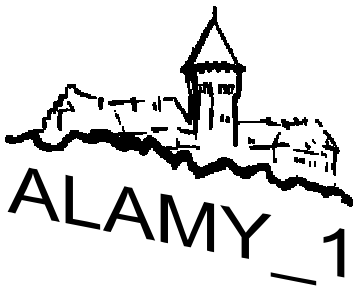

1ST SYMPOSIUM ON THE ALPHA-AMYLASE FAMILY

SMOLENICE CASTLE, SLOVAKIA, SEP 30 – OCT 4, 2001

PROGRAMME AND ABSTRACTS

Programme and abstracts of the
1st Symposium on the Alpha-Amylase Family
held in Smolenice Castle, Slovakia, 30 Sep – 4 Oct, 2001

Edited by
Štefan Janeček

Scientific Programme Committee:
Richard Haser, Štefan Janeček, Takashi Kuriki, E. Ann MacGregor,
Kwan-Hwa Park, Jack Preiss, Yuzuru Suzuki & Birte Svensson

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Kwan-Hwa Park (Suwon, Korea)
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INVITATION

The objective of the **1st Symposium on the Alpha-Amylase Family** is to provide a forum for the presentation and discussion of research on enzymes and proteins belonging to the α-amylase family. The effort to organise this conference has been evoked by the rapidly growing knowledge on this family and its enormous scope. It is expected that the participants are presenting their most recent data in the form of oral talks and posters. The aim is to attract both established researchers as well as PhD-students working in the field of the α-amylase family in its widest sense.

This is the **FIRST** symposium on the α-amylase family. It is our aim to make the Symposium a platform for informal discussions about the most recent results and international collaborations. The scientific programme of **1st Symposium on the Alpha-Amylase Family** is focused but not restricted on new primary and tertiary structures, specificity and evolution, protein engineering, catalytic and binding mechanisms, and stability and stabilisation. The research oriented on non-catalytic modules, e.g. the raw starch-binding domain, is also welcome.

The Symposium is held at the Congress Centre of the Slovak Academy of Sciences in a charming castle situated on a hill above the small city of Smolenice which lies at the foot of Small Carpathian Mountains 60 km North-east of Bratislava, the capital of Slovakia. All the participants are also accommodated in the **Smolenice Castle**. We hope that beatiful natural surrounding and attractive interiors of the Castle will create a special atmosphere for scientific discussions, relaxation and meeting friends.

We wish you a very nice stay in Slovakia !

Smolenice Castle, 30 September 2001 Štefan JANEČEK

PROGRAMME

SUNDAY – 30 September 2001

14.00	Registration desk open Poster set up
16.00 – 16.30	Conference buses from Bratislava and Vienna arrive at Smolenice Castle
18.00	**Opening words – Invitation**
18.10 – 19.30	**Keynote Lecture** Chairman: E. Ann MacGregor
	Birte Svensson (Denmark): Fascinating facets of function and structure of amylolytic enzymes of glycoside hydrolase family 13.
19.30 – 22.00	**Opening Reception**

MONDAY – 1 October 2001

07.30 – 08.30	*Breakfast*
08.25	Announcements
08.30 – 12.00	**Session 1: New primary structures** Chairman: Kwan-Hwa Park
08.30	**Garabed Antranikian** (Germany): Branching and debranching enzymes from hyperthermophilic and thermoalkaliphilic microorganisms.
09.15	**Tadayuki Imanaka** (Japan): An archaeal cyclodextrin glucanotransferase with a structurally novel C-terminal domain necessary for glucanotransferase activity.
10.00 – 10.15	*Coffee Break*
10.15	**Duncan Stanley** (New Zealand): Characterisation of putative alpha-amylases from apple (*Malus domestica*).
10.40	**Hiroshi Akanuma** (Japan): Hepatic alpha-amylase in rat.
11.05	**Michael O'Donohue** (France): Expression and characterisation of the catalytic domain of an archaeal family 57 pullulanase type II: probing of its intriguing catalytic functionality.
11.30	**Johan F.T. van Lieshout** (The Netherlands): Identification of active-site residues of an archaeal alpha-galactosidase, a unique member of glycosyl hydrolase family 57.
12.00 – 13.30	*Lunch*
13.30 – 14.30	**Poster Session**
14.30 – 18.00	**Session 2: Specificity and evolution** Chairman: E. Ann MacGregor
14.30	**Takashi Tonozuka** (Japan): Crystal structures and substrate specificities of two alpha-amylases hydrolyzing cyclodextrins and pullulan from *Thermoactinomyces vulgaris* R-47.

15.15 **Jean-Luc Da Lage** (France): Diversity and evolution of alpha-amylase genes in Animals.

16.00 – 16.15 *Coffee Break*

16.15 **Takashi Kuriki** (Japan): Macromolecule recognition of *Bacillus stearothermophilus* neopullulanase.

16.45 **Gerard Pujadas** (Spain): Independent folding of the A and B domains in alpha-amylase family enzymes deduced from the geometrical constraints on the evolution of the TIM-barrel beta sheet.

17.10 **Štefan Janeček** (Slovakia): How many conserved sequence regions are there in the alpha-amylase family?

17.35 **Daniel Sellos** (France): Lost, shifted or shuffled introns during evolution of alpha-amylase genes.

18.30 – 20.00 *Supper*

TUESDAY – 2 October 2001

07.30 – 08.30 *Breakfast*

08.25 Announcements

08.30 – 12.00 **Session 3: Catalytic and binding mechanism**
Chairman: Birte Svensson

08.30 **Bauke W. Dijkstra** (The Netherlands): Structural investigations of the catalytic mechanism of cyclodextrin glycosyltransferase.

09.15 **Yoshiki Matsuura** (Japan): A possible mechanism of catalysis involving three essential residues in alpha-amylase family enzymes.

10.00 – 10.15 *Coffee Break*

10.15 **Nushin Aghajari** (France): Cold-active alpha-amylase: structural analyses, molecular adaptation, mechanistic aspects and a remarkable tool for novel applications.

10.40 **Kwan-Hwa Park** (Korea): Effect of N-terminal and C-terminal regions on the oligomerization properties of cyclodextrin-/pullulan degrading enzymes.

11.05 **Guy Marchis-Mouren** (France): Mechanism of porcine pancreatic alpha-amylase (PPA): inhibition of amylose and maltopentaose hydrolysis by various inhibitors.

11.30 **Kirill N. Neustroev** (Russia): Amylolytic activity of IgG and sIgA immunoglobulins from human milk.

12.00 – 13.30 *Lunch*

13.30 – 14.30 **Poster Session**

14.30 – 17.30 **Session 4: New three-dimensional structures**
Chairman: Takashi Kuriki

14.30 **Byung-Ha Oh** (Korea): Crystal structures of maltogenic amylase and cyclomaltodextrinase.

15.15 **Jack Preiss** (MI, USA): X-Ray crystallographic studies of *Escherichia coli* branching enzyme.

16.00 – 16.15 *Coffee Break*

16.15 **Richard Haser** (France): Structural basis for substrate recognition and specificity differences of barley alpha-amylase isozymes.

16.40 **Hironori Hondoh** (Japan): Three-dimensional structure of *Bacillus stearothermophilus* neopullulanase.

17.05 **Anni Linden** (Germany): Crystal structure of an alpha-amylase from the hyperthermophilic archaeon *Pyrococcus woesei*.

