Oligoszacharidok előállítása kemoenzimatikus szintézissel

Doktori (Ph.D.) értekezés tézisei

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Synthesis of oligosaccharides by chemoenzymatic methods

Ph.D. Theses

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1. Introduction

The goal of our work was to develop enzymatic methods for the synthesis of oligosaccharides.

Enzyme catalysed synthesis of oligosaccharides is a very attractive method because it allows the formation of well defined oligosaccharides selectively without using any protection of hydroxyl groups. In addition, on the basis of the advances in genetic engineering, it is becoming possible to produce a wider range of enzymes on a large scale, expanding the number of enzymes available for synthetic reactions.

Our potential limitation of the approach however, is that the products of transglycosylation may themselves by hydrolysed by the enzyme during the course of reaction. In our laboratory considerable effort has been made to study the application of transglycosylation ability of retaining glycosidases first of all α-amylases, as an alternative approach for the synthesis of chromogenic oligosaccharide substrates.

Despite the increasing work carried out with glycosidases, little is known about the structural requirements for the binding of a sugar acceptor to the enzyme, and the role of subsites poorly understood, which are essential to improve the synthetic utility of this methodology.

In order to study the nature of the binding site and the process of hydrolysis and trans-glycosylation of α-amylases homologous 2-chloro-4-nitrophenyl ß-maltooligosaccharides were prepared.

Another motivation of our research was to design and synthesise novel, efficient human α-amylase inhibitors.

Disorders of carbohydrate uptake may cause severe health problems such as diabetes, obesity or caries – all of which threaten an increasing population. Therefore there is an obvious need for novel agents or therapeutic strategies that act on the physiological regulation of sugar uptake, blood sugar levels and prevention of oral diseases.

2. Applied methods

For the synthesis of α-amylase substrates and inhibitors classical chemical, chemoenzymatic and enzymatic glycosylation methods were applied. Reactions were monitored by thin layer chromatography (TLC), HPLC and MALDI-TOF MS analysis. Purification and separation of the transfer products were carried out by HPLC on different semipreparative column. Protein engineering was used for the preparation of modified enzymes.

Subsite maps of α-amylases were evaluated by a computer program. Kinetic constants were calculated by classical kinetic methods and different computer programs.

NMR spectroscopy and mass spectrometry (MALDI-TOF ESI-TOF MS) were applied for the identification and characterization of the compounds prepared. Complete assignments of ^{1}H – and ^{13}C-spectra were achieved by the combined analysis of various 1D and 2D measurements such as ^{1}H-^{1}H COSY, TOCSY and ^{13}C-^{1}H HSQC.

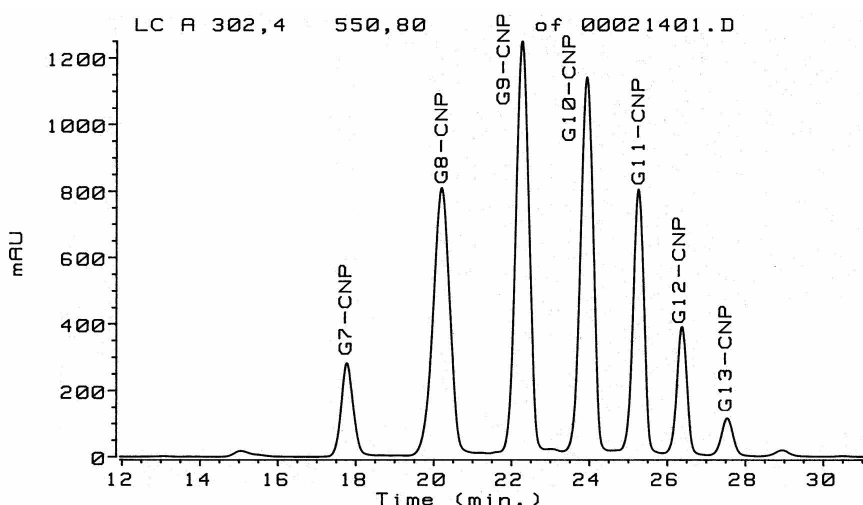
3. New scientific results

3.1. Preparation of CNP ß-maltooligosides

We described a new chemoenzymatic procedure for the synthesis of CNP-maltooligosides as a promising alternative to their multistep chemical synthesis using rabbit skeletal muscle glycogen phosphorylase b (EC 2.4.1.1.).

The primer in the enzymatic reaction was 2-chloro-4-nitrophenyl ß-maltopheptaoside (G_7-CNP), synthesised from ß-cyclodextrin using a convenient chemical method.
CNP-maltooligosaccharides of longer chain length, in the range of DP 8-11, were obtained by a transglycosylation reaction using α-D-glucopyranosyl-phosphate (G-1-P) as a donor.

More than 90% conversion of G_7-CNP was achieved through a 10:1 donor:acceptor ratio. Transglycosylation at 37°C for 30 min with 10 U enzyme resulted in $G_{8\text{-}12}$-CNP oligomers in the ratio of 22,8, 26,6, 23,2, 16,5 and 6,8 % respectively. The reaction pattern was investigated using an HPLC system. The preparative scale isolation of $G_{8\text{-}11}$-CNP glycosides was achieved on semipreparative HPLC column. The productivity of the synthesis was improved by yields up to 70-75%. The structures of the oligomers were confirmed by their chromatographic behaviors and MALDI-TOF MS data (Scheme 1).



