Studies on some carbohydrolases with nutrition potential

PhD thesis

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2003

PhD School/Program

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1. Introduction

In recent years, consumers worldwide have met so-called functional food products, which are claimed to promote health and well-being. They are known as pro-, pre- and symbiotics in human nutrition. Among them prebiotics are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in colon. Fructooligosaccharides (FOS), which selectively stimulate the growth of bifidobacteria are considered to be prebiotics. The market of FOS grows dynamically from the middle of the eighties, especially in Japan, but in the United States of America too, because FOS are now listed as "Generally Recognised as Safe" (GRAS) by Food and Drug Administration (FDA) of USA. In European countries consumers also find them more and more in fermented dairy products. Nowadays, FOS are produced commercially by applying transfructosylating enzymes from the filamentous fungus *Aspergillus niger*. Numerous studies are published dealing with enzymes that have transfructosylating activity, but some questions are still disputed. The yields of FOS production by β-fructofuranosidase remain low, thus the cost of production is relatively high. Another aspect is that the commercially available FOS products contain only 50-60 % of FOS, the rest is made up of 20-30 % of glucose, 10-15 % of unconverted sucrose and small amount of other compounds. The primary sequences of some β-fructofuranosidase enzymes are available in protein databases, but the three-dimensional structure with real conformation is unpublished yet. *Aspergillus niger* which produces numerous enzymes with GRAS status should be an ideal source to study β-fructofuranosidase. The FOS produced by this enzyme can be readily used in food as prebiotics.

Starch is the second biggest polymer, found in the plant kingdom, after cellulose. This is one of the renewable and environmental friendly energy sources available for the humanity in all over the world. The demand for modified and/or converted starch products have increased more and more

over native starch, especially in the industrial production of bioethanol, high fructose and high maltose syrups as well as thiosugars. The latter ones are also claimed to promote health and well-being. Nowadays, microbial amylolytic enzymes have replaced the acid hydrolysis of starch in industry with great success. Most of them are produced commercially by *Bacillus*, *Aspergillus* and *Rhizopus* species. α-Amylases from *Bacillus* species are stable and active between 90-95^{o}C and are used widely in food industry and for other purposes. Main drawbacks of these enzymes are the need Ca^{++} ion and that they work at relatively high and in narrow range of pH (6.2-6.5). Currently, the glucoamylase from *Aspergillus* is used in industrial scale, but this enzyme is stable and active only up to 55-60 ^{o}C and between pH 4.0-6.0. Due to the differences in properties of the two important amylolytic enzymes, the starch bioconversion technology takes a long time (15-95 h) with various technological steps. The "one step" hydrolysis technology would be realisable, if two main amylolytic enzymes were able to work under approximately the same conditions. Taking into consideration that bacteria do not produce adequate amounts of glucoamylase, the thermophilic fungus *Thermomyces lanuginosus*, which is reported to produce extracellularly thermostable α-amylase and glucoamylase appeared to be an ideal source to develop of an economically attractive procedure for the industrial starch processing. Other applications of amylases are also described in the production of some oligosaccharides and cyclodextrins. Due to promising biological properties of these oligosaccharides, in the past years they have gained substantial attention on the fields of medicine and analytical chemistry. New developments, especially in the synthesis and medical chemistry of thiosugars have become important for carbohydrate drug design.

In the present thesis *Aspergillus niger* was selected to investigate the properties of β-fructofuranosidase as well as the production and purification of fructooligosaccharides synthesised by this enzyme. For research dealing with thermostable amylolytic enzymes thermophilic fungus *Thermomyces lanuginosus* was applied.

2. Aims of the thesis

The objectives of the PhD study were to get insights into the properties of β-fructofuranosidase from *Aspergillus niger* and into the properties of amylolytic enzymes from *Thermomyces lanuginosus*. The aims of the thesis were the followings.

β-Fructofuranosidase from Aspergillus niger

- Development of submerse fermentation technology for the production of β-fructofuranosidase by filamentous fungus *Aspergillus niger*.
- Purification and characterisation of β-fructofuranosidase from *Aspergillus niger*.

Fructooligosaccharides

- Production of fructooligosaccharides applying β-fructofuranosidase from *Aspergillus niger*.
- Development of method for the purification of fructooligosaccharides.