17.30 – 18.00 *Refreshment Break*

18.00 – 19.20 **Special Banquet Lecture**
Chairman: Štefan Janeček

Bernard Henrissat (France): Comparative genomics and the metabolism of starch.

19.30 – 22.00 **Symposium Banquet**

WEDNESDAY – 3 October 2001

07.30 – 08.30 *Breakfast*

08.25 Announcements

08.30 – 12.00 **Session 5: Protein engineering**
Chairman: Richard Haser

08.30 **Norbert Sträter** (Germany): Structural basis of the synthesis of large cycloamyloses by amylomaltase.

09.15 **Ryota Kuroki** (Japan): Structure determination and engineering to explore the function of glycosyltrehalose trehalohydrolase from the hyperthermophilic archaeum *Sulfolobus solfataricus*.

10.00 – 10.15 *Coffee Break*

10.15 **Tasuku Nakajima** (Japan): Characterization of the functional module responsible for low temperature optimum of a rice alpha-amylase (Amy 3E).

10.40 **Carsten Andersen** (Denmark): Designing the product specificity of alpha-amylases.

11.05 **Cécile Albenne** (France): Site-directed mutagenesis of key amino acids in the active site of amylosucrase from *Neisseria polysaccharea*.

11.30 **Roy Russell** (UK): Glucan binding domain of streptococcal glucosyltransferases.

12.00 – 13.00 *Lunch*

13.00 – 14.00 **Session 6: Raw starch binding and degrading**
Chairman: Štefan Janeček

13.00	**Brian K. Tibbot** (CA, USA): Functional studies on the barley alpha-amylase raw starch-binding domain.
13.20	**Nathalie Juge** (UK): C-Terminal fusion of barley alpha-amylase isozyme 1 with the starch binding domain of glucoamylase from *Aspergillus niger*.
13.40	**Eva Hostinová** (Slovakia): Molecular cloning and functional expression of a non-extra-SBD-domain type of raw starch degrading glucoamylase from *Saccharomycopsis fibuligera* IFO 0111.
14.15 – 14.30	Conference buses leave for trip to Bratislava
15.30 – 18.00	Sightseeing tour Bratislava with visiting the Devin Castle
18.45 – 19.45	Slovak Folk Artistic Ensemble performance
20.00 – 22.30	*Dinner in the capital*
23.30	Arrival at Smolenice

THURSDAY – 4 October 2001

07.30 – 08.30	*Breakfast*
08.00 – 09.00	Vacation of the rooms Poster removing
08.55	Announcements
09.00 – 12.00	**Session 7: Stability and stabilisation** Chairman: Jack Preiss
09.00	**Wolfgang Liebl** (Germany): Maltosyltransferase of the hyperthermophilic bacterium *Thermotoga maritima*: crystal structure and its implications for the novel transfer specificity and thermostability of the enzyme.
09.45	**Nathalie Declerck** (France): Engineering the thermostability of *Bacillus licheniformis* alpha-amylase.
10.30 – 10.45	*Coffee Break*
10.45	**Mischa Machius** (TX, USA): Stabilization of *Bacillus licheniformis* alpha-amylase through introduction of hydrophobic residues at the surface.
11.10	**Ali-Akbar Saboury** (Iran): Stability, activity and binding properties study of alpha-amylase upon interaction with Ca^{2+} and Co^{2+}.
11.35	**Georges Feller** (Belgium): Structural determinants of cold adaptation and stability in a psychrophilic alpha-amylase.
12.00	**Closing words**
12.15 – 13.00	*Lunch*
13.15 – 13.30	Conference buses leave for Bratislava and Vienna

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INVITED LECTURES AND ORAL PRESENTATIONS ABSTRACTS

KEYNOTE LECTURE OF THE SYMPOSIUM

Fascinating facets of function and structure of amylolytic enzymes of glycoside hydrolase family 13

Birte SVENSSON

Department of Chemistry, Carlsberg Laboratory, Gamle Carlsberg Vej 10, DK-2500 Copenhagen Valby, Denmark; e-mail: bis@crc.dk

The glycoside hydrolase family 13 currently comprises 27 enzyme specificities. Three-dimensional structures are available of 12 different enzyme categories. A major shortcoming is lack of oligosaccharide complexes illustrating in particular interactions with α-1,6-glucosidic linkages in substrates, respectively, with substrate binding regions at a distance from the catalytic site. Modeling has served to provide some information, long substrates, however, are envisaged to interact with enzymes in more than one binding mode and occasionally at more than one binding region − including binding to separate domains. Mutational studies coupled with kinetics analysis contribute to improve insight into the relationship between structure and the mechanisms of binding and catalysis in selected structural models. Another limitation is lack of insight into roles of extra domains in amylolytic enzymes or, where extra domains have been ascribed a function, in the understanding of how these domains interact with substrate and co-operate with the catalytic domain.

Rational design in glycoside hydrolase family 13 has not been straight-forward. Even though conserved sequence motifs correlate with enzyme specificities, this does not suffice as full specificity signature reflecting in detail how various substrates and enzymes interact. Indeed manipulations at motifs of the identified β→α connecting segments creating extended substrate binding sites influence on specificity. One challenge consists in engineering a given enzyme without loosing the level of activity. The industrial application of several family 13 members furthermore motivates design of altered pH activity dependence, temperature stability, substrate specificity, and Ca^{2+} requirement. Such goals are often extremely difficult to achieve. Recently it has been demonstrated that repeated cycles of protein engineering or combination of irrational and rational mutagenesis strategies can lead to enzyme variants with improved properties. Therefore combination of thoughtful in vitro evolution and screening procedures currently appears highly attractive. Another facet of the family 13 mechanism is the reverse reaction. Thus while some members are transglycosylases, others mainly function as hydrolases, which under particular conditions can transglycosylate leading to novel compounds. The elegant glycosynthase approach developed for retaining β-glycoside hydrolases has not yet been demonstrated to be applicable for glycoside hydrolase family 13.

Sensitivity to proteinaceous inhibitors is a feature known for relatively few family 13 members. This property shows great species diversity and cases are known also of closely related isozymes being discriminated by natural inhibitors. Insight into this behaviour has practical application in food and feed sciences, and in plant defence. Relevant enzymes may be manipulated to acquire or suppress sensitivity to inhibitors. In plant defence, systems of inhibitors acting on enzymes from pathogens without effect on endogenous enzymes need to be developed.

Examples will be given using α-amylase isozymes and the debranching enzyme limit dextrinase from barley malt. Focus will be on i) mutational analysis disclosing the impact of individual subsites in binding different substrates and on enzyme specificity, ii) the advantage of combination of irrational and rational mutagenesis strategies to achieve functional enzyme variants with altered properties, iii) transglycosylation reactions, iv) communication between a starch binding domain and the catalytic domain in an α-amylase fusion protein as well as in glucoamylase, the natural enzyme model having a catalytic domain of family 15, and finally v) the engineering of the interaction between barley α-amylase and the endogenous inhibitor BASI (Barley α-Amylase/Subtilsin Inhibitor).

