Study of reverse hydrolysis and transglycosylation for production of oligosaccharides

Theses of Ph.D. Dissertation

GABRIELLA STYEVKÓ

Budapest
2015

PhD School/Program

Name: PhD School of Food Science

Field: Food Science

Head: **Prof. József Felföldi PhD**
Department of Physics and Control
Faculty of Food Science
CORVINUS University of Budapest

Supervisors: **Prof. Quang D. Nguyen PhD**
and
Prof. Ágoston Hoschke CSc.
Department of Brewing and Distilling
Faculty of Food Science
CORVINUS University of Budapest

The applicant met the requirement of the PhD regulations of the Corvinus University of Budapest and the thesis is accepted for the defence process.

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Signature of Head of School **Signatures of Supervisors**

1. Background

It is well known that carbohydrates play an important role in living organisms. These biomolecules act as energy supply (*e.g.* glucose), nutrient store (*e.g.* starch, glycogen, *etc.*), building materials (*e.g*. cellulose, chitin), important components of nucleotides, coenzymes *etc*., and have big diversity in both composition and structure. Based on polymerisation degree, they can be generally classified into four groups: monosaccharides, disaccharides, oligosaccharides (3-10 units) and polysaccharides (the number of units is larger than 10). Oligosaccharides play many significant roles in biological systems. They participate in recognition processes, have a role in organizational and protective mechanisms or they could have effect on biological function of proteins *etc*. Additionally, they are used widespread in food industry because of their favourable physicochemical and physiological features. Non-digestible oligosaccharides - due to their specific glycosidic bonds - are not degradable by enzymes in the upper part of digestive system, and are useful in production of diabetic products or products with low calorie. They may also have prebiotic effect by stimulation of growth and activity of beneficial microorganisms resident in the colon.

Different techniques are available for the synthesis of oligosaccharides: chemical, physical or enzymatic ways. Oligosaccharides may simply be produced by extraction from natural sources. Generally, the chemical synthesis is very complicated and expensive, as well as has numerous limitations such as difficult to scale up, high risk of by-product formation *etc.* The disadvantage of physical oligosaccharide production methods is the non-specificity, therefore the use of these techniques are limited. The main advantages of enzymatic synthesis are that the product spectrum can be controlled by regio- and stereospecificity, while the possibility of by-product formation is minimal and variable conditions can be used for bioconversions. Because of these reasons the enzymatic synthesis is preferred. Recently, many commercial prebiotic oligosaccharides (e.g. GOS, FOS) has been produced by enzymatic technology. Different ways are applied: enzymatic hydrolysis of polysaccharide, transglycosylation and reverse hydrolysis reactions. Several enzymes (hydrolases, glycosyltransferases, glycosynthases) are able to catalyse synthesis reactions, whereby oligosaccharides with specific composition/structure can be produced in either mono- or bisubstrate systems. In the last century due to the intensive development of biotechnology, comprehensive and complex information became available in the literature about catalytic mechanism of enzymes. But on the other hand due to the diversity of biological systems/processes there is still lack of knowledge about the properties of many biocatalysts. It is well known that hydrolases

generally have glycosyltransferase activities under certain conditions (e.i. low water activity), but effects of other parameters on catalytic mechanisms of glycosyltransferase are still unknown. Substrate specificity as well as substrate systems of individual enzymes are also less known especially in transglycosylation and glycosynthesis. No doubt that such scientific knowledge is essential for the development of new, innovative technologies for production of novel oligosaccharides with special functions. New results from this field will contribute to understand the mechanism of enzymes, biological functions (operation and control), and to the elaboration of enzymatic technologies that can provide products with the required function or structure.

2. Objectives

My doctoral research focused on the study of the transglycosylation and reverse hydrolysis activities of enzyme preparations from different sources using both mono- and bisubstrate systems for production of oligosaccharides with new possibilities.

The following tasks were aimed:

- Investigation of some activities of concomitant enzymes of a commercial pectolytic preparation, Pectinex ultra (from *Aspergillus aculeatus*), on different substrates for synthesis of oligosaccharide
    - transglycosylation on disaccharide substrates
    - reverse hydrolysis on monosaccharide substrates
- Study of synthesis of mannose based (di- and oligo-)saccharides with the use of Pectinex ultra enzyme preparation:
    - effect of substrate concentration
    - effect of pH and temperature
    - optimal ratio of enzyme:substrate
    - analysis of Maillard inhibition
    - separation and characterisation of mannose based oligosaccharides
- Investigation of transglycosylation activity of enzyme preparation from *Bifidobacterium longum* Bb-46 on different disaccharide substrates.
- Investigation of bisubstrate systems:
    - reverse hydrolysis of Pectinex ultra enzyme preparation on different combinations of monosaccharides
    - transglycosylation of Pectinex ultra enzyme preparation on different combinations of disaccharides
    - transglycosylation of recombinant levansucrase from *Bacillus megaterium* DSM 319 in bisubstrate systems
    - transglycosylation reactions of *Bifidobacterium longum* Bb-46 in bisubstrate systems

3. Materials and methods

An industrial Pectinex ultra enzyme preparation (from Sigma-Aldrich), a recombinant levansucrase enzyme from *Bacillus megaterium* and an enzyme preparation from *Bifidobacterium longum* were applied for synthesis of oligosaccharides.

