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Experimental and molecular modeling-aided subsite mapping of α-amylases and studies on their transglycosidase activity

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1. Introduction

1.1 Importance of α-amylases

α-Amylases are widespread enzymes and they can be found almost in all living organisms: in *Archea* species, in bacteria, fungi, plants, animals and human where they play a dominant role in carbohydrate metabolism. Plant amylases are responsible for the hydrolysis of stored starch and related polysaccharides, animal and human amylases are responsible for the digestion of food poly- and oligosaccharides.

α-Amylases recently comprise about 30 % of the world's enzyme production. Mainly microbial amylases are used for liquefaction and saccharification of starch in the production of maltodextrin, modified starch and glucose syrups. They are widely used enzymes in several starch-based industries, play important role in processes of food and paper industry, textile desizing, detergent applications, sanitary waste treatment and used to increase the digestibility of animal feed. The pattern of products released by the hydrolysis of starch depends on the source, specificity, temperature and pH optimum of the appropriate enzyme. Therefore, in the last few years the industrially important enzymes were engineered with the aim to modify directly their properties and product pattern. Barley (*Hordeum vulgare*) α-amylase izozymes (AMY1 és AMY2) are necessary in process of brewing. Both human salivary (HSA) and pancreatic α-amylases (HPA) are extensively studied enzymes from the viewpoint of clinical chemistry because of their importance in diagnosis of pancreatic and salivary glands disorders. The serum and urine level of amylases is very importnant in the diagnosis of disorders of producing glands and in the diagnosis of some other diseases. α-Amylase inhibitors are used in the treatment of sugar metabolic disorders to decrease the level of blood sugar. Therefore amylases are the targets of drug design to decrease the amount of absorbed carbohydrates in attempts to treat *hyperglycaemia*, diabetes and obesity.

1.2 Action of α-amylases

α-Amylases (α-1,4-glucan-4-glucanohydrolases; EC 3.2.1.1) constitute a family of endo-amylases catalyzing the cleavage of α-(1-4) glycosidic bonds in starch and related carbohydrates with retention of α-anomeric configuration in the products. α-Amylases belong to glycoside hydrolase family 13 (GH 13) and together with GH70 and GH77 families constitute the clan H of glycoside hydrolases (GH-H), which is called as the α-amylase family of enzymes.

α-Amylases catalyze the endohydrolysis of the α-(1→4) glycosidic linkages of the starch and related poli- and oligosaccharides with the retention of the α-anomeric configuration of the glycosidic bond. Namely, the enzyme releases mono- or oligosaccharides with α-anomeric configuration from the hydrolysis of α-(1→4) glycosidic linkages. The action of amylases is based on the double displacement mechanism. The hydrolysis of glycosydic bonds is a reversible process, therefore retaining glycoside hydrolases have the capacity to catalyze transglycosylation. Many different oligosaccharides have been obtained by chemoenzymatic transglycosylation, which is a very attractive synthetic method because it allows the formation of well-defined oligosaccharides without use of chemical protection of hydroxyl groups.

Barley amylase isoenzymes, AMY1 and AMY2 were previously modified by direct or random mutagenezis to study the structure-function relationships or to modify their activity, specificity or product pattern. Previously, AMY1 and its mutants showed transglycosylation on MOSs and 4-nitrophenyl (PNP) glycoside substrates, however, this ability for the synthesis of oligosaccharides was not further explored.

1.3 Structure of α-amylases

The three-dimensional structures of the single polipeptide chain of α-amylases mostly contain three domains. The catalytic domain (domain A) adopts a central $(\beta/\alpha)_8$-barrel structure where the barrel of eight parallel β-strands are surrounded by eight helices. Domain B is a small protruding loop located between the third β-strand and the third α-helix in the $(\beta/\alpha)_8$-barrel. The substrate binding site is located in the cleft at the interface of domain A and B and substrate binding subsites are made from the side chains of residues located on the loops at the C-terminal ends of β-strands of barrel structure. The architecture of the domain B varies between amylases and it is the major determinant of differences of substrate specificities. Most members of the family carry domain C. The non-catalytic C-terminal anti-parallel β-sheet domain plays an important role in stability/folding of the protein and in substrate binding.

1.4 Subsite architecture of α-amylases and subsite mapping

Each subsite of glycoside hydrolases interacts with one glucose residue of the oligo- or polysaccharide substrate. The nomenclature for sugar-binding subsites of glycosyl hydrolases was introduced by Davies and co-workers: subsites are labeled from the catalytic site, with negative numbers for subsites to the left (non-reducing end side, glycone binding sites) and positive numbers to the right (reducing end side, aglycone binding sites). Key active site

residues showed strong similarity between the members of GH13 family but the β-α loops of domain A shows great variety between α-amylases, therefore the architecture of the substrate binding cleft (the number and nature of substrate binding subsites) is a specific feature of the particular enzymes. Subsite mapping is the process of quantifying the subsite model. Modified, low-molecular weight oligosaccharides are appropriate substrates for amylases to elucidate the number of subsites in the active site of amylases. 2-chloro-4-nitrophenyl maltooligosaccharides (CNP-β-D-MOS) are such chromogenic molecules. Oligomeric substrates can interact productively or non-productively with the subsites. The productive binding mode means that a susceptible bond lies over the catalytic site, in which case the bond is cleaved. Endo-acting enzymes form several productive binding complexes resulting in a given product pattern. The relative rate of formation of each product is called bond cleavage frequency (BCF), which can be used to determine the subsite-binding energies. Computer program SUMA (Subsite Mapping of α-Amylases) developed by Gyémánt and co-workers uses BCFs of a series of oligomeric substrates to calculate the position of the cleavage site, the number of subsites and the binding energy of each subsite. Negative energy values indicate bindings between the enzyme and aligned glucopyranosyl residues, while positive values indicate repulsion. Substitution of amino acid sidechains affect the product pattern, therefore mutations cause the alteration of subsite maps calculated from BCFs. The subsite mapping procedure based on products analysis have been used successfully in case of several wild type and mutant α-amylases.