SESSION 1
NEW PRIMARY STRUCTURES

Branching and debranching enzymes from hyperthermophilic and thermoalkaliphilic microorganisms

Costanzo BERTOLDO, Volker THIEMANN & Garabed ANTRANIKIAN

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A number of hyperthermophilic and thermoalkaliphilic microorganisms belonging to bacteria and archaea were investigated in respect to their ability to produce heat-stable debranching and branching enzymes. Pullulanase, also called debranching enzyme, hydrolyzes α-1,6 glycosidic linkages in pullulan and branched substrates forming maltotriose and linear oligosaccharides, respectively. The branching enzyme on the other hand catalyzes the formation α-1,6 glucosidic linkages in starch (plants) and glycogen (animal and bacteria). From the biotechnological point of view, both enzymes are potential candidates for the production of valuable defined oligosaccharides, which can be used as fat replacers, food texturizers and sweeteners. Here we report on the molecular characteristics of three different pullulanases from hyperthermophilic anaerobic bacteria and archaea and the branching enzyme from a novel thermoalkaliphilic bacterium, *Anaerobranca gottschalkii*, isolated from Lake Bogoria, Kenya. The thermophilic anaerobic bacterium *Fervidobacterium pennivorans Ven5* produces a specific debranching enzyme (*pul*A), whose gene was cloned and sequenced. The recombinant thermostable pullulanase type I is optimally active at 80°C and broad pH range and catalyzes specifically the debranching of polysaccharides such as amylopectin. In order to obtain large amounts of the *pul*A for crystallization trials, we succeeded in the cloning, and expression of *pul*A in *Bacillus subtilis*. Unlike the *E. coli* clone, in *B. subtilis* the enzyme is secreted in the supernatant at high yields. The genes encoding pullulan hydrolyzing enzymes from the hyperthermophilic anaerobic archaea *Desulfurococcus mucosus* and *Thermococcus aggregans* were also cloned and expressed in *E. coli*. By sequence comparison, the enzymes shared 45.4% identity and contained the four conserved regions of amylolytic enzymes. The enzyme from *D. mucosus* (pul*Dm)* was also expressed in *B. subtilis*. Pul*Dm* behaves like pullulanase type II converting pullulan to maltotriose. Interestingly, the enzyme from *T. aggregans* (pulh*Ta*) represents a new class of pullulan hydrolases since it attacks simultaneously α-1,4- and α-1,6-glycosidic linkages in pullulan forming maltotriose, panose, maltose and glucose. Very recently, the complete genome of *Anaerobranca gottschalkii* has been sequenced. The gene encoding the branching enzyme was cloned and expressed in a mesophilic host. The enzyme has been purified from the culture supernatant and data on its physico-chemical properties will be presented.

An archaeal cyclodextrin glucanotransferase with a structurally novel C-terminal domain necessary for glucanotransferase activity

Tadayuki IMANAKA

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We proposed a general concept for an enzyme family, the α-amylase family including most of the amylases and related enzymes in 1992 (TAKATA et al., 1992), based on the structural similarity and the common catalytic mechanisms. The study on neopullulanase was the key to open the door for the formulation of the concept (KURIKI et al., 1988, 1991; IMANAKA & KURIKI, 1989). We discovered a new enzyme, neopullulanase, and proved that the enzyme catalyzes both hydrolysis and transglycosylation at α-1,4- and α-1,6-glucosidic linkages by one active center. Results from a series of experiments using neopullulanase indicated that the four reactions mentioned above could be catalyzed in the same mechanism (KURIKI & IMANAKA, 1999).

I would like to show an archaeal cyclodextrin glucanotransferase as a member of α-amylase family in this symposium. A gene encoding cyclodextrin glucanotransferase (CGTase) from a hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1 (FUJIWARA et al., 1996) was cloned and analyzed. The nucleotide sequence revealed that the gene (*Tk-cgt*) was composed of 2,139 nucleotides corresponding to a protein of 713 amino acid residues. Four conserved regions found in all members of the α-amylase family were present in the sequence, and many residues specifically conserved in CGTases were found. However, overall similarity with other enzymes was not high, and the most similar protein was the archaeal CGTase from a *Thermococcus* sp. B1001 (TACHIBANA et al., 1999) with 40% identity. We found that the C-terminal region of *Tk*-CGT, particularly the region corresponding to domain E, displayed a completely distinct primary structure compared to other previously reported CGTases. *Tk-cgt* was over-expressed in *Escherichia coli*, and the recombinant enzyme was purified by anion-exchange chromatography. The enzyme displayed bonafide CGTase activity, producing mainly β-cyclodextrin along with small amounts of α- and γ-cyclodextrins when soluble starch was used as a substrate. The optimum temperature and pH for the starch-degrading activity were found to be 80° and 5.5-6.0, respectively. Presence of Ca^{2+} enhanced the enzyme activity along with broadening and elevating the optimum temperature to 85-90°. In the presence of 8 mM Ca^{2+}, the enzyme showed extreme thermostability with only a slight loss of enzymatic activity after 80 min of incubation at 85° and a half-life of 30 min at 100°. *Tk*-CGT could hydrolyze soluble starch and glycogen while it failed to hydrolyze pullulan. Deletion of C-terminal 23 amino acid residues rendered the protein inactive in cyclodextrin synthesis, whereas the starch-degrading activity of the mutant was enhanced, indicating a major role of the structurally distinct C-terminal region of *Tk*-CGT in the cyclization reaction.

References

TAKATA, H., KURIKI, T., OKADA, S., TAKESADA, Y., IIZUKA, M., MINAMIURA, N. & IMANAKA, T. 1992. *J. Biol. Chem.* **267**, 18447-18452.

KURIKI, T., OKADA, S. & IMANAKA, T. 1988. *J. Bacteriol.* **170**, 1554-1559.

IMANAKA, T. & KURIKI, T. 1989. *J. Bacteriol.* **171**, 369-374.

KURIKI, T., TAKATA, H., OKADA, S. & IMANAKA, T. 1991. *J. Bacteriol.* **173**, 6147-6152.

KURIKI, T. & IMANAKA, T. 1999. *J. Biosci. Bioeng.* **87**, 557-565.

FUJIWARA, S., OKUYAMA, S. & IMANAKA, T. 1996. *Gene* **179**, 165-170.

TACHIBANA, Y., KURAMURA, A., SHIRASAKA, N., SUZUKI, Y., YAMAMOTO, T., FUJIWARA, S., TAKAGI, M. & IMANAKA, T. 1999. *Appl. Environ. Microbiol.* **65**, 1991-1997.

Characterisation of putative α-amylases from apple (*Malus domestica*)

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Starch metabolism is essential for maintaining carbon balance in plants, and α-amylases are known to play a role in starch degradation in a number of plants and tissues (BECK & ZIEGLER, 1989). α-Amylases have been well studied in monocotyledonous plants, particularly cereals, where α-amylases are secreted from aleurone cells during germination, to break down starch in the endosperm of the grain. In contrast, apple starch is stored within the plastids of living cells, and therefore we would expect to find an α-amylase isozyme localised within plastids.

We have previously isolated a cDNA from apple fruit, termed *Mdamy*8, which encodes an α-amylase-like protein that is up-regulated during cold-treatment of fruit (WEGRZYN et al., 2000). The predicted protein is atypical of plant α-amylases; it does not have an N-terminal signal peptide sequence, and displays major differences within both its B-domain and C-domain. These differences would be expected to alter the subcellular localisation and/or the enzymatic properties of the protein.

A second α-amylase-like cDNA, *Mdamy*9, has been amplified from apple. *Mdamy*9 displays a very high degree of similarity to *Mdamy*8, and appears to have arisen via gene duplication. Both genes share the same intron/exon structure, which is distinct from the structure found in other plant α-amylase genes. This includes an intron within the 5' untranslated region (UTR) that is alternately spliced in *Mdamy*8, and may be involved in post-transcriptional regulation.