The levansucrase enzyme from *Bacillus megaterium* was produced using recombinant technology through *Escherichia coli* BL21 (DE3) host. The production of enzyme was implemented at the Department of Technical Chemistry of Technical University of Braunschweig with the following conditions: 50 mL inoculate was added to 500 mL LB broth containing 0.5 mM ampicillin, then it was incubated for 3 hours (at 37 ^{o}C, with agitation of 120 rpm). 0.5 mM IPTG (isopropil β-D-tiogalactoside) was added to the broth for induction of protein expression. Then the temperature was decreased to 30 ^{o}C and it was incubated for 21 hours. Ultrasonic cell disruption was used for preparation of the enzyme.

Bifidobacterium longum Bb-46 probiotic strain from Christian-Hansen was used for the production of crude bacterial enzyme preparation. TPY medium containing lactose (instead of glucose) was applied for the fermentation (for induction of β-galactosidase synthesis). The fermentation started by adding of 1 V/V % inoculum that had been cultivated for 24 hours. The fermentation was carried out for 21 hours. French Press (with 800 psi pressure) was applied for cell disruption that was performed in three consecutive cycles.

Chromoforic artificial substrates (p-nitrophenyl-β-D-galactopiranoside, p-nitrophenyl-α-D-galactopiranoside, p-nitrophenyl-α-D-glucopiranoside, p-nitrophenyl-β-D-glucopiranoside, p-nitrophenyl-α-D-mannopiranoside) were used for assaying α-mannosidase, β-galactosidase, α-galactosidase, α-glucosidase and β-glucosidase activities. Generally, one unit (U) of enzyme activity is defined as the amount of enzyme that releases 1 μM p-nitrophenol in one minute under reaction conditions. Activities were calculated from the released p-nitrophenol content. The activity of β-fructosidase/levansucrase was assayed using sucrose as substrate. BCA and DNS methods were applied for determination of reducing sugar content. One unit of β-fructosidase/levansucrase enzyme activity is defined as the amount of enzyme that releases 1 μM reducing sugar in one minute under reaction conditions.

In the case of Pectinex ultra enzyme preparation, the synthesis of oligosaccharide on monosubstrate systems was realised in 1 mL volume of 30 g/100ml substrate concentration (arabinose, fructose, glucose, mannose, rhamnose, sorbose, xylose, cellobiose, lactose, maltose, maltulose, melibiose, palatinose, trehalose, turanose) at 60 °C and on pH 5.5. The enzyme reactions were started by adding 0.15 mg enzyme protein/g carbohydrate (13 μL)

doses. The bioconversion was carried out for 96 hours and daily sampling were done. Enzymes were inactivated by boiling for 10 minutes in water bath.

Effect of different parameters were studied: substrate concentration in the range of 10-80 g/100 mL, temperature in the range of 50-80 ^{o}C, pH in the range of pH 3.0-7.0, and ratios of enzyme:substrate in the range of 0.8-3.9 mg enzyme protein/g carbohydrate.

Effect of Maillard inhibiton was investigated at 15 mM concentration of inhibitors.

The transglycosylation reaction catalysed by the preparation was investigated at the following conditions: 5 mL volume, 10 g/100 mL substrate concentration, 60 °C, pH 5.5. Sucrose and lactose were combined with different carbohydrates (sucrose, lactose, maltose, fructose, galactose, glucose, mannose and xylose) acting as bisubstrate systems. Reaction was started by adding 0.16 mg enzyme protein/g carbohydrate enzyme dose. Effects of ratio of substrates to substrates were investigated in the range of 1:9-9:1, effect of carbohydrate concentration was studied in the range of 10-70 g/100 mL.

Bioconversion with levansucrase from *Bacillus megaterium* was realised by combination of sucrose with lactose or maltose at ratio of 2:1 and 1:2. Two substrate concentrations were tested: 30.75 and 61.5 g/100ml. pH6.6 and 37 °C parameters were used for assays. Enzyme amount was 5 U/mL. Experiments were performed at the Department of Technical Chemistry of Technical University of Braunschweig, Germany.