Scheme 1. The preparative scale isolation of $G_{8\text{-}13}$-CNP glycosides

3.2. Subsite mapping of α-amylases

A subsite map shows the number of subsites, the position of the catalytic site and the apparent binding energies between subsite-substrate monomer unit. For subsite map calculation a computer program was developed in our laboratory. The program runs in Windows and uses the experimentally determined BCF data. The program called SUMA and is freely available via internet for research and educational purproses.

3.2.1. Subsite maps of *Bacillus licheniformis* α-amylase (BLA) at elevated temperatures (50, 80 and 100°C)

Bacillus licheniformis is a mesophilic bacterium, but produces a highly thermostable α-amylase BLA. It is widely used in alcohol, sugar and brewing industries for the initial hydrolysis of starch to dextrins, which are then converted to glucose by glucoamylases. However, its function on starch and oligosaccharides is poorly understood. Therefore, we were encouraged to study the action pattern of BLA at different temperature and evaluate subsite maps (Scheme 2).

Substrate	−5 −4 −3 −2 −1	+1 +2 +3	BCF (%) T (°C): 50	80	100
G_4	G—G—G	G—CNP	37	50	0
	G—G	G—G—CNP	35	33	79
	G	G—G—G—CNP	28	17	21
G_5	G—G—G—G	G—CNP	18	27	0
	G—G—G	G—G—CNP	34	31	50
	G—G	G—G—G—CNP	48	40	43
	G	G—G—G—G—CNP	0	2	7
G_6	G—G—G—G—G	G—CNP	68	70	0
	G—G—G—G	G—G—CNP	7	11	59
	G—G—G	G—G—G—CNP	25	16	36
	G—G	G—G—G—G—CNP	0	3	5
G_7	G—G—G—G—G—G	G—CNP	5	12	0
	G—G—G—G—G	G—G—CNP	84	68	62
	G—G—G—G	G—G—G—CNP	11	20	25
	G—G—G	G—G—G—G—CNP	0	0	6
	G—G	G—G—G—G—G—CNP	0	0	7
G_8	G—G—G—G—G—G—G	G—CNP	1	1	0
	G—G—G—G—G—G	G—G—CNP	9	9	27
	G—G—G—G—G	G—G—G—CNP	88	88	62
	G—G—G—G	G—G—G—G—CNP	2	2	7
	G—G—G	G—G—G—G—G—CNP	0	0	3
	G—G	G—G—G—G—G—G—CNP	0	0	1
G_9	G—G—G—G—G—G—G—G	G—CNP	3	1	0
	G—G—G—G—G—G—G	G—G—CNP	10	9	19
	G—G—G—G—G—G	G—G—G—CNP	83	75	68
	G—G—G—G—G	G—G—G—G—CNP	4	12	10
	G—G—G—G	G—G—G—G—G—CNP	0	3	2
	G—G—G	G—G—G—G—G—G—CNP	0	0	1
G_{10}	G—G—G—G—G—G—G—G—G	G—CNP	0	3	0
	G—G—G—G—G—G—G—G	G—G—CNP	6	10	17
	G—G—G—G—G—G—G	G—G—G—CNP	83	65	64
	G—G—G—G—G—G	G—G—G—G—CNP	6	15	14
	G—G—G—G—G	G—G—G—G—G—CNP	5	6	4
	G—G—G—G	G—G—G—G—G—G—CNP	0	1	1

Scheme 2. Action pattern of BLA with CNP modified maltooligosaccharides at 50°C, 80°C and 100°C. G, glucosyl residues; CNP, 2-chloro-4-nitrophenyl groups (CNP is connected to the reducing end in β-form); BCFs are expressed as percentages of total cleavage events calculated from the reaction products. ↓, catalytic site [situated between subsite (-1) and (+1)].

BLA exhibited a unique pattern of action at 50°C on CNP-maltooligosaccharides by cleaving maltopentaose units as main products, 68, 84 and 88 % from the non-reducing end of CNP-G_6, CNP-G_7 and CNP-G_8 respectively, and leaving CNP-glycosides. As the chain length increases, the maximum frequency of attack shifts toward the reducing and of the chain and CNP-G_3 becomes the major product; 88, 83 and 83% from CNP-G_8, CNP-G_9 and CNP-G_{10}, respectively. This favourable release of CNP-G_3 was also observed for the pentamer glycoside (CNP-G_5).

