommended the dosage to be 2 capsules even though 1 capsule already reduced the experienced gastrointestinal symptoms. This suggests that the enzyme activity from one capsule is insufficient for meals with high fructose content. Therefore it is recommended to either increase the enzyme activity of the pellets or to increase the capsule size before the XI containing pellets are marketed.

Contrary to the observational study the randomised clinical trial for efficacy determination did not yield the intended result. The null hypothesis cannot be falsified meaning that no difference between verum and placebo could be observed when analyzing the primary outcome of the study (AUC and hydrogen peak). Furthermore there is a statistically significant increase in the symptoms when comparing verum to placebo seemingly suggesting an increase of gastrointestinal symptoms by xylose isomerase supplementation. This is virtually impossible as the placebo and verum were chemically identical and there is no known possibility for the enzyme activity to increase the experienced symptoms in the study scenario.

So what was the reason for this result? An important point is the high number of participants failing to reach 20ppm breath hydrogen during the HBT with placebo capsules. Since a diagnosed FM was an inclusion criteria all of these participants should have a hydrogen peak of at least 20ppm which is the cut-off for routine measurements. Unfortunately an impact of the placebo treatment cannot be ruled out. For exactly this reason the original study design included 3 hydrogen breath tests where one HBT was without intervention. This HBT was designed to reproduce the initial FM diagnosis to ensure that only participants with a positive breath test are included. This test was rejected by the ethics committee for they ruled that the initial diagnosis by an accepted diagnostic test should be enough. This data would now give invaluable information with the comparison of the HBTs with or without placebo.

If these 24 participants failing to reach the cut-off during the HBT with placebo are excluded from the study the results change dramatically. A statistically not significant but still large decrease of the AUC by 1105ppm*min or 15% caused by verum application can be observed. Furthermore the peak hydrogen value is decreased by the verum by 10.7ppm. This reduction is very close to the statistical significance with a p of 0.062. The increase in symptoms with the verum treatment also loses the statistical significance when these participants are excluded.

It is nearly impossible that the placebo treatment has a positive effect on fructose absorption that cannot be seen with the verum. Again, the chemical identity would ensure a similar effect for both placebo and verum. Therefore a significantly higher AUC after verum when compared to placebo is suspicious.11 participants (17%) had an increase of the over 200%(!) in the HBT with verum when compared to placebo treatment. This effect cannot be explained by any action of the tested substance. Also it is important to note that the standard deviation of the difference of the AUC between verum and placebo treatment was higher than the average AUC of the treatments. This high fluctuation of the measured values nearly excludes the possibility for statistical significance.

This data suggests that the hydrogen breath test may not be as reproducible as generally believed. Only two publications [RUMESSEN and GUDMAND-HOYER, 1990; TRUSWELL et al, 1988] could be found that analysed the reproducibility of the HBT and only one of these [TRUSWELL et al, 1988] examined the HBT with a fructose challenge and then only in healthy subjects. Unfortunately this variation in the hydrogen breath tests makes a definite answer on the influence of xylose isomerase on the AUC impossible.

The recorded symptoms were only secondary outcome because of two reasons. First, recording symptoms in a clinical trial is always problematic because of the artificial nature of the test. Second, no study so far could show a correlation between quantitative hydrogen breath test results and the severity of symptoms although a qualitative correlation exists. And third, provocation with a concentrated fructose solution is very different to symptom induction by normal diet in amount, matrix and gastric emptying. But this was known at the start of the study and the symptoms only included for completeness.

Although generally used for β-galactosidase supplementation [LIN et al, 1993; ONWULATA et al, 1989; RAMIREZ et al, 1994; SANDERS et al, 1992] HBTs might not be optimal for the assessment of the efficacy of enzyme supplementation for the treatment of fructose malabsorptions. Although there were a few peculiar results with HBT studies of lactase supplementation most studies yielded expected results. Also lactulose as a non-absorbable carbohydrate yields reproducible breath tests [RUMESSEN et al, 1990] whereas fructose yields slightly less, but still acceptable reproducibility in healthy subjects[TRUSWELL et al, 1988]. The only major difference that has not been addressed so far is the identity of the sugar and the mechanism of its absorption. In this aspect lactulose, lactose and fructose all differ significantly. So a major influence on HBT reproducibility might be the fructose absorption of the intestine. For example some patients claim to be able to tolerate more fructose on vacation when they feel relaxed and calm. This has not been addressed so far in clinical studies and remains controversial since several explanations can be found to explain the reduced gastrointestinal symptoms in a relaxed state. Nonetheless it might suggest a variability of the absorptive capacity of fructose which

would cause low reproducibility of HBTs in patients with FM. Therefore another method for the assessment of the efficacy of xylose isomerase supplementation might be needed.

The efficacy of the xylose isomerase supplementation for the treatment of FM remains unclear. One observation study yielded a very positive assessment of the efficacy. Unfortunately this study allows no definite answer since it was designed for dosage finding only. The clinical study designed to assess the efficacy yielded no definite result. Although the null hypothesis of the clinical study could not be falsified the problems associated with the study data allow no definite answer.