1.5 Substrate binding sites of AMY1

The substrate binding cleft located on the surface of AMY1 consists of 12 subtrate binding subsites, there are 8 glycone and 4 aglycone subsites. Barrier subsites show unfavorable binding energies located at the internal subsites −3 and −5 and at outer subsites −8 and +3/+4. Y105 makes stacking interaction with the glucose ring of the substrate at the high affinity subsite −6 alhelyen (-12,4 kJ/mol). T212 located at the outer subsite +4. The V47 together with Y105 serves as an „entrance” to the active site for the substrate. The substrate adopts a half-circle conformation centered on V47 (and S48 sidechains). Therefore the V47 and S48 amino acids play important role in subtrate binding at several glycone-binding subsites.

Amylolytic enzymes acting on starch and on related poly- and oligosaccharides could make interactions with carbohydrates not only at the substrate binding cleft but also at binding sites situated on the surface of the enzyme far from the active site. AMY1 has two secondary

surface binding sites: the starch granule-binding site and the so-called “a pair of sugar tongs”. These sites don’t have own catalytic activity but play important role in substrate binding and in the action of the enzyme. W278 and TW279 located at the starch granule-binding site make stacking interaction with the glucose residues of the substrate, Y380 and H395 are the key amino acids of the “a pair of sugar tongs” located at domain C.

There are several methods to study the substrate binding sites of amylases and to explore the structure-function relationships. Importance of amino acids can be studied by site-directed or random mutagenesises or by covalent modifications. After modifications the effects can be detected for example by enzyme kinetic and activity measurements and results can give information about the role of appropriate amino acids. Experimental procedure of subsite mapping is also a useful method to study the structure-function relationships. Differences between wild type and modified enzymes can be detected by determination of products patterns and the binding energies of the individual subsites. Computer-aided analysis of X-ray crystallographic structures of enzymes complexed with substrate-analogue or inhibitor molecule is an effective way to examine enzyme-substrate interactions. The analyses of X-ray structures could be supplemented by molecular mechanical/dynamical calculations to predict the interactions in a quantitative manner. Bioinformatical devices have been used in several cases to study the structure-function relationships of α-amylases. Nowadays, there are no available procedures with which the subsite binding energies of these enzymes can be calculated based on the use of the crystallographic structures or homologous models by a molecular modeling-aided procedure.

2. Goals

In this work, we aimed to

I.) study the secondary substrate binding site mutants of barley amylase 1 (AMY1) located at the surface of the protein far from the active site and establish the effect of mutations of key amino acids (W278, W279, Y380 and H395) using the method of product analysis based subsite mapping

II.) study the effect of mutations of V47 and S48 amino acids located at the active site of AMY1 using the method of product analysis based subsite mapping

III.) study the increased transglycosylation activity of AMY1 mutants observed during product analysis; explore application of AMY1 and the subsite mutants in enzymatic synthesis of 4-methylumbelliferyl-maltooligomers; use of synthesized substrates in a fast and simple fluorometric assay for α-amylases

IV.) develop a computer-aided procedure for subsite mapping of α-amylases to predict the subsite binding energies and the bond cleavage frequencies of wild type and mutant α-amylases based on the use of simple molecular mechanical calculations and the use of the crystallographic structures and homologous models of the enzymes

3. Materials and methods

3.1 Materials

The maltooligosaccharides (MOS) and some acceptor molecules were purchased from SIGMA, few acceptors were generous gift from our co-workers. A 2-chlolo-4-nitrophenyl-β-D-maltooligosaccharides (CNP-β-D-MOS) were synthesized chemo-enzymatically based on previously published methods. 4-methyl-umbelliferyl-maltooligosaccharides (MU-α- and β-D-MOS) were synthesized chemo-enzymatically according to the method described in the dissertation. Synthesis of CNP-MOS substrates was performed with the use of glycogen phosphorylase *b* enzyme. Wild type barley α-amylase 1 izozyme (AMY1) and its mutants were generous gift from Prof. Birte Svensson (Enzyme and Protein Chemistry, Department of Systems Biology, Technical University of Denmark): AMY1, Y105A, Y380A, Y105A/Y380M, H395A, Y380A/H395A, W279A, W278A/Y380A, W279A/Y380A, V47A, V47F, V47D, S48Y, V47K/S48G, V47G/S48D, V47L/S48A and V47I/S48I. *Bacillus licheniformis* (BLA) and human pancreatic α-amylases (HSA) were purchased from SIGMA. A *Bacillus stearothermophilus* maltogenic α-amylase (BSMA) was generous gift from our co-workers.

