3RD SYMPOSIUM ON THE ALPHA-AMYLASE FAMILY

SMOLENICE CASTLE, SLOVAKIA, SEP 23 – 27, 2007

PROGRAMME AND ABSTRACTS

Programme and abstracts of the
3rd Symposium on the Alpha-Amylase Family
held in Smolenice Castle, Slovakia, 23 – 27 Sep, 2007

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Štefan Janeček

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3rd Symposium on the Alpha-Amylase Family

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Foreword

Smolenice Castle (Slovakia), the Congress Centre of the Slovak Academy of Sciences, welcomes all the participants of the ALAMY_3!

This is the third symposium in the established series of the symposia on the α-amylase family. The effort to organise the ALAMY_3 has been based on the success of the preceding two symposia, ALAMY_1 and ALAMY_2, which were evoked by the rapidly growing knowledge on this enzyme family and its enormous scope. It has been our aim to make these symposia platform for informal discussions about the most recent results and international collaborations in the field of the α-amylase family in its widest sense.

The scientific programme of the ALAMY_3 is focused again to various aspects of the α-amylase enzyme family including the topics from the clan GH-H (families GH13, GH70 and GH77) as well as the families GH31 and GH57. A space has been provided for lectures about the regulation of metabolism of starch and glycogen as well as the various families of starch-binding domains (i.e. the CBM families) including the applied aspects. The lectures consist of 14 Invited Lectures and 13 Oral Talks. The latter were carefully selected from the submitted abstracts by the members of Scientific Programme Committee. The results of the remaining 36 abstracts will be presented in the form of posters that will be on display during the entire symposium.

The Scientific Programme Committee decided also to establish a Lifetime Achievement Award for a significant and worldwide recognised contribution to the knowledge on the α-amylase family. It is a moral prize in the form of a glass prism in which the three-dimensional picture of an α-amylase model is engraved by laser. The Committee has arrived at the conclusion that this year the Award will be given to Dr. Ann MacGregor.

The Local Organising Committee made every effort to prepare also a pleasant social programme for all participants. It includes the Opening Reception, Banquet with Poster Awards as well as the trip to Bratislava with a short guided tour in the historical centre and a dinner joined with tasting the wines of Slovak provenance.

We hope to have also a gossamer weather that together with the attractive Castle atmosphere will enable us to spend again a few days of inventive work and fruitful relaxation.

I, on behalf of the organisers, wish all of you a nice stay and an inspiring meeting!

Smolenice Castle, 23 September 2007 Štefan JANEČEK

3rd Symposium on the Alpha-Amylase Family

PROGRAMME

SUNDAY – 23 September 2007

15:00	Registration Desk Open Mounting of Posters
17:00 – 18:00	Conference buses from Bratislava and Vienna arrive at Smolenice Castle
19:45	**Opening words - Štefan Janeček**
20:00 – 22:00	**Opening Reception**

MONDAY – 24 September 2007

07:30 – 08:30 *Breakfast*

08:55 Announcements

09:00 – 12:25 **Chairmen: Birte Svensson and Kwan-Hwa Park**

09:00 – 10:00 Keynote ALAMY_3 Lecture - L1

Bauke W. Dijkstra (*Groningen, The Netherlands*): **Structural and functional characterization of a glucansucrase (ΔN-GTF180) from *Lactobacillus reuteri* 180, a GH family 70 enzyme.**

Session: The clan GH-H

10:00 – 10:40 L2

Pedro M. Coutinho (*Marseille, France*): **Division of α-amylase-related families and its importance for genomic analysis.**

10:40 – 11:10 *Coffee/Tea Break and Posters*

11:10 – 11:35 L3

Wataru Saburi (*Fuji, Japan*): **Structure-function relationship and engineering of dextran glucosidase from *Streptococcus mutans*.**

11:35 – 12:00 L4

Nushin Aghajari (*Lyon, France*): **Insights into sucrose isomerization from various sucrose isomerase structures.**

12:00 – 12:25 L5

Natarayan Ramasubbu (*Newark, NJ, USA*): **Unique molecular features of human salivary α-amylase for its multiple functions in the oral cavity.**

12:25 – 14:00 *Lunch Break*

14:00 – 16:00 **Poster Session I.**

16:00 – 18:10 **Chairmen: John F. Robyt and Carsten Andersen**
Session: The families GH31 and GH57

16:00 – 16:40 L6
Tadayuki Imanaka (*Kyoto, Japan*): **New GH57 branching enzyme from *Thermococcus kodakaraensis*.**

16:40 – 17:20 L7
Leila Lo Leggio (*Copenhagen, Denmark*): **Structure and substrate binding by the GH31 α-glucosidase MalA from *Sulfolobus solfataricus*.**

17:20 – 17:45 L8
Duncan Stanley (*Norwich, U.K.*): **Probing the role of α-glucosidase (GH31) in the endosperm of germinating barley (*Hordeum vulgare*).**

17:45 – 18:10 L9
Eva Nordberg Karlsson (*Lund, Sweden*): **Differences and similarities between neopullulanase-like enzymes isolated from thermophilic bacteria.**

19:00 – 22:00 *Supper at the court of the Castle with country music*

TUESDAY – 25 September 2007

07:30 – 08:30 *Breakfast*

08:55 Announcements

09:00 – 12:05 **Chairmen: E. Ann MacGregor and Jack Preiss**
Session: The clan GH-H

09:00 – 09:40 L10
Rachel M. van der Kaaij (*Haren, The Netherlands*): **What the annotators could not know; novel family GH13 enzymes in fungi and their unexpected functions in cell wall metabolism.**

09:40 – 10:20 L11
Yoann Brison (*Toulouse, France*): **How to synthesize the rare α-1,2 glucosidic linkage with an enzyme derived from a GH70 family glucansucrase?**

10:20 – 10:50 *Coffee/Tea Break and Posters*

10:50 – 11:15 L12
Kazuhisa Sugimoto (*Osaka, Japan*): **Sucrose phosphorylases catalyze novel transglycosylation reaction on carboxylic compounds.**

11:15 – 11:40 L13
Maher Abou Hachem (*Kgs. Lyngby, Denmark*): **Calcium binding to barley α-amylase isozymes and its implications on stability and activity.**

11:40 – 12:05 L14
Slavko Kralj (*Haren, The Netherlands*): **Determinants for glucosidic bond specificity in glucansucrase enzymes of glycoside hydrolase family 70 (GH70).**

12:05 – 14:00 *Lunch Break*

14:00 – 23:00 **Trip to Bratislava**
(conference buses leave for Bratislava)

19:00 – 22:00 *Dinner in the Vinum Galeria Bozen (Matyšák) in Pezinok*

~ 23:00 Arrival at the Smolenice Castle

WEDNESDAY – 26 September 2007

07:30 – 08:30 *Breakfast*

08:55 Announcements

09:00 – 12:05 **Chairmen: Takashi Kuriki and Bauke W. Dijkstra**
Session: Metabolism of starch and glycogen

09:00 – 09:40 L15
Kwan-Hwa Park (*Seoul, Korea*): **Role of carbohydrate-hydrolyzing and -debranching enzymes in sugar utilization of *Bacillus subtilis*.**

09:40 – 10:20 L16
Andreas Blennow (*Copenhagen, Denmark*): **Modulation of the degradability of the starch granule.**

10:20 – 10:50 *Coffee/Tea Break and Posters*

10:50 – 11:15 L17
John F. Robyt (*Ames, IA, USA*): **Mechanisms involved in the biosynthesis of starch.**

11:15 – 11:40 L18

Jack Preiss (*East Lansing, MI, USA*): **Crystal structure of the closed conformation of *Escherichia coli* glycogen synthase: critical amino acids involved in substrate binding and catalysis.**

11:40 – 12:05 L19

Jean-Luc Da Lage (*Gif sur Yvette, France*): **Where do animal α-amylases come from? An interkingdom trip.**

12:05 – 13:30 *Lunch Break*

13:30 – 15:30 **Poster Session II.**

15:30 – 16:35 **Chairman: Marc J.E.C. van der Maarel**
Session: Applied aspects of starch hydrolysis

15:30 – 16:10 L20

Carsten Andersen (*Bagsvaerd, Denmark*): **Amylase development for industrial applications.**

16:10 – 16:35 L21

Margaret Slupska (*San Diego, CA, USA*): **An evolved archaeal α-amylase with performance and mode of action tailored for the ethanol industry.**

16:35 – 17:00 *Coffee/Tea Break*

17:00 – 18:00 **Chairman: Štefan Janeček**
Special ALAMY_3 Lecture - L22

Manuel Palacín (*Barcelona, Spain*): **The structure of human 4F2hc ectodomain provides a model for homodimerization and electrostatic interaction with plasma membrane.**

18:00 – 19:00 *Refreshment Break*

19:00 – 20:00 **Chairman: Birte Svensson**
Banquet ALAMY_3 Lecture - L23

Richard Haser (*Lyon, France*): **When structural biology helps to understand sugar-processing enzymes and to stay in touch with the sweet α-amylase family.**

20:00 – 24:00 **Symposium Banquet with Awards**

THURSDAY – 27 September 2007

07:30 – 08:30 *Breakfast*
Vacation of the rooms; Removing of Posters

08:55 Announcements

09:00 – 10:45 **Chairman: Richard Haser**
Session: Starch-binding domains as CBM families

09:00 – 09:40 L24
David Stapleton (*Parkville, VIC, Australia*): **New insights into the structure and function of the AMPK glycogen-binding domain.**

09:40 – 10:20 L25
D. Wade Abbott (*Victoria, BC, Canada*): **Structural insights into the recognition of α-glucans by carbohydrate-binding modules.**

10:20 – 10:45 L26
Margaret Dah-Tsyr Chang (*Hsinchu, Taiwan, China*): **Correlation and characterization of functions and secondary/tertiary structures of carbohydrate binding modules.**

10:45 – 11:15 *Coffee/Tea Break*

11:15 – 12:05 **Chairman: Magali Remaud-Simeon**
Closing ALAMY_3 Lecture - L27

Birte Svensson (*Kgs. Lyngby, Denmark*): **An enzyme family reunion – similarities, differencies and eccentricities in actions on α-glucans.**

12:05 – 12:15 **Closing words - Štefan Janeček**

12:15 – 13:30 *Lunch and Farewell*

12:30 – 13:00 The 1st Conference Bus leaves for Bratislava and Vienna
13:30 – 14:00 The 2nd Conference Bus leaves for Bratislava and Vienna

LECTURE ABSTRACTS

Structural and functional characterization of a glucansucrase (ΔN-GTF180) from *Lactobacillus reuteri* 180, a GH family 70 enzyme

Bauke W. DIJKSTRA[1], Andreja VUJIČIĆ-ŽAGAR[1], Tjaard PIJNING[1], Slavko KRALJ[2] & Lubbert DIJKHUIZEN[2]

[1] *Laboratory of Biophysical Chemistry, University of Groningen, The Netherlands*
[2] *Department of Microbiology, University of Groningen, The Netherlands*

Bacterial α-glucans are synthesized by glucansucrase enzymes (glucosyltransferases), using sucrose as substrate. Glucansucrase enzymes cleave the glycosidic bond of their substrate sucrose and couple a glucose unit to a growing glucan (polyglucose) chain (transglucosylation), water (hydrolysis), or to other acceptor substrates (acceptor reaction). Depending on the main glucosidic linkages present in their glucan four different types of α-glucans synthesized by lactic acid bacteria are recognized [1,2]: dextran [α-(1→6)], mutan [α-(1→3)], alternan [α-(1→3)/α-(1→6)] and reuteran [α-(1→4)].

Due to the vast potential industrial applications of these α-glucans, glucansucrases have been extensively studied [1,2]. To date, 47 different glucansucrases have been classified in Glycoside Hydrolase family 70, but no 3D structure is yet known for them.

In order to investigate the molecular basis of the reaction mechanism of GH70 family enzymes and their linkage type specificity we have purified, crystallized and solved the structure of a 117 kDa N-terminally truncated form of *Lactobacillus reuteri* glucansucrase ΔN-GTF180 [3], synthesizing a glucan containing α-(1→6) and α-(1→3) glucosidic linkages. Also complexes of ΔN-GTF180 with sucrose (substrate) and maltose (acceptor) at 2.3 and 2.0 Å resolution, were solved. Moreover, two native crystal forms, a triclinic and an orthorhombic one (1.6 Å and 2.0 Å resolution, respectively) show that large conformational changes might play an important role in modulating linkage type specificity. The 3D structure revealed that the predicted linear domain organisation of glucansucrases [1,2] is erroneous. Instead, a surprising "U-fold" domain structure is observed, in which for 4 of the 5 domains an N- and a C-terminal part of the polypeptide chain combine to form the domain. Our crystallographic results could be fully validated by both biochemical and mutagenesis experiments.

1. Monchois V., Willemot R.M. & Monsan P. 1999. *FEMS Microbiol. Rev.* **23:** 131-151.
2. Van Hijum S.A., Kralj S., Ozimek L.K., Dijkhuizen L. & Van Geel-Schutten G.H. 2006. *Microbiol. Mol. Biol. Rev.* **70:** 157-176.
3. Kralj S., Van Geel-Schutten G.H., Dondorff M.M., Kirsanovs S., Van der Maarel M.J. & Dijkhuizen L. 2004. *Microbiology* **150:** 3681-3690.

Invited Lecture - L2

Division of α-amylase-related families and its importance for genomic analysis

Pedro M. COUTINHO[1], Mark R. STAM[1,2], Etienne G. J. DANCHIN[1], Corinne RANCUREL[1], Thomas BERNARD[1] & Bernard HENRISSAT[1]

[1] UMR6098, AFMB - CNRS/Aix-Marseille Universités, Case 932, 163 Avenue de Luminy, 13288 Marseille, France
[2] UMR314 INRA-CEA, Laboratoire de Radiobiologie et d'étude du Génome, 78352 Jouy en Josas, France

The Glycoside Hydrolase family classification comprises a number of families dedicated to starch and glycogen degradation and modification [1]. Several of these families comprise a large number of activities, mostly acting as glycosidases and/or transglycosidases. The resulting family multispecificity is at the origin of difficulties in predicting enzyme activities on the simple basis of family attribution, a major issue in this genomic era. The principles of the ongoing subdivision of starch- and glycogen-related families into subfamilies, based on overall sequence similarities [2], will be shown. Most of the resulting subfamilies present a significant homogeneity of the activities already described. However some subfamilies are uncharacterized or reveal low levels of biochemical characterization. The application of the resulting subfamilies in genome annotation will be highlighted.

MRS and EJGD thank Novozymes, A/S and the EU (FungWall STREP), respectively, for financial support.

1. CAZy. 1998-2007. Carbohydrate-Active enZymes database; http://www.cazy.org.
2. Stam M.R., Danchin E.G.J., Rancurel C., Coutinho P.M. & Henrissat B. 2006. *Protein Eng. Des. Sel.* **19:** 555-562.

Structure-function relationship and engineering of dextran glucosidase from *Streptococcus mutans*

Wataru SABURI[1,2], Hironori HONDOH[2], Haruhide MORI[2], Masayuki OKUYAMA[2] & Atsuo KIMURA[2]

[1] *Nihon Shokuhin Koko Co., Ltd., Fuji 417-8530, Japan*
[2] *Research Faculty of Agriculture, Hokkaido University, Sapporo 060-8589, Japan*

Dextran glucosidase (DG; EC 3.2.1.70) hydrolyzes α-1,6-glucosidic linkage at the non-reducing end of dextran and isomaltooligosaccharides. The primary structure of DG shows high similarity to those of bacterial α-glucosidases including oligo-1,6-glucosidases, members of GH family 13, although the specificities for substrate chain length of these enzymes are clearly different. DG has the high activity toward long-chain substrate, but oligo-1,6-glucosidase and α-glucosidase prefer short-chain substrate. In these enzymes, the three-dimensional structure of *Bacillus cereus* oligo-1,6-glucosidase (BCOG) has been solved, but the substrate binding structure is not available. Therefore, we could not discuss the structure-function relationship of substrate binding of GH 13 α-glucosidases.

In this study, we solved the crystal structures of free and substrate binding forms of DG. The overall structure of DG was very similar to that of BCOG except for length of β→α loop 4. This loop of DG was clearly shorter than that of BCOG, suggesting that DG has the space to accommodate long chain substrate in this position. In addition, DG has a bulky amino acid, Trp238, in subsite +2 unlike other bacterial α-glucosidases. We focused on β→α loop 4 and Trp238 of DG as the important structural elements for high activity toward long-chain substrate. Trp238-mutated DGs (W238A/N/P) and chimeric DG, in which β→α loop 4 of DG was replaced by that of oligo-1,6-glucosidase, showed the lower specificity to long-chain substrate than wild type as implied by our structure analysis [1].

Next, we engineered DG to provide a new approach for production of oligosaccharides. An oxidized cysteine was introduced as the catalyst to the position of the catalytic nucleophile of DG (Asp194), displaying the remarkably high transferring activity. At first, we replaced Asp194 by Cys (D194C). The enzymatic activity of D194C was descended to 8.1×10^{-4} % of that of the parent enzyme, but the activity was increased up to 0.27% by the thiol group oxidation with KI. The oxidized form of Cys194 was identified as cysteine sulfinic acid ($-SOO^-$) by mass spectrometric analysis of lysyl endopeptidase digest of the oxidized D194C (D194C-OX). D194C-OX showed similar pH profile and substrate specificity to the parent enzyme. Interestingly, D194C-OX exhibited much higher transglucosylation activity than the wild-type enzyme. D194C-OX efficiently transferred a glucosyl moiety from not only *p*-nitrophenyl α-glucoside having a good leaving group but also isomaltooligosaccharides.

1. Saburi W., Mori H., Saito S., Okuyama M. & Kimura A. 2006. *Biochim. Biophys. Acta* **1764:** 688-698.

Insights into sucrose isomerization from various sucrose isomerase structures

Stéphanie RAVAUD[1], Xavier ROBERT[1], Hildegard WATZLAWICK[2], Richard HASER[1], Ralf MATTES[2] & Nushin AGHAJARI[1]

[1] Laboratoire de BioCristallographie, Institut de Biologie et Chimie des Protéines, CNRS et Université de Lyon, UMR 5086, IFR 128 "BioSciences Lyon-Gerland", F-69367 Lyon Cedex 07, France
[2] Universität Stuttgart, Institut für Industrielle Genetik, Allmandring 31, D-70569 Stuttgart, Germany

The structural isomers of sucrose (α-D-glucosylpyranosyl-1,2-β-D-fructofuranoside), trehalulose (α-D-glucosylpyranosyl-1,1-D-fructofuranose) and isomaltulose (α-D-glucosylpyranosyl-1,6-D-fructofuranose) are very attractive sugar substitutes as they have similar taste profiles and very similar physical and organoleptic properties to sucrose.

Various diseases related to the over-consumption of sugar make a growing need for sugar substitutes. Sucrose is an inexpensive and readily available D-glucose donor, and the industrial potential for enzymatic synthesis of the sucrose isomers trehalulose and/or isomaltulose from sucrose is large. The product specificity of sucrose isomerases which catalyze this reaction depends essentially on the possibility for tautomerization of sucrose which is required for trehalulose formation.

A number of three-dimensional structures of native and mutant complexes of a trehalulose synthase from *Pseudomonas mesoacidophila* MX-45 which mimic successive states of the enzyme reaction have been solved by x-ray crystallography methods. Combined with mutagenesis studies they give for the first time thorough insights into substrate recognition and processing, and reaction specificities of these enzymes. Amongst the important outcomes of this study is the discovery of an aromatic clamp defined by Phe256 and Phe280 playing an essential role in substrate recognition and in controlling the reaction specificity, which is further supported by mutagenesis studies. Furthermore, this study highlights essential residues for binding the glucosyl- and fructosyl-moieties.

Unique molecular features of human salivary α-amylase for its multiple functions in the oral cavity

Narayanan RAMASUBBU, Chandran RAGUNATH, Suba GA MANUEL & Hameetha BM SAIT

Department of Oral Biology, University of Medicine and Dentistry of New Jersey, Newark, NJ 07103 USA

Human salivary α-amylase (HSAmy) has three distinct functions relevant to oral health: 1) hydrolysis of starch; 2) binding to hydroxyapatite; and 3) binding to bacteria (e.g. viridans streptococci). Oral bacteria utilize the starch hydrolyzing activity to derive their nutrients from dietary starch. Localized acid production by bacteria, through the metabolism of maltose generated by HSAmy, can lead to the dissolution of tooth enamel, a critical step in dental caries formation. HSAmy is a component of the acquired enamel pellicle and is used by *S. gordonii* to colonize the oral cavity. Although the active site of HSAmy for hydrolysis of starch is well characterized, the regions responsible for the enamel binding and bacterial binding are not. The molecular surface of HSAmy possesses a cluster of twelve acidic residues, which provide a large negative electrostatic potential. We hypothesized that the twelve acidic residues might play a significant role in enamel binding ability of HSAmy. To test the role of this cluster of twelve acidic residues in HA binding, we mutated these acidic residues to alanine and generated three multiple mutants containing the mutations Glu76Ala-Asp77Ala-Glu78Ala (mutant 3A); Glu76Ala-Asp77Ala-Glu78Ala, Asp173Ala, Glu181Ala, Asp188Ala, Asp212Ala, Glu224Ala (mutant 6A) and Asp363Ala, Glu369Ala, Asp375Ala and Asp381Ala (mutant 4A). The HA binding abilities of purified HSAmy and the mutants were compared using a HA binding assay. Significant reduction in the HA binding ability was observed for the mutants (11% of HSAmy was unbound compared to 25% for 3A, 71% for 6A and 35% for 4A). Mutation had minimal effect on enzymatic activity (oligosaccharide hydrolysis) and structural aspects (CD spectroscopy). Our results support the hypothesis that the negative electrostatic potential surface created by the 12 acidic residues is critical in the binding of HSAmy to enamel.