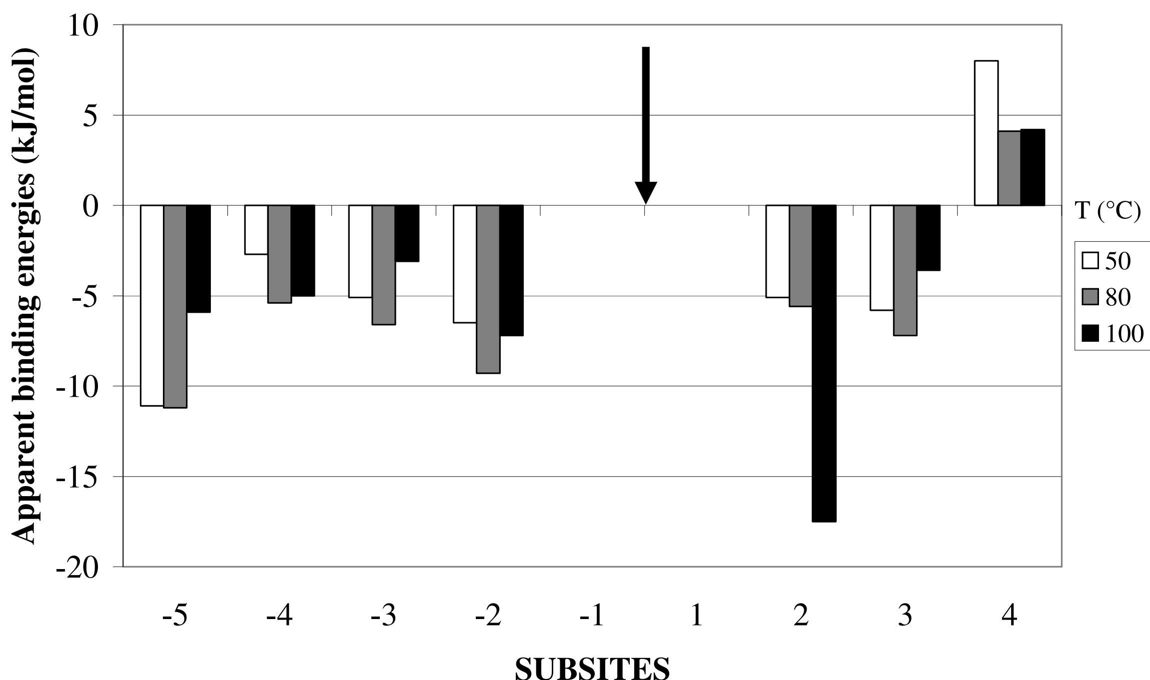
Our results strongly suggest the presence of at least eight subsites in BLA, five glycone sites (-5, -4, -3, -2, -1) and three aglycone sites (+1, +2, +3) and the catalytic site is

located between subsites (-1) and (+1). In the ideal arrangement subsite (+3) was filled by a glucopyranosyl unit and the aglycon sites (-5, -4, -3, -2, -1) were also occupied by glucose residues which resulted in an interesting dual product specificity for the dominant formation of CNP-G_3 and maltopentaose.

BCFs were evaluated for chain length of 4-10 of CNP ß-maltooligosides and these quantitative data were used to calculate the subsite map for BLA.

Subsite (+4) has positive free-energy of binding and will be referred to as “barrier subsite”. This barrier subsite resulted in the dual product specificity of BLA. Results confirm that the eight subsites originally assumed for our experimental data are correct and BCFs are predicted correctly.

To elucidate how temperature effects subsite map of BLA, comparative studies were performed at 50°C, 80°C and 100°C. Subsite map at 80°C indicates more favourable bindings compared to the hydrolyses at 50°C. Hydrolysis at 100°C resulted in a clear shift in the product pattern and suggests significant differences in the active site architecture. Two preferred cleavage modes were seen for all substrates in which subsite (+2) and (+3) were dominant, but CNP-G_1 was never formed. In the preferred binding mode of short oligomers, CNP-G_2 serves as the leaving group (79%, 50%, 59% and 62% from CNP-G_4, CNP-G_5, CNP-G_6 and CNP-G_7, respectively). While CNP-G_3 is the dominant hydrolysis product from CNP-G_8, CNP-G_9 and CNP-G_{10} (62%, 68% and 64% respectively). The high binding energy value (-17 kJ/mol) found at subsite (t+2) is consistant with the significant formation of CNP-G_2. Subsite mapping at 80°C and 100°C cofirms that there are no further binding sites despite the presence of longer products (Scheme 3).