3.2 Methods

3.2.1 Preparation of CNP group containing maltooligosaccharide substrate series

CNP-MOS substrate series were synthesized chemoenzymatically using glycogen phosphorylase *b* enzyme based on the previously published methods. CNP-MOS of degree of polymerization 3−6 (DP 3−6) were synthesizd by a phosphorolysis reaction, DP 8−11 series by transglycosylation. Both DP 3−6 (Supelcosil LC-18 semipreparative column (5 μm, 250*10 mm)) and DP 8-11 substrates (Hypersil NH_2 APS semipreparative column (5 μm, 250*10 mm)) were separated and purified by HPLC (Hewlett-Packard 1090 Series II HPLC). CNP-glycosides were monitored by diode array detector at 302 nm wavelength.

3.2.2 Bond cleavage frequency analysis

Bond cleavage frquencies (BCF) were determined using CNP-β-D-MOS of DP 3−11 substrates. CNP-glycosides released by hydrolysis or transglycosylation were separated by HPLC (ZORBAX Eclipse XDB-C18 reversed phase column (5 μm, 150*4,6 mm)). To

minimize influence of secondary hydrolysis on transfer products, reactions were observed at <10% substrate conversion. Percentage of products was determined based on HPLC chromatograms usind values of peak areas.

3.2.3 Calculation by SUMA software

Calculation of both subsite maps and bond cleavage frequencies was performed using the Windows compatible computer program SUMA with unified parameters. Apparent subsite binding energies (E_{SUMA}) were calculated based on the bond cleavage frequncies. Bond cleavage frequencies were calculated using the subsite binding energies as input data.

3.2.4 Transglycosylation reactions

Parameters were optimized for transglycosylation. The transglycosidase activity of AMY1 was studied systematically, using several donors and acceptors. Release of products was followed at 302 and 317 nm for PNP- and MU-glycosides, respectively, using ZORBAX Eclipse XDB-C18 column (5 μm, 4.6 mm, 150 mm) and/or Supelcosil C18 (3 μm, 4,6 mm, 150 mm). The percentage of conversion of the acceptor was used to compare the transglycosidase activity between the different reactions.

3.2.5 Preparation of MU group containing maltooligosaccharide substrate series

MU-α-D-MOS molecules were synthesized chemoenzymatically, using MU-α-D-G1 acceptor and G7 donor in the V47F AMY1 catalyzed transglycosylation reaction. MU-α-D-MOS molecules were separated and purified by HPLC using YMC-Pack Polyamine II. column (5 μm, 250*4,6 mm).

3.2.6 Activity measurements using MU-MOS substrates

MU-α-D-MOSs were used to investigate the activity of HSA, BLA and BSMA. Activity of HSA was measured using MU-α-D-G3 as substrate. Activity measurement of BLA was performed using MU-α-D-G5 while MU-α-D-G2 was used in case of BSMA. Fluorescence was measured in microtiter plate at room temperature using Wallac VICTOR^2 1420 fluorimeter-luminometer (PerkinElmer Inc.) at 355 nm excitation (λ_{ex}) and 460 nm emission (λ_{em}) wavelengths. Kinetic parameters were determined using GRAFIT software (Erithacus Software Ltd.).

3.2.7 ^{1}H and ^{13}C NMR

Structural parameters of MU-MOSs were determined by ^{1}H and ^{13}C NMR spectroscopy. Spectra were recorded with Bruker DRX-500 spectrometer. Measurements and evaluation of spectra were performed by Prof. Gyula Batta (Department of Organic Chemistry, Faculty of Science and Technology, University of Debrecen).

3.2.8 MALDI-TOF MS

The MALDI-TOF MS measurements were performed at the Department of Applied Chemistry (Faculty of Science and Technology, University of Debrecen) using Bruker Biflex MALDI-TOF mass spectrometer.

3.2.9 Subsite mapping by molecular modeling

Crystal structures of α-amylases were downloaded from the Protein Data Bank and were used as templates for building of 3D structures of wild type and mutant enzymes using Sybyl program package (Tripos International). Sequences were derived from UniProt Database. Enzyme-substrate interaction energy calculations and visualizations were performed on Silicon Graphics Fuel workstations (Silicon Graphics International). Figures were created using Sybyl and VMD („Visual Molecular Dynamics") softwares.

Molecular modeling-aided subsite binding energy calcualtion procedure consists of the following steps.

Structures of the maltooligosaccharide substrates were modeled based on the crystal structures of maltose, maltoheptaose and a substrate-analogue inhibitor acarbose complexed with various alpha-amylases (Step 1.). Structure of the substrate was refined by a short energy minimization procedure (number Powell iterations: 20, dielectric constant: 1, AMBER7_FF99 force field). Initial structure of an enzyme-substrate complex was generated by merging the substrate into the binding cleft of the enzyme and deleting the inhibitor if it existed in the original crystal structure (Step 2.). Appropriate amino acid sidechains were modified to build the homologous models of mutant enzymes, the spatial position of the side chain of the modified residue was predicted by a short molecular dynamics run. To refine the position of the substrate and the mutated residues, a short minimization procedure was performed by Sybyl (100 Powell iterations, dielectric constant 1, AMBER7_FF99 force field) on the 8 Å surround of the oligosaccharide chain and the mutated residues. The resulted complex was further

energy-minimized without any fixed atoms by Sybyl using the following parameters: AMBER7_FF99 force field, non-bonded cutoff 8 Å, 100 Powell iterations and dielectric constant 1. Interaction energies were calculated between the protein and the carbohydrate residues of each subsite (E_{Sybyl}) (Step 3.). During the evaluation of data (Step 4.) these values were compared to those calculated based on the experimentally determined action patterns (E_{SUMA}). Linear regression analysis was performed between E_{SUMA} and E_{Sybyl} values. After regression analysis, E_{Sybyl} data were linearly transformed to the scale of E_{SUMA} data with the values of intercept and slope of the best fit line to get E_{Transf} using Microsoft Excel (Microsoft Corporation) and SigmaPlot (Systat Software Inc.) softwares. Models of studied enzymes were aligned using Sybyl program on Silicon Graphics Fuel workstation.