We are investigating the subcellular targeting of Mdamy8 using Green Fluorescent Protein (GFP) as a marker of protein localisation. The GFP gene has been spliced to several cDNA sequences derived from *Mdamy*8, and the resulting chimaeric genes have been transformed into plant cells. A variety of fusion constructs have been produced, to overcome the possibility that addition or insertion of the GFP gene may disrupt targeting signals encoded within *Mdamy*8. The localisation of the GFP/Mdamy8 fusion proteins will be determined by examining transformed cells using fluorescence or confocal microscopy. The enzymatic properties of the Mdamy8 and Mdamy9 proteins are also being studied, by expressing both of the genes in the yeast *Pichia pastoris*.

References

BECK, E. & ZIEGLER, P. 1989. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 95-117.

WEGRZYN, T., REILLY, K., CIPRIANI, G., MURPHY, P., NEWCOMB, R., GARDNER, R. & MACRAE, E. 2000. *Eur. J. Biochem.* **267**, 1313-1322.

Hepatic α-amylase in rat

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α-Amylase activity and malto-oligosaccharides have long been described without isolation of any relevant cytosolic enzyme in mammalian liver. We present the purification and primary structure of a neutral α-amylase from rat liver, the full coding sequence of its mRNA (accession number: AB057450), the enzyme's unique properties, and possible functions of the expected hepatic amylolysis.

References

KAMETANI, S., SHIGA, Y. & AKANUMA, H. 1996. *Eur. J. Biochem.* **242**, 832-838.

HIRANO, K., ZIAK, M., KAMOSHITA, K., SUKENAGA, Y., KAMETANI, S., SHIGA, Y., ROTH, J. & AKANUMA, H. 2000. *Glycobiology* **10**, 1283-1289.

Expression and characterisation of the catalytic domain of an archaeal family 57 pullulanase type II - probing of its intriguing catalytic functionality

Florent CHANG PI HIN, Marta ERRA-PUJADA, Philippe DEBEIRE, Francis DUCHIRON & Michael J. O'DONOHUE

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Pullulanases type I (EC 3.2.1.41) specifically catalyse the hydrolysis of α-1,6 glucosidic linkages in pullulan. Pullulanases type II also possess this ability but, in addition, hydrolyse α-1,4 glucosidic bonds in other polysaccharides. In recent years, many pullulanases have been identified and studied, since these enzymes are potentially useful in biotechnological applications. Indeed, pullulanases are already employed along with other amylolytic enzymes for the transformation of starch to glucose, maltose or fructose syrups.

More recently, several archaeabacteria have been described as pullulanase-producers. Among these microorganisms, the Thermococcales family is particularly well represented and the genes encoding pullulanases type II from *Pyrococcus woesi* (RÜDIGER et al., 1995), *P. furiosus* (DONG et al., 1997) and *Thermococcus hydrothermalis* (ERRA-PUJADA et al., 1999) have now been cloned and successfully expressed in *E. coli*. These three enzymes are highly homologous, although the pullulanase from *T. hydrothermalis* is much bigger. Furthermore, the primary sequences of these pullulanases, which have been grouped within family 57 of the glycoside hydrolase classification, do not exhibit the conserved consensus sequences which characterise the functionally closely-related family 13 enzymes.

In our laboratory we are studying the pullulanase from *T. hydrothermalis*. This enzyme, which in its native form is composed of 1339 amino acids, is highly thermostable (active at 105°C) and, as one would expect of a type II pullulanase, displays the dual capacity to cleave both α-1,6 and α-1,4 glucosidic linkages. Preliminary studies of this latter feature have indicated that this pullulanase, like that of *P. furiosus*, possess a single catalytic site which is responsible for both activities. The cloning and expression of a truncated protein (the first 769 amino acids of this pullulanase) has revealed that, whatever the actual number of active sites, all of the catalytic determinants of this enzyme are contained within the N-terminal region. In addition, this finding indicates that the C-terminal sequence (which contains several remarkable features) must fulfil another, as yet undetermined, function.

In this report we will present data concerning the characterisation of the truncated catalytically-active pullulanase. So far, we have employed a variety of analytical approaches in order to obtain the first clues concerning the catalytic mechanism and the structural architecture of this pullulanase. In particular, we have concentrated on the dual catalytic functionality of this enzyme, and have performed comparative experiments which have provided insight into this phenomenon. Similarly, using a variety of substrates and inhibitors, we have attempted to refine the description of this enzyme's specificity and to reveal its mode of action.

References

DONG, G., VIEILLE, C. & ZEIKUS, J.G. 1997. *Appl. Environ. Microbiol.* **63**, 3577-3584.
ERRA-PUJADA, M., DEBEIRE, P., DUCHIRON, F. & O'DONOHUE, M.J. 1999. *J. Bacteriol.* **181**, 3284-3287.
RÜDIGER, A., JORGENSEN, P.L. & ANTRANIKIAN, G. 1995. *Appl. Environ. Microbiol.* **61**, 567-575.

Identification of active-site residues of an archaeal α-galactosidase, a unique member of glycosyl hydrolase family 57

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We have previously identified a galactoside utilization cluster on the genome of *Pyrococcus furiosus*. One of the genes in this cluster codes for a putative glycoside hydrolase. Low but significant similarity was found with the thermophilic α-amylases classified in family 57 of glycoside hydrolases. We have functionally overexpressed the *P. furiosus* gene in *E. coli* and characterized the purified enzyme as the first archaeal α-galactosidase (GalA). Remarkably, the enzyme displayed no amylase or (amylo)pullulanase activity. GalA shows highest activity towards *p*-nitrophenyl-α-galactopyranoside (pNP-α–gal) at an optimal pH of 5.0-5.5. The enzyme is also able to hydrolyze melibiose and raffinose with an activity of 9% and 4 % compared to pNP-α–gal. With an optimal temperature of 115 ^{o}C and a half-life time of 15 hrs at 100 ^{o}C GalA is the most thermo-active and thermo-stable α-galactosidase described to date. Moreover, based on amino acid sequence homology it concerns a novel type of α-galactosidase.

By site-directed mutagenesis of conserved glutamate residues we have tried to identify the putative catalytic residues. Addition of external nucleophiles like azide or formate to some extent restored the activity of one inactive mutant. Comparison with the distantly related α-mannosidases of family 38 strongly suggests that this residue is the catalytic nucleophile. This would be the first identified catalytic residue of a family 57 member. By identifying this catalytic residue of GalA we hope to get more insight into the unknown mechanism of the family 57 amylases. The nucleophile mutant may be useful for improved synthesis of oligosaccharides by GalA.

SESSION 2
SPECIFICITY AND EVOLUTION

Crystal structures and substrate specificities of two α-amylases hydrolyzing cyclodextrins and pullulan from *Thermoactinomyces vulgaris* R-47

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Thermoactinomyces vulgaris R-47 α-amylases, TVA I and TVA II, hydrolyze specific (α1→4)-glucosidic linkages of pullulan to produce panose and also hydrolyze cyclodextrins. Although the properties of TVA I and TVA II are similar, TVA I strongly hydrolyzes starch while TVA II shows outstanding kinetic values for small oligosaccharides.