HSAmy possesses several secondary saccharide-binding sites in which aromatic residues act as binding platforms. We tested the hypothesis that the secondary saccharide sites harboring the aromatic residues may play role in bacterial binding. A mutant of HSAmy in which aromatic residues at 4 secondary saccharide-binding sites were replaced with alanine was expressed and purified. The wild type and the mutant enzymes were characterized for their abilities to exhibit enzyme activity, starch binding, bacterial binding and hydroxyapatite binding. The ability of the two enzymes to bind to the recombinant amylase binding protein AbpA from *S. gordonii* was also tested. Our results clearly showed that 1) the mutant amylase was impaired in starch binding, bacterial binding but not hydroxyapatite binding; 2) the bacterial binding mediated by AbpA occurs at a site other than the active site. Collectively, our results suggest that the aromatic residues at the secondary saccharide binding sites may have an impact in bacterial colonization and provide a molecular basis for the functions of HSAmy in the oral cavity.

This work was supported by the USPHS Grant DE12585.

New GH57 branching enzyme from *Thermococcus kodakaraensis*

Tadayuki IMANAKA

Department of Synthetic Chemistry & Biological Chemistry, Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510, Japan

We have proposed a general concept for the α-amylase family in 1992 [1,2], based on structural similarity and common catalytic mechanisms of the amylases and related enzymes. This family includes enzymes that catalyze the hydrolysis and transglycosylation of α-1,4- and α-1,6-glycosidic bonds, such as α-amylase, pullulanase, isoamylase, neopullulanase, branching enzyme (BE). Members of the α-amylase family mostly overlap with proteins in the family 13 of the glycoside hydrolases (GH-13 family) according to Henrissat's classification.

BE catalyzes the formation of branch points in glycogen and amylopectin by cleavage of α-1,4-glucosidic bonds and their subsequent transfer to α-1,6-positions. BEs identified to date all belong to the α-amylase/GH-13 family. We have identified a novel BE encoded by an uncharacterized ORF (TK1436) of the hyperthermophilic archaeon, *Thermococcus kodakaraensis* KOD1. TK1436 had been annotated as a conserved protein of unknown function showing similarity to members of glycoside hydrolase family 57 (GH-57 family). At the C-terminus of the TK1436 protein, two copies of a helix-hairpin-helix (HhH) motif were found. TK1436 orthologs are distributed in the Thermococcales of the Archaea, cyanobacteria, some actinobacteria and a few other bacterial species. When recombinant TK1436 protein was incubated with amylose as the substrate, a product peak was detected by high-performance anion exchange chromatography (HPAEC), eluting slower than the substrate. Isoamylase treatment of this reaction mixture significantly increased the level of short-chain α-glucans, indicating that the reaction product contained many α-1,6-branching points. TK1436 protein showed an optimal pH of 7.0, an optimal temperature of 70 °C, and thermostability up to 90 °C as determined by the iodine-staining assay. These properties were maintained in a truncated protein devoid of the HhH motifs (TK1436ΔH protein). The average molecular weight of branched glucan after the reaction with TK1436ΔH protein was over 100 times larger than that of the starting substrate. These results clearly indicate that TK1436 encodes a structurally novel BE.

The present identification of BE specificity in the GH-57 family is significant as this finding proves the existence of all four basic activities in the GH-57 family pertaining to the synthesis and decomposition of α-glucans. It thus seems that the GH-57 family can be regarded as a second "α-amylase family" with a structural background distinct from that of the GH-13 family [3].

1. Takata H., Kuriki T., Okada S., Takesada Y., Iizuka M., Minamimura N. & Imanaka T. 1992. *J. Biol. Chem.* **267:** 18447-18452.
2. Kuriki T. & Imanaka T. 1999. *J. Biosci. Bioeng.* **87:** 557-565.
3. Murakami T., Kanai T., Takaha H., Kuriki T. & Imanaka T. 2006. *J. Bacteriol.* **188:** 5915-5924.

Structure and substrate binding by the GH31 α-glucosidase MalA from *Sulfolobus solfataricus*

Heidi A. ERNST[1], Sine LARSEN[1,2] & Leila LO LEGGIO[1]

[1] *Biophysical Chemistry Group, Department of Chemistry, Faculty of Science, University of Copenhagen,Universitetsparken 5, DK-2100, Denmark*
[2] *European Synchrotron Radiation Facility (ESRF), Grenoble 38043, France*

The GH31 family is very diverse in terms of substrate specificity and mechanism, including hydrolases, transglycosidases as well as glucan lyases. The catalytic function common to all members of GH31 is the cleavage of a terminal carbohydrate, while their substrates vary in size from small disaccharides to large storage polymers, such as starch and glycogen, and glycoproteins. The majority of GH31 enzymes are α-glucosidases which play essential roles in carbohydrate metabolism or in glycoprotein processing and quality control. The crystal structure of the 693 residue α–glucosidase MalA from the hyperthermophile *Sulfolobus solfataricus* was determined at 2.5 Å resolution [1,2]. The structure was solved by molecular replacement using the *Escherichia coli* α-xylosidase YicI [3] as search model. Due to the low sequence identity (~23%) between the two enzymes, exploitation of non-crystallographic symmetry was crucial for structure determination. A complex with β-octyl-glucopyranoside was also determined at the same time [2], while more recently complexes with the α-glucosidase inhibitors deoxynojirimycin and acarbose were determined at 3.3 and 2.8 Å resolution, respectively.

MalA and YicI represent two distinct subgroups in GH31 which differ in sequence characteristics as well as substrate specificities. Despite low sequence similarities in the terminal regions, the overall folds of the four major domains are conserved between these two distant members of the family. Although both enzymes form homohexamers their structures differ significantly in quaternary organization: MalA is a dimer of trimers, YicI a trimer of dimers. MalA has a clear preference for maltose (Glc-α1,4-Glc) whereas YicI prefers isoprimeverose (Xyl-α1,6-Glc) as substrate. Comparative structural analysis of MalA and a covalent glycosyl:YicI intermediate [3] reveals possible structural determinants of substrate specificity in the -1 subsite, and the structures of MalA in complex with β-octyl-glucopyranoside and acarbose further elucidate the mode of substrate recognition in the + subsites. MalA belongs to the major subgroup in GH31, which also includes the α-glucosidases from higher organisms, and its structure provides the first molecular model for this large group of biologically important enzymes.

This research was supported by the EU as part of the NEPSA project, and the Danish Natural Science Research Council.

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2. Ernst H.A., Lo Leggio L., Willemoës M., Leonard G., Blum P. & Larsen S. 2006. *J. Mol. Biol.* **358:** 1106-1124.
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Probing the role of α-glucosidase (GH31) in the endosperm of germinating barley (*Hordeum vulgare*)

Duncan STANLEY[1], Martin REJZEK[1], Henrik NAESTED[2], Simone DEDOLA[1], Birte SVENSSON[2], Rob A. FIELD[1], Kay DENYER[1] & Alison M. SMITH[1]

[1] *John Innes Centre, Colney Lane, Norwich NR4 7UH, UK*
[2] *Enzyme and Protein Chemistry, BioCentrum-DTU, Søltofts Plads, Building 224, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark*

The endosperm of barley is packed with starch during development, and this starch is mobilised during the germination of the seed. A number of enzymes have been implicated in this process, including α-amylases, debranching enzymes and α-glucosidase [1]. The exact role of barley α-glucosidase (GH31) in the process is unclear. *In vitro* studies have shown that α-glucosidase is able to attack native starch granules and act synergistically with α-amylases [2,3].

To probe the role of α-glucosidase in starch degradation, we tested a series of potential inhibitor compounds against recombinant barley α-glucosidase, prepared by the method of Naested et al. [4]. We established that the chemical 1-deoxynojiromycin (DNJ) and a number of its derivatives were potent inhibitors of barley α-glucosidase *in vitro*. Germinating barley seeds treated with DNJ displayed severely stunted root development, when compared to seeds grown in water alone. Analysis of carbohydrates in the endosperm of 6-day old seedlings revealed high levels of starch and soluble glucans (both 1.5-fold higher than water-treated seed) and maltose (3-fold higher), and a 2-fold decrease in the level of glucose in DNJ-treated seeds. The same results were obtained with the DNJ derivative *N*-hydroxyethyl-1-deoxynojiromycin (miglitol). However, treatment of barley seeds with another derivative of DNJ, *N*-butyl-deoxynojiromycin, led to reductions in root and shoot development equivalent to those seen in DNJ-treated seed, but did not dramatically alter the endosperm carbohydrate profile compared to water-grown seed. This evidence suggests that DNJ is able to inhibit α-glucosidase in germinating endosperm, slowing down starch degradation and glucose release, as well as other enzymes involved in growth of embryonic tissues, and that more specific inhibitors of α-glucosidase may be found amongst compounds related to DNJ. We are currently screening an expanded library of inhibitor compounds, and searching for specific inhibitors of other enzymes in the starch degradative pathway.

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Differences and similarities between neopullulanase-like enzymes isolated from thermophilic bacteria

Eva NORDBERG KARLSSON[1], Antje LABES[2], Pernilla TURNER[1], Olafur H. FRIDJONSSON[3], Christina WENNERBERG[1], Gudmundur O. HREGGVIDSON[3], Olle HOLST[1], Jakob K. KRISTJANSSON[3] & Peter SCHÖNHEIT[2]

[1] *Department of Biotechnology, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden*
[2] *Institute for General Microbiology, Christian-Albrechts-University Kiel, Am Botanischen Garten 1-9, DE-24118 Kiel, Germany*
[3] *Prokaria Ltd., Gylfaflöt 5, IS-112 Reykjavik, Iceland*

A number of thermophilic glycoside hydrolase family 13 members have been isolated, from bacterial strains or directly from environmental DNA, using a consensus primer strategy utilizing two of the four conserved regions present in GH13. Six genes, encoding enzymes with highest sequence similarities to neopullulanase-like enzymes (neopullulanases, maltogenic amylases and cyclodextrinases) were selected for expression in *E. coli* and characterization.

The sequences encoding three of the enzymes were isolated from the genomic DNA of three thermophilic bacterial strains originating from Icelandic hot springs and belonging to the genera *Anoxybacillus, Thermoactinomyces,* and *Geobacillus,* respectively. Three enzymes were encoded by genes isolated from environmental DNA, collected from Icelandic hot springs. The typical domain structure of neopullulanase-like enzymes, including an N-terminal domain, the catalytic module composed of the A- and B-domain, and a C-terminal domain, were found in five of the enzymes. One enzyme (isolated from environmental DNA) was lacking the N-terminal domain.

Significantly different activity profiles were found for the enzymes, and specificity differences will be discussed. Three of the enzymes harboured cyclodextrinase, maltogenic amylase, as well as neopullulanase activity. One of these enzymes, from *Thermoactinomyces*, exhibited the NABC-domain structure but was shown to be monomeric, an unusual feature among cyclodextrin degrading enzymes. Two of the enzymes were lacking neopullulanase activity. One enzyme was lacking activity on cyclodextrins, and was the enzyme that also lacked the N-terminal domain. This moderately thermophilic enzyme was mainly showing neopullulanase activity, and had an amino acid sequence with the highest similarity to enzymes from *Thermus thermophilus* and *Synechocystis sp.* Despite the availability of similar gene sequences, there are to our knowledge no activity data available for comparison. Based on our results, the N-terminal domain appears to be necessary for activity on cyclodextrins, while oligomerization is not.

Invited Lecture - L10

What the annotators could not know; novel family GH13 enzymes in fungi and their unexpected functions in cell wall metabolism

Rachel M. VAN DER KAAIJ[1,2], Xiao-Lian YUAN[3], Arthur F.J. RAM[3], Štefan JANEČEK[4], Marc J.E.C. VAN DER MAAREL[1,2,5] & Lubbert DIJKHUIZEN[1,2]

[1] *Centre for Carbohydrate Bioprocessing, TNO-University of Groningen, Kerklaan 30, 9751 NN, Haren, The Netherlands*
[2] *Microbial Physiology Research Group, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Kerklaan 30, 9751 NN, Haren, The Netherlands*
[3] *Fungal Genetics Research Group, Institute of Biology, Leiden University, Wassenaarseweg 64, 2333 AL, Leiden, The Netherlands.*
[4] *Institute of Molecular Biology, Slovak Academy of Sciences, SK-84551 Bratislava, Slovakia*
[5] *Business Unit Food and Biotechnology Innovations, TNO Quality of Life, Rouaanstraat 27, 9723 CC, Groningen, The Netherlands*

Aspergillus niger is a non-pathogenic filamentous soil fungus used for various biotechnological applications, such as the production of citric acid and enzymes for the food industry. We searched for putatively starch degrading enzymes in the genome of *A. niger* and studied their expression, aiming to unveil all components of its starch degrading network. The regulation of most of the genes encoding family GH13, 15 and 31 enzymes, however, suggested they play a role in other physiological processes. Among them, we identified two novel groups of family GH13 enzymes, with homologues in other fungi, and studied their biochemical characteristics and physiological role.

Enzymes from the first group were annotated as extracellular α-amylases due to their high similarity with known fungal α-amylases. However, biochemical studies on two *A. niger* proteins showed that they have α-glucanotransferase activity on maltooligosaccharides and starch, and negligible hydrolyzing activity [1]. Analysis of an *A. niger* knockout for one of the proteins suggested it plays a role in the formation of cell wall α-glucan.

The second novel group contains intracellular enzymes belonging to subfamily GH13_5, as opposed to all fungal extracellular α-amylases of subfamily GH13_1. One previous study [2] suggested a role for these predicted intracellular enzymes in cell wall α-glucan formation. We expressed one of the *A. niger* proteins and showed that it is an α-amylase, albeit with very low specific activity.

Our studies show that annotation of novel family GH13 enzymes is not always straightforward, even after the useful subdivision of family GH13 in subfamilies [3]. In addition, association of these enzymes with metabolism of starch/glycogen should sometimes be abandoned to reach new insights into their role in (microbial) physiology.

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Invited Lecture - L11

How to synthesize the rare α-1,2 glucosidic linkage with an enzyme derived from a GH70 family glucansucrase?

Yoann BRISON, Emeline FABRE, Sophie BOZONNET, Pierre MONSAN & Magali REMAUD-SIMEON

Laboratoire d'Ingénierie des Systèmes Biologiques et des Procédés, UMR CNRS/INSA 5504, UMR INRA/INSA 792, INSA, 135 avenue de Rangueil, 31077 Toulouse cedex 4, France

Among the glucansucrases from GH70 family, DSR-E glucansucrase, an enzyme from *L. mesenteroides* NRRL B-1299 is the unique enzyme able to synthesize from sucrose a glucan polymer containing both α-1,6 and α-1,2 glucosidic linkages. In the presence of sucrose donor and maltose acceptor, DSR-E keeps its specificity and produces glucooligosaccharides that also contain α-1,2 linkages. The presence of the rare α-1,2 linkage confers to these GOS prebiotic properties and a potential to prevent Type II diabete in mice [1].

With a molecular weight of 313 kDa, DSR-E is the biggest glucansucrase found in family 70. It possesses a very original structure due to the presence of two catalytic domains, CD1 and CD2 separated by a glucan binding domain. Both catalytic domains belong to GH70 family [2]. Constructions of truncated forms of DSR-E revealed that CD1 is responsible for α-1,6 linkage synthesis and CD2 is a specific domain, responsible for the α-1,2 linkage synthesis [3].

In order to better understand and control the α-1,2 linkage mode of synthesis, the truncated variant GBD-CD2 was characterized in detail. Notably, it is a pure α-1,2 branching enzyme that catalyses α-1,2 transglucosylation from sucrose donor to dextran and α-1,6 GOS. Rational engineering enabled us to localize regions involved in the enzyme specificity and the kinetic analysis revealed that the transglucosylation reaction follows a ping-pong bi-bi model (k_{cat} = 460 s^{-1}) and competes with a weak sucrose hydrolase activity (k_{cat} = 46 s^{-1}). By adjusting the reaction conditions, a nice panel of α-1,2 branched dextrans harbouring different and controlled degrees of branching can be synthesized.

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Sucrose phosphorylases catalyze novel transglycosylation reaction on carboxylic compounds

Kazuhisa SUGIMOTO[1], Koji NOMURA[1], Hiromi NISHIURA[1], Kohji OHDAN[1], Takahisa NISHIMURA[1], Hideo HAYASHI[2] & Takashi KURIKI[1]

[1] *Biochemical Research Laboratory, Ezaki Glico Co., Ltd., Osaka, Japan*
[2] *Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Japan*

Glycosylation is an important method for the structural modification of bioactive compounds. It can be used to improve physicochemical and biological properties of many compounds. For example, glycosylation of hesperidin greatly improved its solubility in water, and glycosylation of arbutin significantly improved its inhibitory effect on human tyrosinase [1]. There are many reports on enzymatic glycosylation of aglycones having glycosyl residues, alcoholic OH group, and phenolic OH group [2]. However, there had been no report on glycosylation of carboxylic groups in various aglycones using transglycosylating reaction of carbohydrate active enzymes. Here, we present the first report for glycosylation of carboxylic compounds by sucrose phosphorylase, an α-amylase family enzyme [3]. Detailed mechanism and the structure of the products using benzoic acid as a model of carboxylic compounds is also described [4].

Two sucrose phosphorylases were employed for glycosylation of carboxylic group. *Streptococcus mutans* sucrose phosphorylase showed remarkable transglycosylating activity, especially under acidic conditions. *Leuconostoc mesenteroides* sucrose phosphorylase also showed transglycosylating activity, however, the efficiency of the reaction was much lower than that of *S. mutans* sucrose phosphorylase. Three main products were detected from the reaction mixture using benzoic acid and sucrose as an acceptor and a donor molecule, respectively. These compounds were identified as 1-*O*-benzoyl α-D-glucopyranoside, 2-*O*-benzoyl α-D-glucopyranose, and 2-*O*-benzoyl β-D-glucopyranose based on the analyses of the products as well as the acetylated products using 1D- and 2D-NMR following the isolation of each product. Time-course analyses proved that 1-*O*-benzoyl α-D-glucopyranoside was initially produced by the transglycosylation reaction of the enzyme. 2-*O*-Benzoyl α-D-glucopyranose and 2-*O*-benzoyl β-D-glucopyranose were produced from 1-*O*-benzoyl α-D-glucopyranoside by intramolecular acyl migration reaction. *S. mutans* sucrose phosphorylase showed broad acceptor-specificity. This sucrose phosphorylase catalyzed transglycosylation to various carboxylic compounds such as short-chain fatty acids, hydroxy acids, and dicarboxylic acids.

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Calcium binding to barley α-amylase isozymes and its implications on stability and activity

Maher ABOU HACHEM[1], Wei-Feng XUE[2], Adiphol DILOKPIMOL, Bent W. SIGURSKJOLD[3] & Birte SVENSSON[1]

[1] Enzymes and Protein Chemistry, BioCentrum, Technical University of Denmark, Søltofts Plads building 224, DK-2800 Kgs. Lyngby, Denmark
[2] Astbury Centre for Structural Molecular Biology, Institute of Molecular and Cellular Biology, University of Leeds, Woodhouse Lane 7823, 7486, Leeds, U.K.
[3] Department of Biochemistry, August Krogh Institute, University of Copenhagen, Universitetsparken 13, DK-2100 Copenhagen, Denmark

α-Amylases, which catalyse hydrolysis of α-1,4-linked glucosidic linkages in starch and glycogen, play a significant role in utilising this abundant source of energy and carbon. Structural studies have demonstrated that calcium binding is almost conserved in the α-amylase family (glycoside hydrolase family 13) with at least one conserved Ca^{2+} binding site that bridges the A and B domains of these enzymes together. In germinating barley seeds, two α-amylase isozymes referred to as AMY1 and AMY2 are expressed for starch degradation. The structures of these two homologous isozymes (80% sequence identity), including their three Ca^{2+} binding sites, are virtually identical with only minor local differences [1]. Two calcium ions, co-ligated via an acidic side chain, are virtually solvent inaccessible while the third is solvent exposed and bound in a long loop distant from the conserved Ca^{2+} binding site. Although Ca^{2+} is crucial for α-amylases, its precise role is still ill-understood. Hence, this study discerns the contribution of Ca^{2+} binding to the biochemical and biophysical properties of barley α-amylases. An isothermal titration calorimetry procedure was developed to determine the macroscopic Ca^{2+} binding constants in these α-amylases, and the major advantages of this method is that it circumvents the need to prepare calcium-free enzymes and it can handle enzymes with multiple binding sites. In addition to wild type enzymes, the study involves a set of AMY1 domain B mutants targeting either Ca^{2+} ligands or resulting in surface charge alterations close to the calcium binding sites. The results show that the different sites have different impact on the structural integrity and activity of the enzyme, with the double Ca^{2+} system being crucial for activity, while the isolated third Ca^{2+} having little or no effect on the activity of the enzyme. However, this latter ion had a dramatic effect on the thermostability of AMY1 as judged by the loss of 10 °C in unfolding temperature when an acidic Ca^{2+} ligand is exchanged with an alanine. Interestingly, we have demonstrated that mutants with charge alterations close to the double calcium binding sites seemed to modulate thermal stability. Hence a single mutation resulted in a 4 fold increased stability in the absence of any added Ca^{2+}. These results suggest that charge manipulation close to the Ca^{2+} binding sites in domain B offers a tool for engineering both the stability and calcium dependence of these enzymes and similar systems.