4. Results and discussion

4.1 Studies on secondary substrate binding site mutants of AMY1

The secondary substrate binding sites located far from the active site play important role in the action of the enzyme leading to multi-site substrate interactions. Therefore, mutations of these sites have effect both on the enzyme-substrate interactions and the bond cleavage frequencies. Altered subsite binding energies can be calculated from the altered BCFs, therefore subsite mapping is a useful method to study the secondary substrate bindig sites.

W279A, W278A/Y380A, W279A/Y380A, Y380A, H395A, H395A/Y380A and Y105A/Y380M single and double mutants of AMY1 contained the mutations of key amino acids of "a pair of sugar tongs" and the „starch granule binding site". Subsite maps were calculated for 8 aglycone and 4 glycone-binding subsites based on the BCFs of CNP-MOS substrate series. Bond cleavage frequencies of mutants closely resembled that of the wild type. The dominant role of Tyr105 was shown in case of Y105A/Y380M mutant, because the subsite map and BCF of this double mutant closely resembles the values of Y105A mutant. Previously transglycosylation activity was detected for AMY1. We also observed transglycosylation by AMY1 during CNP-G4 hydrolysis: 8% of products were released by transfer reaction, observed at $\leq$ 10% CNP-G4 conversion. All of mutant enzymes showed enhanced transglycosylation activity during the BCF analysis, not only on the CNP-G4 substrate, but also on shorter and longer substrates. The calculated subsite maps of Y380A and H395A mutants revealed a striking gain and a striking loss in the total subsite binding energy determined to be -39.7 and -12.6 kJ/mol, respectively, compared to the wild-type AMY1 value of -22.6 kJ/mol. The binding energy gain in Y380A was fairly evenly distributed along the active site subsites. Subsite maps of W279A, W278A/Y380A and W279A/Y380A starch granule binding site mutants resembled that of the wild type without the affinity gain characteristic of the Y380A mutant.

In this work we studied the W279A, W278A/Y380A, W279A/Y380A, Y380A, H395A, H395A/Y380A and Y105A/Y380M secondary substrate binding site mutants of AMY1 and we evaluated the effects of mutations based on the results of experimental subsite mapping procedure.

4.2 Studies on active site mutants of AMY1

S48Y, V47A, V47F, V47D, V47K/S48G, V47G/S48D, V47I/S48I and V47L/S48A active site mutants of AMY1 were prepared by random mutagenesis to study the role of V47 and S48 amino acids in substrate binding. We mapped the active site of these enzymes and subsite maps were calculated for 12 subsites based on the determined BCF-s.

We observed the remarkable changes of preferred binding modes which can be explained by the altered subsite maps. Subsites of mutant enzymes with higher or lower affinities compared to the wild type enzyme lead to the alteration of product pattern. AMY1 mutants were found to show enhanced transglycosylation activity compared to the wild type enzyme. Each active site AMY1 mutants showed enhanced transglycosylation activity compared to the wild type enzyme and formed more transfer product using both CNP-G4 and longer and shorter CNP-MOS also.

Active site mutants showed remarkably changes in subsite binding energies and in total binding energy values. Almost all mutant enzyme showd decrease in subsrtate binding affinity at −7 and −6 subsite. High affinity subsite −6 (-12,40 kJ/mol) showed dramatic decrease of binding energy (-1,13 kJ/mol) in case of S48Y. Subsite binding energy of +2 and −2 subsites also increased almost in all cases. These effects caused the change of substrate specificity of mutant enzymes. The high-affinity subsites −2 through +2 control the shorter substrates, while longer ones are anchored at −6. The high- and low-affinity subsites together with the barrier subsites determine the formation of productive complexes and therefore the rate of hydrolysis of substrates. In case of wild type enzyme the binding modes of longer substrates are co-ordinated by the high affinity −6 and +2 subsites. Mutations decrease the anchoring effect of the −6 subsite, therefore the velocity of hydrolysis changes in case of substrates of different DP. Because of the more preferred binding mode the AMY1 catalyzed hydrolysis of CNP-G7 substrate is faster than the hydrolysis of shorter ones ($v_{CNP\text{-}G4}/v_{CNP\text{-}G7}$ = 0,08). The rate of product formation by hydrolysis was higher in case of shorter substrates because of the altered binding modes in mutant enzymes. Affinity of V47F for CNP-G4 also increased ($v_{CNP\text{-}G4}/v_{CNP\text{-}G7}$ = 22) because of the lower subsite binding affinity of subsite −6.