Crystal structure of TVA II complexed with its substrate. We have already determined the unliganded crystal structure of TVA II (KAMITORI et al., 1999). To determine the crystal structure of TVA II-substrate complex, its catalytic residues, Asp421, Glu354, and Asp421, were modified. The activities of these mutated enzymes were markedly reduced (less than 0.05% of the activity of wild-type enzyme) but traces of activities were still remained (ICHIKAWA et al., 2000). The structures of the mutated TVA II complexed with β-cyclodextrin (β-CD) and maltohexaose were determined (KONDO et al., 2001; YOKOTA et al., 2001a). We have already reported that the structure of TVA II was composed of domains A, B, C, and N, and TVA II was observed as a dimeric form in the crystal. β-CD and maltohexaose bound domain A of TVA II and this region was located close to domain B. In the α-amylase family enzymes, the structure of cyclodextrin glycosyltransferase (CGTase) complexed with β-CD was reported. The overall structures of TVA II and CGTase are quite different because the similarity of domains except domains A, B, and C of these enzymes are not found. However, the position of β-CD for the crystal structure of CGTase resembled that of TVA II.

Crystal structure of TVA I and analyses of roles of domain N for both enzymes. Crystals of TVA I were obtained using polyethylene glycol 10,000 (KONDO et al., 2000). The crystal structure of TVA I was determined by multiple isomorphous replacement at 2.5 Å using a Rigaku R-AXIS IIc imaging plate system. Like TVA II, the structure of TVA I was composed of domains A, B, C, and N. However, unlike TVA II, TVA I was observed as a monomeric form in the crystal, and the positions and directions of domain N of both enzymes were quite different. Also, about a half of amino acid residues located on the catalytic center were not conserved between TVA I and TVA II. To investigate the roles of domain N for both enzymes, domain N of TVA I and TVA II was deleted (YOKOTA et al., 2001b). The activities of these mutated enzymes were greatly reduced.

References

ICHIKAWA, K., TONOZUKA, T., YOKOTA, T., SHIMURA, Y. & SAKANO, Y. 2000. *Biosci. Biotechnol. Biochem.* **64**, 2692-2695.

KAMITORI, S., KONDO, S., OKUYAMA, K., YOKOTA, T., SHIMURA, Y., TONOZUKA, T. & SAKANO, Y. 1999. *J. Mol. Biol.* **287**, 907-921.

KONDO, S., OHTAKI, A., TONOZUKA, T., SAKANO, Y. & KAMITORI, S. 2001. *J. Biochem.* **129**, 423-428.

KONDO, S., KAJI, A., YUGUCHI, K.,TONOZUKA, T., SAKANO, Y. & KAMITORI, S. 2000. *Protein Peptide Lett.* **7**, 197-200.

YOKOTA, T., TONOZUKA, T., SHIMURA, Y., ICHIKAWA, K., KAMITORI, S. & SAKANO, Y. 2001a. *Biosci. Biotechnol. Biochem.* **65**, 619-626.

YOKOTA, T., TONOZUKA, T., KAMITORI, S. & SAKANO, Y. 2001b. *Biosci. Biotechnol. Biochem.* **65**, 401-408.

Diversity and evolution of α-amylase genes in Animals

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Many living organisms show a tendency to have multiple amylase genes. It is also true in Animals. In Drosophilids, the number of *Amy* copies is from two to at least seven, including the divergent paralog *Amyrel*. Comparisons between copies show that the divergence, the gene arrangement and the intron/exon structure are variable among copies within species. In addition, there are often differential expressions of the different copies. A survey of a number of animal *Amy* sequences shows that the number and position of introns is highly variable, between zero and nine introns. The inferred protein sequences also suggest some evolutionary events which could have adaptative or functional significance, such as the loss or gain of some amino acid stretches: for example, a motif of nine amino acids has been found in some species but not in others, independently from the phylogenetic relationships. Also, additional cysteins may create new disulfide bridges in some amylases.

Macromolecule recognition of *Bacillus stearothermophilus* neopullulanase

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Neopullulanase was the key enzyme to open the door for the formulation of the concept of α-amylase family (TAKATA et al., 1992, KURIKI & IMANAKA, 1999). The enzyme catalyzes both hydrolysis and transglycosylation at α-1,4- and α-1,6-glucosidic linkages by one active center (KURIKI et al., 1991; 1996). The different recognition of the neopullulanase toward amylose and amylopectin was preliminary reported previously (KURIKI et al., 1988). We describe here the mechanism of macromolecule recognition of the enzyme. The mixture of synthetic amylose and amylopectin from various sources was used as the substrate. Neopullulanase completely hydrolyzed amylose, but scarecely did amylopectin. Although the molecular weight of amylopectin (approx. 10^8) slightly deteriorated, the degradation of amylopectin completely halted at the molecular weight of approx. 10^7. This unique macromolecule recognition was also found in cyclomaltodextrinase from *Bacillus* sp. A2-5a (OHDAN et al., 2000) and maltogenic amylase from *Bacillus licheniformis* (KIM et al., 1992). The neopullulanase from *Bacillus megaterium*, which has quite similar primary structure of *Bacillus polymyxa* neopullulanse (YEBRA et al. 1999), Novamyl (maltogenic α-amylase from *Bacillus stearothermophilus*; DAUTER et al., 1999), α-amylases, and β-amylase did not exhibit this substrate-selectivity toward amylose and amylopectin at all. The mechanism of macromolecule recognition of the neopullulanase will be discussed correlating with the structures of amylose, amylopectin, and cyclomaltodextrin.

References

DAUTER, Z., DAUTER, M., BROZOZOWSKI, A.M., CHRISTENSEN, S., BORCHERT, T.V., BEIER, L., WILSON, K.S. & DAVIES, G.J. 1999. *Biochemistry* **38**, 8385-8392.

KIM, I.C., CHA, J.H., KIM, J.R., JANG, S.Y., SEO, B.C., CHEONG, T.K., LEE, D.S., CHOI, Y.D. & PARK K.H. 1992. *J. Biol. Chem.* **267**, 22108-22114.

KURIKI, T. & IMANAKA, T. 1999. *J. Biosci. Bioeng.* **87**, 557-565.

KURIKI, T., KANAKO, H., YANASE, M., TAKATA, H., SHIMADA, J., HANDA, S., TAKADA, T., UMEYAMA, H. & OKADA, S. 1996. *J. Biol. Chem.* **271**, 17321-17329.

KURIKI, T., OKADA, S. & IMANAKA, T. 1988. *J. Bacteriol.* **170**, 1554-1559.

KURIKI, T., TAKATA, H., OKADA, S. & IMANAKA, T. 1991. *J. Bacteriol.* **173**, 6147-6152.

OHDAN, K., KURIKI, T., TAKATA, H. & OKADA, S. 2000. *Appl. Microbiol. Biotechnol.* **53**, 430-434.

TAKATA, H., KURIKI, T., OKADA, S., TAKESADA, Y., IIZUKA, M., MINAMIURA, N. & IMANAKA, T. 1992. *J. Biol. Chem.* **267**, 18447-18452.

YEBRA, M.J., BLASCO, A. & SANZ, P. 1999. *FEMS Micobiol. Lett.* **170**, 41-49.