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Oral Talk - L14

Determinants for glucosidic bond specificity in glucansucrase enzymes of Glycoside Hydrolase family 70 (GH70)

Slavko KRALJ[1,2], Wieger EEUWEMA[1,2] & Lubbert DIJKHUIZEN[1,2]

[1] *Centre for Carbohydrate Bioprocessing, TNO-University of Groningen, PO Box 14, 9750 AA Haren, The Netherlands*
[2] *Microbiology Department, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands*

Glucansucrase enzymes of lactic acid bacteria convert sucrose into different α-glucans that strongly differ in glucosidic bonds present (e.g. dextran, mutan, alternan and reuteran) [1]. Our studies aim to identify structural features that determine glucosidic linkage specificity. Previously, a region in the catalytic domain of reuteransucrase (GTFA) from *Lactobacillus reuteri* 121 has been identified strongly determining product specificity. Conversion of GTFA from a mainly α-(1→4) (~45%, reuteran) to a mainly α-(1→6) (~80%, dextran) synthesizing enzyme was achieved [2].

Detailed mutational analysis of this conserved region, immediately next to the catalytic Asp1133 (putative transition state stabilizer) showed that residue Asn1134 is the main determinant for glucosidic bond product specificity in this reuteransucrase [3].

In GTF180 of *L. reuteri* 180, synthesizing a dextran (~50% α-(1→6) linkages), the similar region was targeted. Exchange of the 1137-SNA-1139 triplet with NNS (present in GTFA) resulted in introduction of ~5% α-(1→4) glucosidic linkages (linkage type not present in glucan made by wild type GTF180). Introduction of up to 15% α-(1→4) glucosidic linkages was achieved by additional introduction of mutation V1027P (residue present in GTFA). Clear understanding of the structural features in glucansucrase enzymes (3D strucuture elucidation of GTF180 underway in group of Prof. B.W. Dijkstra) that determine the nature and ratio of glucosidic linkages synthesized eventually may allow production of tailor-made poly- and oligosaccharide products suitable for diverse applications.

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Role of carbohydrate-hydrolyzing and - debranching enzymes in sugar utilization of *Bacillus subtilis*

Jae-Hoon SHIM[1], Jong-Tae PARK[1], Tae-Joo YANG[1], Myung-Ok KYUNG[1], Kyung-Mo SONG[1], Hyunju CHA[1], Jung-Wan KIM[2] Winfried BOSS[3] & Kwan-Hwa PARK[1]

[1] *Center for Agricultural Biomaterials and Department of Food Science & Biotechnology, Seoul National University, Korea*
[2] *Department of Biology, University of Incheon, Korea*
[3] *Department of Biology, University of Konstanz, D-78457 Konstanz, Germany*

A search for the sequences of bacterial genomes involved in sugar utilization indicated that bacterial enzymes for the degradation of maltodextrin and glycogen belonged to glucosidases/transglucosidases and glucosyltransferases, possessing either a complete or a minimal set of corresponding enzymes. To investigate the functions of the enzymes, hydrolyzing (YvdF) or debranching (AmyX) carbohydrates in sugar utilization of *Bacillus*, three *B. subtilis* 168 mutants defective in *yvdF*, *amyX*, or both were constructed and their physiological properties were examined *in vivo* and *in vitro*. Wild type *B. subtilis* could uptake maltoheptaose and β-cyclodextrin via two distinct transporters, MdxE and YvfK/CycB, respectively, and YvdF localized close to the cell membrane hydrolyzed them immediately to small linear maltodextrins. The role of YvdF and AmyX in glycogen utilization was also investigated using cell extracts of the mutants. Breakdown of glycogen increased in the order of wild type > the *yvdF* mutant > the *amyX* mutant > the *amyX/ yvdF* double mutant. The preference of debranching enzymes for side chain length of glycogen was found to play an important role in shaping glycogen during synthesis and degradation. The chain length specificity of debranching enzymes from various bacteria was tested by incubating the enzymes with branched β-cyclodextrin. AmyX showed specificity for the hydrolysis of side chains of 3-5 glucosyl residues. TreX (*Sulfolobus solfataricus*) showed high specificity toward a wide range of side chain lengths 3-7 and GlgX (*E. coli*) toward 3-4 glucosyl residues. Interestingly, pullulanase from *Nostoc* hydrolyzed side chains of long maltooligosaccharides with DP 9-10, exclusively. Based on the results, we propose a debranching mechanism of glycogen breakdown in bacteria.

Invited Lecture - L16

Modulation of the degradability of the starch granule

Andreas BLENNOW

Plant Biochemistry Laboratory, Center for Molecular Plant Physiology (PlaCe), Department of Plant Biology, The Royal Veterinary and Agricultural University, 40 Thorvaldsensvej, DK-1871 Frederiksberg C, Copenhagen, Denmark

Starch is by far our major dietary energy intake. This is problematic in the Western world where over-weight and life-style related diseases can be directly related to high carbohydrate intake. In the plant the starch granule is constructed in a manner to provide a regulated degradation by specific enzyme systems. Learning from how the plant self-regulates starch degradability starch can be modulated in the plant to reduce its dietary degradability leading to strategies to make starch a more resistant, slower and healthier carbohydrate. When used in the diet the resistant starch, RS, is fermented in the colon producing protective fermentation products.

In the plant, starch is deposited as self-organised, semi-crystalline granules. The lengths and clustered positions of the α-glucan side chains direct starch granule by assembly to form the organised starch granule particle. From recent data we highlight general principles by which regulated disintegration of the starch granule is achieved in the plant. Degradation is moderated by the chain length of the amylopectin. Cereal storage starches form smaller crystalline units forming a dense crystalline A-type polymorph being efficiently attacked by hydrolytic enzymes as demonstrated *in vitro*. On the contrary, the more hydrated but more stable B-type polymorph formed by longer chains in tuberous and photosynthetic starch types is more resistant towards enzymatic attack.

To permit degradation in the plant starch granules of the B-type polymorph are therefore phosphorylated [1]. For that purpose, starch phosphorylating enzymes (glucan water dikinase, GWD) parallels that of the evolvement of starch granule structures in Nature. GWD catalyzes phosphorylation of starch in a dikinase type reaction where the β-phosphate of ATP is transferred to either the C-6 or C-3 position of the glycosyl residue of amylopectin. The detailed reaction mechanism of GWD involves reduction and oxidation induced structural transitions providing an enzyme sensitive to redox state of the plant. Interestingly, redox state also directs enzyme affinity to the starch granule *in vivo* [2]. Engineering of GWD can improve enzyme activity as well as changing the substrate preference. Homologues of GWD contain specific carbohydrate binding modules of the family CBM 45 [3] (and http://www.cazy.org/). CBM45 is also present in plastidial α-amylases like the *Arabidopsis* AMY3 and seems to be unique for regulated plastidial starch metabolism.

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Mechanisms involved in the biosynthesis of starch

John F. ROBYT & Rupendra MUKERJEA

Laboratory of Carbohydrate Chemistry and Enzymology, Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, IA 50011 USA

In 1940, Hanes reported the enzymatic synthesis of amylose and maltodextrins by the reaction of potato phosphorylase with α-glucose-1-phosphate (α-Glc-1-P) and a maltodextrin primer. This reaction, however, rapidly slowed down as the concentration of inorganic phosphate (P_i) was formed. It was found that the synthetic reaction with the putative primers will only occur when the ratio of P_i to α-Glc-1-P is less than the equilibrium value of 3.1 at pH 7. The ratio *in vivo* is 20- to 40-fold higher and phosphorylase is exclusively acts as a degradative enzyme *in vivo*. In 1960, Leloir, et al. found that ADPGlc was the high energy donor for the biosynthesis of starch and that active starch synthesizing enzymes were entrapped in the starch granules. They, however, assumed that the synthesis was by the primer nonreducing-end mechanism. Because of a number of problems and unanswered questions that arose about this mechanism, we have investigated the mechanism of starch biosynthesis. Three sets of experiments have been performed. The first were a pulse and chase study with ADP-[^{14}C]Glc and nonlabeled ADPGlc, respectively, with 8 different kinds of starch granules. The pulsed granules gave starch that on reduction with $NaBH_4$, followed by hydrolysis, gave ^{14}C-glucitol and -glucose, and the chase reaction gave a significant decrease in ^{14}C-glucitol. These experiments indicated that the starch was being elongated from the reducing-end rather than from the nonreducing-end. The second involved the addition of the putative primers, maltose, maltotriose, and amylodextrin (d.p. 12) with 3 kinds of starch granules and ADPGlc. The putative primers inhibited starch biosynthesis in increasing amounts, as the concentrations were increased, contrary to what would be expected for primers. They did undergo reaction in which ^{14}C-Glc was added to their nonreducing-ends in exponentially decreasing amounts, with the next higher homologue the major product. The third was the reaction of pulsed and chased granules with the exo-acting enzymes, glucoamylase and beta-amylase. The two enzymes gave approximately equal amounts of ^{14}C-products, glucose and maltose, respectively. If the addition of Glc from ADPGlc had been to the nonreducing-ends of primers, the chased starch should not have given any or much less ^{14}C-products, because nonlabeled Glc would have been added to the nonreducing-ends. A two-site insertion mechanism is proposed in which the addition of Glc from ADPGlc is to the reducing-end of a growing starch chain. Glc and the growing starch chain are covalently attached to the two sites; the amylose chain is transferred to the C4-OH group of Glc by a transglycosylation reaction, forming an α-1→4 linkage and the elongation of the chain.

Crystal structure of the closed conformation of *Escherichia coli* glycogen synthase: critical amino acids involved in substrate binding and catalysis

Fang SHENG[1], Alejandra YEP[2], Miguel BALLICORA[2], Jack PREISS[2] & James H. GEIGER[1]

[1] *Department of Chemistry, Michigan State University, East Lansing, MI 48824 USA*
[2] *Department of Biochemistry & Molecular Biology, Michigan State University, East Lansing, MI 48824 USA*

At present 2 crystal structures of glycogen synthase have been reported. One is from *Agrobacterium tumefaciens* [1] and one from *Pyrococcus abyssi* [2]. However, both of these enzymes were crystallized in their catalytically inactive "open" conformations. We have crystallized and have determined the crystal structure of the substrate-bound closed form of the *E. coli* glycogen synthase. In addition we have determined the structure of the ADP-glucose-bound, enzymatically inactive E377A mutant glycogen synthase [3]. Glu residue 377 is important in binding of the glucose moiety of the ADP-Glc substrate. Asp 137, His 161, Arg300 and Lys 305 are suggested by the structure to be critical catalytic residues in the reaction. Indeed, mutagenesis of these amino acids to Ala causes a decrease of Vmax, respectively, of 8,140-, 710-, 2590-, and 1240-fold [4]. The change in Km for ADP-glucose is slight for the Lys305 and Asp 137 mutants and only about 10-fold for the Arg300 and His161 mutants. The structure and mutagenesis studies indicate that the active site of the bacterial glycogen synthase is similar to the active sites of retaining GT-B glycosyltransferases such as maltodextrin phosphorylase and trehalose-6-P synthase.

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Oral Talk -L19

Where do animal α-amylases come from? An interkingdom trip

Jean-Luc DA LAGE[1], Etienne G. J. DANCHIN[2] & Didier CASANE[1]

[1] Laboratoire Evolution, génomes et spéciation (LEGS), CNRS, 91198 Gif sur Yvette cedex, France
[2] AFMB-UMR 6098, CNRS, Universités Aix-Marseille I et II, Case 932, 163 Avenue de Luminy, 13288 Marseille cedex 09, France

α-Amylases are widely found in Eukaryotes and Prokaryotes. Few amino acids are conserved among these organisms, but at an intra-kingdom level, conserved protein domains exist. In animals, numerous conserved stretches are considered as typical of animal α-amylases. Searching databases, we found no animal-type α-amylases outside the Bilateria. Instead, we found in the sponge *Reniera sp*. and in the sea anemone *Nematostella vectensis*, α-amylases whose most similar cognate was that of the amoeba *Dictyostelium discoideum*. We found that this "Dictyo-type" α-amylase was shared not only by these non-Bilaterian animals, but also by other Amoebozoa, Choanoflagellates, and Fungi. This suggested that the Dictyo-type α-amylase was present in the last common ancestor of Unikonts. The additional presence of the Dictyo-type in some Ciliates and Excavates, suggests that horizontal gene transfers may have occurred among Eukaryotes. We have also detected putative interkingdom transfers of amylase genes, which obscured the historical reconstitution. A possibility is that the animal-type α-amylase originated in a small group of γ-Proteobacteria, and was then transferred to an ancestor of Bilateria.

Invited Lecture - L20

Amylase development for industrial applications

Carsten ANDERSEN, Anders VIKSO-NIELSEN, Sven PEDERSEN, Henrik BISGAARD-FRANTZEN, Peter SKAGERLIND, Lilian E. T. BALTSEN, Allan SVENDSEN & Torben V. BORCHERT

Novozymes A/S, Krogshojvej 36, DK-2880 Bagsvaerd, Denmark

The α-amylase (EC 3.2.1.1) is a key enzyme for the industrial production of high-fructose corn syrups and bioethanol, as well in detergent and textile industries to remove starch from textile and surfaces. But the conditions for these amylase applications are highly diverted and in order to optimize the processes, specific amylases have been developed for each application.

When bacterial α-amylases operating under industrial relevant conditions (>100 °C) were introduced in 1973, it was a revolution for the starch processing industry. The traditional acid hydrolysis at high temperature was environmentally unfriendly and led to significant levels of byproducts, while the enzymatic process is highly efficient. Two basic problems, however, still remained with the first generation amylases in this new process: pH of the starch slurry had to be adjusted from about 4.0 to 6.0 and calcium had to be added to stabilize the liquefying bacterial α-amylase. Further, when insight in the relationship between the structure of various bacillus α-amylases and the product specificity was obtained, the issue of panose formation in the process was resolved [1]. A more simple process with fewer unit operations conducted at a lower temperature would be the next breakthrough for the starch processing industry. By finding very efficient enzymes for raw starch hydrolysis below its gelatinization temperature, we have demonstrated how the current process can be turned into a "one-step" starch hydrolysis process [2].

As starch is also a significant part of our food, starch containing stains will be an inevitable target for disc and laundry cleaning. Unlike for the starch liquefaction, the optimal amylase for cleaning processes has to be active and stable at alkaline pH and further compatible with the chemicals in the detergent matrix. These demands have lead to development of new amylases for the detergent application, starting from wild type amylases isolated from alkaline bacillus species.

With the availability of 3-dimensional structures of a bacillus derived α-amylase from the mid-nineties [3,4], the above challenges could be addressed by combined screening for new wild type amylases and protein engineering, resulting in a number of new and improved commercial enzyme products [5]. This lecture will review the development during the last decade, with special emphasis on relationship between α-amylase structure and application properties of the enzymes.

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Oral Talk - L21

An evolved archaeal α-amylase with performance and mode of action tailored for the ethanol industry

Margaret SLUPSKA

Verenium Corporation, 4955 Directors Place, San Diego, CA 92121 USA

State of the art enzyme discovery and evolution technologies were used at Verenium Corporation (formerly Diversa) to develop a new high performance α-amylase enzyme with a unique mode of action on starch, and pH and temperature tolerance ideally suited to the dry mill ethanol process. High throughput screening of biodiverse environmental DNA libraries was employed to identify a novel set of genes encoding highly thermostable α-amylases. Three α-amylases originating in extremophile bacteria belonging to the order *Thermococcales (Euryarchaea)*, and contained in biomass samples collected from the floor of the Pacific Ocean, the Sea of Cortez and the Mediterranean Sea respectively were selected for evolution using Verenium's DirectEvolutionTM technologies, resulting in an optimized hybrid α-amylase stable at high temperatures (over 100 ºC) in the absence of added calcium, and optimally active at pH 4.8 [1]. The new hybrid enzyme displayed a more random multichain mode of action on starch, when compared with thermophilic α-amylases originating from Gram positive bacteria of the genus *Bacillus* [2]. In multiple trials at ethanol plants, this unique mode of action resulted in exceptionally rapid grain mash thinning during liquefaction, enabling higher solids throughput, and increased ethanol production.

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The structure of human 4F2hc ectodomain provides a model for homodimerization and electrostatic interaction with plasma membrane

Joana FORT[1,2], Laura R DE LA BALLINA[1], Hans E BURGHARDT[1], Carles FERRER-COSTA[3], Javier TURNAY[4], Cristina FERRER-ORTA[2], Isabel USÓN[5], Antonio ZORZANO[1], Juan Fernández RECIO[3,6], Modesto OROZCO[3], María Antonia LIZARBE[4], Ignacio FITA[2] & Manuel PALACÍN[1]

[1] Institute for Research in Biomedicine, Barcelona Science Park, Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Barcelona, and CIBERER, Barcelona, Spain
[2] Instituto de Biología Molecular de Barcelona (IBMB-CSIC), Institute for Research in Biomedicine, Barcelona Science Park, Spain
[3] Molecular Modeling and Bioinformatics, Institute for Research in Biomedicine, Barcelona, Spain
[4] Departamento de Bioquímica y Biología Molecular I, Facultad de Ciencias Químicas, Universidad Complutense, Madrid, Spain
[5] ICREA at Instituto de Biología Molecular de Barcelona (IBMB-CSIC), Spain
[6] Barcelona Supercomputing Center, Barcelona, Spain

4F2hc (CD98hc) is a multifunctional type II membrane glycoprotein involved in amino acid transport and cell fusion, adhesion and transformation. The structure of the ectodomain of human 4F2hc has been solved using monoclinic (2DH2) and orthorhombic (2DH3) crystal forms at 2.1 and 2.8 Å, respectively. It is composed of a $(\beta\alpha)_8$ barrel and an antiparallel β8 sandwich related to bacterial α-glycosidases, although lacking key catalytic residues and consequently a conventional catalytic activity. 2DH3 is a dimer with Zn^{2+} coordination at the interface. Human 4F2hc expressed in several cell types resulted in cell surface and Cys109 disulfide bridge-linked homodimers with major architecture features of the crystal dimer, as demonstrated by crosslinking experiments. The monomer and homodimer of the ectodomain of 4F2hc have a polarized charged surface. Our results provide the first crystal structure of Heteromeric Amino acid Transporters and suggest a dynamic interaction of 4F2hc ectodomain with plasma membrane. The role of 4F2hc homodimers in cell transformation will be discussed.

When structural biology helps to understand sugar-processing enzymes and to stay in touch with the sweet α-amylase family

Richard HASER

Laboratoire de BioCristallographie, Institut de Biologie et Chimie des Protéines, UMR 5086-CNRS/Université de Lyon I and IFR128 «BioSciences Gerland-Lyon Sud», 7 Passage du Vercors, F-69367 Lyon cedex 07, France

Recent results will be discussed on enzymes from various origins (plants, bacteria…) involved in sugar recognition and processing, on the basis of their high resolution structures in the presence and absence of ligands of interest (natural substrates, inhibitors, protein partners…) and of appropriate mutants. In this context we are studying, for example, the structure/function relationships of two major α-amylase isozymes produced in the aleurone layer of barley seeds. These enzymes in combination with limit dextrinase, β-amylases, are of pivotal importance for starch degradation and embryo growth during seed germination. Coupled enzymatic and structural analysis using site directed mutagenesis, gene shuffling, and X-ray crystallography have provided the essential data that enables to contribute to the fundamental understanding of the catalytic hydrolytic cleavage of α-1,4-linked carbohydrates, in starch and related oligosaccharides.

A number of bacterial amylases are also known in terms of detailed 3D architectures. Our contribution to the structure/function relationships of amylases from psychrophilic microorganisms led to clarify the features which control molecular adaptation, recognition of sugars and high catalytic efficiency at low temperatures.

Characterization of highly efficient sucrose isomerases have also been reported from isomaltulose-and trehalulose-producing bacteria. The first three-dimensional structures (native and complexes) of a sucrose isomerase producing predominantly trehalulose (a nutritional sugar with high health advantages for diabetics and nondiabetics) were recently established, and help to elucidate the mechanism of isomerase action.

Finally, a number of new and unexpected insights into the action of enzymes belonging to the α-amylase family at the molecular level have been added in the recent years. They contribute a lot to the understanding on how these enzymes tackle the processing of the different substrates they act with in nature. Ultimately, technological processes as well may benefit from the improved insight into the conversion of starch and related sugars, and for example into the industrial biosynthesis of sugars with significant health advantages.

Invited Lecture - L24

New insights into the structure and function of the AMPK glycogen-binding domain

David STAPLETON, Ann KOAY & Paul GOOLEY

Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Parkville, Victoria, Australia

The AMP-activated protein kinase (AMPK) is heterotrimer consisting of α catalytic subunit and β/γ regulatory subunits and acts as a critical focal point for whole body and cellular mechanisms maintaining energy homeostasis by regulating carbohydrate metabolism, food intake, gene transcription and protein synthesis. The AMPK β subunit contains a mid-molecule glycogen-binding domain (GBD) that is conserved across all eukaryotic species and although promotes the association between AMPK and glycogen particles in intact cells, its physiological function remains unclear. To further understand the function of the GBD we determined its structure in complex with β-cyclodextrin. The structure reveals a carbohydrate binding pocket with extensive contact between several residues and five glucose units - consistent with in vitro data showing that the five-sugar maltopentaose is the smallest oligosaccharide to prevent AMPK from binding to glycogen - held in a pincer-like grasp with two tryptophan residues cradling two β-cyclodextrin glucose units and a leucine residue piercing the β-cyclodextrin ring. The critical protein-carbohydrate residues have been confirmed by mutational analysis, resulting in the partial or complete inhibition of GBD/glycogen association. To further investigate carbohydrate recognition and binding in AMPK β1 GBD we have utilized NMR spectroscopy the results of which will be discussed together with new insights into the association of AMPK with purified glycogen particles.