Remarkable changes observed in case of mutation of V47 and S48 amino acids can be explained by their importance in substrate binding. They are participated in substrate binding almost in all aglycone-binding sites because the substrate adopts a half-circle conformation centered on these sidechains. Furthermore, V47 together with Y105, may be considered as the substrate “entrance” to the active site cleft.

We performed the subsite mapping of S48Y, V47A, V47F, V47D, V47K/S48G, V47G/S48D, V47I/S48I and V47L/S48A novel AMY1 mutants. Our data, together with the results of previous experiments are important to understand the structure-function relationships of AMY1. We used these results also in case of studies on transglysosylation activity of AMY1 and to prepare a test set of data to test the computer-aided subsite mapping procedure.

4.3 Examination of hte transferase activity of AMY1 and its mutants

Druing the bond cleavage frequency analysis increased transglycosylation activity of AMY1 mutants was observed on CNP-MOS substrates. Barley amylase isoenzymes, AMY1 and AMY2 previously showed transglycosylation on MOSs and 4-nitrophenyl (PNP) glycoside substrates, but this ability for the synthesis of oligosaccharides was not further explored. The increased transglycosylation activity encouraged us to use these mutants for enzymatic synthesis.

4.3.1 Effect of AMY1 subsite mutation on transferase activity

The transglycosidase activity of AMY1 was studied systematically, using several donors and chromophore-group containing acceptors to demonstrate the effect of the monosaccharide unit for transglycosylation. The results confirm that the equatorial position of 4-OH is critical since monosaccharides with axial OH at position 4 were not acceptors for AMY1. The anomeric configuration is not so important; β- and α-glycosides are similarly efficient acceptors. Aglycon part of acceptor effects on transferase efficiency, as well. MOSs of different degree of polymerization (DP 2−7) were used as donors. Noticeably reverse hydrolysis did not occur in the presence of glucose and maltose, maltotetraose and maltopentaose were good donors for all tested enzymes (AMY1, Y105A, Y105A/T212Y, V47F, S48Y, V47K/S48G and V47G/S48D). Each active site AMY1 mutants formed more transfer product at the same reaction conditions compared to the wild type.

4.3.2 Enzymatic synthesis by V47F AMY1 – optimization of reaction conditions

MU-MOSs are potentially good substrates for α-amylases but they are not commercially available up to now, with the exception of MU-α- and β-D-glucopyranosides. Therefore the chemoenzymatic synthesis of MU-α-D-MOS substrates was necessary for fluorometric assay.

We have tested both MU-α- and β-D-G1 as acceptors for enzymatic transglycosylation to synthesize MU group-containing MOSs as new, fluorogenic substrates for α-amylase assays. Each steps of optimization procedure of the transglycosylation reaction was performed using MU-β-D-G1 as acceptor to avoid loss by hydrolysis of chromophore group, because the enzyme is not capable to cleave the β-glycosidic bond. Enhanced transglycosylation activity of each active site AMY1 mutant was observed during the subsite mapping experiments compared to the wild type enzyme. Although the mutant enzymes produce higher amounts of transfer products than AMY1, in the presence of acceptor the difference was not so remarkable. Enzymatic synthesis was performed with the use of V47F AMY1 mutant, the parameters for synthesis reaction were optimized. We tested different concentrations of MU-β-D-G1 acceptor (4, 8, 12 and 15 mM) and G7 donor (10−40 mM), the effect of pH (pH 4.5−6.5) and concentration of DMSO (10−40 %) on transglycosylation reaction. ^{1}H and ^{13}C NMR spectroscopy were used to determine the anomeric configuration and the interglycosidic bond type of MU-β-D-MOSs and spectra revealed that the mutated enzyme preserved the stereo- and regioselectivity.

4.3.3 Enzymatic synthesis by V47F AMY1 – preparative reaction

Enzymatic transglycosylation was catalyzed by V47F AMY1 active site mutant. MU-α-D-MOS products of DP 2−6 were separated and purified by HPLC. MU-α-D-G2 was the main product, might be generated also from the secondary hydrolysis of initial products. Longest product MU-α-D-G6 was formed only in trace amount (0.6 %). Products were characterized and NMR and used as substrates for enzyme activity assay. MU-α-MOS products of DP 2−5 were identified by MALDI-TOF MS according to the good agreement between the calculated and the measured molecular masses. The results show that enzymatic transglycosylation by AMY1 V47F is a usable method for the synthesis of MU-α-D-MOSs. Although MU-MOSs could be good fluorogenic substrates for α-amylase assay they are not commercially available up to now. Enzymatic synthesis described in dissertation may be the solution for preparation of amylase substrates.

4.3.4 Activity measurements using the fluorogenic substrate

To test the utility of the synthesized fluorogenic MU-α-D-MOS substrates activities were investigated for BSMA, HSA and BLA. The distribution of the high and low affinity binding subsites and the barrier subsites within the active site determines the hydrolytic

efficiency on series of substrates with various length: high-affinity subsites next to the cleavage sites (−2 through +2) allow effective cleavage of shorter substrates, while distal high-affinity subsites control the action on longer substrates. Therefore, the substrates for activity measurements were selected on the basis of the subsite maps. When the fluorophore group-anchoring α-glycosidic linkage lies over the cleavage site hydrolysis occurs and this is the only productive binding mode for the split of the fluorophore. Calibration using resulted in excellent correlation (r^2 = 0.999) between the concentration of 4-methylumbelliferone and the measured fluorescence. We proved that enzymes liberate the methylumbelliferone (MU) exclusively from the substrates. Decrease in peak area of the MU substrate without the release of other products was observed on HPLC chromatogram.