Thermostable amylolytic enzymes from Thermomyces lanuginosus

- Screening of *Thermomyces lanuginosus* strains for the production of amylolytic enzymes.
- Optimisation of medium composition for the production of amylolytic enzymes.
- Purification of amylolytic enzyme in mg quantity.
- Determination of the physicochemical properties of amylolytic enzymes as well as effects of pH, temperature, metal ions and other compounds on activity and stability of amylolytic enzymes.
- Determination of kinetic parameters of amylolytic enzymes on different substrates.
- Analysis of the amino acid sequence of amylolytic enzymes from *Thermomyces lanuginosus*.

3. Materials and Methods

- Filamentous fungi *Aspergillus niger* and *Thermomyces lanuginosus* strains were from different culture collections such as ATCC, CBS, DSM or IMI. All other chemicals were analytical grades and purchased from Sigma, Reanal, Merck, Pharmacia, BioRad etc.
- Transfructosylating activity of β-fructofuranosidase was assayed applying Somogyi/Nelson and GOD/POD methods. One unit of transfructosylating activity (U) was expressed as the amount of enzyme that transfers 1 μmol of fructose from donors to acceptors in 1 minute under the relevant conditions.
- The α-amylase activity was assayed based on starch estimation using iodine method. One unit (U) of α-amylase activity was defined as the amount of enzyme that hydrolyses 1 mg soluble starch in 1 minute under the relevant conditions.
- Glucoamylase activity was assayed based on measurement of glucose using GOD/POD method. One unit (U) of glucoamylase activity was defined as the amount of enzyme that releases 1 μmol glucose in 1 minute under the relevant conditions.
- The cultivation for enzyme production was carried out using submerse technology. The optimisation of medium composition and investigation of the thermostability of amylolytic enzymes were carried out using Response Surface Method. The SPSS 10.0 statistical software package was applied for evaluation of the working hypotheses.
- Low-pressure liquid chromatographical steps were applied in purification of the enzymes. The columns with different resins were connected to FPLC or GradiFrac (Pharmacia) systems.
- Gelfiltration on BioGel P-2 was used for purification of fructooligosaccharides.

- Various methods such as Buiret, Lowry, modified Lowry, Bradford and BCA were used for estimation of proteins.
- The analyses of sugars were carried out using chromatographical (HPAELC and HPLC) and other analytical methods such as BCA, Somogyi/Nelson and GOD/POD.
- Determination of protein molecular mass was done using sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with different staining methods. The isoelectric points were estimated by electric focusing on agarose or polyacrylamide gel.
- The purified α-amylase enzyme was applied onto Applied BioSystem 476A pulsed liquid sequenator (Foster City, Ca, USA) to determine the primary sequence of the peptide chain. The primary sequence was analysed using the BLAST database (http://pbil.univ-lyonl.fr/BLAST/).

4. Results

β-Fructofuranosidase from Aspergillus niger

- β-fructofuranosidase activity of some *Aspergillus niger* strains was screened and the IMI 303386 strain was selected for future studies. This strain was able to produce higher levels of intra- and extracellular β-fructofuranosidase and inulinase on inulin than on sucrose. Inulinase hydrolysed inulin in an exo-fashion and released mainly fructose.
- These enzymes are stable in frozen forms for at least 2 weeks, but after precipitation with ammonium sulphate the stability of the enzymes decreased for 4-5 days.
- The intracellular β-fructofuranosidase from *A. niger* IMI 303386 was purified to homogeneity according to SDS-PAGE by various steps by $(NH_4)_2SO_4$ precipitation, DEAE Sepharose Fast Flow ion-exchange chromatography and Ultrogel AcA44 gelfiltration. This protocol gave fifty-fold purification and 42 % recovery of enzyme activity.