Invited Lecture - L25

Structural insights into the recognition of α-glucans by carbohydrate-binding modules

D. Wade ABBOTT, Alicia LAMMERTS VAN BUEREN & Al BORASTON

Biochemistry and Microbiology, University of Victoria, Victoria BC, Canada V8W 3P6

Carbohydrate-binding modules (CBMs) are discreet modules with carbohydrate binding activity that are most often found within the context of multi-modular carbohydrate active enzymes. There are currently 49 families of CBMs, which are classified on the basis of amino acid sequence identity. Of these 49 families, seven are known to contain starch specific CBMs, often referred to as starch-binding modules. In the last two years the number of structures representing families previously uncharacterized at the structural level has tripled. This has allowed the analysis of the general molecular determinants of α-1,4-glucan recognition by carbohydrate-binding modules. In general, the binding sites contain three aromatic sidechains which impart a distinct concave contour. This elegantly accommodates the convex surface presented by the α-faces of glucose residues in the helical polysaccharide chain. The binding energy imparted by substantial van der Waals interactions between these complementary surfaces is supplemented by only a handful of hydrogen bonds between the protein and carbohydrate. Given the quite rigid and distinct structure of the helix adopted by α-1,4-linked glucose residues we suggest that this mode of α-glucan recognition is likely to be an evolutionary theme among non-catalytic α-1,4-glucan binding proteins. In addition to this structural information I will also introduce our current understanding of starch recognition and utilization within the invasive lung pathogen *Streptococcus pneumoniae*.

Correlation and characterization of functions and secondary/tertiary structures of carbohydrate binding modules

Wei-I CHOU[1], Tun-Wen PAI[2], Ping-Chiang LYU[3], Yuh-Ju SUN[3], Shi-Hwei LIU[1], Chia-Chin SHEU[1] & Margaret Dah-Tsyr CHANG[4]

[1] Simpson Biotech Co., Ltd, Taoyuan Country, Taiwan, People's Republic of China
[2] Department of Computer Science and Engineering & Center for Marine Bioscience and Biotechnology, National Taiwan Ocean University, Keelung, Taiwan, People's Republic of China
[3] Institute of Bioinformatics and Structure Biology & Department of Life Science, National Tsing Hua University, Hsinchu, Taiwan, People's Republic of China
[4] Institute of Molecular and Cellular Biology & Department of Life Science, National Tsing Hua University, Hsinchu, Taiwan, People's Republic of China

Members of a protein family often have highly conserved sequences; most of these sequences carry identical biological functions and possess similar three-dimensional (3-D) structures.

The glucoamylase produced by *Rhizopus oryzae* is a commonly used glycoside hydrolase in industry. It comprises a *C*-terminal catalytic domain and an *N*-terminal starch-binding domain which belongs to carbohydrate-binding module (CBM) family 21 rather than family 20. Extensive structural and biochemical investigations of family 20 CBMs have been reported; however, those of family 21 CBMs are quite limited. Although sequence identity between the starch-binding domain of CBM20 and that of CBM21 is as low as 13.5%, a molecular model of the family 21 CBM from *R. oryzae* glucoamylase (*Ro*GACBM21) was constructed according to progressive secondary structure correlation (PSSC) modified structure-based sequence alignment. We have first predicted the model of *Ro*GACBM21 [1] employing combination of novel bioinformatics methodology in the absence of resolved three-dimensional structural information. In addition, we have recently published the three dimensional structure of *Ro*GACBM21 [2] resolved by nuclear magnetic resonance spectroscopy (NMR), indicating that our novel bioinformatic tools are applicable and practicable in characterization of structure-function relationship of CBMs.

1. Chou W.I., Pai T.W., Liu S.H., Hsiung B.K. & Chang M.D. 2006. *Biochem. J.* **396:** 469-477.
2. Liu Y.N., Lai Y.T., Chou W.I., Chang M.D. & Lyu P.C. 2007. *Biochem. J.* **403:** 21-30.

Closing ALAMY_3 Lecture - L27

An enzyme family reunion – similarities, differencies and eccentricities in actions on α-glucans

Birte SVENSSON

Enzyme and Protein Chemistry, BioCentrum-DTU, Technical University of Denmark, Søltofts Plads, Building 224, DK-2800 Kgs. Lyngby, Denmark

α-Glucans in general, starch, glycogen and their derived oligosaccharides are processed by a host of more or less closely related enzymes that represent wide diversity in structure, mechanism, specificity and biological role. Structural sophistication continues to emerge hand-in-hand with eye-opening analysis on modes of action. Indeed the "test of time" blends with remaining questions and new relations. Information from inside and outside of ALAMY_3 will provide examples on what the family contains and some future directions. This year a quantum leap crowned the structural biology of these enzymes by the glucansucrase structure opening the unmasking of the mystery on the "robotics mechanism". This central issue is further reflected in the focus on carbohydrate binding domains and their long underrated potential. What share families 13 and 31 more than figures "one" and "three" and is the lid finally lifted off the disguise of the starch lyase - or ? Is family 57 holding back more secret specificities, will the different families be sporting new "eccentric" functions, are there new families out there and why are structures of "simple" enzymes still missing ? Indeed new understanding and discovery of biological roles continuous to add to the value of the collection of enzyme models, sequences and evolution, and design for useful and novel applications.

LIST OF POSTERS

P1 Jahan ALIKHAJEH, Khosro KHAJEH, Mehdi NADERI-MANESH, Bijan RANJBAR & Hossein NADERI-MANESH: Kinetic analysis, inhibition investigation and prediction of pKa values of *Bacillus* KR-8104 α-amylase: the determinants of pH-activity profile.

P2 Annabel BIJTTEBIER, Hans GOESAERT & Jan A. DELCOUR: Action pattern of different amylases on amylose and amylopectin.

P3 Yoann BRISON, Emeline FABRE, Sophie BOZONNET, Pierre MONSAN & Magali REMAUD-SIMEON: GBD-CD2: an α-1,2 transglucosidase originally from DSR-E glucansucrase.

P4 Camilla CHRISTIANSEN, Maher ABOU HACHEM, Martin J. BAUMANN, Esben FRIIS, Anders VIKSØ-NIELSEN, Andreas BLENNOW & Birte SVENSSON: Characterization of CBM20 starch binding domains from three different origins.

P5 Juanita Yazmin DAMIÁN-ALMAZO, Alina MORENO, Fernando GONZÁLEZ-MUÑOZ, Agustin LÓPEZ-MUNGUÍA, Xavier SOBERÓN & Gloria SAAB-RINCÓN: Protein engineering of a thermostable α-amylase to enhance alcoholysis reactions.

P6 Véronique DESSEAUX, Caroline SANCHEZ, Jean-MICHEL BRUNEL, Pierre STOCKER, Nathalie JUGE & El Hassan AJANDOUZ: Inhibition of porcine pancreatic α-amylase activity by derivatives of naturally occurring polyamine and dietary compounds.

P7 Stéphane EMOND, Isabelle ANDRÉ, Kais JAZIRI, Gabrielle POTOCKI-VÉRONÈSE, Philippe MONDON, Khalil BOUAYADI, Hakim KHARRAT, Pierre MONSAN & Magali REMAUD-SIMEON: Directed evolution to investigate and increase amylosucrase thermostability.

P8 Marek GABRIŠKO, Ľubica URBÁNIKOVÁ & Štefan JANEČEK: The relatedness between the GH13 oligo-1,6-glucosidase subfamily and heteromeric amino acid transporters.

P9 Andrej GODÁNY, Barbora VIDOVÁ & Štefan JANEČEK: The evidence that a catalytic triad is enough for a functional amylolytic enzyme from the α-amylase family.

P10 Daniel GUILLÉN, Monserrat SANTIAGO, Larissa LINARES, Ricardo PÉREZ, Beatriz RUIZ, Sergio SÁNCHEZ & Romina RODRÍGUEZ-SANOJA: Cooperative binding to raw-starch of the five modules starch-binding domain from *Lactobacillus amylovorus* α-amylase.

P11 Haider M. HAMZAH: Production, purification and immobilization of amylase enzyme produced from local isolate of *Bacillus subtilis*.

P12 Khomaini HASAN, Yandi ANDIYANA, Susanti KUSUMAWIDJAYA, Safri ISHMAYANA, Idar KARDI, Rinrin I. RACHMAWATY, Wangsa T. ISMAYA, Dessy NATALIA, Toto SUBROTO & Soetijoso SOEMITRO: Domain organization of a raw starch-digesting α-amylase from *Saccharomycopsis fibuligera* R-64.

P13 Noomen HMIDET, Jean-Guy BERRIN, Véronique DESSEAUX, Nathalie JUGE & Moncef NASRI: Functional characterisation and expression in *Escherichia coli* of a novel thermostable α-amylase from *Bacillus licheniformis* NH1.

P14 Jana HÝBLOVÁ, Jan HUBERT, Radek ŠINDELKA, Miloslav ŠANDA, Martin HORN, Iva KŘÍŽKOVÁ, Ivan KLUH, Lucie MAREŠOVÁ, Zdeněk VOBURKA, František KOCOUREK & Michael MAREŠ: *In vivo* and *in vitro* interactions of α-amylase inhibitor αAI-1.

P15 Štefan JANEČEK, Birte SVENSSON & E. Ann MACGREGOR: Remote homologies between the clan GH-H and family GH31.

P16 Lili KANDRA, Gyöngyi GYÉMÁNT, Narayanan RAMASUBBU, Gyula BATTA & Ágnes ZAJÁCZ: Structure-function correlation studies on human salivary amylase (HSA) using tannins as inhibitors.

P17 Slavko KRALJ, Wieger EEUWEMA & Lubbert DIJKHUIZEN: Tailoring glucosidic bond specificity in glucansucrase enzymes of family 70.

P18 Young-Su LEE, Sung-Hee KIM, Myoung-Hee LEE, Jae-Hoon SHIM, Hee-Seob LEE, Tae-Joo YANG, Myung-Ok KYUNG, Kyung-Mo SONG, Ju-Yuel BEAK, Kwan-Soo KIM & Kwan-Hwa PARK: Enzymatic synthesis of novel α-amylase inhibitors using the transglycosylation activities of glucosidase and maltogenic amylase.

P19 Shu-Chuan LIN, Wei-I CHOU, Chia-Chin SHEU & Margaret Dah-Tsyr CHANG: Stable linker peptides for recombinant fusion protein expression in *E. coli*.

P20 Shi-Hwei LIU, Wei-I CHOU, Shu-Chuan LIN, Chia-Chin SHEU & Margaret Dah-Tsyr CHANG: Economic process model for high performance purification of recombinant protein comprising starch binding domain.

P21 Martin MACHOVIČ & Štefan JANEČEK: Domain evolution in the GH13 pullulanase subfamily with focus on the carbohydrate-binding module family CBM48.

P22 Nasrin MOLLANIA, Khosro KHAJEH, Mehdi NADERI-MANESH & Marzieh GHOLLASI: Isolation, purification, biochemical characterization and cloning of a new extracellular α-amylase from *Geobacillus thermodenitrificans* LH8 which is insensitive to phytic acid.

P23 Claire MOULIS, Gabrielle POTOCKI-VERONESE, Pierre MONSAN & Magali REMAUD-SIMEON: How to control dextran polymerization?

P24 Romila MUKERJEA, Rupendra MUKERJEA & John F. ROBYT: An enzymatic mechanism for how starch granules grow *in vivo*.

P25 Dessy NATALIA, Cahyu PURNAWAN, Iman PRAWIRA, Sri PUDJIRAHARTI, Nur A. OCTAVIANI, Anastasia RIANY, Rukman HERTADI, Muhammad B. AMRAN, Zeily NURACHMAN & Sarwono HADI: Properties of thermostable recombinant α-amylase of *Bacillus licheniformis* SITH.

P26 Dessy NATALIA, Fernita PUSPASARI, Keni VIDILASERIS, Zeily NURACHMAN, Ocky K. RADJASA, Debbie S. RETNONINGRUM, Achmad S. NOER, Marc VAN DER MAAREL & Lubbert DIJKHUIZEN: Raw starch degrading α-amylases from Indonesian marine microorganisms.

P27 Dessy NATALIA, Anna SANAWATI, Fernita PUSPASARI, Khomaini HASAN, Toto SUBROTO, Zeily NURACHMAN, Bauke W. DIJKSTRA, Lubbert DIJKHUIZEN & Soetijoso SOEMITRO: Improving thermal stabilization of *Saccharomycopsis fibuligera* α-amylase by introduction of new disulphide bond between A and C domains.

P28 Morten M. NIELSEN, Eun-Seong SEO, Joakim ANDERSEN, Adiphol DILOKPIMOL, Sophie BOZONNET, Maher ABOU HACHEM, Henrik NÆSTED, Andreas BLENNOW & Birte SVENSSON: Mutational analysis of individual roles of carbohydrate binding surface sites in barley α-amylase 1.

P29 Marta PALOMO REIXACH, Slavko KRALJ, Marc J.E.C. VAN DER MAAREL & Lubbert DIJKHUIZEN: Structure-function relationship of bacterial glycogen branching enzymes.

P30 Wataru SABURI, Haruhide MORI, Masayuki OKUYAMA & Atsuo KIMURA: Conversion of *Streptococcus mutans* dextran glucosidase to transglycosylase through replacement of catalytic nucleophile by oxidized cysteine.

P31 Natsuko SATO, Hikaru TOBASE, Mamoru NISHIMOTO, Syun TAKEURA, Jintanart WONGCHAWALIT, Haruhide MORI, Masayuki OKUYAMA & Atsuo KIMURA: Characterization of an allosteric α-glucosidase from bumblebees (*Bombus terrestris*) and expression of its gene.

P32 Eun-Seong SEO, Maher ABOU HACHEM, Morten M. NIELSEN, Adiphol DILOKPIMOL, Kenji FUKUDA & Birte SVENSSON: Mutational analysis of sugar tongs in barley α-amylase 2.

P33 David STAPLETON, Paul O'DONNELL, Ryan Gilbert WILSON, Richard E.H. WETTENHALL & Glendon PARKER: The glycogen-associated proteome.

P34 Marc J.E.C. VAN DER MAAREL, Jan J. JORE, Alwin ALBERS & Peter J. PUNT: Overproduction of the raw starch binding domain of *Aspergillus niger* glucoamylase.

P35 Malene Bech VESTER-CHRISTENSEN, Henrik NÆSTED, Maher ABOU HACHEM & Birte SVENSSON: Production of recombinant barley limit dextrinase.

P36 Shekufeh ZAREIAN, Marzieh GHOLLASI, Khosro KHAJEH, Saman HOSSEINKHANI & Bijan RANJBAR: Isolation, purification and biochemical analysis of a new pullulanase from *Geobacillus stearothermophilus*.

POSTER ABSTRACTS

Poster - P1

Kinetic analysis, inhibition investigation and prediction of pKa values of *Bacillus KR-8104* α-amylase: the determinants of pH-activity profile

Jahan ALIKHAJEH, Khosro KHAJEH, Mehdi NADERI-MANESH, Bijan RANJBAR & Hossein NADERI-MANESH

Department of Biochemistry and Biophysics, Faculty of Science, Tarbiat Modares University, P.O. Box 14115-175, Tehran, Iran

A native α-amylase capable of activity at low pH was isolated from *Bacillus sp. KR-8104* (KRA) naturally occurred in rhizoplane zone of potato cultured soils. An investigation was set out to account some evidence for its specific properties. Kinetic parameters of KRA with respect to *Bacillus licheniformis* α-amylase (BLA) and *Bacillus amyloliquefaciens* α-amylase (BAA), showed a shift in acidic limb for KRA pH-profile. After isolation of KRA gene and determination of its nucleotide and protein sequences, the three dimensional structure of KRA was simulated based on BLA as template. Using PROPKA program the pKa values of titrable groups in KRA model together with other few members of this family were predicted either from simulated model or available structure at protein data bank. A correlation between catalytic residue pKa values and the optimal pH-activity profile was seen. Both our experimental data and prediction studies indicated a shift in pKa value of catalytic residues to acidic pH. Moreover alignment studies raised amino acid substitutions in KRA compare to BLA which may affect the KRA putative active site leading to the formation of an extra hydrogen bond between Glu261 and Arg229 and in turn shifts the pH-activity profile to lower amount. Also, while *Bacillus subtilis* α-amylase and *Bacillus amyloliquefaciens* α-amylase did not show any inhibition with two different types of inhibitors (Acarbose and proteinaceous inhibitor type 3 from wheat kernel), KRA completely lost it's activity in <10 μg/ml concentration of inhibitor. Apparent inhibitory constants (K_I) for carbohydrate type and protein type inhibitor were 1.94 mmol and 2.08 μg/ml respectively as evaluated by Dixon plot and further confirmed by using spectroscopic method. It is interesting that all these amylase in present study did not show decrease in their activity in presence of a bifunctional α-amylase/subtilisin inhibitor from rice (RASY). This specific inhibition pattern implies critical differences at least in active site region of KRA relative to other commercial available α-amylases.

Action pattern of different amylases on amylose and amylopectin

Annabel BIJTTEBIER, Hans GOESAERT & Jan A. DELCOUR

Laboratory of Food Chemistry and Biochemistry, Katholieke Universiteit Leuven, Kasteelpark Arenberg 20, B-3001 Leuven, Belgium

Amylases degrade the linear substrate amylose through a multiple attack mechanism, in which an amylase cleaves several glycosidic bonds after a first random hydrolytic attack and before dissociating from the substrate. However, amylose typically represents only a relatively small fraction of starch. The main fraction consists of the branched polymer, amylopectin, which hence is the main substrate in starch hydrolysis. It is rather unclear to what degree the action pattern of an amylase on the side chains of amylopectin is restricted by the branched structure. The main objective of the study was therefore to investigate the amylase action pattern on amylopectin and to compare it with the multiple attack action on amylose.

In this work, the action pattern of five different amylases [porcine pancreatic α-amylase (PPA); fungal α-amylase from *Aspergillus oryzae* (TAKA); *Bacillus amyloliquifaciens* α-amylase (BAA); *B. stearothermophilus* α-amylase (BStA), *B. cereus* β-amylase (BCB)] was studied at three different temperatures (35 °C, 50 °C and 70 °C) using potato amylose and waxy maize starch.

For amylose, the action pattern and the level of multiple attack (LMA) was deduced from the relation between the drop in relative blue value (620 nm) and the increase in the total reducing value during amylolysis. The different α-amylases had a different level of multiple attack (LMA) on amylose with BStA and PPA having a high LMA, TAKA a low LMA and BAA having an intermediate LMA. For all α-amylases but BStA, LMA increased with temperature to a degree depending on the amylase.

For amylopectin, the action pattern was analysed based on the relation between the drop in relative blue value (525 nm) and the increase in the total reducing value during amylolysis on the one hand and the analysis of the structure of the residual waxy maize starch on the other hand. Like for amylose, the amylases display a different LMA on amylopectin, with BStA having the highest LMA, followed by PPA and with BAA and TAKA having the lowest LMA. Furthermore, the LMA was reflected in the molecular weight and side chain distribution of the amylopectin residue. Our results indicate that branch points and amylase structural properties impact LMA on amylopectin. Consequently, amylases can not be considered as hydrolyzing starch in a truly random manner. Finally, no significant differences in LMA at various temperatures were found, except for BStA.

Poster - P3

GBD-CD2: an α-1,2 transglucosidase originally from DSR-E glucansucrase

Yoann BRISON, Emeline FABRE, Sophie BOZONNET, Pierre MONSAN & Magali REMAUD-SIMEON

Laboratoire d'Ingénierie des Systèmes Biologiques et des Procédés, UMR CNRS/INSA 5504, UMR INRA/INSA 792, INSA, 135 avenue de Rangueil, 31077 Toulouse cedex 4, France

DSR-E glucansucrase from *L. mesenteroides* NRRL B-1299 is a unique enzyme able to synthesize from sucrose a glucan polymer containing both α-1,6 and α-1,2 glucosidic linkages. In the presence of sucrose donor and maltose acceptor, DSR-E keeps its specificity and produces glucooligosaccharides that also contain α-1,2 linkages. The presence of the rare α-1,2 linkage confers to these GOS prebiotic properties and a potential to prevent Type II diabete in mice [1].

The DSR-E enzyme, the biggest glucansucrase (313 kDa) found in family 70 possesses a very original structure due to the presence of two catalytic domains, CD1 and CD2 separated by a glucan binding domain. Both catalytic domains belong to GH70 family [2]. Constructions of truncated forms of DSR-E revealed that CD1 is responsible for α-1,6 linkage synthesis and CD2 is specific of the α-1,2 linkage synthesis [3].

In order to better understand and control the α-1,2 linkage mode of synthesis, the truncated variant GBD-CD2 was characterized in detail. Notably, it is a pure α-1,2 branching enzyme that catalyses α-1,2 transglucosylation from sucrose donor to dextran and α-1,6 GOS acceptors. The optimal parameters for GBD-CD2 activity were 40 °C and 5.5 for temperature and pH respectively. Rational engineering enabled us to localize regions involved in the enzyme specificity and the kinetic analysis revealed that the transglucosylation reaction follows a ping-pong bi-bi model (k_{cat} = 460 s^{-1}) and competes with a weak sucrose hydrolase activity (k_{cat} = 46 s^{-1}). By adjusting the reaction conditions, a nice panel of α-1,2 branched dextrans harbouring different and controlled degrees of branching can be synthesized.