Independent folding of the A and B domains in α-amylase family enzymes deduced from the geometrical constraints on the evolution of the TIM-barrel β-sheet

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The α-amylase family of enzymes have different activities but a common ancestor. Their polypeptide chain has always a multi-domain arrangement (although the number of domains usually depends on the enzyme activity). The domains that we call A and B are always found in all of these structures. The A domain, which is the catalytic domain, is always a TIM-barrel fold. The function of the B domain, which lies between the third β-strand and the third α-helix of the A domain, has not been fully established. Its sequence length and fold are variable (JANECEK et al., 1996). No one has yet studied how the variability of the B domain affects the folding of the A domain. This is the aim of our poster. To do this, we have studied the geometrical characteristics of the eight-stranded β-sheet at the core of the A domain. Some authors have described the packing of side chains within the closed β-sheet as one of the most important factors for maintaining the TIM-barrel structure (LESK et al., 1989). This packing may be described as a scaffold made up of a set of layers that are perpendicular to the axis of the barrel (PUJADAS et al., 1996). All crystallized enzymes from family 13 have a four-layered scaffold inside their TIM barrel. Each layer is made up of four residues that belong to alternate strands (one residue for each strand). The side chains of these residues face the inside of the barrel (layers 2 and 3 are responsible for the side-chain packing inside the β-sheet). The carbon atoms in the α position (CA) of the four residues that form a layer are roughly located in the same plane. Each β-strand has two alternate residues in two layers, whereas the side chain of the middle residue faces the external helices or coils. The parameters for describing the geometry of a layer are: (i) the distance between opposite CA atoms, (ii) the angle between the two segments that connect opposite CA atoms, and (iii) the area (which becomes elliptical). Our results show that these geometrical parameters do not depend on the length or fold of the B domain, and support the idea of an independent folding pathway for the A and B domains in α-amylase enzymes. Unwanted mutations that produce a different barrel geometry may be recognised by molecular chaperones and discarded as functional molecules. Preliminary results suggest that family 77 enzymes have the same barrel geometry as family 13 enzymes, which supports the hypothesis that they have a common origin.

Acknowledgment
With this talk, Gerard PUJADAS would like to pay tribute to Prof. PALAU, who, with these results, made his final contribution to science.

References

JANECEK, S., SVENSSON, B. & HENRISSAT, B. 1996. *Chemicke Listy* **45**, 322-331.
LESK, A.M., BRÄNDÉN, C. I. & CHOTHIA, C. 1989. *Proteins* **5**, 139-148.
PUJADAS, G., RAMÍREZ, F.M., VALERO, R. & PALAU, J. 1996. *Proteins* **25**, 456-472.

How many conserved sequence regions are there in the α–amylase family?

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The α-amylase family as the family of enzymes catalysing the hydrolysis and/or synthesis of α-1,4- and/or α-1,6-glucosidic bonds was defined in 1992 (TAKATA et al., 1992). However, it was clear a few years ago that such a family exists, e.g., as new enzyme specificities were sequenced: cyclodextrin glucanotransferase (BINDER et al., 1986), isoamylase (AMEMURA et al., 1988), neopullulanase (KURIKI & IMANAKA, 1989), or also by comparative studies (HENRISSAT, 1991; JESPERSEN et al., 1991). Now it has become evident that the α-amylase family includes the enzymes acting on sucrose and trehalose, i.e. the saccharides containing the α-1,5 and α-1,1-glucosidic bonds, respectively (MACGREGOR et al., 2001). Moreover, there exists a group of proteins having transport and antigenic functions with sequences remarkably closely related to the enzymes from the α-amylase family (JANEČEK, 2000a).

One of the most interesting features of the α-amylase family is that its members contain a few very well conserved sequence regions despite the overall low sequence similarity (below 10%). It seems that only 3 residues (catalytic β4-Asp, β5-Glu and β7-Asp) may be totally invariantly conserved throughout the family (JANEČEK, 2000b). Concerning the conserved sequence regions 4 regions covering the catalytic and/or substrate-binding residues have been established (NAKAJIMA et al., 1986) and accepted for the definition of the α-amylase family (TAKATA et al., 1992). Historically, three of the four regions were known in 1985 for the α-amylases from bacteria, fungi, plants and animals: firstly pointed out by TODA et al (1982) for Taka-amylase A and pig pancreatic α-amylase, later by FRIEDBERG (1983) who added *Bacillus amyloliquefaciens* α-amylase, and finally by ROGERS (1985) by adding the barley α-amylase.

Among the conserved sequence regions or similarities additional to the four ones established by NAKAJIMA et al. (1986), two cover roughly the strands β2 and β8 of catalytic $(\beta/\alpha)_8$-barrel and one is located near the C-terminus of domain B (JANEČEK, 2000b). Thus, for instance, the region covering the strand β2 may be helpful in discriminating the true α-amylases from cyclodextrin glucanotransferases, whereas the short stretch from domain B may indicate the calcium binding requirements by an enzyme from the α-amylase family. All the three additional conserved sequence regions contain the amino acid residues specifically connected to a given enzyme specificity. This knowledge could be used in the rational protein design focused on tailoring the new properties of enzymes from the α-amylase family according to industrial requests.

References

AMEMURA, A., CHAKRABORTY, R., FUJITA, M., NOUMI, T. & FUTAI, M. 1988. *J. Biol. Chem.* **263**, 9271-9275.
BINDER, F., HUBER, O. & BÖCK, A. 1986. *Gene* **47**, 269-277.
FRIEDBERG, F. 1983. *FEBS Lett.* **152**, 139-140.
HENRISSAT, B. 1991. *Biochem. J.* 280, 309-316.
JANEČEK, Š. 2000a. *Trends Glycosci. Glycotechnol.* **12**, 363-371.
JANEČEK, Š. 2000b. In: *Glycoenzymes* (OHNISHI, M., HAYASHI, T., ISHIJIMA, S. & KURIKI, T., eds.), pp. 19-54, Japan Scientific Societies Press, Tokyo.
JESPERSEN, H.M., MACGREGOR, E.A., SIERKS, M.R. & SVENSSON, B. 1991. *Biochem. J.* **280**, 51-55.
KURIKI, T. & IMANAKA, T. 1989. *J. Gen. Microbiol.* **135,** 1521-1528.
MACGREGOR, E.A., JANEČEK, Š. & SVENSSON, B. 2001. *Biochim. Biophys. Acta* **1546**, 1-20.
NAKAJIMA, R., IMANAKA, T. & AIBA, S. 1986. *Appl. Microbiol. Biotechnol.* **23**, 355-360.
ROGERS, J.C. 1985. *Biochem. Biophys. Res. Commun.* **128**, 470-476.
TAKATA, H., KURIKI, T., OKADA, S., TAKESADA, Y., IIZUKA, M., MINAMIURA, N. & IMANAKA, T. 1992. *J. Biol. Chem.* **267**, 18447-18452.
TODA, H., KONDO, K. & NARITA, K. 1982. *Proc. Japan Acad.* **B58**, 208-212.

Lost, shifted or shuffled introns during evolution of α-amylase genes

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α-Amylase is present in all groups of animals and characterized by a β/α eight-barrel three dimensional structure. Phylogeny inferred from cDNAs or aminoacid sequences revealed some interesting results especially concerning the position of *Drosophila*. Moreover, the structure of their genes with one or absence of introns is particular. To understand better these particularities, different hypothesis could be enphasized such as the disparition of introns during evolution of insect group or the specific and (or) rapid adaptation to new substrates.

Intron position were determined in crustaceans and other groups such as molluscans. Among Arthropoda, Crustacea penaeides are very ancient as already present in Devonian. The structure of their amylase genes revealed the presence of nine introns. Position and phase of some of these introns are recovered in other insects such as *Ostrinia* where 4 of them are still present. Two are also present in *Ceratitis* and in drosophila family, the second one (according to *Penaeus* numbering) is present in most of drosophilae and the fifth, present in Amyrel from *D. melanogaster,* with the same intron phase. These results are in favor of the disappearance of introns during evolution of arthropoda.