Bioconversions of crude enzyme preparation from *Bifidobacterium longum* Bb-46 were carried out in 1 mL volume with 30 g/100mL carbohydrate concentration, at 40 °C and on pH 6.6. Reactions were started by adding 1.08 enzyme protein/g carbohydrate (100 μL) doses.

Carbohydrates were detected by HPLC-RID, HPAEC-PAD, TLC and MALDI-TOF-MS methods.

The protein content was determined by Bradford method.

To separate carbohydrates FPLC equipment containing BioGel-P2 filled column was applied.

4. Results

The following significant results were achieved:

- The Pectinex ultra preparation has several activities to synthesize oligosaccharides. The preparation catalysed transglycosyl reaction on cellobiose, maltose, maltulose, palatinose, trehalose and turanose. Interestingly, it was able to hydrolyse both alpha-galactoside and beta-galactoside bonds, as well as to transfer galactose from one to another substrate molecule. Reverse hydrolysis reaction was observed on glucose and mannose substrates resulting carbohydrate products with higher polymerization degree. On the other hand neither disaccharides nor oligosaccharides were detected on arabinose, fructose, rhamnose, sorbose and xylose.
- Taking into consideration the importance of mannooligosaccharides, synthesis of mannose based saccharides was selected for detailed study. The effects of different conditions on the production of oligosaccharides were investigated. The highest oligosaccharide yield was obtained at 60 g/100ml mannose concentration, pH 5.0, 70 °C, 3.1 mg protein/g product. Three *Maillard* inhibitors (*o*-phenilene-diamine-dihydrochloride, semicarbazide-hydrochloride and aminoguanidine-hydrochloride) were tested to enhance the synthesis of oligosaccharide. Addition of aminoguanidine-hydrochloride increases the yield of product compared to control run (without inhibitor). The other two inhibitors had no effects on the production of oligosaccharides.
- The mannose-based products were separated using gel filtration with BioGel-P2 column, and the collected fractions were analysed by MALDI-TOF-MS technique (at the Department of Inorganic and Analytical Chemistry, University of Debrecen, Hungary). The results showed that at least five types of oligosaccharides with polymerisation degree of DP_2 to DP_6 were synthesized on mannose substrate *via* reverse hydrolysis reaction. Structural analyses of these products are in progress.
- Transglycosylation activities of Pectinex ultra were also investigated in different bisubstrate systems. In case maltose and sucrose were present in the system, an oligosaccharide was detected that did not appear in monosubstrate (sucrose or maltose) systems used as controls (according to TLC method). The optimal ratio of maltose:sucrose and the optimal substrate concentration were determined to be 1:9 and 60 g/100ml, respectively. Double oligosaccharide yield was achieved if optimal condition was applied. Based on the changes of carbohydrate composition and

concentration, the synthesis of oligosaccharide on the maltose:sucrose bisubstrate system may be due to a transfructosyl reaction in which the sucrose acts as a donor and the maltose as an acceptor.

- Production of oligosaccharides by levansucrase from *Bacillus megaterium* was also investigated. Two kinds of bisubstrate (sucrose:maltose and sucrose:lactose) were applied. This enzyme can catalyse transfructosyl reaction in both systems, and both maltose and lactose can be acceptors of levansucrase. While in the case of sucrose: maltose the highest yield was achieved at 2:1 substrate ratio and 61.5 g/100ml substrate concentration, respectively, whereas in the case of sucrose: lactose at 1:2 ratio and 61.5 g/100ml substrate concentration.
- Transglycosylation by crude enzyme preparation of *Bifidobacterium longum* on bisubstrate system was also investigated. Lactose and lactulose were combined with maltose and sucrose. In the cases of lactulose:sucrose and lactose:sucrose bisubstrate systems, the yields of oligosaccharides were significantly higher than the ones observed in the cases of monosubstrates. The optimal substrate ratio and substrate concentration of the combination of lactose:sucrose were 61:39 and 25 g/100ml, respectively. In the case of lactose: maltose, these values were changed to be 33:67 and 40 g/100ml. In both cases of bisubstrate systems, a product with different retention (on TLC) was detected, which are not produced in monosubstrate systems. According to the change of carbohydrate composition, the synthesis of these products may be caused by transgalactosylation reaction. In these reactions lactose can act as donor, maltose and sucrose as acceptor molecules.

Results that were generated in my doctoral research are fundamental, but I believe they contribute to the better understanding of catalytic mechanisms, and biological functions (control and operation). Such basic knowledge will also contribute to the elaboration of enzymatic technologies that can provide products with the required function or structure.

The perspective in case of Pectinex ultra enzyme preparation is the study and improvement of the production of newly detected enzymes by strain development or by molecular biological methods.