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Poster - P4

Characterization of CBM20 starch binding domains from three different origins

Camilla CHRISTIANSEN[1], Maher ABOU HACHEM[1], Martin J. BAUMANN[1], Esben FRIIS[2], Anders VIKSØ-NIELSEN[2], Andreas BLENNOW[3] & Birte SVENSSON[1]

[1] *Enzyme and Protein Chemistry, BioCentrum, Technical University of Denmark, Kgs Lyngby, Denmark*
[2] *Novozymes A/S, Bagsvaerd, Denmark*
[3] *Plant Biology Laboratory, Faculty of Life science, University of Copenhagen, Frederiksberg, Denmark*

Starch binding domains (SBDs) have been found in carbohydrate binding module (CBM) families 20, 21, 25, 26, 34, 41, 45 and 48 (http://www.cazy.org/). SBDs from CBM family 20 display high phylogentic diversity, being encountered in archae, bacteria, fungi, and plants. Members of this family are typically found in amylolytic and related enzymes and contain around 100 amino acid residues folded as a well conserved anti parallel β-barrel [1]. A major function of the SBD is to bind onto granular starch thereby increasing the local substrate concentration, however, the precise impact of SBD on starch degradation remains to be elucidated. Despite the high number of sequences and the diversity in family CBM20, it is not entirely clear if functional or specificity differences exist within the family. Hence, this study aims at casting light on this question by a comparison of representatives of CBM20 from various sources. To date, no CBM20 of plant origin has been characterised, but recently, we identified a putative CBM20 in *Arabidopsis thaliana* glucan water dikinase 3 (GWD3), an enzyme responsible for starch phosphorylation [2], and its starch binding function has been demonstrated. The GWD3-SBD will be compared with another CBM20 member, the SBD from the human glycogen phosphatase laforin [3]. The SBD from glucoamylase of *Aspergillus niger* is included as a reference. The three SBDs were produced as GST-fusion proteins in *Escherichia coli* and the interaction with soluble carbohydrates will be probed by surface plasmon resonance to determine K_d values and describe the structural requirements of the ligands for the molecular recognition. Binding to insoluble supramolecular substrates will be evaluated using an array of starches including phosphorylated starches. Pivotal residues in the binding activity of the SBDs will be explored by site directed mutagenesis. Finally, the binding onto starch granules of different botanical sources and genotypes will be examined using confocal laser scanning microscopy.

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Protein engineering of a thermostable α-amylase to enhance alcoholysis reactions

Juanita Yazmin DAMIÁN-ALMAZO, Alina MORENO, Fernando GONZÁLEZ-MUÑOZ, Agustin LÓPEZ-MUNGUÍA, Xavier SOBERÓN & Gloria SAAB-RINCÓN

Departamento de Ingeniería Celular y Biocatálisis. Instituto de Biotecnología, UNAM, Apartado Postal 510-3 Cuernavaca, Morelos 62260, Mexico

The production of alkyl-glucosydes by enzymatic means has been a major goal for several research groups in the last decades, using a wide variety of substrates such as cellulose, xilobiose, cellobiose, sacarose and nucleotide activated sugars, among others. The focus of our work is to produce alkyl-glucosydes from an abundant and cheap substrate such as starch and a well known enzyme in terms of structure-function relationship as α-amylase. In particular our efforts have been addressed to engineer thermostable amylases in order to produce alkyl-glycosides (from starch) through a single enzymatic step. In order to optimize the alcoholysis reaction we selected a thermostable, saccharifying amylase from *T. maritima* and designed several mutants aiming at a net reduction in the hydrolytic reaction (transfer of the glycosyl moiety to water) in favor of the transferase reaction (transfer to an acceptor, an alcohol in this case), by specific modifications introduced in the acceptor binding site. In particular we obtained a double mutant with an eight-fold increase in the alcoholysis/hydrolysis ratio when compared to the wild-type enzyme, yielding 16 mg/mL of methyl-glucoside. This is the highest yield reported in the literature for such reactions.

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Poster - P6

Inhibition of porcine pancreatic α-amylase activity by derivatives of naturally occurring polyamine and dietary compounds

Véronique DESSEAUX[1], Caroline SANCHEZ[2], Jean-MICHEL BRUNEL[1], Pierre STOCKER[1], Nathalie JUGE[1,3] & El Hassan AJANDOUZ[1]

[1] *Biosciences FRE-CNRS 3005 Faculté des Sciences de St Jérôme – Université Paul Cézanne Aix Marseille III -Service 342 Av. Escadrille N. Niemen 13397 Marseille Cedex 20, France*
[2] *Laboratoire d'Hématologie-CHU Timone, Inserm U626, 13385 Marseille Cedex 5, France*
[3] *Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, U.K.*

Amylase inhibition has gastrointestinal and metabolic effects, such as the induction of carbohydrate tolerance, satiety, and weight loss and delayed gastric emptying, which may aid in the treatment of diabetes and obesity. The aim of this study was to evaluate *in vitro* (1) the effect of new compounds as inhibitors of pancreatic α-amylase (PPA) and (2) the ability of starch preparations to resist hydrolysis by PPA.

Inhibitors targeting pancreatic α-amylase and intestinal α-glucosidases delay glucose production following digestion and are currently used in the treatment of Type II diabetes. Examples of such inhibitors which are in clinical use are acarbose, miglitol and voglibose. The polyamine spermidine was previously found to bind to α-amylase from barley and its molecular interactions inside the active site were identified. In the present study, polyamine compounds and derivatives were tested as potential new inhibitors of α-amylase activity using PPA and amylose and maltopentaose as substrates. Although spermine, spermidine, cadaverine and putrescine were found to be poor natural inhibitors of the porcine enzyme with Ki in the millimolar range, the synthesised N-acetyl-naphta-spermine was a potent competitive inhibitor with Ki = 0.1 mM. Other polyamine derivatives containing polyphenol rings were also found to be inhibitors of PPA. As a consequence, we tested phenolic compounds containing gallic acid such as cathechine gallate, epicatechine gallate and epigallocatechin gallate. All of these compounds were found to have a marked inhibitory effect on PPA using the long and the short-chain substrates, amylose and maltopentaose.

On the other hand, several samples of modified starches derived from various technological treatments such as stabilized and reticulated corn starch (Waxy) and potato starch, widely consumed by humans, were found to highly resist to the hydrolysis by PPA.

In conclusion, we have shown that a reduction of PPA activity can be achieved using endogenous compounds (polyamines) or dietary compounds (polyphenols) and that a proportion of dietary starches is resistant to the hydrolysis by PPA. Taken together these findings may be useful for the prevention and management of conditions associated with the metabolic syndrome such as diabetes and obesity.

Poster - P7

Directed evolution to investigate and increase amylosucrase thermostability

Stéphane EMOND[1], Isabelle ANDRÉ[1], Kais JAZIRI[1], Gabrielle POTOCKI-VÉRONÈSE[1], Philippe MONDON[2], Khalil BOUAYADI[2], Hakim KHARRAT[2], Pierre MONSAN[1] & Magali REMAUD-SIMEON[1]

[1] *UMR5504, UMR792, Ingénierie des Systèmes Biologiques et des Procédés, CNRS, INRA, INSA. 135, avenue de Rangueil, F-31400 Toulouse, France*
[2] *MilleGen, Immeuble BIOSTEP - Bâtiment A. Rue Pierre et Marie Curie – BP38183, 31681 Labège cedex, France*

Amylosucrase from *Neisseria polysaccharea* (AS, EC 2.4.1.4) is a glucansucrase from Glycoside Hydrolase (GH) family 13 that catalyses the *de novo* synthesis of a water-insoluble amylose-like polymer from sucrose [1]. Since this substrate is a readily available and cheap agroresource, this enzyme is an attractive biocatalyst for the industrial synthesis of modified polysaccharides and amylose-like polymers displaying a wide range of specific properties [2]. However, the development of an industrial process involving AS is limited by its low catalytic efficiency on sucrose alone (k_{cat} = 1 s^{-1}) and its low thermostability ($t_{1/2}$(50 °C) ~ 3 min).

The present study describes the identification of thermostable derivatives of AS using a combinatorial engineering approach based on the construction of random mutant libraries, subsequently selected and screened for the desired trait. Screening of a fraction of active clones selected out of these libraries led to the identification of three AS variants (two double mutants and one single mutant) displaying 10 to 30-fold increased half-lives at 50 °C compared to the parent wild-type enzyme. Sequence and structural analysis revealed that these variants contained mutations scattered all over the protein structure. Comparative analysis of *in silico* mutants with the wild type AS offered the possibility to highlight structural features that could contribute to the enzyme thermostability enhancement. One of the AS mutant identified during the screening is the most thermostable amylosucrase characterized to date ($t_{1/2}$(50 °C) ~ 32 min). Moreover, this new variant was able to catalyze at 50 °C the high yield synthesis of amylose chains possessing higher length compared to those produced by wild-type AS at 30 °C.

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Poster - P8

The relatedness between the GH13 oligo-1,6-glucosidase subfamily and heteromeric amino acid transporters

Marek GABRIŠKO, Ľubica URBÁNIKOVÁ & Štefan JANEČEK

Institute of Molecular Biology, Slovak Academy of Sciences, Bratislava, Slovakia

Heteromeric amino acid transporters (HATs) are composed proteins consisting of a light subunit and heavy subunit (rBAT or 4F2hc), connected by a disulfide bridge [1]. The heavy subunit is a type II membrane *N*-glycoprotein with single transmembrane domain, an intracellular NH_2 terminus and a large extracellular COOH terminus. Previous studies have shown similarity between the extracellular C-terminal domain of both rBAT and 4F2hc and the enzymes from the α-amylase family [2,3]. From the sequence-structure point of view, the basic difference between rBAT and 4F2hc is that rBAT possesses the segment that corresponds with domain B of GH13 enzymes protruding out of the catalytic TIM-barrel in the place of loop 3 (between the strand 3 and helix 3), whereas the 4F2hc does not have it [4]. In order to investigate further this resemblance we have undertaken a bioinformatics study focused on sequence alignment of rBAT and 4F2hc proteins from various organisms and eight enzymes specificities from the α-amylase family that constitute the so-called oligo-1,6-glucosidase subfamily [5] that has been recognized to be the α-amylase-family enzyme group most closely related to HATs [3]. The recently solved three-dimensional structure of the extracellular domain of 4F2hc [1] enables one to compare in a detail the situation within the active site of oligo-1,6-glucosidase [6], a representative of the α-amylase family, and the corresponding part of the structure in 4F2hc. With regard to catalytic triad of the oligo-1,6-glucosidase (Asp199, Glu255 and Asp329), only the catalytic nucleophile, Asp199, positioned at the strand β4 of the TIM-barrel, could have its counterpart in the 4F2hc, Asp248, indicating thus the 4F2hc probably lost the glucosidase activity. As far as the rBAT is concerned, it is possible to suggest the correspondences for all the three oligo-1,6-glucosidase catalytic residues in its sequence. This closer similarity has also been reflected in the evolutionary tree (calculated including the gaps from the alignment), where the enzymes from the α-amylase family cluster together with rBATs. Overall the taxonomy is respected in both rBAT and 4F2hc parts of tree; the insect representatives seem to represent an intermediary group between them.

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Poster - P9

The evidence that a catalytic triad is enough for a functional amylolytic enzyme from the α-amylase family

Andrej GODÁNY, Barbora VIDOVÁ & Štefan JANEČEK

Institute of Molecular Biology, Slovak Academy of Sciences, Bratislava, Slovakia

The α-amylase family [1] consists of three glycoside hydrolase (GH) families, GH13, GH70 and GH77, forming thus the clan GH-H [2]. One of the essential characteristics of the α-amylase family has been the presence of the absolutely conserved catalytic triad, i.e. the aspartic acid, glutamic acid and aspartic acid residues positioned near the C-termini of the catalytic TIM-barrel strands β4, β5 and β7, respectively [3,4]. In addition to these three catalytic residues that play the role of catalytic nucleophile (β4-Asp206; numbering from the Taka-amylase A), proton donor (β5-Glu230) and transition-state stabiliser (β7-Asp297), the arginine (β4-Arg204) positioned two residues before the catalytic nucleophile was considered to be the fourth indispensable residue conserved invariantly within the entire α-amylase family [5]. This functionally important arginine is involved, for example, in the Cl-binding site of chloride-dependent α-amylases [6]. The eventual loss of the invariance of the above-mentioned arginine was first demonstrated by Machovic & Janecek [7] who found a lysine in the corresponding position in the putative GH77 amylomaltase present in the genome of the Lyme disease spirochaete *Borrelia burgdorferi* [8]. The family GH77 contains prokaryotic amylomaltases and plant disproportionating enzymes (both possessing the 4-α-glucanotransferase activity; EC 2.4.1.25). Bioinformatics analysis revealed that the putative GH77 amylomaltase (malQ) from *B. burgdorferi* (BB0166) contains several amino acid substitutions in the positions that are important and conserved in all GH77 amylomaltases. In order to confirm the exclusive sequence features and to verify the eventual enzymatic activity, the *malQ* gene from *B. burgdorferi* was amplified using PCR. A ~1.5 kb amplified DNA fragment was sequenced, cloned and expressed in *Escherichia coli*, and the resulting recombinant protein was preliminary characterised for its activity towards a series of maltooligosaccharides (G1-G7). This study confirmed that the remarkable substitution of the arginine really exists and the GH77 malQ protein from *B. burgdorferi* is a functional amylolytic enzyme although its exact enzyme specificity has still to be determined. It is nevertheless reasonable to conclude that the catalytic triad alone is enough for a member of the α-amylase family to be an active enzyme.

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Poster - P10

Cooperative binding to raw-starch of the five modules starch-binding domain from *Lactobacillus amylovorus* alpha-amylase

Daniel GUILLÉN, Monserrat SANTIAGO, Larissa LINARES, Ricardo PÉREZ, Beatriz RUIZ, Sergio SÁNCHEZ & Romina RODRÍGUEZ-SANOJA

Departamento de Biología Molecular y Biotecnología. Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México A. P. 70228., Ciudad Universitaria, Coyoacán 04510. México, D. F.

The *Lactobacillus amylovorus* α-amylase has an unusual starch binding domain (SBD) composed of five tandem modules [1]. To investigate the importance of the modules in the adsorption phenomena, the peptides corresponding to 1, 2, 3, 4RU's and the entire SBD were expressed and purified. Adsorption assays with the recombinant proteins were conducted on corn raw-starch and the correspondent adsorption isotherms were analyzed with the Langmuir equation. We notice that the Kad of the complete SBD is higher than the Kad of the α-amylase (catalytic domain plus SBD), this result suggests not all the modules are participating at the same time in the interaction with the insoluble substrate, perhaps as a consequence of a steric hindrance produce by the catalytic domain. On the other hand, the adsorption capacity (measured as the adsorption constant "Kad") increases as a function of the number of modules presents in the peptide, this correlation reveals a cooperative effect in the binding to raw-starch of the modules that constitute the SBD. The cooperative-binding effect has been described in other glycoside hydrolases like cellulases [2], xylanases [3,4] and in one amylase [5] but to our knowledge this is the first description of a five tandem module cooperative binding in glycoside hydrolases [6].

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Poster - P11

Production, purification and immobilization of amylase enzyme produced from local isolate of *Bacillus subtilis*

Haider M. HAMZAH

Department of Biology, College of Science, University of Sulaimani, Iraq

Sixteen isolates of amylase producing *Bacillus* spp. were isolated from different sources (soil, water, fruits, and vegetables) in Sulaimani city, and they were identified in all samples. Using starch-agar medium, the isolate No. 9 was of highest productivity of amylase enzyme. This isolate was diagnosed as *B. subtilis*.

The liquid state fermentation method for this isolate gave a 12.4 U/ml enzyme activity and 11.34 U/mg protein specific activity, whereas the solid state fermentation (wheat bran) gave 18.8 U/ml enzyme activity and 14.22 U/mg protein specific activity.

Amylase enzyme produced from *B. subtilis* was purified by two main steps included precipitation with ammonium sulfate 40-60% saturation and gel filtration chromatography on Sephadex column (G-100). The obtained purification fold and recovery were 67.92 and 43.9% respectively.

The enzyme was immobilized by entrapment in calcium alginate beads. The enzyme retained 53% of its original activity after a month of storage at 4 ^{o}C. On the other hand, free enzyme lost its activity completely in less than 20 days.

The results of this study indicate the possibility of using wheat bran in solid state fermentation to produce industrial levels of amylase. Results also indicate the efficiency of immobilization technique by calcium alginate beads and preserving of the enzyme activity for a relatively long period of time.

Poster - P12

Domain organization of a raw starch-digesting α-amylase from *Saccharomycopsis fibuligera* R-64

Khomaini HASAN[1], Yandi ANDIYANA[1], Susanti KUSUMAWIDJAYA[1], Safri ISHMAYANA[1], Idar KARDI[1], Rinrin I. RACHMAWATY[1], Wangsa T. ISMAYA[1], Dessy NATALIA[2], Toto SUBROTO[1] & Soetijoso SOEMITRO[1]

[1] Biochemistry Laboratory, Department of Chemistry, Faculty of Mathematics and Natural Sciences, Padjadjaran University, Jalan Singaperbangsa No. 2, 40133, Bandung, Indonesia
[2] Biochemistry Laboratory, Department of Chemistry, Bandung Institute of Technology, Jalan Ganesa No. 10, 40132, Bandung, Indonesia

An α-amylase from *Saccharomycopsis fibuligera* R-64 was successfully isolated and separated from the glucoamylase by means of hydrophobic interaction chromatography. The purified enzyme has demonstrated attractive properties potential for industrial applications. We have been performing structural studies, in the absence of the enzyme structure, which is aimed to engineer the enzyme to meet the requirement for such applications. Proteolytic digestion of the enzyme has produced two major fragments which most likely representing the canonical catalytic and C domains. PEGylation of the enzyme has resulted not only in the increase of enzyme stability upon incubation 50 ^{o}C, also the enzyme resistance against proteolytic digestion. When the two fragments were separated, the presumably catalytic domain has shown similar activity to the undigested enzyme. While the K_{cat} value was similar, the K_M of this presumably catalytic domain was 2.5 times higher which indicated that the affinity of this fragment toward substrate is lower. In addition, the thermostability studies showed that the half-life time of this fragment was also lower than the undigested enzyme. Therefore, it is very tempting to propose that the domain C plays a role in the substrate binding affinity and/or supporting the integrity of the enzyme. Similar to the undigested enzyme, the presumably catalytic domain was able to digest various raw starches (maize, tapioca, sago, and potato). However, the fragments have interestingly not demonstrated adsorption onto raw starch. These results suggested that *S. fibuligera* R-64 α-amylase belongs to the raw starch-digesting, but not adsorbing, α-amylase and the domain organization of the enzyme resembles that of α-amylase family.

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Poster - P13

Functional characterisation and expression in *Escherichia coli* of a novel thermostable α-amylase from *Bacillus licheniformis* NH1

Noomen HMIDET[1], Jean-Guy BERRIN[2], Véronique DESSEAUX[2], Nathalie JUGE[2,3] & Moncef NASRI[1]

[1] *Laboratoire de Génie Enzymatique et de Microbiologie, Ecole Nationale d'Ingénieurs de Sfax,B.P. "W" 3038 Sfax, Tunisia*
[2] *Biosciences FRE-CNRS 3005 Fac. des Sciences de St Jérôme - Univ. Paul Cézanne Aix Marseille III -Service 342 Av. Escadrille N. Niemen 13397 MARSEILLE Cedex 20, France*
[3] *Institute of Food Research, Norwich Research Park, Colney, Norwich, Norfolk NR4 7UA, U.K.*

Thermostable α-amylase, catalyzing the hydrolysis of starch to dextrin, maltose and glucose at high temperature, is one of the most industrial important enzymes. Several species of *Bacillus* have been found and genetic improved to produce thermostable α-amylases. In present study, a novel α-amylase (AmyN) was purified to homogeneity from *Bacillus licheniformis* NH1 crude supernatant by ammonium sulphate precipitation, gel filtration and anion exchange chromatography. The enzyme has a relative molecular mass of ~58 kDa, as estimated by SDS-PAGE. With regards to amylolytic activity, AmyN degrades soluble starch and produces maltopentaose as end product, as also confirmed using G7 and G6 maltooligosaccharides. AmyN remained highly active over a wide range of pH, retaining more than 90% activity from 5.0 to 10.0, with the optimal reaction temperature and pH at 90 ^{o}C and 6.5, respectively. The thermostability of AmyN at 90 ^{o}C was enhanced in the presence of Ca^{2+}. Inhibition kinetic parameters using acarbose as inhibitor and amylose as substrate were in agreement with an incompetitive type.

The gene encoding *amy*N was isolated by PCR and heterologously expressed in *E. coli* BL21 cells using the pDEST17 expression system. The recombinant $(His)_6$ tag-enzyme was purified in one affinity chromatography step and displayed biochemical properties and kinetic parameters similar to the native enzyme. The deduced amino acid sequence showed 92% identity with the *B. licheniformis* NCIB 8061 α-amylase (BLA) of known three-dimensional structure (PDB 1BLI), differing by 21 amino acids in the mature sequence. Protein modelling and structural predictions at the substitution sites suggest critical candidates for the stabilisation properties of *B. licheniformis* α-amylases.

Poster - P14

In vivo and *in vitro* interactions of α-amylase inhibitor αAI-1

Jana HÝBLOVÁ[1,2], Jan HUBERT[3], Radek ŠINDELKA[4], Miloslav ŠANDA[1], Martin HORN[1], Iva KŘÍŽKOVÁ[3], Ivan KLUH[1], Lucie MAREŠOVÁ[1], Zdeněk VOBURKA[1], František KOCOUREK[3] & Michael MAREŠ[1]

[1] *Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, v.v.i., Flemingovo nam. 2, Prague 6, CZ 166 10, Czech Republic*
[2] *Czech University of Agriculture Prague, Kamycka 129, Prague 6, CZ 16521, Czech Republic*
[3] *Research Institute of Crop Production, Drnovska 506, Prague 6, CZ 16106, Czech Republic*
[4] *Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, v.v.i., Videnska 1083, Prague 4, CZ 14220, Czech Republic*

α-Amylase inhibitor αAI-1 from seeds of common bean *Phaseolus vulgaris* belongs to the lectin family of defense proteins. The inhibitory specificity of αAI-1 was screened in vitro with a panel of digestive α-amylases extracted from 30 species. This analysis showed a selective inhibition of α-amylases from three orders of insect (Coleoptera, Hymenoptera and Diptera) and from an annelid worm. To understand how αAI-1 discriminates among the tested enzymes, the α-amylases sensitive and insensitive to αAI-1, respectively, were compared by molecular modeling. Based on the screening, the model coleopteran species *Tribolium castaneum*, expressing the sensitive α-amylases, was selected for a detailed analysis of the in vitro and in vivo interaction with αAI-1. Two digestive α-amylase isoenzymes were isolated from this stored grain pest, identified by peptide fingerprinting, and their inhibition by αAI-1 was investigated. Insecticidal activity of αAI-1 was demonstrated by suppression of the development of the *T. castaneum* larvae in bioassay where αAI-1 was supplied in the diet. The physiological response to αAI-1 was monitored using qRT-PCR, which showed that the expression of digestive α-amylases is inducible. The observed dynamics of amylolytic system may help to compensate for the antinutritional effect of ingested α-amylase inhibitors.