- The molecular mass of β-fructofuranosidase monomer from *A. niger* was estimated to be in the range between 115 and 135 kDa. This enzyme shows two different monomers on isoelectric focusing with agarose gel. The pI value of the major and the minor monomers was calculated to be 5.4 and 4.4, respectively.
- The β-fructofuranosidase showed maximal transferase activity when it was incubated at 55 ^{o}C and pH 5.5. Among to the investigated metal ions and other compounds Ba^{++}, Mg^{++}, Ca^{++} and EDTA are activators, while Hg^{++}, Ag^{+} and Ni^{++} are strong inhibitors of β-fructofuranosidase activity.
- The enzyme is completely stable in the pH range from 4 to 8 and up to 55 ^{o}C. More than 90 % of residual activity was recovered after 5-hour incubation. The enzyme lost its activity drastically when it was incubated at temperature higher than 60 ^{o}C.
- β-Fructofuranosidase from *A. niger* IMI 303386 is a glycoprotein and its carbohydrate content was calculated to be approximately 17 %.
- Intracellular β-fructofuranosidase from sucrose-based medium catalysed the best transfructosylating reaction. The concentration of fructooligosaccharides reached a maximum using 25 % (w/v) sucrose as substrate at 72 h.
- The purification procedure was carried out applying gelfiltration using BioGel P-2 columns. The main fructooligosaccharide components were kestose and nystose. These components were purified to homogeneity according to HPLC analysis.

Thermostable amylolytic enzymes from Thermomyces lanuginosus

- *Thermomyces lanuginosus* strains were cultured on different media. On potato-dextrose agar their colony have reached diameter of 6-8 cm at 45°C after three-day incubation. The structure and the colours of their surface were different. Deviations of colony morphology are

observed among the various strains. During the growth some strains showed segregation of their fructification properties.

- A new rapid method was developed to screen *Thermomyces lanuginosus* strains for production of amylolytic enzymes. The screening was carried out applying this method on solified medium containing soluble starch. The utilisation of starch was detected by the iodine vapour method. The ratios of clear zone to colony were calculated and according to these values the strains were ranked. These results were confirmed in fermentation trials assaying amylolytic activities of the individual strains. The ATCC 34626 strain, which secretes appreciable amount of both amylolytic enzymes (α-amylase and glucoamylase), was selected for further studies.
- The pH of ferment broth has significant influences on amylolytic enzyme activities during fermentation. The maximal enzyme activities were measured when applying fermentation media prepared with 100 mM citrate buffer pH 4.9.
- *Thermomyces lanuginosus* grew very well on all tested carbon sources such as soluble starch, native starch, glucose, maltodextrin, dextrin, maltose, dextran and amylopectin. In the case of α-amylase the maltodextrin, in the case of glucoamylase dextrin substrates proved to be good for enzyme production by *T. lanuginosus*, but the fungus also secreted high levels of amylolytic enzymes on native and soluble starch.
- Among the tested inorganic and organic nitrogen sources, L-asparagine was the best. Cultivating the *T. lanuginosus* ATCC 34626 strain on yeast extract the gained enzyme activities were half of that, which were detected on L-asparagine.
- To determine the optimal concentration of carbon and nitrogen sources, the Response Surface Method (RSM) was applied. The optimal concentration of soluble starch and L-asparagine for α-amylase were 6.5 % and 0.75 %, for glucoamylase were 2.0 % and

0.75 %, respectively. Calculated values were confirmed experimentally.

- ❖ The concentration of K_2HPO_4 and KH_2PO_4 in fermentation media were also optimised and confirmed.
- ❖ The following media were suggested for the production of amylolytic enzymes (α-amylase and glucoamylase) using *Thermomyces lanuginosus* ATCC 34626 strain:
  - o α-amylase: soluble starch 6.5%, L-asparagine 0.75%, KH_2PO_4 0.15%, K_2HPO_4 0.1 %, $MgSO_4.7H_2O$ 0.05%, Vogel's trace element solution 0.1 mL
  - o glucoamylase: soluble starch 2.0 %, L-asparagine 0.75%, KH_2PO_4 0.15%, K_2HPO_4 0.1 %, $MgSO_4.7H_2O$ 0.05%, Vogel's trace element solution 0.1 mL

  Both media should be prepared with 100 mM citrate buffer pH 4.9.
- ❖ The extracellular amylolytic enzymes from *T. lanuginosus* were purified to homogeneity according to SDS-PAGE applying multi-steps procedures. Different techniques were applied such as precipitation with ammonium sulphate, ion-exchange chromatography with DEAE Sepharose Fast Flow, gelfiltration with Sepharose CL-6B or Superose 12, ion-exchange chromatography with Q Sepharose Fast Flow. Applying these protocols approximately 17-fold purification and 27 % of recovery in the case of α-amylase, and 8.6-fold purification and 23 % of recovery in the case of glucoamylase were achieved.
- ❖ The molecular mass of purified α-amylase and glucoamylase were estimated to be 61 kDa and 75 kDa, respectively, according to SDS-PAGE using 1-D-gelanalysis software v2.4b (Signum/Biotech Fisher GMBH, Germany). Their pI values were calculated to be 3.5 - 3.6 and 4.1 - 4.3, respectively.
- ❖ The amylolytic enzymes from *T. lanuginosus* exhibit pH optima in the range between 4.6 and 6.6 in the case of α-amylase and 4.4 - 5.6 in the case of glucoamylase. Both purified enzymes have temperature optima at 70 ^{o}C. Effects of numerous metal ions and compounds on