6 Summary

A suitable enzyme for the treatment of fructose malabsorption, namely xylose isomerase, was identified during the course of this work and a purification step developed to obtain xylose isomerase in a highly active and stable form which could be easily processed further.

As a pharmaceutical dosage form enteric coated micropellets were used and a formulation developed that allowed the production of highly active pellets with very good acid resistance and almost perfect dissolution characteristics. The only drawback was an activity loss of 33% during production which probably can be decreased by the use of other equipment and larger batch sizes.

The developed preparation proved to be non-toxic in a toxicology study and the supplementation of xylose isomerase containing pellets efficacious in reducing fructose-induced symptoms when tested in a not-placebo-controlled observational study with 70% rating the efficacy of the product with good. It might be advantageous to further increase the amount of enzyme per dose since one capsule was not sufficient in some cases and the intake of two capsules recommended by all participants of the observational study who reported an efficacy.

Although generally used for the assessment of the efficacy of enzyme supplementation for carbohydrate malabsorptions, the trial based on hydrogen breath tests proved not to be optimal in my case. The placebo-controlled clinical trial based on HBTs yielded no definite result and further assessment of the efficacy of xylose isomerase supplementation for the treatment of FM is necessary.

7 Literature

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8 Addendum

8.1 English Abstract

Fructose malabsorption is a common digestive disorder resulting from incomplete absorption of ingested fructose. Excess fructose is degraded by bacteria in the large bowel into hydrogen, methane, carbon dioxide and short chain fatty acids leading to symptoms like bloating, wind, abdominal pain or diarrhea. Currently the only treatment is a fructose-free diet which can lead to nutritional imbalances. The aim of this work is to develop a new treatment of fructose malabsorption based on the in vivo elimination by enzymatic conversion of excess fructose in the small intestine which would prevent the generation of symptoms and allow the affected persons to eat a normal diet.

A suitable enzyme for the treatment of fructose malabsorption, namely xylose isomerase, was identified during the course of this work and a purification step developed to obtain xylose isomerase in a highly active and stable form which could be easily processed further. As a pharmaceutical dosage form enteric coated micropellets were used and a formulation developed that allowed the production of highly active pellets with very good acid resistance and almost perfect dissolution characteristics.

The developed preparation proved to be non-toxic in a toxicology study and the supplementation of xylose isomerase containing pellets efficacious in reducing fructose-induced symptoms when tested in a not-placebo-controlled observational study with 70% rating the efficacy of the product with good. It might be advantageous to further increase the amount of enzyme per dose since one capsule was not sufficient in some cases and the intake of two capsules recommended by all participants of the observational study who reported an efficacy.

Although generally used for the assessment of the efficacy of enzyme supplementation for carbohydrate malabsorptions, a placebo-controlled clinical trial based on hydrogen breath

tests proved not to be optimal. The clinical trial conducted during this work yielded no definite result because of a high fluctuation in the measured values.

8.2 German Abstract

Fruktose Malabsorption ist eine häufige vorkommende Verdauungsstörung, bei der nicht vollständig resorbierte Fruktose gastrointestinale Symptome auslöst. Diese überschüssige Fruktose wird im Dickdarm von Bakterien anaerob in kurzkettige Fettsäuren, Methan, Kohlendioxid und Wasserstoff abgebaut. Diese Abbauprodukte verursachen Symptome wie Blähungen, Bauchschmerzen und Durchfall. Als Behandlung steht derzeit nur eine Fruktose-freie Diät zur Verfügung, die allerdings zu Nährstoffmangel führen kann. Das Ziel dieser Arbeit ist die Entwicklung einer neuen Behandlungsmethode, die auf einer enzymatischen Umwandlung der überschüssigen Fruktose im Darm basiert. Dies würde die Entstehung von Symptomen verhindern und den Einschluss von Fruktose-haltigen Speisen in die Diät erlauben.

In der Xylose Isomerase konnte im Rahmen dieser Arbeit ein passendes Enzym gefunden werden. Für diese Enzym wurde eine Reinigungsmethode entwickelt, die ein hochaktives und stabiles Enzympulver liefert, das für die Weiterverarbeitung sehr gut geeignet ist. Für die pharmazeutische Formulierung wurden enterisch gecoatete Micropellets mit sehr guter Säureresistenz und fast perfekten Dissolutionseigenschaften entwickelt.

Die Enzymsupplementation zeigte keine toxischen Folgen in einer 90Tage Toxizitätsstudie in Ratten. Bei einer nicht Placebo-kontrollierte Beobachtungsstudie bescheinigten 70% der Probanden der Supplementation mit Xylose Isomerase hältigen Pellets eine gute Wirkung bei der Reduktion von Fruktose-induzierten Symptomen. Eine Erhöhung der Enzymaktivität pro Kapsel könnte die Wirkung noch verbessern, da alle Probanden für eine optimale Wirkung die Einnahme von 2 Kapseln empfahlen.

Obwohl Sie der Standard für Wirksamkeitsstudien von Enzymsupplementationen für Kohlenhydratmalabsorptionen sind, brachte eine Placebo-kontrollierte klinische Studie auf der Basis von Wasserstoffatemtests aufgrund von stark schwankenden Messwerten kein eindeutiges Ergebnis.

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