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Poster - P15

Remote homologies between the clan GH-H and family GH31

Štefan JANEČEK[1], Birte SVENSSON[2] & E. Ann MACGREGOR[3]

[1] *Institute of Molecular Biology, Slovak Academy of Sciences, Bratislava, Slovakia*
[2] *Enzyme and Protein Chemistry, BioCentrum-DTU, Technical University of Denmark, Kgs. Lyngby, Denmark*
[3] *2 Nicklaus Green, Livingston, West Lothian, U.K.*

Although both the α-amylase family, i.e. the glycoside hydrolase (GH) clan GH-H (the GH families 13, 70 and 77), and family GH31 share some characteristics [1,2] such as (i) $(\beta/\alpha)_8$-barrel catalytic domain; (ii) retaining reaction mechanism; and (iii) β4-strand aspartic acid as catalytic nucleophile - their overall different catalytic machinery prevents to classify the family GH31 into the clan GH-H [3]. Based on the idea that residues equivalent in the primordial catalytic GH-H/GH31 $(\beta/\alpha)_8$-barrel may not be found in the present-day GH-H and GH31 structures at strictly equivalent positions, the so-called remote sequence homologies have been identified [4] in a sequence alignment of GH-H and GH31 representatives (31 and 7 enzyme specificities, respectively). The novelty of this approach is that the three-dimensional structure correspondences are not taken strictly into account since the priority is to maximise sequence similarity (identity). Although some functionally important and conserved residues in GH13 (or clan GH-H) may still have their counterparts in GH31 and *vice versa*, these residues do not necessarily obey the structure-based sequence comparison, i.e. they need not occupy the corresponding positions in the aligned remote homologies. Moreover, due to very large divergence, these analogous residues may adopt different functions in the present-day clan GH-H and GH31 enzymes. The remote sequence homologies are proposed to cover the strands β3, β4, β7 and β8 of the GH-H and GH31 $(\beta/\alpha)_8$-barrels. An evolutionary tree common for 31 GH-H and 7 GH31 enzyme specificities was constructed from their sequence alignment that was based on the structure comparison of GH13 α-amylase from *Aspergillus oryzae* and GH31 α-xylosidase from *Escherichia coli*. With regard to the relatedness of the $(\beta/\alpha)_8$-barrels of GH-H and GH31 enzymes, the N-terminal $(\beta/\alpha)_4$-half of the barrels exhibited a closer relationships than it was observed for the C-terminal half [5]. Of the three GH-H families, the family GH77 displayed the closest relationship to family GH31.

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Poster - P16

Structure-function correlation studies on human salivary amylase (HSA) using tannins as inhibitors

Lili KANDRA[1], Gyöngyi GYÉMÁNT[1], Narayanan RAMASUBBU[2], Gyula BATTA[1] & Ágnes ZAJÁCZ[1]

[1] Department of Biochemistry, Faculties of Sciences and Arts, University of Debrecen, PO Box 55. 4010 Debrecen, Hungary
[2] Department of Oral Biology, University of Medicine and Dentistry of New Jersey, Newark, NJ 07103 USA

Dental caries is a multifactorial disease in which diet, nutrition, microbial infection, and host response play important roles. It has been shown that human salivary amylase (HSA) takes part in the formation of dental plague and subsequent caries formation.

Therefore there is an obvious need for novel agents or therapeutic strategies that could reduce the cariogenic potential of starch-containing foods.

We first reported the effectiveness and specifity of tannins on HSA. Tannins were gallotannins isolated from the gallnuts of oaks. Structural analysis was carried out by MALDI-TOF MS, ESI MS and NMR studies. In acidum tannicum (*Quercus robur*) quinic acid, while in Aleppo tannin glucose was esterified by gallic acids (DP 2-6).

Kinetic studies revealed that tannins were very efficient inhibitors of HSA [1]. It was interesting, that the Aleppo tannin was a more efficient inhibitor than acidum tannicum (from *Quercus*) in spite of the very similar structure and composition. Structural explanation was found for this observation. Post Source Decay (PSD) measurements revealed that the pentagalloyl-quinic acid and pentagalloyl-glucose (PGG) gave different fragments indicating different arrangement of gallic acids around the polyol. The free OH groups of gallotannins are able to participate in hydrogen bonding with the amino acids of the active site cleft. Furthermore, the aromatic rings of gallic acids can provide stacking interactions with aromatic residues Trp59, Trp62 and Tyr151 located near subsites (-3)/(-2), (-1)/(+1) and (+2), respectively. Replacement of aromatic residues by side directed mutagenesis reduced significantly the inhibitor activity. Binding of PGG to HSA was also tested in Saturation Transfer Difference (STD) experiments. STD effect of aromatic residues confirms that PGG must bind to HSA most likely at the hydrophobic areas of the protein.

This work was supported by OTKA T047075 and OTKA T033499 grants.

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Poster - P17

Tailoring glucosidic bond specificity in glucansucrase enzymes of family 70

Slavko KRALJ[1,2], Wieger EEUWEMA[1,2] & Lubbert DIJKHUIZEN[1,2]

[1] *Centre for Carbohydrate Bioprocessing, TNO-University of Groningen, PO Box 14, 9750 AA Haren, The Netherlands*
[2] *Microbiology Department, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands*

Bacterial α-glucans are synthesized by glucansucrase enzymes, using sucrose as substrate [1]. The products of these enzymes find numerous applications in the food as well as in the non-food industries (e.g. bread, prebiotics). The food grade bacteria involved, particularly (probiotic) lactobacilli, have a clear potential as food additives and as functional food ingredients with both health and economic benefits.

In the past, four different glucansucrase genes, *gtf180*, *gtfML1, gtfA* and *gtfO* have been isolated from four different *Lactobacillus reuteri* strains, LB 180, LB ML1, LB 121 and LB ATCC 55730. These genes encoded a dextransucrase [dextran, α-(1→6) glucosidic linkages], a mutansucrase [mutan α-(1→3) glucosidic linkages], a reuteransucrase containing 50% α-(1→4) linkages and a reuteransucrase with 70% α-(1→4) linkages, respectively. These four glucansucrase enzymes from four different *Lb. reuteri* strains are highly similar at the amino acid level (65% identity and 70% similarity) but nevertheless synthesize very different α-glucan products [2,3].

Rational design, based on comparisons of the putative sugar-binding acceptor subsites in the catalytic core of different glucansucrases, allowed conversion of GTFA of *Lb. reuteri* 121 from a mainly α-(1→4) (~45%, reuteran) to a mainly α-(1→6) (~80%, dextran) synthesizing enzyme [4,5]. The reverse, introduction of up to 15% α-(1→4) glucosidic linkages, was achieved with rational mutation of *gtf180*. Construction of (active) hybrid enzymes between GTFA and GTFO gave insights that regions beyond the catalytic site are of importance in determining glucosidic bond specificity in glucansucrases of family 70.

Clear understanding of the structural features in glucansucrase enzymes that determine the nature and ratio of glucosidic linkages synthesized eventually may allow production of tailor-made poly- and oligosaccharide products suitable for diverse (food) applications.

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Poster - P18

Enzymatic synthesis of novel α-amylase inhibitors using the transglycosylation activities of glucosidase and maltogenic amylase

Young-Su LEE[1], Sung-Hee KIM[1], Myoung-Hee LEE[1], Jae-Hoon SHIM[1], Hee-Seob LEE[2], Tae-Joo YANG[1], Myung-Ok KYUNG[1], Kyung-Mo SONG[1], Ju-Yuel BEAK[3], Kwan-Soo KIM[3] & Kwan-Hwa PARK[1]

[1] Center for Agricultural Biomaterials and Department of Food Science & Biotechnology, Seoul National University, Korea
[2] Department Food Science and Nutrition, University of Pusan, Korea
[3] Department of Chemistry, Yonsei University, Korea

To develop novel inhibitors with hypoglycemic activity, a maltogenic amylase from *Therms sp.* (ThMA) and a glucosidase from *Thermotoga maritima* (TMG) were employed for the transglycosylation reaction using acarviosine-glucose as a donor and 1-propenyl-1-deoxy-α-D-glucopyranoside (for ThMA) or glucose (for TMG) as acceptors. The two major compounds produced by ThMA were determined as acarviosine-α-(1,6)-(1-propenyl-1-deoxy-α-D-glucopyranoside) and acarviosine-α-(1,4)-(1-propenyl-1-deoxy-α-D-glucopyranoside). The results indicated that acarviosine was transferred to C-6 or C-4 of 1-propenyl-1-deoxy-α-D-glucopyranoside forming an α-(1,6) or an α-(1,4)-glycosidic linkage. TMG also produced two major transfer products via transglucosylation of acarviosine-glucose to glucose. Analysis of their structures indicated that acarviosine was transferred to glucose at either C-6 forming an α-(1→6) glycosidic linkage or at C-3 forming an α-(1→3) glycosidic linkage. Inhibition kinetics of the transfer products were investigated using an α-amylase from porcine pancreas and an α-glucosidase from rat intestine. All the inhibitors were found to be mixed-type inhibitors for the α-amylase. Among them, acarviosine-α-(1,6)-(1-propenyl-1-deoxy-α-D-glucopyranoside) was the most potent inhibitor for the α-amylase, inhibiting the enzyme 6.7 times higher than acarbose. K_i of acarviosine-α-(1,6)-(1-propenyl-1-deoxy-α-D-glucopyranoside) against the α -glucosidase was 2.6 times higher than acarbose.

Poster - P19

Stable linker peptides for recombinant fusion protein expression in *E. coli*

Shu-Chuan LIN[1], Wei-I CHOU[2], Chia-Chin SHEU[2] & Margaret Dah-Tsyr CHANG[1]

[1] *Institute of Molecular and Cellular Biology & Department of Life Science, National Tsing Hua University, Hsinchu, Taiwan 300, People's Republic of China*
[2] *Simpson Biotech Co., Ltd., Taoyuan Country, Taiwan 333, People's Republic of China*

Rhizopus oryzae glucoamylase (*Ro*GA) is a multi-domain protein consists of an *N*- terminal raw starch-binding domain (*Ro*SBD), a glycosylated linker peptide, and a *C*-terminal catalytic domain. The 36 amino acids linker region (residues 132-167) has been characterized to be highly glycosylated to facilitate secretion of functional domains of recombinant GA in yeast [1]. Here the function of the linker region in protein expression in prokaryotic system such as *Escherichia coli* is further investigated. *Ro*SBD belongs to the carbohydrate-binding module (CBM) family 21 and possesses a high binding affinity towards raw starch such [2] that it is used to recovery and isolation of recombinant SBD-fused enhanced green fluorescent protein (eGFP) in this study. Several linker variants including the original linker region from *Ro*GA were engineered in order to investigate the effects of different linker peptides on the expression and purification of the SBD-eGFP fusion protein. The practical application of the linker peptides in terms of spatially separation of *Ro*SBD from the target proteins, efficiency of expression and purification, as well as maintenance of the stability of fusion protein will be reported.

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Poster - P20

Economic process model for high performance purification of recombinant protein comprising starch binding domain

Shi-Hwei LIU[1], Wei-I CHOU[1], Shu-Chuan LIN[2], Chia-Chin SHEU[1] & Margaret Dah-Tsyr CHANG[2]

[1] *Simpson Biotech Co., Ltd, Taoyuan Country, Taiwan 333, People's Republic of China*
[2] *Institute of Molecular and Cellular Biology & Department of Life Science, National Tsing Hua University, Hsinchu, Taiwan 300, People's Republic of China*

The starch-hydrolysing enzyme glucoamylase from *Rhizopus oryzae* (*Ro*) is a commonly used glycoside hydrolase in industry. It consists of a *C*-terminal catalytic domain and an *N*-terminal starch-binding domain (SBD), which belongs to the carbohydrate-binding module family 21 (CBM21). Comparison of the binding parameters of different SBDs reveals that *Ro*SBD possesses 40-fold higher binding capacity and 2-fold higher binding affinity than SBD derived from *Aspergillus niger* [1]. In general, production of proteins by expression in microbial systems has become a significant source of high value, medically important proteins. Purification and recovery of recombinant proteins are major considerations in the design of a fermentation process. A recombinant protein comprising the SBD can be produced and purified using starch matrix. Thus, we have developed a set of high efficiency recombinant protein expression [2] and purification system HERPEPS^{TM} to produce recombinant protein at industrial scale. HERPEPS^{TM} uses a very strong starch-binding affinity fusion protein (AFP) as the affinity tag to purify the recombinant protein from selected host fermentation broth. In our cases, purity and optimized recovery rate of AFP-tagged proteins AFP-eGFP, AFP-phytase and AFP-lipase are quite high up to 95% and 70%, respectively. Fast one-step recovery of recombinant protein without using chromatographic column can be achieved. Pure recombinant protein can be achieved up to 99% in laboratory scale using chromatographic column. The inexpensive starch as the adsorption substrate and the generally regarded as safe (GRAS) grade affinity tag make the purification process not only very efficient but also extremely economic and safe. HERPEPS^{TM} is not only good for production of recombinant proteins but also ideal for many industrial enzymes when the cost control is a major concern.

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Poster - P21

Domain evolution in the GH13 pullulanase subfamily with focus on the carbohydrate-binding module family CBM48

Martin MACHOVIČ[1] & Štefan JANEČEK[1,2]

[1] *Institute of Molecular Biology, Slovak Academy of Sciences, Bratislava, Slovakia*
[2] *Department of Biotechnologies, Faculty of Natural Sciences, University of SS. Cyril and Methodius, Trnava, Slovakia*

The α-amylase family, i.e. the glycoside hydrolase (GH) clan GH-H, consists within the CAZy classification of three GH families: GH13, GH70 and GH77 [1]. It contains the enzymes with more than 30 different specificities [2]. Although several subfamilies were identified previously [3,4], the entire main α-amylase GH family, GH13, has been divided into the subfamilies only recently [5]. This work deals with the so-called pullulanase subfamily [6], corresponding with seven CAZy GH13 subfamilies GH13_8 to GH13_14, that covers four specificities: branching enzyme (BE; EC 2.4.1.18), pullulanase (PUL; EC 3.2.1.41), isoamylase (ISOA; EC 3.2.1.68) and maltooligosyltrehalose hydrolase (MOTH; EC 3.2.1.141). The studied sample of selected proteins counts 84 enzymes: 40 BEs, 16 PULs, 14 ISOAs and 8 MOTHs plus 6 enzymes exhibiting amylopullulanase and/or α-amylase-pullulanase activities. All these enzymes possess in their structures a domain that has been recognised as a motif related to the starch-binding domains of carbohydrate-binding module (CBM) families CBM20 and CBM21 [7,8] and subsequently classified within CAZy as a family CBM48. Since three-dimensional structure is available for a representative of all specificities, it was possible to investigate the evolutionary relationships within the individual domains and to compare the structures of CBM48 with each other as well as with those of CBM20 and CBM21. The evolutionary trees were constructed for catalytic part (TIM-barrel + domain B), domain C, CBM48 module, conserved sequence regions and complete amino acid sequences. All the trees (except for the one reflecting the domain C) were found as similar containing two main groups of enzymes: (i) BEs and MOTHs; and (ii) PULs and ISOAs. The tree for domain C was changed substantially; only the BEs retained their single cluster not mixed with other specificities. Taxonomy was respected only within clusters with pure specificity, i.e. the evolution of CBM48 reflects the evolution of specificities rather than evolution of species. This is a feature different from what was observed for the starch-binding domain of the family CBM20 [9].

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Poster - P22

Isolation, purification, biochemical characterization and cloning of a new extracellular α-amylase from *Geobacillus thermodenitrificans* LH8 which is insensitive to phytic acid

Nasrin MOLLANIA, Khosro KHAJEH, Mehdi NADERI-MANESH & Marzieh GHOLLASI

Department of Biochemistry and Biophysics, Faculty of Sciences, Tarbiat Modares University, Tehran, Iran

α-Amylases (1,4-α-D-glucanohydrolase; EC 3.2.1.1) from different microorganisms have a wide range of properties and action patterns on starch substrate. Thermostable α-amylases are of great industrial importance in the production of corn syrup or dextrose. In this study a new α-amylase from a recently found strain LH8 was purified by ion-exchange and hydrophobic chromatographies. Sodium dodecyl sulphat polyacrilamid gel electrophoresis showed a band for the purified enzyme with an apparent mass of 64 kDa. The optimum temperature and pH range of the enzyme were 80˚C and 5, respectively. K_m and V_{max} values were 3 mg/ml and 6.5 µmol/min, respectively. The enzymes was activated by Mn^{2+}, Ca^{2+}, K^+, Cr^{3+} and Al^{3+}, whereas decreased by Mg^{2+}, Ba^{2+}, Ni^{2+}, Zn^{2+} and Fe^{3+} ions , EDTA and Cu^{2+}. The irreversible thermoinactivation data suggest that this enzyme can be considered as a thermophilic enzyme. Phytic acid in 2 mM and 5 mM concentration couldn't inhibit this enzyme. 16S rDNA sequencing studies carried out on the bacterium and phylogenic relationships determined. The results indicated 99% identity with *Geobacillus thermodenitrificans*. According to this identification the primer for gene of alpha-amylase was designed and the gene was isolated and cloned into *Escherichia coli* DH5α by inserting DNA fragments between the SacI and XhoI sites of PET 24d. Subsequently, we sent plasmid DNA fragment to *Escherichia coli* BL21 for expression. We confirmed our cloning with PCR and double digestion with restriction enzymes. Analysis and translation of the nucleotide sequence were performed with the tools available at the ExPASy Molecular Biology Server (www.expasy.ch). The structure of the *Geobacillus thermodenitrificans* LH8 α-amylase (GTA) was modeled with the protein homology modeling SWISS-MODEL server using the crystal structure of alpha-amylase (PDB code 1HVX) as template (swissmodel. expasy.org). When suitably aligned, the deduced amino-acid sequence of LH8 exhibits 83% identity to *Geobacillus stearothermophilus* α-amylase. The sequence comparison suggested that the amino acid sequence of LH8 included four conserved regions (I through IV) that were previously identified in α-amylase family: (I) 135DVVFNH140, (II) 264GFRLDAVKH272, (III) 294FTVGEYW300, (IV) 361VDNHD365. ASP 268, GLU 298 and ASP 365 are catalytic residues. Lysine 237 of the catalytic 4 loop from *Bacillus licheniformis* α-amylase (BLA) changes to ASP in GTA. It has been suggested that this change is important in the resistance of GTA to phytic acid. Moreover, in this report we present the kinetic parameters and inhibition constants of phytic acid on BLA and discuss about the mechanism of this inhibition.

How to control dextran polymerization?

Claire MOULIS, Gabrielle POTOCKI-VERONESE, Pierre MONSAN & Magali REMAUD-SIMEON

UMR5504, UMR792 Ingénierie des Systèmes Biologiques et des Procédés, CNRS, INRA, INSA, 135 avenue de Rangueil, F-31077 Toulouse cedex 4, France

Dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F (DSR-S, GH-70 family) has been used for more than 50 years to produce dextran, an homopoysaccharide of D-glucosyl units containing about 95% of α-1,6 bonds, from sucrose. The combination of biochemical and kinetic analyses clearly demonstrated that the polymerization mechanism did not involve two catalytic sites, as previously suggested [1], but only one. Two molecules can act as D-glucopyranosyl unit acceptor and initiate the polymerization process [2]: sucrose itself and D-glucose resulting from sucrose hydrolysis, the latter being preferred when produced in sufficient amount. Glucan chain elongation then occurs by transfer of D-glucosyl units at the non-reducing end of the acceptor chain.

The structure of the dextransucrase molecule consists in a variable zone located at the N-terminal end, followed by the catalytic domain, containing the two acid residues involved in the catalytic mechanism, and a glucan-binding domain (GBD) at the C-terminal end. This GBD contains repeated amino acid sequences, which were found to play a key role in dextran chain elongation. Based on these results, a semi-processive mechanism of polymerization was proposed, and preparation of several truncated forms demonstrated the possibility to control the size of the dextran chain from isomaltooligosaccharides to high molecular weight dextrans by controlling the size and structure of the GBD. Using sucrose as substrate, it was thus possible to directly synthesize dextrans of 10 and 40 kDa with a very high yield [3]. This one-step process constitutes a promissing alternative pathway to their production by acid hydrolysis of high molecular weight dextran followed by precipitation fractionation.

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Poster - P24

An enzymatic mechanism for how starch granules grow *in vivo*

Romila MUKERJEA, Rupendra MUKERJEA & John F. ROBYT

Laboratory of Carbohydrate Chemistry and Enzymology, Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, IA 50011 USA

Starch granules are known to contain active starch synthesizing enzyme entrapped within the granules. They are also known to be composed of alternating layers of crystalline and amorphous rings, known as growth rings. Layers of starch granules can be successively and linearly peeled from the surfaces of the granules by aqueous-dimethylsulfoxide [1]. The amount of starch synthesized in 7−8 successive peeled layers was determined by reacting the granules with ADP−[^{14}C]Glc and then measuring the amount of ^{14}C in each layer. Four types of starches had starch synthesis throughout the granules, showing that synthesis did not exclusively occur at the surface of the granules, as previously proposed [2]. The starch synthase activity was also assayed in each of the layers and it was found to alternate between high and low activities. From these experiments and our previous studies on the mechanism of starch chain elongation, we proposed that starch granules are initiated by the aggregation of starch synthase enzymes that synthesize starch chains by the two-site insertion mechanism and the chains are extruded outward from their active sites. The chains are hydrolyzed from the active sites and new chains synthesized. The synthesis stops when the supply of ADPGlc is diminished, resulting in a small granule. Eventually new starch synthesizing enzymes are adsorbed onto the small granules and synthesize new starch chains. As the starch chains are being extruded outward in three dimensions, and being branched by branching enzymes, a new layer of crystalline and amorphous starch is formed. Those parts of the starch chains where they are first synthesized are relatively close together and form intermolecular bonds to give the crystalline area and as the chains get longer, they are in larger volumes and are father apart, giving less interaction and the amorphous areas. These processes repeat themselves a number of times, giving successive growth rings, composed of alternating crystalline and amorphous areas. The alternating starch synthase activities that were observed in the peeling of the granules appear to be a reflection of the crystalline and amorphous layers.