amylolytic enzyme activities were investigated. Zn^{++} ions strongly inhibit both enzyme activities. Mn^{++} and Fe^{++} ions are activators in the case of glucoamylase; Ca^{++} and Ba^{++} are activators of α-amylase.

- ❖ The accepted hypothesis of the work was that enzymes with half-life times longer than 12 hours at 60 °C are regarded as thermostable. Based on this hypothesis both amylolytic enzymes are thermostable in the pH range between 4.5 and 8.5. The amylolytic enzymes from *T. lanuginosus* loose activities rapidly when incubated at temperature higher 80 °C or at pH lower than 4.0. In the presence of 10 mM Ca^{++} ion the half-life time of α-amylase was five-times longer than in the absence of it. The half-life time of glucoamylase was three-times longer in the presence of 10 mM α-cyclodextrin.
- ❖ Both enzymes are found to be glycosylated; α-amylase contains 8.5 % carbohydrate and glucoamylase 3.3 %. The major monosaccharides were mannose, glucosamine and galactosamine.
- ❖ The K_m and V_{max} of α-amylase on soluble starch were 0.68 mg/ml and 45.19 U/mg, respectively. The K_m values of glucoamylase on maltose, maltotriose, maltotetraose, maltopentaose and soluble starch were 6.5 mM, 3.5 mM, 2.1 mM, 1.1 mM and 0.8 mg/ml, respectively.
- ❖ The first 37 residues of *N*-terminal of the purified α-amylase of *T. lanuginosus* ATCC 34626 were determined. Almost complete homology (95 %) with the α-amylase from *Aspergillus oryzae* and *Emericella nidulans* was observed. The sequence alignment was done using BLAST protein database (http://pbil.univ-lyonl.fr/BLAST/). The results of sequence analysis suggested that α-amylase from *Thermomyces lanuginosus* ATCC 34626 should belong to glycohydrolase class 13.

5. New scientific results

β-Fructofuranosidase from Aspergillus niger

- A method was developed for extraction and purification to homogeneity of β-fructofuranosidase from *Aspergillus niger*. The physicochemical properties of purified β-fructofuranosidase were also determined. The enzyme consists of at least two monomers. Molecular mass of each was 115-135 kDa according to SDS-PAGE and the major and minor monomers have isoelectric point 5.4 and 4.4, respectively.
- The bioconversion time (72 hours) was determined and fixed to reach maximum amount of fructooligosaccharides during transfructosylation applying β-fructofuranosidase from *Aspergillus niger*.
- A method for purification of fructooligosaccharides to homogeneity was developed. For gelfiltration two BioGel P-2 columns connected in series were applied using distilled water as eluent.

Thermostable amylolytic enzymes from Thermomyces lanuginosus

- New fermentation media were developed by applying experimental design methods to production of α-amylase and glucoamylase enzymes by *Thermomyces lanuginosus* ATCC 34626 strain. Applying these media in the case of α-amylase five-times and in the case of glucoamylase ten-times higher activities were detected than when the original medium was used.
- Methods were developed for the purification of α-amylase and glucoamylase enzymes from *Thermomyces lanuginosus*. The molecular mass and isoelectric point of α-amylase was calculated to be 61 kDa and 3.5-3.6, respectively, while molecular mass of glucoamylase was 75 kDa and its isoelectric point was in the range between 4.1 and 4.3.
- New concept for the evaluation of a thermostability of thermostable enzyme was introduced. Investigating the effects of pH and temperature as well as their interaction and selecting the half-life time

of the enzyme as dependent factor, model can be constructed using Response Surface Method. Application of this methodology makes possible the objective characterisation and comparison of enzyme from different sources.