In molluscans, the position and the phase of intron 2 and 3 are conserved while in vertebrates intron 2 and 4 are also at the same position. A shift was determined with the position of some introns, this shift could be very short, such as for intron 3 in human, or longer. These changes were reported mainly in the 5'of the genes suggesting inplication of polymerases. Many introns appeared later in evolution at a non specific position in the end of the gene, where the stuctural components are of less importance.

The position of these structural elements has been of importance, not only for the protein function but also in the structuration of the genes. More observation however are necessary to confirm these hypotheses, taking into account more primitive eukaryots in the different groups such as poriferes, cnidaires, ecdysozoa and lophotrozoa, based on the latest classification and other members of α-amylase family.

SESSION 3
CATALYTIC AND BINDING MECHANISM

Structural investigations of the catalytic mechanism of cyclodextrin glycosyltransferase

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Cyclodextrin glycosyltransferase (CGTase) is an enzyme which belongs to the α-amylase family. It catalyzes the formation of circular α-(1,4)-linked oligoglucosides called cyclodextrins from linear α-(1,4)-linked glucans such as starch. CGTases are typically 75-kDa bacterial proteins consisting of 5 domains (A-E). Domains A and B constitute the catalytic domains, whereas C and E are specialized in binding to raw starch granules. The reaction mechanism is a double displacement process, which proceeds in two steps. In the first step CGTase cleaves an α-(1,4)-glycosidic bond in its substrate, and forms a covalent β-(1,4)-linked glycosyl intermediate. In the second step, the covalent bond of the intermediate is cleaved, and an α-(1,4)-glycosidic bond is reformed with an acceptor, such as water or the 4-OH group of another sugar residue. We have determined X-ray structures of CGTase in complex with an intact substrate, and of CGTase with a covalently bound reaction intermediate (UITDEHAAG et al., 1999; 2000). These structures provide the first structural evidence for the occurrence of a covalent intermediate in the reaction mechanism of the α-amylase family. Furthermore, they give evidence for substrate distortion and show in atomic detail how catalysis in the α-amylase family proceeds by the concerted action of all active site residues.

CGTases are unique in that they can use the 4-OH group at the non-reducing end of the covalently bound sugar intermediate as the acceptor in the reaction. However, the X-ray structure of the covalent intermediate shows that the sugar chain is bound in a linear way and that the non-reducing end is about 23 Å away from the active site. To analyze how the enzyme makes the non-reducing end move to the active site, we determined the X-ray structure of CGTase in complex with a γ-cyclodextrin, and applied molecular dynamics to simulate the transition from the linear intermediate to the cyclic product (UITDEHAAG et al., 2001). The circularization occurs at the surface of the enzyme molecule, and several residues are engaged to catalyze this process. Site-directed mutagenesis of these residues demonstrates their importance for the cyclization reaction, and thus supports our simulation of the circularization pathway.

References

UITDEHAAG, J.C.M., MOSI, R., KALK, K.H., VAN DER VEEN, B.A., DIJKHUIZEN, L., WITHERS, S.G. & DIJKSTRA, B.W. 1999. *Nature Struct. Biol.* **6**, 432-436.

UITDEHAAG, J.C.M., VAN ALEBEEK, G.-J.W.M., VAN DER VEEN, B.A., DIJKHUIZEN, L. & DIJKSTRA, B.W. 2000. *Biochemistry* **39**, 7772-7780.

UITDEHAAG, J.C.M., VAN DER VEEN, B.A., DIJKHUIZEN, L., ELBER, R. & DIJKSTRA, B.W. 2001. *Proteins: Struct. Funct. Genet.* **43**, 327-335.

A possible mechanism of catalysis involving three essential residues in α-amylase family enzymes

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The α-amylase family enzymes always carry strictly conserved three essential catalytic residues; Glu230, Asp206 and Asp297 in terms of TAA (Taka-amylase A) numbering. The elucidation of the functional roles of these residues has been a focus of attention in the structure-function studies on the family enzymes. Of these three residues, the roles of Glu230 and Asp206 have been generally accepted as working for acid (proton donor) and base (nucleophile) catalyst, respectively. The catalytic mechanism has been discussed mostly on the basis of these two residues. However, the critical role of the third residue Asp297 seems to be still undefined and under dispute (SVENSSON, 1994), except the facts that it is regularly involved in the substrate binding (QIAN et al., 1994; STROKOPYTOV et al., 1996; YOSHIOKA et al., 1997; FUJIMOTO et al., 1998).

In the structure analyses of *P. stutzeri* maltotetraose-forming amylase, we have determined the structures of maltotetraose complexed (though we used maltopentaose in cocrystallization) five enzymes in each of which single catalytic residue was mutated (YOSHIOKA et al., 1997; HASEGAWA et al., 1999). The mutants used were E219Q, E219G, D193N, D193G and D294N; the respective residue numbers 219, 193 and 294 corresponding to 230, 206 and 297 in TAA. The bound maltotetraose was found to occupy the sub-sites –1 to –4 in any case. The differences in the complexed structures were most prominent at the –1 site (reducing end of maltotetraose). In the mutants of E219Q, E219G, D193N and D193G, the glucose ring at this site are deformed adopting half-chair form, however, in the D294N complex the deformation disappeared. This glucose unit is firmly bound by about ten hydrogen bond pairings from surrounding polypeptide and waters, which is necessary for suffering bond cleavage. However, the fact that the deformation was not observed in D294N mutant cannot be overlooked. It is an indication that the strength of bonds from the surroundings in this mutant is not sufficient to impose deformation. This is supported by the fact that the occupancy of the bound maltotetraose was decreased in D294N than in the other mutants. Thus we may draw conclusion that the conversion of Asp294 to Asn considerably lowered the binding constant of substrate to enzyme, resulting in loss of the excess force to deform the glucose ring.

These findings lead us to the understanding on the role of Asp297 (TAA numbering) as follows: It is indispensable for activity by causing the deformation of the glucose ring at –1 site by firmly binding the substrate, and we may regard this residue playing a role to deserve to be called as "fixer" in the catalytic process in hydrolyzing amylose (HASEGAWA et al., 1999).

In the talk, the author will also refer to the intramolecular transglycosylation mechanism in maltooligosyl trehalose synthase whose structure we have determined recently.

References

FUJIMOTO, Z., TAKASE, K., DOUI, N., MOMMA, M., MATSUMOTO, T. & MIZUNO, H. 1998. *J. Mol. Biol.* **277**, 393-407.
HASEGAWA, K., KUBOTA, M. & MATSUURA, Y. 1999. *Protein Eng.* **12**, 819-824.
QIAN, M., HASER, R., BUISSON, G., DUEE, E. & PAYAN, F. 1994. *Biochemistry* **33**, 6284-6294.
STROKOPYTOV, B., KNEGTEL, R., PENNINGA, D., ROZEBOOM, H., KALK, K., DIJKHUIZEN, L. & DIJKSTRA, B. 1996. *Biochemistry* **35**, 4241-4249.
SVENSSON, B. 1994. *Plant Mol. Biol.* **25**, 141-157.
YOSHIOKA, Y., HASEGAWA, K., MATSUURA, Y., KATSUBE, Y. & KUBOTA, M. 1997. *J. Mol. Biol.* **271**, 619-628.