5. Novel scientific results

1. Transglycosylation and reverse hydrolysis reactions were successfully realized with Pectinex ultra SP-L enzyme preparation. The preparation catalyses transglucosylation on cellobiose, maltose, maltulose, palatinose, trehalose and turanose; transgalactosylation on melibiose as well. Reverse hydrolysis reaction was detected on glucose and mannose substrate.
2. The optimal parameters of mannose based oligosaccharide synthesis by Pectinex ultra were determined: 60 g/100 ml substrate concentration, pH5.0, 70 °C, 3.1 mg enzyme protein/g carbohydrate. It was established that DP_2-DP_6 mannooligomers can be synthesized on this substrate.
3. Pectinex ultra SP-L enzyme preparation catalyses transglycosylation reaction on maltose:sucrose bisubstrate. In this case an oligosaccharide was detected that has different retention than malto- and fructooligosaccharides (according to TLC method).
4. New acceptors of recombinant levansucrase from *Bacillus megaterium* were observed. The enzyme can catalyse transfructosyl reaction in which the fructose transferred from sucrose to maltose and lactose.
5. Crude enzyme preparation from *Bifidobacterium longum* Bb-46 was produced. It was proved that this enzyme can catalyse transglycosylation reaction on lactose, lactulose, maltose and sucrose substrates. In some bisubstrate systems (lactose:maltose and lactose:sucrose) the oligosaccharide synthesis was optimised from the point of oligosaccharide content. Optimum substrate ratios and concentrations are: in case of lactose:maltose 33:67 and 40 g/100 mL, in case of lactose:sucrose 61:39 and 25 g/100 mL, respectively.

6. Conclusion and suggestions

β-galactosidase and fructosyltransferase enzymes of Pectinex ultra SP-L were already applied for synthesis of galacto- and fructooligosaccharides. Further enzymes of the preparation with different activities were proved. With use of the preparation oligosaccharides can be synthesised by transglucosylation on cellobiose, maltose, maltulose, palatinose, turanose and trehalose disaccharides, and by transgalactosylation on melibiose. In addition the preparation can catalyse reverse hydrolysis reaction on mannose and glucose monosaccharides as well. The newly proved activities of the preparation can further broaden possibilities of production of different oligosaccharides.

It was established that in case of mannose based synthesis five carbohydrates with different polymerisation degree can be formed, from DP_2 to DP_6. In previous studies the highest polymerisation degree of synthesized oligomers was up to DP_4. Therefore α-mannosidase from *Aspergillus aculeatus* could be useful for production of mannooligosaccharides with high degree of polymerisation.

During the analysis of the preparation in bisubstrate systems an oligosaccharide was detected in case of maltose:sucrose combination that had different structure. According to the change of carbohydrate composition the synthesis may be a transfructosylation reaction, in which the sucrose participated as a donor and maltose is an acceptor. New acceptors of levansucrase from *Bacillus megaterium* were observed (maltose and lactose). Oligosaccharides were synthesized by crude enzyme preparation from *Bifidobacterium longum* on lactose:maltose and lactose:sucrose bisubstrates.

My results of bisubstrate systems could open new possibilities for the development of production technique of novel oligosaccharides. Studies of their structures are necessary for application of synthesized products. From this point of view, some important research directions are opened such as determination of chemical structures of oligosaccharides or *in vitro* and *in vivo* investigation the prebiotic or other beneficial physiological effects of saccharides synthesized, studies of catalytic mechanisms/properties of enzyme cocktails or individual enzymes after purification process. Characterisation of individual enzymes is also necessary for development of enzymatic technology.

8. Publications

Articles in journals

Journals with impact factor

Styevkó G., Styevkó Cs., Hoschke Á., Nguyen D. Q. (2013) Novel oligosaccharide synthesizes by glycosyltransferase activity from Pectinex ultra SP-L enzyme preparation. *Acta Alimentaria,* 42: 99-106. (IF: 0,427, 2013)

Havas P., Kun Sz., **Styevkó G.**, Slacanac V., Hardi J., Rezessy-Szabó J. (2014) Fruit and Vegetable juice fermentation with *Bifidobacteria*. *Acta Alimentaria,* 43: 64-72. (IF: 0,274, 2014)

Journal without impact factor, in hungarian language

Styevkó Cs., **Styevkó G.**, Hoschke Á., Nguyen Q. D. (2014) Pectinex ultra enzimkészítmény transzglikozil aktivitása. *Élelmiszer Tudomány Technológia,* 2: 22-27.

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Styevkó G., Nguyen V. D., Hoschke Á., Nguyen Q. D. (2014) Oligosaccharide synthesis on different substrates by commercial enzyme preparation from *Aspergillus aculeatus*. Conference of Chemical Engineering ’14, Veszprém, Conference proceeding, 167.

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