Enzyme kinetic parameters (K_M and v_{max}) were determined based on Michaelis-Menten kinetics by non-linear regression, furthermore kcat and kcat/K_M values were calculaterd also. Assay based on the use of MU-α-D-MOS susbtrates may provide a fast and simple fluorometric method for activity measurements of α-amylases.

4.4 Computer-aided subsite mapping of α-amylases

We developed a computer-aided procedure which may supplement the experimental subsite mapping methods and helps to predict and understand the characteristic features of α-amylases. The computer-aided analysis of X-ray crystallographic structures and homologous models is an effective way to examine enzyme-substrate interactions in a quantitative manner. Our working hypothesis was that the subsite binding energies could be predicted from enzyme-substrate interaction energies calculated by a molecular mechanical program. Therefore in this work, we aimed to develop a computer-aided procedure for subsite mapping of α-amylases to calculate the subsite binding energies by molecular mechanical calculations using Sybyl program and to predict primary experimental data (BCFs) using the computer program SUMA.

4.4.1 Data sets

Two sets of data were used to optimize and test the computational procedure. Training set was used to optimize the parameters of subsite binding energy calculations, experimentally determined BCFs were collected from the literature and for each enzyme and based on them, subsite maps were calculated by SUMA. Test set was used the test the computational procedure, action patterns of these enzymes were determined experimentally and subsite maps were calculated by SUMA.

The training set consisted of the subsite binding energies of all wild-type enzymes and some mutants of human salivary amylase (HSA) and barley amylase 1 (AMY1) enzymes. Wild-type enzymes were chosen based on the existence of experimental BCF values as well as available crystal structure. Enzyme groups were formed within the sets based on the wild-type enzymes and each mutant belonged to the group of the corresponding wild-type enzyme. *Bacillus amyloliquefaciens* α-amylase (BAA), *Aspergillus oryzae* α-amylase (TAA), AMY2 and pig (*Sus scofa*) pancreatic α-amylase (PPA) groups contained only the wild type enzymes, while HSA group contained also the data of Y151M and W58L mutants, AMY1 group consisted of the data of wild type and Y105A, Y105F, Y105W, T212Y and Y105A/T212Y mutants. Published subsite maps of all wild-type enzymes were recalculated by SUMA from the previously published action patterns because of the difference between the number of subites in published subsite maps and the degree of polymerization of structural models of substrates. The original and the recalculated binding energies were in excellent agreement, square of the correlation coefficients (r^2) from the linear regression analysis were between 0.942 and 0.999. The training set consisted of total 13 wild-type and mutant enzymes and 97 subsite binding energy values.

The test set was created from subsite binding energies of novel mutants of AMY1: S48Y, V47A, V47D, V47F, V47I/S48I, V47K/S48G, V47G/S48D and V47L/S48A. The test set consisted of total 8 mutant enzymes and 73 subsite binding energy values.

4.4.2 Structural and sequence alignment

Sequence identities of the studied enzymes were determined based on structural alignment. In contrast to the various degree of sequence identity (11−86 %) between the wild-type enzymes, they were all classified into the glycoside hydrolase family 13 and showed high degree of structural conservation. The β-strands of $(\beta/\alpha)_8$-barrels, the catalytic amino acids and the ione binding sites showed high structural conservation. The structural conservation of these enzymes were much stronger than the sequence conservation which give the hope that our procedure can be successfully applied for wide range of α-amylases of other glycoside hydrolases.

4.4.3 Steps of calculation

Homologous models of enzyme-substrate complexes of the training set were built and interaction energies were calculated between the protein and the carbohydrate residues of each

subsite (E_{Sybyl}). These values were compared to those calculated based on the experimentally determined action patterns (E_{SUMA}). To reach highest correlation between E_{SUMA} and E_{Sybyl} data, parameters of the energy-minimization procedure were optimized. Several force fields available in Sybyl program: Kollman_All_Atom, Amber95 and Amber7_FF99 were tested, together with an in-house implementation of Glycam06 force field. Amber7_FF99 was chosen because its performance was the best in our hand. Furthermore, wide-variety of values of dielectric constant (1, 2, 3, 4 and 8), non-bonded cutoff (8, 10 and 12), number of iterations (20-1000) and the width of minimized shell (1-12 angstrom) of atoms around the substrate and the mutated residues in the initial energy minimization were also probed to find a good combination of parameters. Our aim was to build a relatively fast procedure, therefore explicit molecules for bulk solvent and extensive minimization were avoided. Linear regression analysis was performed between E_{SUMA} and $E_{Transf.}$ values. Calculated E_{Sybyl} and $E_{Transf.}$ values of training enzymes showed good correlation with E_{SUMA} values: r^2 were in the range of $0.827-0.929$. The procedure was applied for the test set to assess the predictive potential of the model. Calculated E_{Sybyl} and $E_{Transf.}$ values of the test set showed correlation with E_{SUMA} values: r^2 was 0.502 which was lower than the corresponding value of 0.827 of AMY1 group of the training set, as expected. However, the error distribution was odd: all values out of 95% prediction interval were belonged to V47D, V47K/S48G and V47G/S48D mutants. All these mutants and only these mutants contained a substitution from neutral to charged residue. If all values of these mutants were omitted from the plot, the r^2 value rose up to 0.638.