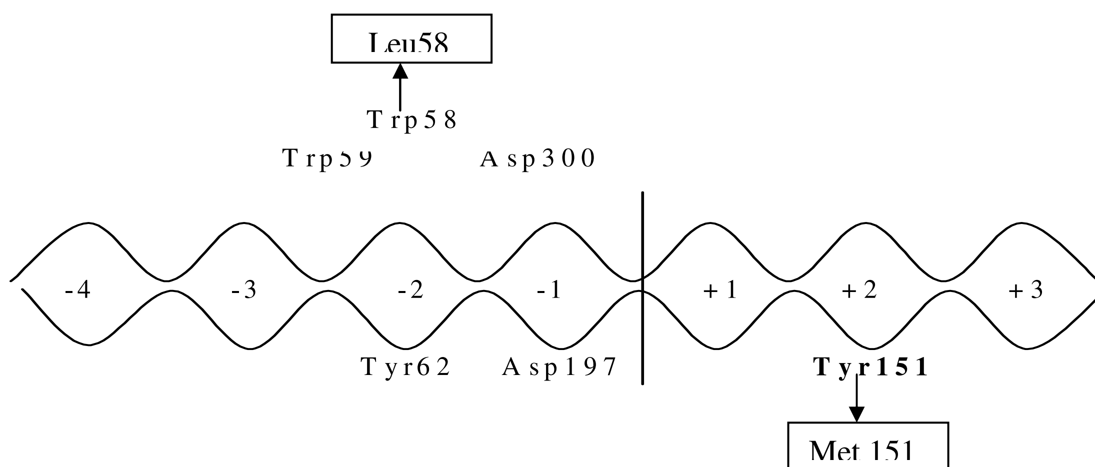
Scheme 3. Subsite maps for BLA. The white bars depict the subsite map at 50°C, grey bars and black bars are related to the subsite maps of 80°C and 100°C, respectively. The arrow indicates the location of hydrolysis. The reducing end of maltooligomers situated at the right hand of the subsite map.

3.2.2. Subsite map of human salivary α-amylase (HSA) and its Y151M mutant

Human amylases of both salivary (HSA) and pancreatic origin (HPA) have been extensively studied from the viewpoint of clinical chemistry, because they are important as indicators of pancreatic and salivary glands disorders. Our interest was focused on salivary amylase, which is a multifactorial enzyme involved in distinct biological functions. It has been shown that it may play a significant role in dental plaque formation and subsequent process of dental caries formation and progression.

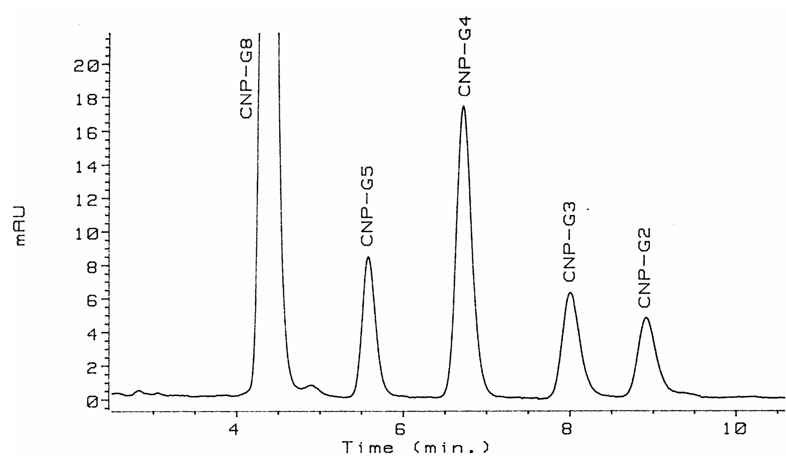
Furthermore, α-amylases are used as targets for drug design in attempts to treat diabetes, obesity and hyperlipermia. Diabetes mellitus is the most common serious metabolic disease in the world; it effects hundreds of millions.

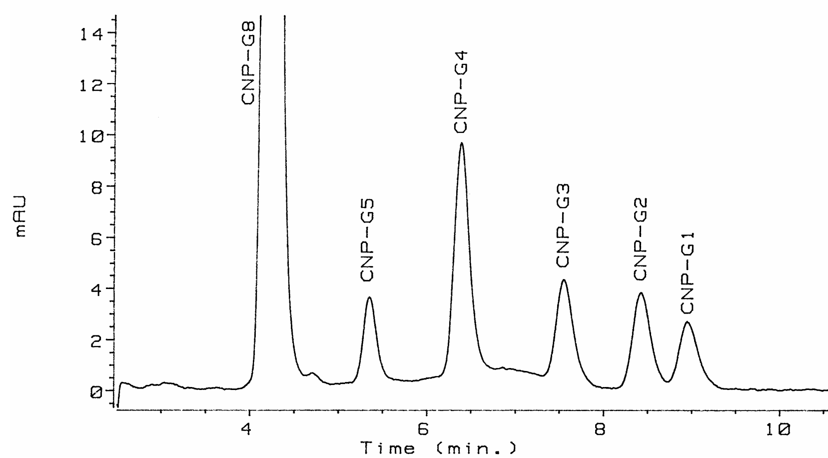
The active site of human amylases harbors three aromatic residues Trp59, Tyr62 and Tyr151, which provide stacking interactions to the bound glucose moieties. It has been shown that Tyr151 occurs at subsite (+2) and may influence the size of the leaving group. To study the role of subsite (+2) in recognition of terminal residue of substrate a mutant was generated in which the tyrosine at position 151 of HSA was replaced by a methionine (Scheme 4).