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Poster - P25

Properties of thermostable recombinant α-amylase of *Bacillus licheniformis* SITH

Dessy NATALIA[1,2], Cahyu PURNAWAN[1], Iman PRAWIRA[1], Sri PUDJIRAHARTI[3], Nur A. OCTAVIANI[3], Anastasia RIANY[3], Rukman HERTADI[1], Muhammad B. AMRAN[1], Zeily NURACHMAN[1] & Sarwono HADI[1]

[1] *Biochemistry Division, Faculty of Mathematics and Natural Sciences, Bandung Institute of Technology, Jl. Ganesha 10, Bandung, Indonesia*
[2] *Centre for Life Sciences, Bandung Institute of Technology, Jl. Ganesha 10, Bandung, Indonesia*
[3] *Chemistry Research Centre, Indonesian Research Institute, Bandung, Indonesia*

Bacillus licheniformis α-amylase is widely used in starch processing because of its thermal stability. A gene encoding α-amylase of Indonesian *Bacillus licheniformis* SITH (*bla*) isolate has been amplified by Polymerase Chain Reaction (PCR) and expressed in *Escherichia coli* (*ec*-bla) [1]. The *ec*-bla purified by nickel affinity chromatography had a pH optimum of 5.5 and maximum activity at 60 ^{o}C in the absence of $CaCl_2$. The calculated half-lives of *ec*-bla in the presence of 10 mM at pH 5.5 were 100 minutes at 60 ^{o}C and 43 minutes at 80 ^{o}C, respectively. The *ec*-bla retains 50% activity in 1.5 M GuCl and has f_D value of 0.5 at 4.2 M GuCl sugesting the two transition mode on its denaturation. Identification of starch hydrolysis product of *ec*-bla by HPLC using Supercogel-Ca column revealed that the hydrolysis product was pentaoligosaccharide (G5). To meet the industrial interest, bla should be secreted into the culture medium. Therefore the *bla* gene was PCR-amplified and then sucloned into *Saccharomyces cerevisiae* expression vector as a fusion with oligonucleotide of invertase signal sequence. The deduced amino acid sequence of the *sc-bla* exhibits 2 amino acid substitutions, namely L134R and S320A compared to the published sequence (Acc. No. E01158). The *sc-bla* expression was regulated by a hybrid promoter of *GAL10* and *PGK1* promoter together with a *PGK1* terminator. The α-amylase activity was in the range of 3200-3700 U/mL without galactose and the activity increased up to 6500 U/mL upon the addition of 4.8% of galactose into the medium. HPLC analysis of starch hydrolysis product of *sc*-bla suggested that oligosaccharides (G5-G7) were predominant, and interestingly some amount of maltose was also observed.

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Poster - P26

Raw starch degrading α-amylases from Indonesian marine microorganisms

Dessy NATALIA[1,2], Fernita PUSPASARI[1,2], Keni VIDILASERIS[1], Zeily NURACHMAN[1], Ocky K. RADJASA[3], Debbie S. RETNONINGRUM[2,4], Achmad S. NOER[1], Marc van der MAAREL[5] & Lubbert DIJKHUIZEN[5]

[1] *Biochemistry Division, Faculty of Mathematics and Natural Sciences, Bandung Institute of Technology, Jl. Ganesha 10, Bandung, 40132, Indonesia*
[2] *Centre for Life Sciences, Bandung Institute of Technology, Jl. Ganesha 10, Bandung, 40132, Indonesia*
[3] *Centre for Tropical Coastal and Marine Studies, Diponegoro University, Semarang, Indonesia*
[4] *School of Pharmacy, Bandung Institute of Technology, Jl. Ganesha 10, Bandung, 40132, Indonesia*
[5] *Centre for Carbohydrate Bioprocessing, University of Groningen, Groningen, The Netherlands*

α-Amylases share a common three dimensional structure and mechanism, however their properties differ and in many cases are related to the producing organism milieu. One potential source for screening of novel α-amylase with and novel activity is marine microorganism. There are only a few reports on α-amylase from marine microorganisms. Indonesia as the largest archipelagic country in the world will provide enormous biodiversity of α-amylase producing microorganisms. Microbial isolates producing starch degrading α-amylase were screened from collection of Indonesian marine biosphere obtained by selective procedures and potential isolates were identified using 16S RNA gene sequence. One isolate from sea water of Cilaut Eureun, West Java, identified as *Bacillus subtilis* ALSHL3, secretes raw starch degrading α-amylase. α-Amylase produced by *B. subtilis* ALSHL3 shows high activity within the pH range of 5.0-8.0 and the temperature range of 55- 65 °C. Two major protein bands with molecular weight of 135 and150 kDa were detected on activity staining of culture supernatant. Further identification using Scanning Electron Microscope reveals the ability of the α-amylase(s) of *B. subtilis* ALSHL3 to degrade various starch granules. Since coral surface is more nutrient rich than seawater, α-amylase producing bacteria were also screened from this unique ecological environment. *B. aquimaris* isolated from soft coral *Sinularia* sp. from Merak Kecil Island, West Java, produces several amylolytic proteins with molecular weight varying from 60 kDa up to 160 kDa based on activity staining of culture supernatant. Characterization of the *B. aquimaris* α-amylase showed that the optimum activity was at pH 9 and 37 °C and the enzymes are capable of degrading various starch granules. Taken together our data has enriched the knowledge on α-amylase particularly from marine microorganism. We are currently employing genetic approach to isolate the gene encoding α-amylase from the later microorganism. By comparing with known α-amylases which had been found, it is expected that the α-amylase of *B. aquimaris* will have new domain and/or activity.

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Poster - P27

Improving thermal stabilization of *Saccharomycopsis fibuligera* α-amylase by introduction of new disulphide bond between A and C domains

Dessy NATALIA[1,2], Anna SANAWATI[1], Fernita PUSPASARI[1,2], Khomaini HASAN[3], Toto SUBROTO[3], Zeily NURACHMAN[1], Bauke W. DIJKSTRA[4], Lubbert DIJKHUIZEN[4] & Soetijoso SOEMITRO[3]

[1] *Biochemistry Division, Faculty of Mathematics and Natural Sciences, Bandung Institute of Technology, Jl. Ganesha 10, Bandung, Indonesia*
[2] *Centre for Life Sciences, Bandung Institute of Technology, Jl. Ganesha 10, Bandung, Indonesia*
[3] *Biochemistry Laboratory, Department of Chemistry, University of Padjajaran, Jl. Singaperbangsa No. 2, Bandung, Indonesia*
[4] *Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Groningen, The Netherlands*

The gene encoding *Saccharomycopsis fibuligera* R64 α-amylase (*ALP1*) had been cloned and highly expressed in *Pichia pastoris*. As an attempt to improve the *S. fibuligera* R64 α-amylase (ALP1) thermostability, a new disulphide bond predicted using The SS BOND programme is introduced between the A and C domains. The new disulphide bond is located in the position of S314C/S415C which is generated by employing site directed mutagenesis. Recombinant α-amylases (ALP1 and alp1) produced in *P. pastoris* show optimal pH and temperature of 5.5 and 55 ^{o}C, respectively. Interestingly, the alp1 mutant retains 96% of its activity at 60 ^{o}C while the ALP1 activity is only 78% of the optimal activity. Disulphide bond will stabilize the native conformation of the protein and based on the *in silico* study it is found that the total energy of the alp1 is -20270.9 kJ/mol while the ALP1 total energy is -20179.1 kJ/mol.

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Poster - P28

Mutational analysis of individual roles of carbohydrate binding surface sites in barley α-amylase 1

Morten M. NIELSEN[1], Eun-Seong SEO[1], Joakim ANDERSEN[1], Adiphol DILOKPIMOL[1], Sophie BOZONNET[1], Maher ABOU HACHEM[1], Henrik NÆSTED[1], Andreas BLENNOW[2] & Birte SVENSSON[1]

[1] Enzyme and Protein Chemistry, BioCentrum-DTU, Technical University of Denmark, Søltofts Plads, Building 224, DK-2800 Kgs. Lyngby, Denmark
[2] Plant Biochemistry Laboratory, Center of Molecular Plant Physiology (PlaCe), Faculty of Life Sciences, University of Copenhagen, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark

α-Amylases (EC. 3.2.1.1) catalyze the hydrolysis of internal 1,4-α-glucosidic linkages in starch and related oligo- and polysaccharides with retention of the anomeric configuration. Structural studies of both barley α-amylase 1 (AMY1) and the catalytically inactive D180A AMY1 with substrate and substrate analogues have shown that AMY1 comprises two secondary oligosaccharide binding sites (Starch binding site 1 and 2, SBS1 and SBS2) in addition to the substrate binding cleft [1,2]. First, SBS1 on the catalytic domain A contains two contiguous tryptophans (Trp278 and Trp279). SBS2 is located in the C-terminal domain, also called "a pair of sugar tongs", where an essential tyrosine residue (Tyr380) has a key role in binding oligosaccharides. Surface plasmon resonance showed a 7-fold increase in apparent K_d to β-cyclodextrin for the mutant Y380A and the pseudotetrasaccharide acarbose did not bind to SBS2 in the crystal structure of Y380A [2]. The joint role of SBS1 and SBS2 with the substrate binding cleft for the hydrolysis of starch in barley α-amylases is not known. The aim of this project is to study a series of relevant double mutants that combine mutations at subsite –6 (Tyr105), SBS1 (Trp278 and Trp279), and SBS2 (Tyr380). Surface plasmon resonance analysis of all dual site mutants including Y380A showed consistently increased K_d towards β-cyclodextrin. The dual SBS1 and SBS2 mutants lost the ability to adsorb onto barley starch granules. The dramatic decrease in affinity was accompanied by a similar decrease in the efficiency to hydrolyze starch granules. The affinity of AMY1 for starch granules was found to depend on the botanical source, genotype and ratio between amylose and amylopectin. AMY1 showed the highest affinity for the waxy-type and the lowest affinity for high amylose maize starch granules. The affinity to starch granules will be further analysed for surface site mutants.

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Poster - P29

Structure-function relationship of bacterial glycogen branching enzymes

Marta PALOMO REIXACH[1,2], Slavko KRALJ[1,2], Marc J.E.C. van der MAAREL[1,2,3] & Lubbert DIJKHUIZEN[1,2]

[1] *Centre for Carbohydrate Bioprocessing, TNO-University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands*
[2] *Microbial Physiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Haren, The Netherlands*
[3] *Business Unit Food and Biotechnology Innovations, TNO Quality of Life, Groningen, The Netherlands*

Glycogen is an energy reserve polymer of many animals and bacteria. It is composed of a backbone of glucose residues linked by α-1,4 glucosidic bonds with α-1,6 linked side chains. The linear α-1,4-glucan is synthesized from UDP-glucose by the enzyme glycogen synthase. The side chains are introduced by the glycogen branching enzyme (GBE) or 1,4-α-glucan:1,4-α-glucan 6-glycosyl transferase (E.C. 2.4.1.18). This enzyme catalyzes the formation of α-1,6 branch points by cleaving an α-1,4 glucosidic linkage in the donor substrate and transferring a terminal fragment of 6 to 7 glucose residues to the C6 hydroxyl position of an internal glucose residue that acts as the acceptor substrate. Even though a GBE from family GH 57 has been identified recently, most of the GBEs belong to family 13 of glycoside hydrolases, to which also enzymes such as the α-amylase belong. GBE contain three major domains, a central $(\beta/\alpha)_8$ barrel catalytic domain (shared with the rest of the family), an N-terminal domain and a C-terminal domain. The only 3D structure resolved until now is that of an N-terminally truncated GBE of *Escherichia coli*. However, there is little insight into the structure-function relationship of GBE.

We intend to study GBE in more detail to understand how these enzymes work. With this objective, the GBE of the extremophilic bacteria *Deinococcus radiodurans* and *Deinococcus geothermalis* were overproduced in *Escherichia coli*, purified to homogeneity and biochemically characterized. The branched amylose produced by these two enzymes differed significantly to those described before.

Poster - P30

Conversion of *Streptococcus mutans* dextran glucosidase to transglycosylase through replacement of catalytic nucleophile by oxidized cysteine

Wataru SABURI[1,2], Haruhide MORI[2], Masayuki OKUYAMA[2] & Atsuo KIMURA[2]

[1] *Nihon Shokuhin Koko Co., Ltd., Fuji 417-8530, Japan*
[2] *Research Faculty of Agriculture, Hokkaido University, Sapporo 060-8589, Japan*

Dextran glucosidase (DG, EC 3.2.1.70), belonging to glycoside hydrolase family 13 (GH 13), hydrolyzes an α-1,6-glucosidic linkage at the non-reducing end of dextran. The three catalytic acids of GH 13 enzymes are completely conserved in the region II, III, and IV of GH 13. The catalytic Asp in the region II, which corresponds to Asp194 of DG, acts as the catalytic nucleophile.

Glycosynthase, which is the catalytic nucleophile mutant of glycosidase with no hydrolyzing activity, efficiently transfers a glycosyl moiety of glycosyl fluoride having the opposite anomeric configuration of the original substrates. Glycosynthase technique is very useful for oligosaccharide synthesis, but the synthesized substrate (fluoride substrate) was required, and HF was produced as byproduct from glycosyl fluoride, giving the possible difficulty in utilization of glycosynthase-approach for the industrial production of oligosaccharides. In this study, we focused on introduction of unusual amino acid, oxidized cysteine, as the catalyst into the position of the catalytic nucleophile to provide a novel technique for efficient production of oligosaccharides.

The catalytic nucleophile mutant of DG (D194C) showed much lower activity toward *p*-nitrophenyl α-glucoside (*p*NPG) than the parent enzyme (8.1×10^{-4} %). However, the activity of this mutant enzyme was increased up to 0.27% of the parent enzyme by oxidation of thiol group with KI. The oxidized form of introduced Cys was determined by mass spectrometric analysis of lysl endopeptidase (Lys-C) digest of the oxidized mutant enzyme. The Lys-C-digested peptide bearing Cys194 was isolated by reverse phase HPLC, and its mass, measured by MALDI-TOF-MS, was higher than theoretical value by about 32, indicating that introduced cysteine was converted to cysteine sulfinic acid (SOOH). The enzymatic property of the oxidized D194C (D194COX) was examined and compared with that of parent DG. Almost no differences were displayed in pH profile, stabilities of pH and temperature, pI and substrate specificity. However, D194COX much more efficiently catalyzed transglucosylation toward not only *p*NPG having a good leaving group but also isomaltooligosaccharides. In the initial stage of the reaction toward *p*NPG and phenyl α-glucoside, D194COX catalyzed only transglucosylation unlike the parent enzyme. The replacement of the catalytic nucleophile by oxidized Cys was a novel technique to convert glycosidase to transglycosidase without using fluoride substrate. Therefore, we think that this technique can be applied in the industrial production of oligosaccharides.

Poster - P31

Characterization of an allosteric α-glucosidase from bumblebees (*Bombus terrestris*) and expression of its gene

Natsuko SATO, Hikaru TOBASE, Mamoru NISHIMOTO, Syun TAKEURA, Jintanart WONGCHAWALIT, Haruhide MORI, Masayuki OKUYAMA & Atsuo KIMURA

Division of Applied Bioscience, Research Faculty of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

α-Glucosidase (EC 3.2.1.20) catalyzes the splitting of α-glucosyl residue from non-reducing end of substrate having an α-glucosidic linkage to liberate α-glucose. There were three kinds of α-glucosidase isozymes (abbreviated as HBG-I, HBG-II, and HBG-III) in European honeybees (*Apis mellifera*) [1-3]. HBG-I is found in the ventriculus of honeybee to contribute to the digestion of sugar. HBG-II is in both of ventriculus and haemolymph to hydrolyze the sugar. HBG-III, a honey-producing enzyme, is in hypopharyngeal gland to be secreted into nectar corrected by bees and to degrade sucrose, a main component of the nectar, to glucose and fructose [4]. We have cloned the genes of HBG-I, HBG-II, and HBG-III, certifying that three isozymes were α-amylase family enzymes (GH-13 α-glucosidases). Gene expression was tried for those three isozymes using *Pichia pastoris*-system, which succeeded the production of recombinant HBG-II and HBG-III, but HBG-I gene was not expressed [5]. HBG-I was an allosteric enzyme to display a negative cooperativity for sucrose, maltose, and aryl α-glucosides [6]. This interesting allosteric enzyme was also found in Asian honeybees (*Apis cerana japonica*; enzyme being abbreviated as JBG-I) [7], implying that the HBG-I-type α-glucosidase was important in flower bees (genera of *Apis* and *Bombus*).

It was found that bumblebees (*Bombus terrestris*) had the same type of allosteric α-glucosidase (BIG-I), so that we purified BIG-I as an electrophoretically homogeneous protein and investigated its properties [molecular mass, 80 kDa; sugar contents, 7.6% as a mannose; pH-optimum, pH 5.5; pH-stability (treatment at 4 ^{o}C for 24 h), pH 5.0 to 10.0; temperature-stability (treatment for 15 min), less than 47 ^{o}C]. Substrate specificity of BIG-I was characterized by a negative cooperativity toward sucrose, maltose, and *p*-nitrophenyl α-glucoside with Hill coefficients of 0.69, 0.72, and 0.48, respectively. We cloned cDNA of BIG-I, a deduced amino-acid sequence of which showed identities of 59 and 60% to those of HBG-I and JBG-I, respectively. Recombinant BIG-I, expressed using a *Pichia pastoris*-system, exhibited the identical properties to original enzyme, including the negative cooperativity to above-described three substrates.

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Poster - P32

Mutational analysis of sugar tongs in barley α-amylase 2

Eun-Seong SEO, Maher ABOU HACHEM, Morten M. NIELSEN, Adiphol DILOKPIMOL, Kenji FUKUDA & Birte SVENSSON

Enzyme and Protein Chemistry Group, BioCentrum-DTU, Technical University of Denmark, Søltofts Plads, Bldn. 224, DK-2800 Kgs. Lyngby, Denmark

α-Amylases (EC. 3.2.1.1) catalyze the hydrolysis of internal 1,4-α-glucosidic linkages in starch and related oligo- and polysaccharides with retention of the anomeric configuration. Structural studies of both barley α-amylase 1 (AMY1) and the catalytically inactive D180A AMY1 with substrate and substrate analogues have shown that AMY1 comprises two surface carbohydrate binding sites in addition to the substrate binding cleft [1,2]. In the recently discovered "sugar tongs" site on domain C of AMY1, Tyr^{380} captures oligosaccharide ligands [2]. The "sugar tongs" are reported to ensure binding onto starch and assist in disentangling the α-glucan chains facilitating the enzymatic attack, to guide the polysaccharide chains to the active site, and to be candidates for allosteric regulation of enzyme function through carbohydrate interactions. However, the "sugar tongs" site is empty in the crystal structure of the AMY2/acarbose complex [3], resulting in the proposal that $Pro^{376}{}_{AMY2}$ corresponding to $Ser^{278}{}_{AMY1}$, hinders a conformational shift of $Tyr^{378}{}_{AMY2}$ by rigidifying the preceding loop thus preventing oligosaccharide binding [2]. M6 (A42P) was made to solve a poor secretory expression problem of wild-type rAMY2. The mutant M6 was virtually identical with AMY2 wild-type in stability and enzymatic properties and was secreted from *Pichia pastoris* in 20-fold higher amounts than rAMY2 [4].

Tyr^{380} of the "sugar tongs" surface binding site was investigated in single Y380A, Y378M, and Y378F mutants. In addition P376S was examined as an AMY1 mimic. Y378F and Y378M showed similar and reduced activity compared to M6. P376S showed about 40% reduced activity on insoluble blue starch compared to M6. K_d of β-cyclodextrin binding was determined by surface plasmon resonance to 1.0 mM for P376S compared to values of 0.2 mM for AMY1, 1.1 mM for AMY2 and 1.8 mM for M6.

In conclusion, the "sugar tongs" site in barley α-amylase 2 also has a role in the mechanism of degradation of polysaccharide substrates. Characterizations of other mutants are currently in progress.

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Poster - P33

The glycogen-associated proteome

David STAPLETON[1], Paul O'DONNELL[1], Ryan Gilbert WILSON[1], Richard E.H. WETTENHALL[1] & Glendon PARKER[2]

[1] Bio21 Molecular Science and Biotechnology Institute, University of Melbourne, 30 Flemington Road, Parkville, Victoria, Australia
[2] Department of Medicine, University of Utah, School of Medicine, Salt Lake City, UT 84132 USA

Glycogen, a branched polymer of glucose, is a cellular energy store that is crucial for whole body energy metabolism, metabolic regulation and exercise performance. Maintenance and utilization of glycogen occurs through the concerted action of a defined group of enzymes. In addition, the glycogen particle is intimately connected with other metabolic pathways and targeted to specific loci within the cell. We hypothesize that the tight regulation and targeting of the glycogen particle requires the binding of other regulatory proteins to the particle, if only at a much lower stoichiometry compared to the glycogen metabolic enzymes. Using a series of centrifugation and sucrose steps together with gel filtration we isolated purified glycogen particles from rat liver tissue. Glycogen particles were freeze-dried and digested with trypsin and the resulting peptides identified by mass spectrometry. We identified glycogen metabolic enzymes together with many proteins known to be associated with subcellular organelles and genethonin-1, a protein of unknown function that contains a predicted transmembrane domain and carbohydrate-binding domain (CBM20).