It seems that our simple procedure was only moderately able to model this combined effect as the correlation coefficient was dropped from 0.827 to 0.638 (neglecting mutants having substitutions to charged residues). Large changes in charge distribution of the mutated position turned out to be also problematic, as in cases of V47D, V47K/S48G and V47G/S48D mutants: the correlation coefficient was further dropped to 0.502. However, special care is needed when neutral residues was changed to charged one (and probably *vice versa*) in the substrate binding site of an α-amylase. Some mutants containing amino acid changes in the secondary binding sites were also tested, but our procedure failed to correctly predict changes caused by these distant sites, as expected.

4.4.4 Prediction of action pattern

Bond cleavage frequencies were calculated using the $E_{Transf.}$ values as input data in SUMA and the predicted BCFs were correlated with the experimentally determined values. To confirm the usability of SUMA program for BCF prediction we correlated the BCFs calcualted from E_{SUMA} values to the experientally detemined ones. Calculations resulted in excellent correlations between the BCFs (r^2 = 0.852−0.996). Linear regression analysis between predicted and published BCFs of enzymes of the training set resulted in good correlation (r^2 = 0.727−0.835). In case of mutant AMY1 enzymes of the test set, the correlation coefficient was similarly low to that of binding energy analysis: r^2 = 0.538. Not unexpectedly, the odd error distribution was also present here: if values of mutant enzymes having substitution to charged residues (V47D, V47K/S47G and V47G/S48D) were omitted, the value of r^2 rose up to 0.728.

Our procedure was successfully adopted for α-amylases belonging to different kingdoms: we studied bacterial, fungal, plant and mammalian enzymes.

The computer-aided subsite mapping may support the studies on structure-function relationships and the examination of the role of substrate binding residues. Our method can supplement the experimental subsite mapping procedure by prediction of subsite maps and action patterns in a fast and cheap way. It can be used to supply protein engineers in design of modified enzymes with altered action pattern.

5. Summary

In this work we used the method of subsite mapping to examine the W279A, W278A/Y380A, W279A/Y380A, Y380A, H395A, H395A/Y380A and Y105A/Y380M secondary substrate binding site mutants of AMY1. We mapped the active site of S48Y, V47A, V47F, V47D, V47K/S48G, V47G/S48D, V47L/S48A and V47I/S48I active site mutants of barley α-amylase 1 (AMY1). 2-Chloro-4-nitrophenyl-β-D-maltooligosaccharides of degree of polymerizaion 3−11 (CNP-β-D-MOS of DP 3−11) were used to determine the bond cleavage frequencies (BCF). Subsite binding energies were calculated using the computer program for subsite mapping of α-amylases (SUMA).

Enhanced transglycosylation activity of several mutants of AMY1 was observed during the BCF analysis and this ability was further explored and studied systematically. After optimization of reaction conditions, 4-methylumbelliferyl-α-D-maltooligosaccharides (MU-α-D-MOS) of DP 2−5 have been successfully synthesized using V47F active site mutant of AMY1. MU-α-D-MOS were used efficiently as fluorogenic substrates in a fast and simple fluorometric α-amylase assay to determine the kinetic parameters of *Bacillus stearothermophilus* maltogenic α-amylase, *Bacillus licheniformis* α-amylase and human salivary α-amylase. Described method may be the solution for preparation of fluorogenic α-amylase substrates which are not commercially available up to now.

We developed a computer-aided subsite mapping procedure for α-amylases. A training set of data was collected from the literature using published data, to prepare a test set of data we determined experimentally the BCF-s and calculated the subsite maps of V47/S48 single and double mutants of AMY1. Parameters of the calculations were set up to get correlation between the calculated and the experimental binding energies of enzymes of a training set. Calculations on an independent test set also resulted in good correlation, except mutants containing neutral to charged residue substitution. Developed method was adopted for several α-amylases and is useful to calculate subsite binding energies of homologous models of wild-type and mutant enzymes by molecular mechanical program using Sybyl molecular modeling software as well as the primary experimental data (BCFs) with the use of computer program SUMA. Our computer-aided procedure may help to understand structure-function relationship of α-amylases and to design enzymes with new features.

6. Publications

6.1 Publications related to the thesis

6.1.1 Published articles

Nielsen M. M., Bozonnet S., Seo E. S., **Mótyán J. A.**, Andersen J. M., Dilokpimol A., Abou Hachem M., Gyémánt G., Næsted H., Kandra L., Sigurskjold B. W., Svensson B. (2009) „Two secondary carbohydrate binding sites on the surface of barley alpha-amylase 1 have distinct functions and display synergy in hydrolysis of starch granules" Biochemistry **48** (32) 7686-7697.
IF 3.226

Mótyán, J.A., Gyémánt, G., Harangi, J., Bagossi, P. (2011) „Computer-aided subsite mapping of α-amylases" Carbohydrate Research **346** (3): 410-415.
IF 2.025 (2009)

6.1.2 Article before review

Mótyán, J. A., Fazekas, E., Mori, H., Svensson, B., **Bagossi**, **P.**, Kandra, L., Gyémánt, G. „Transglycosylation by barley α-amylase 1"
Submitted to Journal of Molecular Catalysis B - Enzymatic (2010.12.09.), under review
IF 2.4 (2009)

6.2 Conference presentations related to the thesis

6.2.1 Oral presentations

Kandra, L., **Mótyán**, **J. A.**, Farkas, E., Svensson, B., Gyémánt, G. „Mapping of barley amylases - Effect of mutation on subsite maps and activities" *Carbohydrate research in Hungary Conference 2008 - Annual Meeting of the Working Group for Carbohydrate Chemistry of the Hungarian Academy of Sciences,* Mátrafüred, Hungary, 2008.05.29−30.