Scheme 4. Subsite model of human α-amylases

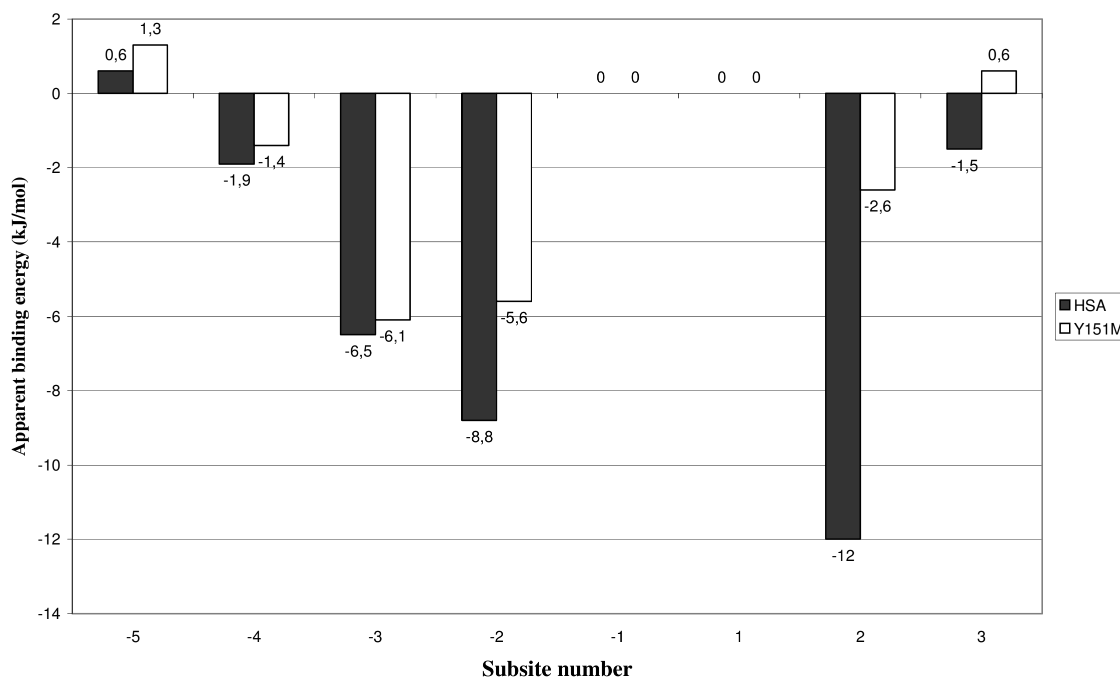
The product distribution for Y151M mutant, on the same oligosaccharide series, was very interesting and markedly different from that of the HSA. The moiety CNP-G_1 is the major product of hydrolysis when CNP-G_3 and CNP-G_4 were used as substrates and was significantly released in the hydrolysis of longer oligomers (DP 6-10) as well whereas this monomer glycoside was not recognisable as a product in the hydrolysis of the corresponding substrates by HSA. These results can be explained by the presence of methionine at subsite (+2) which is not advantageous to the polar glucose residues (Scheme 5).





Scheme 5. HPLC chromatogram of CNP-G8 hydrolysis product catalysed by HAS and Y151M mutant. Injected volume 20 μl.

We characterized the substrate-binding sites of HSA and its Y151M mutant. (Scheme 6).



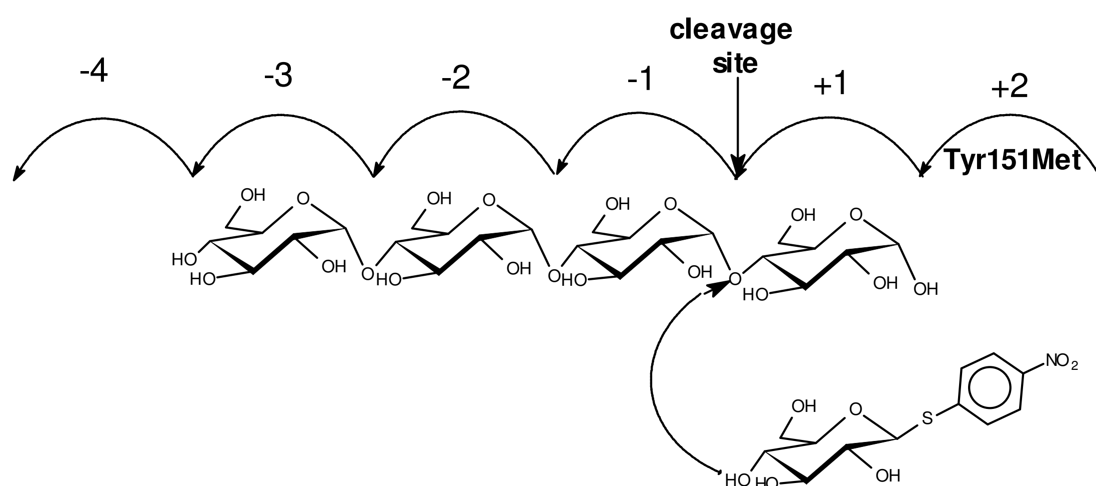
Scheme 6. Subsite maps for human salivary a-amilase and its Y151M mutant

It describes the first subsite map which shows the number of subsites, the position of cleavage sites, and apparent binding energies. The binding region of HSA is composed of four glycone and three aglycone-binding sites, while that of Tyr151Met is composed of four glycone and two aglycone-binding sites. The subsite maps show that Y151M has strikingly decreased binding energy at subsite (+2), where the mutation has occurred (-2,6 kJ/mol), compared to the binding energy at subsite (+2) of HSA (-12,0 kJ/mol). The product distribution revealed that in the mutant maltose changed to glucose as the minimal leaving group. In addition, the mutant showed a significant reduction in its hydrolytic efficiency and a remarkably good increase in its transferase activity. It was envisaged that the structural change at the aglycone binding site could improve the synthetic activity of HSA and PNP-glycosides would be better acceptors for the mutant than for the wild type enzyme.