- The kinetic parameters of α-amylase and glucoamylase from *Thermomyces lanuginosus* on various substrates (soluble starch, maltose, maltotriose, maltotetraose and maltopentaose) were determined.
- The first 37 residues from *N*-terminal of α-amylase from *Thermomyces lanuginosus* ATCC 34626 were determined. Using database search (http://pbil.univ-lyonl.fr/BLAST/) the segment shows strong homology with sequences of α-amylase isolated from *Emericella nidulans* and *Aspergillus species.* Based on these results the α-amylase from *T. lanuginosus* can be classified into carbohydrolase class 13.

6. Aspects of biotechnological exploitation and future research

Aspergillus niger IMI 303386 strain is able to synthesize adequate amount of intracellular β-fructofuranosidase enzyme on medium containing sucrose. This enzyme catalyses the fructosyltransfer to build up fructooligosaccharides that can be used in the production of functional foods for human nutrition. Future works should go to different ways:

- Studies on structure of β-fructofuranosidase enzyme.
- Development of a technology for the immobilisation of β-fructofuranosidase enzyme, even immobilisation of fungus cell.
- Scaling up experiments and design of bioreactor for the production of large amount fructooligosaccharides.

The results of optimisation of media composition can be exploited in the industrial scale production of amylolytic enzymes. Applying these techniques in combination with strain development, a new technology can

be developed for the production of thermostable amylolytic enzymes complex using *Thermomyces lanuginosus*.

The new concept for evaluation of thermostable enzymes gives possibilities to compare thermostable enzymes from different sources.

The *N*-terminal sequence of α-amylase from *Thermomyces lanuginosus* gives preliminary information to further works on studies of the structure of this enzyme.

7. List of publications

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3. **Nguyen DQ** (1997) Termofil gomba amilolitikus enzim termelése és vizsgálata. XXIII. OTDK, Keszthely, április
4. **Nguyen DQ** (1997) Termofil gomba amilolitikus enzim termelése és vizsgálata, Diplomamunka, KÉE, Budapest

The works were carried out in the following laboratories:

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Institute of Food Research, Reading Laboratory
Reading, United Kingdom

Gent University
Department of Biochemistry, Physiology and Microbiology
Laboratory of Biochemistry, Gent, Belgium

Central Institute of Food Research
Division of Biochemistry, Budapest

Curriculum Vitae

Quang D. Nguyen was born on July 14th, 1970 in Nghi Duc, Nghe an, Vietnam. After finishing higher school he took admission to Hanoi Agricultural University. Based on the result of admission and the Cultural and Educational Agreement between Hungarian and Vietnamese Governments he won the state scholarship to Hungary. In 1990 he started his studies at GödöllŖ Agricultural University (Hungary), but in 1991 he moved to University of Horticulture and Food, Faculty of Food (Budapest, Hungary). During the university studies, in 1994 he participated the Competition for students of Hungarian Agricultural Universities about informatics and won second place medal. In 1995 he won the extra-prize medal with his thesis entitled "Modelling of canned heating" at XXII. OTDK. In 1995 he started diploma works at Department of Brewing and Distilling. The results of his works were demonstrated at MÉTE OTDK and the first place medal was awarded to him. One year later in 1997, he took the second place medal at XXIII. OTDK. He obtained Master of Science (MSc) degree in 1997 from University of Horticulture and Food, Faculty of Food. In this year he took admission to PhD programme of Food Engineering and Sciences under supervisor of Prof. Dr. Ágoston Hoschke. His PhD topic is "study of fungal enzymes". In 1998 thanks for INCO-COPERNICUS Programme in frame of exchange of researchers he spent 3 months in Institute of Food Research, Reading Laboratory, UK, and here he has done the research works dealing with β-fructofuranosidase synthesised by *Aspergillus niger*. Three years later, he spent 6 months in Ghent University, Department of Microbiology, Physiology and Biochemistry, Laboratory of Biochemistry (Belgium). There he did the works dealing with kinetics of amylolytic enzymes from *Thermomyces lanuginosus* as well as determination of *N*-terminal sequence of α-amylase.
Since 2001 he works as assistant lecturer at Department of Brewing & Distilling, Szent István University.
The results of his research works were published in various scientific periodicals as well as demonstrated at different international and Hungarian scientific conferences.

Budapest, October 2002