Mótyán, J.A., Gyémánt, G., Kandra, L., Bagossi, P., Harangi, J. „Examination of the substrate binding sites of α-amylase enzymes" *2nd Molecular Cell and Immune Biology Winter School*, Krompachy, Slovakia, 2009.01.06−09.

Mótyán, J.A., Fazekas, E., Svensson, B., Kandra, L., Gyémánt, G. „Transglycosylations by modified barley amylase 1 enzymes - Enzymatic synthesis of 4-methylumbelliferyl-β-D-maltooligosaccharides" *Carbohydrate research in Hungary Conference 2009 - Annual Meeting of the Working Group for Carbohydrate Chemistry of the Hungarian Academy of Sciences,* Mátrafüred, Hungary, 2009.05.28−29.

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Svensson, B., Andersen, J. M., Vester-Christensen, M. B., Jensen, J. M., Seo, E.S., Nielsen, M. M., **Mótyán**, **J. A.**, Kandra, L., Gyémánt, G., Haser, R., Aghajari, N., Hachem, M. A. „New insights into structure/function relationships in plant α-amylase family GH13 members" *Annual Meeting of the Japanese Society of Applied Glycoscience and 17th Symposium on Amylases and Related Enzymes 2009,* (*J. Appl. Glycosci*.,Vol. 56, Suppl., 2009)

Mótyán, J. A., Fazekas, E., Harangi, J., Bagossi, P., Svensson, B., Kandra, L., Gyémánt, G. „Transglycosylations by modified barley amylase 1 enzymes - Enzymatic synthesis of 4-methylumbelliferyl group-containing maltooligosaccharides as new substrates for α-amylases" *Carbohydrate research in Hungary Conference 2010 - Annual Meeting of the Working Group for Carbohydrate Chemistry of the Hungarian Academy of Sciences*, Mátrafüred, Hungary, 2010.05.27−28.

Mótyán, J. A., Gyémánt, G., Kandra, L., Harangi, J., Bagossi, P. „In vitro and in silico studies on α-amylase enzymes" *4th Molecular Cell and Immune Biology Winter School,* Galyatető, Hungary, 2011.01.11−14.

6.2.2 Poster presentations

Mótyán, J. A., Bagossi, P., Harangi, J. „Amiláz enzimek szubsztrátkötô alhelyeinek vizsgálata molekuláris modellezéssel", *Annual meeting of the Hungarian Biochemical Society 2007,* Debrecen, Hungary, 2007.08.26−29.

Mótyán, J. A., Fazekas, E., Harangi, J., Bagossi, P., Svensson, B., Kandra, L., Gyémánt, G. „Másodlagos szubsztrátkötő-helyek vizsgálata az árpa amilázban (AMY1)" *Annual meeting of the Hungarian Biochemical Society 2008,* Szeged, Hungary, 2008.08.31−09.03.

Mótyán, J. A., Gyémánt, G., Bagossi, P., Harangi, J. „Subsite mapping of α-amylase enzymes with molecular modeling & Examination of the substrate binding sites of α-amylase enzymes with molecular modeling" *4th Central European Conference "Chemistry towards Biology"*, Dobogókő, Hungary, 2008.09.08−11.

Svensson, B., Vester-Christensen, M. B., Andersen, J. M., Jensen, J. M., Seo, E. S., Nielsen, M. M., **Mótyán, J. A.**, Kandra, L., Gyémánt, G., Haser, R., Aghajari, N., Henriksen, A., Hachem, M. A. „New insights in structure/function relationships involving calcium ions, proteinaceous inhibitors, and multiple surface sites in glycoside hydrolase family 13" *8th Carbohydrate Bioengineering Meeting*, Ischia, Naples, Italy, 2009.05.10−13.

Svensson, B., Seo, E. S., Andersen, J. M., Nielsen, M. M., **Mótyán, J. A.**, Kandra, L., Gyémánt, G., Hachem, M. A. „Multiple surface sites and active site cross-talk in α-amylase" *VIII. European Symposium of The Protein Society,* Zürich, Switzerland, 2009.06.14−18.

Mótyán, J. A., Gyémánt, G., Bagossi, P., Harangi, J. „Alfa-amiláz enzimek szubsztrátkötő zsebének alhelytérképezése molekuláris modellezéssel" *Annual meeting of the Hungarian Biochemical Society 2009,* Budapest, Hungary, 2009.08.23−26.

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2. Nielsen, M.M., Bozonnet, S., Seo, E.S., **Mótyán, J.A.**, Andersen, J.M., Dilokpimol, A., Abou Hachem, M., Gyémánt, G., Naested, H., Kandra, L., Sigurskjold, B.W., Svensson, B.: Two Secondary Carbohydrate Binding Sites on the Surface of Barley alpha-Amylase 1 Have Distinct Functions and Display Synergy in Hydrolysis of Starch Granules.
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