3.3. Enzymatic glycosylations

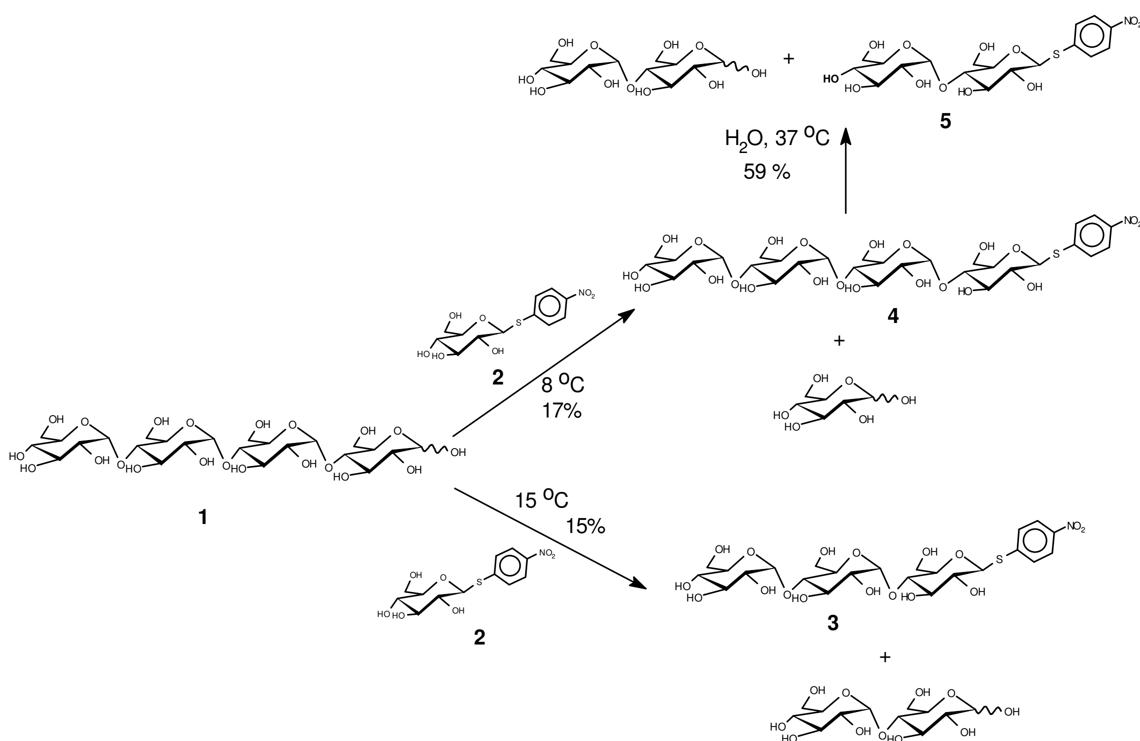
3.3.1. Introducing transglycosylation activity into human salivary α-amlylase (HSA) by mutation

Taking advantage of the transferase activity of the mutant we developed an enzymatic strategy for the synthesis of PNP-glycosides with DP from 2 to 4. Y151M was capable of transferring maltose and maltotriose residues from a maltotetraose donor onto different PNP-glycosides (Scheme 7).



Scheme 7. Transglycosylation procedure catalyzed by Y151M

For preparative purposes 4-nitrophenyl 1-thio-ß-glucoside was selected as an acceptor. The reaction can be characterized by 3 stages (scheme 2): The first is the hydrolysis of donor. Maltotetraose is cleaved to maltose (20%) or maltotriose and glucose (80%). The second is transglycosylation with maltotriose leading to the formation of the tetramer glycoside (at 8°C) or transglycosylation with maltose resulted in the synthesis of trimer glycoside (15°C) - which was followed by the third one, the secondary hydrolysis of the transfer products leading to the accumulation of dimer glycoside (at 37°C) (Scheme 8).