Poster - P34

Overproduction of the raw starch binding domain of *Aspergillus niger* glucoamylase

Marc J.E.C. van der MAAREL[1], Jan J. JORE[2], Alwin ALBERS[2] & Peter J. PUNT[2]

[1] *Department of Innovative Ingredients and Products, Business Unit Food and Biotechnology Innovations, Rouaanstrat 27, 9723 CC, Groningen, The Netherlands*
[2] *Department of Microbiology, Business Unit Food and Biotechnology Innovations, P.O. Box 360, 3700 AJ, Zeist, The Netherlands*

Raw starch binding domains are small, non-catalytic polypeptide molecules present in raw starch degrading enzymes of bacteria and fungi. They belong to the superfamily of carbohydrate binding modules (CBM) which are also present in polysaccharide degrading enzymes such as cellulases and chitinases [1]. CBM bind tightly to the insoluble polysaccharide and can disrupt the organized structure of the polysaccharide in such a way that the catalytic domain can hydrolyse the glycosidic linkages and thus degrade the substrate. A well known example of a raw starch binding domain is that of *Aspergillus niger* glucoamylase. This domain is 107 amino acids in size and is attached to the catalytic domain by a highly O-glycosylated linker region.

To study the binding kinetics of the glucoamylase raw starch binding domain, Paldi et al. [2] successfully expressed the domain in *E. coli*. In this communication we report on the overproduction of the *A. niger* glucoamylase raw starch binding domain in a strain of *A. niger* lacking endogenous extracellular amylases. A proteolytic cleavage site was introduced in the linker region to allow the cleavage of the domain from the glucoamylase as soon as the glucoamylase was excreted into the medium. Subsequently, the raw starch binding domain was partially purified from the growth medium by addition of waxy maize starch granules and subsequently released from the starch by increasing the temperature mildly. This resulted in a sample with almost pure raw starch binding domain as evaluated by SDS-PAGE and Western blotting using antibodies raised against this domain. These results demonstrate that the production of carbohydrate binding modules is certainly feasible using this fungal expression system. Because fungi are excellent hosts for the production of extracellular proteins, it will certainly be possible to produce raw starch binding and other types carbohydrate binding modules in sufficiently large amounts to evaluate their use and subsequent commercial production to functionalize polysaccharides.

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Poster - P35

Production of recombinant barley limit dextrinase

Malene Bech VESTER-CHRISTENSEN, Henrik NAESTED, Maher ABOU HACHEM & Birte SVENSSON

Enzyme and Protein Chemistry, Technical University of Denmark, Søltofts Plads, Building 224, DK-2800, Kgs. Lyngby, Denmark

Limit dextrinase (LD) belongs to the glycoside hydrolase family 13 (GH13). It catalyses hydrolysis of α-1,6-glucoside linkages in limit dextrins, pullulan and amylopectin. Activities of limit dextrinase, α-glucosidase and α- and β-amylase are important for starch degradation and production of glucose during barley seed germination. The concerted action of these enzymes furnishes the oxidative metabolism of the growing embryo during early development thereby making the endosperm-stored energy accessible for plant growth [1]. This study presents the first reported recombinant production of active barley limit dextrinase. The open reading frame of the gene encoding limit dextrinase was initially amplified using RT-PCR [2]. The gene fragment encoding the LD lacking its signal peptide was sub-cloned into the expression vector pPIC9K (Invitrogen) under the control of the AOX1 promoter. We optimized the feed batch protocol [3] which enabled the production of active recombinant barley limit dextrinase in a 5 l Biostat B fermentor. Limit dextrinase was purified by a two-step purification strategy. Affinity chromatography using β-cyclodextrin (β-CD) crossed linked to a Sepharose matrix was followed by size exclusion chromatography (SEC) on a Sephadex-G200 column. The molecular mass of the recombinant limit dextrinase was approximately 98 kDa which corresponds to the molecular mass of the native barley enzyme. The identity of the recombinant LD was verified by MALDI-TOF-MS MS/MS. The kinetics constant of the purified enzyme was determined using pullulan as substrate and they were consistent with the reported value of native LD.

The established system for the recombinant expression of limit dextrinase using *Pichia pastoris* facilitates future investigation of structure/function relationships using site-directed mutagenesis and truncations guided by information of family GH13 domain architecture as retrieved from the CAZy-database.

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Poster - P36

Isolation, purification and biochemical analysis of a new pullulanase from *Geobacillus stearothermophilus*

Shekufeh ZAREIAN, Marzieh GHOLLASI, Khosro KHAJEH, Saman HOSSEINKHANI & Bijan RANJBAR

Department of Biochemistry and Biophysics, Faculty of Sciences, Tarbiat Modares University, Tehran, Iran

A thermophilic strain L14, isolated from Iranian hot springs, produced an extracellular pullulanase upon growth on optimized liquid medium. The strain is likely to be *Geobacillus stearothermophilus* since the analysis of 16S rDNA gene sequence showed highest similarity (99%) with this strain. The enzyme was purified by ammonium sulfate precipitation and anion exchange chromatography. The purified enzyme showed a band on SDS-PAGE with an estimated molecular mass of 130 kDa for monomeric enzyme. The purified enzyme had an optimum pH of 5.5 and an optimum temperature of 65°C. It had good stability at 60-70 ^{o}C. The enzyme could hydrolyze pullulan and starch. The Km value for the enzyme activity on pullulan was 0.044% (w/v) and Km on soluble starch was 0.5% (w/v). The products of enzymatic reaction on pullulan and starch were glucose, maltose and maltotriose as shown by TLC analysis. It has been suggested that the purified pullulanase from *Geobacillus* sp. L14 is classified under pullulan hydrolase type III. To our knowledge this *Geobacillus* pullulanase is the sole pullulan hydrolase type III known in *Bacillus* strains to date.

PRESENTING AUTHORS

LECTURES

POSTERS

LIST OF PARTICIPANTS

Abbott D. Wade
Biochemistry and Microbiology, University of Victoria, Victoria BC, Canada V8W 3P6
E-mail: wabbott@uvic.ca

Abou Hachem, Maher
Enzyme and Protein Chemistry, BioCentrum-DTU, Technical University of Denmark, Søltofts Plads - Building 224, DK-2800 Kgs. Lyngby, Denmark
E-mail: maha@biocentrum.dtu.dk

Aghajari, Nushin
Laboratoire de BioCristallographie, Institut de Biologie et Chimie des Protéines, CNRS et Université de Lyon, UMR 5086, IFR 128 "BioSciences Lyon-Gerland", F-69367 Lyon Cedex 07, France
E-mail: n.aghajari@ibcp.fr

Andersen, Carsten
Novozymes A/S, Krogshojvej 36, DK-2880 Bagsvaerd, Denmark
E-mail: CarA@novozymes.com

Andersen, Joakim
Enzyme and Protein Chemistry, BioCentrum-DTU, Technical University of Denmark, Søltofts Plads - Building 224, DK-2800 Kgs. Lyngby, Denmark
E-mail: joakim@nogas.dk

Baumann, Martin J.
Enzyme and Protein Chemistry, BioCentrum-DTU, Technical University of Denmark, Søltofts Plads - Building 224, DK-2800 Kgs. Lyngby, Denmark
E-mail: mb@biocentrum.dtu.dk

Blennow, Andreas
Plant Biochemistry Laboratory, Center for Molecular Plant Physiology (PlaCe), Department of Plant Biology, The Royal Veterinary and Agricultural University, 40 Thorvaldsensvej, DK-1871 Frederiksberg C, Copenhagen, Denmark
E-mail: abl@life.ku.dk

Brison, Yoann
Laboratoire d'Ingénierie des Systèmes Biologiques et des Procédés, UMR CNRS/INSA 5504, UMR INRA/INSA 792, INSA, 135 avenue de Rangueil, 31077 Toulouse cedex 4, France
E-mail: yoann.brison@insa-toulouse.fr

Chang, Margaret D.T.
Institute of Molecular and Cellular Biology & Department of Life Science, National Tsing Hua University, Hsinchu, Taiwan, People's Republic of China
E-mail: dtchang@life.nthu.edu.tw

Coutinho, Pedro M.
UMR6098, AFMB - CNRS/Aix-Marseille Universités, Case 932, 163 Avenue de Luminy, 13288 Marseille, France
E-mail: Pedro.Coutinho@afmb.univ-mrs.fr

Da Lage, Jean-Luc
Laboratoire Evolution, génomes et spéciation (LEGS), CNRS, 91198 Gif sur Yvette cedex, France
E-mail: Jean-Luc.Da-Lage@legs.cnrs-gif.fr

De Maria, Leonardo
Novozymes A/S, Protein Design, Brudelysvej 26, 2880 Bagsvaerd, Denmark
E-mail: LeDM@novozymes.com

Desseaux, Veronique J.B.
Biosciences FRE-CNRS 3005 Fac. des Sciences de St Jérôme - Univ. Paul Cézanne Aix Marseille III -Service 342 Av. Escadrille N. Niemen 13397 MARSEILLE Cedex 20, France
E-mail: veronique.desseaux@univ.u-3mrs.fr

Dijkstra, Bauke W.
Laboratory of Biophysical Chemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands
E-mail: b.w.dijkstra@rug.nl

Gabrisko, Marek
Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, SK-84551 Bratislava, Slovakia
E-mail: Marek.Gabrisko@savba.sk

Ghollasi, Marzieh
Department of Biochemistry and Biophysics, Faculty of Sciences, Tarbiat Modares University, Tehran, Iran
E-mail: marzieh582003@yahoo.com

Godany, Andrej
Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, SK-84551 Bratislava, Slovakia
E-mail: Andrej.Godany@savba.sk

Goesaert, Hans
Laboratory of Food Chemistry and Biochemistry, Katholieke Universiteit Leuven, Kasteelpark Arenberg 20, B-3001 Leuven, Belgium
E-mail: hans.goesaert@biw.kuleuven.be

Guillen, Daniel
Departamento de Biología Molecular y Biotecnología. Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México A. P. 70228., Ciudad Universitaria, Coyoacán 04510. México, D. F.
E-mail: danielguillenx@yahoo.com.mx

Hamzah, Haider Mousa
Department of Biology, College of Science, University of Sulaimani, Iraq
E-mail: haideralaubaidi@hotmail.com

Hasan, Khomaini
Biochemistry Laboratory, Department of Chemistry, Faculty of Mathematics and Natural Sciences, Padjadjaran University, Jalan Singaperbangsa No. 2, 40133, Bandung, Indonesia
E-mail: lesanrego@yahoo.com

Haser, Richard
Laboratoire de BioCristallographie, Institut de Biologie et Chimie des Protéines, CNRS et Université de Lyon, UMR 5086, IFR 128 "BioSciences Lyon-Gerland", F-69367 Lyon Cedex 07, France
E-mail: r.haser@ibcp.fr

Hyblova, Jana
Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, v.v.i., Flemingovo nam. 2, Prague 6, CZ 166 10, Czech Republic
E-mail: hyblova@uochb.cas.cz

Imanaka, Tadayuki
Department of Synthetic Chemistry & Biological Chemistry, Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510, Japan
E-mail: imanaka@sbchem.kyoto-u.ac.jp

Janecek, Stefan
Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, SK-84551 Bratislava, Slovakia
E-mail: Stefan.Janecek@savba.sk

Kandra, Lili
Department of Biochemistry, Faculties of Sciences and Arts, University of Debrecen, PO Box 55. 4010 Debrecen, Hungary
E-mail: kandra@puma.unideb.hu

Khajeh, Khosro
Department of Biochemistry and Biophysics, Faculty of Science, Tarbiat Modarres University, P. O. Box 14115-175, Tehran, Iran
E-mail: KHAJEH@modares.ac.ir

Kimura, Atsuo
Division of Applied Bioscience, Research Faculty of Agriculture, Hokkaido University, Sapporo 060-8589, Japan
E-mail: kimura@abs.agr.hokudai.ac.jp

Kralj, Slavko
Microbiology Department, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands
E-mail: s.kralj@rug.nl

Kuriki, Takashi
Ezaki Glico Co., Ltd., 4-6-5 Utajima, Nishiyodogawa-ku, Osaka 555-8502 Japan
E-mail: kuriki-takashi@glico.co.jp

Kyung, Myung Ok
Center for Agricultural Biomaterials and Department of Food Science & Biotechnology, Seoul National University, Korea

Lin, Shu-chuan
Institute of Molecular and Cellular Biology & Department of Life Science, National Tsing Hua University, Hsinchu, Taiwan 300, People's Republic of China
E-mail: d938213@oz.nthu.edu.tw

Liu, Shi-Hwei K.
Simpson Biotech Co., Ltd, Taoyuan Country, Taiwan 333, People's Republic of China
E-mail: d874213@life.nthu.edu.tw

Lo Leggio, Leila
Biophysical Chemistry Group, Department of Chemistry, Faculty of Science, University of Copenhagen,Universitetsparken 5, DK-2100, Denmark
E-mail: leila@ccs.ki.ku.dk

MacGregor, E. Ann
2 Nicklaus Green, Livingston, West Lothian, EH54 8RX, U.K.
E-mail: alexander.macgregor2@btinternet.com

Mukerjea, Rupendra N.
Laboratory of Carbohydrate Chemistry and Enzymology, Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, IA 50011 USA
E-mail: mukerjea@iastate.edu

Mukerjea, Ilora
Accompanying person *(Mukerjea, R.N.)*

Natalia, Dessy
Biochemistry Division, Faculty of Mathematics and Natural Sciences, Bandung Institute of Technology, Jl. Ganesha 10, Bandung, Indonesia
E-mail: dessy@mail.chem.itb.ac.id

Nielsen, Morten M.
Enzyme and Protein Chemistry, BioCentrum-DTU, Technical University of Denmark, Søltofts Plads - Building 224, DK-2800 Kgs. Lyngby, Denmark
E-mail: mmn@biocentrum.dtu.dk

Nordberg Karlsson, Eva
Department of Biotechnology, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden
E-mail: Eva.Nordberg_Karlsson@biotek.lu.se

Palacin, Manuel
Institute for Research in Biomedicine, Barcelona Science Park, Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Barcelona, and CIBERER, Barcelona, Spain
E-mail: mpalacin@pcb.ub.es

Palomo Reixach, Marta
Centre for Carbohydrate Bioprocessing, TNO-University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands
E-mail: m.palomo.reixach@rug.nl

Park, Kwan-Hwa
Center for Agricultural Biomaterials and Department of Food Science & Biotechnology, Seoul National University, Korea
E-mail: parkkh@snu.ac.kr

Hee Hyuck, Im
Accompanying person *(Park, K.H.)*

Preiss, Jack
Department of Biochemistry & Molecular Biology, Michigan State University, East Lansing, MI 48824 USA
E-mail: preiss@msu.edu

Preiss, Karen Sue
Accompanying person *(Preiss, J.)*

Putseys, Joke A.
Department of Microbial and Molecular Systems, Catholic University of Leuven, Kasteelpark Arenberg 20, 3000 Leuven, Belgium
E-mail: joke.putseys@hotmail.com

Ramasubbu, Narayanan
Department of Oral Biology, University of Medicine and Dentistry of New Jersey, Newark, NJ 07103 USA
E-mail: ramasun1@umdnj.edu

Remaud-Simeon, Magali
UMR5504, UMR792 Ingénierie des Systèmes Biologiques et des Procédés, CNRS, INRA, INSA, 135 avenue de Rangueil, F-31077 Toulouse cedex 4, France
E-mail: magali.remaud@insa-toulouse.fr

Robyt, John F.
Laboratory of Carbohydrate Chemistry and Enzymology, Department of Biochemistry, Biophysics, and Molecular Biology, Iowa State University, Ames, IA 50011 USA
E-mail: jrobyt@iastate.edu

Robyt, Lois
Accompanying person *(Robyt, J.F.)*

Saab-Rincon, Gloria
Departamento de Ingeniería Celular y Biocatálisis. Instituto de Biotecnología, UNAM, Apartado Postal 510-3 Cuernavaca, Morelos 62260, Mexico
E-mail: gsaab@ibt.unam.mx

Saburi, Wataru
Nihon Shokuhin Koko Co., Ltd., Fuji 417-8530, Japan
E-mail: wataru.saburi@nisshoku.co.jp

Seo, Eun-Seong
Enzyme and Protein Chemistry, BioCentrum-DTU, Technical University of Denmark, Søltofts Plads - Building 224, DK-2800 Kgs. Lyngby, Denmark
E-mail: ess@biocentrum.dtu.dk

Seo, Nam-Seok
Samlip General Foods Co., Ltd., Korea

Kim, Mi-Ok
Accompanying person *(Seo, N.S.)*

Seo, Hye-Ryung
Accompanying person *(Seo, N.S.)*

Slupska, Margaret
Verenium Corporation, 4955 Directors Place, San Diego, CA 92121 USA
E-mail: Margaret.Slupska@verenium.com

Song, Kyung Mo
Center for Agricultural Biomaterials and Department of Food Science & Biotechnology, Seoul National University, Korea

Stanley, Duncan
John Innes Centre, Colney Lane, Norwich NR4 7UH, UK
E-mail: Duncan.Stanley@bbsrc.ac.uk

Stapleton, David
Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Parkville, Victoria, Australia
E-mail: dis@unimelb.edu.au

Stapleton, Viviene S.
Accompanying person *(Stapleton, D.)*

Sugimoto, Kazuhisa
Biochemical Research Laboratory, Ezaki Glico Co., Ltd., 4-6-5 Utajima, Nishiyodogawa-ku, Osaka 555-8502 Japan
E-mail: sugimoto-kazuhisa@glico.co.jp

Svensson, Birte
Enzyme and Protein Chemistry, BioCentrum-DTU, Technical University of Denmark, Søltofts Plads - Building 224, DK-2800 Kgs. Lyngby, Denmark
E-mail: bis@biocentrum.dtu.dk

Urbanikova, Lubica
Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, SK-84551 Bratislava, Slovakia
E-mail: Lubica.Urbanikova@savba.sk

van der Kaaij, Rachel M.
Microbial Physiology Research Group, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, Kerklaan 30, 9751 NN, Haren, The Netherlands
E-mail: r.m.van.der.kaaij@rug.nl

van der Maarel, Marc J.E.C.
Department of Innovative Ingredients and Products, Business Unit Food and Biotechnology Innovations, Rouaanstrat 27, 9723 CC, Groningen, The Netherlands
E-mail: marc.vandermaarel@tno.nl

Vester Christensen, Malene Bech
Enzyme and Protein Chemistry, BioCentrum-DTU, Technical University of Denmark, Søltofts Plads - Building 224, DK-2800 Kgs. Lyngby, Denmark
E-mail: mvc@biocentrum.dtu.dk

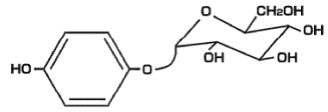
Vidova, Barbora
Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, SK-84551 Bratislava, Slovakia
E-mail: b.vidova@gmail.com

Yang, Tae Joo
Center for Agricultural Biomaterials and Department of Food Science & Biotechnology, Seoul National University, Korea

Glico

α-Arbutin (Alpha-Arbutin)

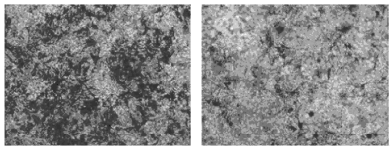
Effective Skin-Lightening Agents for Cosmetic. α-Arbutin suppresses melanin production that causes freckles and suntan by the inhibition of a tyrosinase activity. Ezaki Glico Co., Ltd. was established, for the first time in the world, the manufacturing process of α-Arbutin using their original transglycosylation enzyme that catalyzes alpha-anomer selective transglycosylation reaction.



4-Hydroxyphenyl-α-D-glucopyranoside
$C_{12}H_{16}O_7 = 272$

INCI name: Alpha-Arbutin
CAS number: 84380-01-8
ELINCS number: 440-470-8

We have clarified skin-lightening effects by clinical study, inhibitory study on melanin synthesis in cultured human cells, and inhibitory study on human tyrosinase.



control α-Arbutin
Microscopic Views of Cultured Human Skin Models (X100)

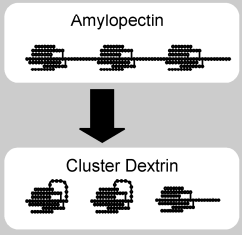
Cluster Dextrin

New Functional Dextrin for Foods. Cluster Dextrin has been on the market in Japan since 2002 as a food material, and used for improvement of taste of foods, as a component of sports drinks, as a spray-drying aid and so on.

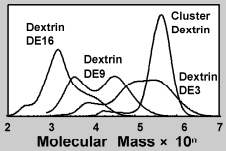
Properties

? Dextrose equivalent is less than 5.
? Cluster Dextrin is highly soluble in water, and the solution is highly stable during storage.
? Cluster Dextrin has relatively long side chains,and these chains are considered to take helix conformation. Such helix structures can form inclusion complexes with guest molecules such as organic acids.
? Cluster Dextrin hardly absorbs moisture, and hardly causes browning reaction. These properties make Cluster Dextrin excellent for spray-drying aid.
? The sweetness of Cluster Dextrin is low compared with those of dextrins with relatively high DE.
? Cluster Dextrin has low unfavorable taste derived from starch compared with other dextrins.

Model of Reaction to Produce Cluster Dextrin



Molecular Weight Distribution of Various Dextrins



Contact to: Ezaki Glico Co., Ltd.
New Material Development Group Phone: +81 6-6477-8281 FAX: +81 6-6477-8267

Notes