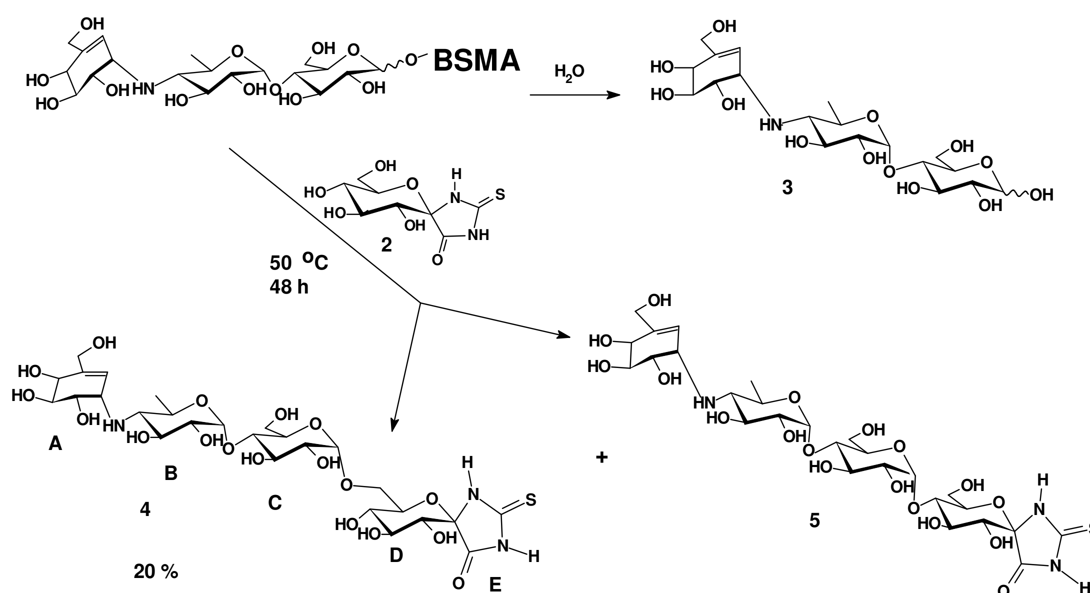
Scheme 8. Transglycosylation procedure catalyzed by Y151M

NMR studies revealed that the mutant retained its regio- and stereoselectivity, in this way the donor fragments were linked to the acceptor by α-1,4 glycosidic linkages, exclusively. Therefore the longer oligomers can be readily hydrolysed and the dimer was accumulated.

3.3.2. Synthesis of acarviosinyl-isomaltosyl-spiro-thiohydantoin (PTS-GTH) catalysed by *Bacillus stearothermophylus* α-amylase (BSMA)

BSMA is a thermostable maltogenic α-amlylase (EC 3.2.1.1.) and exhibits both hydrolytic and transferase activity. It is able to hydrolyse the potent amylase inhibitor acarbose, and transfer the product to different acceptors. We found that BSMA was capable of transferring the acarviosine-glucose residue (PTS) from an acarbose donor onto glucopyranosylidene-spiro-thiohydantoin. [^{1}H and ^{13}C NMR studies revealed that the enzyme reserved its stereoselectivity. Glycosylation took place mainly at C-6 resulting in α-acarviosinyl-isomaltosyl-spiro-thiohydantoin.].

Reaction was followed by TLC, HPLC and MALDI-TOF analysis. Two UV detectable products were separated by HPLC on a reverse phase column. The two newly formed transglycosylated products appeared after 10 hours incubation time and their amounts were significantly increased after 48 hours.
The structure of the main product was analysed by ESI-TOF measurement and NMR spectroscopy.
Suggested reaction between acarbose and GTH can be seen in Scheme 9.



Scheme 9. Transglycosylation procedure catalyzed by BSMA

3.4. PTS-GTH – a new amylase inhibitor

This compound was found to be a much more efficient salivary amylase inhibitor than GTH. Kinetic investigations were carried out on a synthetic and the natural substrate of HSA. The inhibition is a mixed-noncompetitive type on both substrates and only one molecule of inhibitor binds to the enzyme. Kinetic constants calculated from secondary plots are in micromolar range.

4. Summary

We described a new chemoenzymatic procedure for the synthesis of 2-chloro-4-nitrophenyl (CNP) ß-maltooligosaccharides (DP 3-12) as a promising alternative to their multistep chemical synthesis using rabbit skeletal muscle glycogen phosphorylase b (EC 2.4.1.1.). The productivity of the synthesis was improved by yield up to 70-75 %. We dissolved the preparative scale isolation of the glycosides on semipreparative HPLC column. These compounds are independent for subsite mapping of α-amylases.

We desccribed the first subsite maps for the *Bacillus licheniformis* α-amylase (BLA) and human salivary α-amylase (HSA). For subsite mapping a computer program was developed. The subsite maps revealed the number of subsites, the position of the catalytic site and the apparent binding energies between subsites and substrate monomer unit.

Transferase activity was introduced into the Y151M mutant of human salivary amylase. Using maltotetraose as donor and different PNP-glycosides as acceptors transfer products were isolated. Tyr151Met mutant reserved its regio-, and stereospecifity and the donor fragments were linked to the acceptor by α (1,4) glycosidic linkages exclusively.

Synthesis of acarviosinyl-isomaltosyl-spiro-thiohydantoin (PTS-GTH) was carried out by BSMA. Glycosylation took place mainly at C-6 position. The transfer product was found to be a very efficient salivary amylase inhibitor.

The widening interest in the treatment and prevention of sugar metabolic disorders stimulates our work further to study the protein-ligand interaction and design new and efficient drugs as amylase inhibitors.

5. List of publications

Papers related to the subject of the dissertation

1. L. Kandra, **J. Remenyik**, Gy. Gyémánt, A. Lipták
   Effect of temperature on subsite map of Bacillus licheniformis alpha amylase
   Acta Biologica Hungarica (**2006**) 57:367-375. Impakt: 0.636

2. L. Kandra, Gy. Gyémánt, **J. Remenyik**, C. Ragunath, N. Ramasubbu
   Transzglycosylations catalysed by Y151M mutant of human salivary alpha-amylase (HSA)
   Biologia, Bratislava (**2005**) 60:57-64. Impakt: 0.208

3. L. Kandra, **J. Remenyik**, Gy. Batta, L. Somsák, Gy. Gyémánt, Kwan Hwa Park
   Enzymatic synthesis of a new inhibitor of alpha-amylases: acarviosinyl-isomaltosyl-spirothiohidantoin
   Carbohydrate. Research (**2005**) 340:1311-1317. Impakt: 1.533

4. **J. Remenyik**, C. Ragunath, N. Ramasubbu, Gy. Gyémant, A. Lipták, L. Kandra
   Introducing transglycosylation activity to human salivary α-amylase (HSA)
   Organic Letters. (**2003**) 5:4895-4898. Impact: 3.715

5. L. Kandra, Gy. Gyémánt, **J. Remenyik**, C. Ragunath, N. Ramasubbu
   Subsite mapping of human salivary α-amylase and the mutant Y151M
   FEBS Letters (**2003**) 544:194-198. Impakt: 3.64

6. Kandra, L., Gyémánt, G., **Remenyik, J.**, Hovánszki, G., Lipták, A.
   Action pattern and subsite mapping of Bacillus licheniformis alpha-amylase (BLA) with modified maltooligosaccharide substrates
   FEBS Letters (**2002**) 518(1-3):79-82. Impakt: 3.64

Other paper

1. L. Kandra, Á. Zajácz, **J. Remenyik**, Gy. Gyémánt
   Kinetic investigation of a new inhibitor for human salivary α-amylase
   Biochemical and Biophysical Research Communications (**2005**) 334:824-828.
   Impakt: 2.830

2. Tóth, A., **Remenyik, J**., Bajza, I., Lipták, A.
   Synthesis of the methyl ethers of methyl 6-deoxy-3-C-methyl-α-L-talopyranoside and -α-L-mannopyranoside. Examination of the conformation and chromatographic properties of the compounds.
   Arkivoc **2003** (V) 28-45. . Impakt: 0,392

3. Kandra, L., Gyémánt, G., Pál M., Petró, M., **Remenyik, J**., Lipták, A.
   Chemoenzymatic synthesis of 2-chloro-4-nitrophenyl beta-maltoheptaoside acceptor-products using glycogen phosphorylase b
   Carbohydrate Research (**2001**) 333(2):129-136. Impakt: 1,606

5.1. Lectures (L) and posters (P) related to the subject of the dissertation

1. L. Kandra, G. Gyémánt, **J. Remenyik**
   Subsite mapping of α-amylases
   MTA Szénhidrátkémiai Munkabizottság Előadóülése 2002. Mátrafüred (L)

2. Kandra L., Gyémánt Gy., Remenyik J.
   α-amilázok kötőhelyeinek térképezése
   Debreceni Tudományos Napok 2002, Debrecen (L)

3. **Remenyik J.**, Gyémánt Gy., Kandra L., Lipták A.
   A Bacillus licheniformis α-amiláz kötőhelyének vizsgálata
   Magyar Biokémiai Társaság előadóülése, Tihany, 2003 12-15. (P)

4. **J. Remenyik**, G. Gyémánt, L. Kandra, A. Lipták
   Introduction of transglycosdase activity into the human salivary α-amylase
   1st Austrian Hungarian Carbohydrate Conference, Burg Schlaining, Austria, 2003.09.04-06. (L)

5. **Remenyik J.**, Kandra L., Gyémánt Gy., Lipták A.
   Akarbózanalógok szintézise Bacillus stearothermophilus maltogén amilázzal
   MTA Szénhidrát kémiai Munkabizottság Előadóülése 2004.10.05. (L)

6. **Remenyik J.**, Kandra L.
   Oligoszacharidok szintézise a humán nyál amiláz (HSA) Y151M mutáns enzimével
   Kémiai Előadói Napok, Szeged, 2004.10.25-27. (L)

7. **J. Remenyik**, L. Kandra, Gy. Gyémánt, L. Somsák, A. Lipták
   Towards the enzymatic synthesis of alpha-amylase inhibitors
   2nd Austrian Hungarian Carbohydrate Conference, Somogyaszaló, 2005.05.24-26. (L)

8. **Remenyik J**., Kandra L., Gyémánt Gy., Somsák L., Batta Gy., Kwan Park, Lipták A.
   Bacillus stearothermophylus maltogén amiláz (BSMA) katalizálta glikozílezések
   Vegyészkonferencia, Hajdúszoboszló, 2005.06.25-27. (L)

9. L. Kandra, G. Gyémánt, **J. Remenyik**,
   Enzyme catalysed glycosylation reaction
   1st European Chemistry Congress, Budapest, 2006.08.27-31. (P)