Cold-active α-amylase: structural analyses, molecular adaptation, mechanistic aspects and a remarkable tool for novel applications

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Among the extremophile microrganisms, those which operate in very cold environments secrete psychrophilic enzymes, which indeed are working very well under these extreme conditions (RUSSELL, 1998). In fact, these enzymes differ from their mesophilic counterparts by displaying a higher catalytic efficiency at low temperature. They have therefore considerable potential in biotechnological applications. In order to gain a better understanding of their nature and mode of action, we have undertaken X-ray crystallographic studies for several psychrophilic enzymes from Antarctic bacteria. The main goal is to elucidate the various factors which control and govern adaptation to cold of these enzymes, and at the same time allow high catalytic activity in these extreme temperature conditions. Up to now, three types of enzymes have been studied in our laboratory, a calcium/zinc metalloprotease, an α-amylase and a phosphoglycerate kinase (PGK).

In the case of the bacterial α-amylase, its 3-D structure in its native state as well as in complex with various inhibitor substrate analogues have been determined at high resolution. Several structures of mutants have also been established to improve our understanding of the structure/function relationships of this enzyme (AGHAJARI et al., 1998a, 1998b; AGHAJARI & HASER, 1999; AGHAJARI, N., FELLER, G., GERDAY, C. & HASER, R., in preparation). With this extensive structural information, we can now describe at a molecular level the catalytic machinery and process which leads to the degradation of sugar substrate chains, as well as the aspects of inhibition, activation and the transglycosylation events specific to this enzyme. Remarkably this enzyme, in the crystalline state, is able to perform a transglycosylation reaction (AGHAJARI, N., ROTH, M. & HASER, R., submitted) leading to the synthesis of a new type of oligosaccharide, of potential interest for inhibition of α-glycosidases in general and for therapeutic aspects (treatment of diabetes, obesity...).

The same kind of approach has been applied to the bacterial metalloprotease and very recently to PGK. On the basis of the X-ray crystallographic analyses, the detailed comparative studies of these "cold" structures with their mesophilic and thermostable counterparts indeed highlights factors which most probably are crucial for cold adaptation of enzymes, and which confer a higher overall intrinsic flexibility in order to optimize the catalytic efficiency of these molecules.

The perspectives envisioned on the basis of these results point in several directions: in terms of fundamental research more three-dimensional structures of psychrophiles are now on the way in different laboratories to further improve the understanding of determinants of psychrophily and of high activity at low temperatures; further studies of psychrophilic systems in conjunction with mutagenesis experiments will certainly contribute to render them more thermostable and thus accelerate the use of cold loving enzymes in various applications.

References

RUSSELL, N.J. 1998. In: *Advances in Biochemical Engineering Biotechnology* (SCHEPER, T., ed.), pp 1-21, Springer-Verlag, Berlin.

AGHAJARI, N., FELLER, G., GERDAY, C. & HASER, R. 1998. *Protein Sci.* **7**, 564-572.

AGHAJARI, N., FELLER, G., GERDAY, C. & HASER, R. 1998. *Structure* **6**, 1503-1516.

AGHAJARI, N. & HASER, R. 1999. In: *Cold-adapted Organisms: Ecology, Physiology, Enzymology and Molecular Biology* (MARGESIN, R. & SCHINNER, F., eds), pp. 277-296, Springer-Verlag, Berlin.

Effect of N-terminal and C-terminal regions on the oligomerization properties of cyclodextrin-/pullulan degrading enzymes

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Cyclodextrin-/pullulan degrading enzymes catalyze the hydrolysis and transglycosylation reactions with various substrates such as starch, cyclodextrin (CD), and pullulan. Recently, it has been shown that these enzymes exist in equilibria of monomer-dimer and monomer-dimer-tetramer or -dodecamer. In CD-/pullulan degrading enzymes, we have identified two regions involving in oligomerization; one in the N-terminal region and the other in C-terminal region. The three-dimensional structure and deletion mutagenesis experiment revealed that the N-terminal region affected the dimerization properties of the monomeric enzyme. Unlike dimerization, dodecamerization of the dimeric units was related with the C-terminal region of the enzyme. We have also shown that the multisubstrate specificity was highly dependent on the oligomeric state in this group of enzymes. In addition, the oligomerization properties of five different enzymes representing this subgroup are examined by comparison of amino acid sequences in N-terminal and C-terminal regions to illustrate the relationship between the quaternary structure and the specificity of the enzyme.

Mechanism of porcine pancreatic α-amylase (PPA): inhibition of amylose and maltopentaose hydrolysis by various inhibitors

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The effect of the pseudosaccharidic inhibitor acarbose, the substrate analogue, cyclodextrin, and the phaseolus protein inhibitor, α-AI on amylose and maltopentaose PPA catalysed hydrolysis was studied. Statistical analysis of the kinetic data was performed. Acarbose, which mimics the transition state, is a strong inhibitor: the inhibition is of the mixed non-competitive type involving abortive complexes. According to the model proposed two carbohydrate-binding secondary sites (in addition to the active site) are needed for amylose hydrolysis while only one extra site is required for DP-18 and maltopentaose hydrolysis.

Cyclodextrins whose structure is similar to one helical turn of the amylose chain are much weaker inhibitor of the amylose and maltopentaose hydrolysis. The type of inhibition depends on the substrate used. The inhibition of amylose hydrolysis is of the competitive type while the inhibition of maltopentaose is of the mixed non-competitive type, one secondary site is postulated. This site is very likely the same than the acarbose one. Spectrum difference data indicate that one Trp possibly Trp 134 or Tyr 174 is present at each secondary binding site (LARSON et al., 1994; KOUKIEKOLO et al., 2001).

The Phaseolus protein alpha-AI is a strong inhibitor of both amylose and maltopentaose hydrolysis. With both substrates the inhibition is of the mixed non-competitive type involving two secondary binding sites.

X-ray studies of the crystallized amylase-inhibitor/substrate complex (QIAN et al., 1994, 1995; LARSON et al., 1994) also show the secondary carbohydrate binding sites in addition to the active site. These binding sites might play a role either at the substrate entrance or at the product exit, they might also participate to displacement of the amylose chain in the multiple attack mechanism.

References

KOUKIEKOLO, R., DESSEAUX, V., MOREAU, Y., MARCHIS-MOUREN, G. & SANTIMONE, M. 2001. *Eur. J. Biochem.* **268**, 841-848.

LARSON, S.B., GREENWOOD, A., CASCIO, D., DAY, J. & MCPHERSON, A. 1994. *J. Mol. Biol.* **235**, 1560-1584.

QIAN, M., HASER, R., BUISSON, G., DUEE, E. & PAYAN, F. 1994. *Biochemistry* **33**, 6284-6294.

QIAN, M., HASER, R. & PAYAN, F. 1995. *Protein Sci.* **4**, 747-755.

Amylolytic activity of IgG and sIgA immunoglobulins from human milk

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We investigated IgG fractons from patients suffering from multiple sclerosis as well as IgG and sIgA fractions isolated from human milk of healthy women in the hydrolysis of different substrates including maltooligosaccharides with different degree of polymerization (DP), p-nitrophenyl (PNP) maltosides with various DP ranging from 2 to 7. All analyzed electrophoretically homogeneous preparations of IgG and its Fab fragments as well as sIgA antibodies possess α-amylolytic activity. The specific activities of catalytic antibodies from human milk varied in the range from 0.11 up to 0.2 U/mg that is about three orders higher than that for IgGs from the sera of multiple sclerosis patients and one order higher that that for cancer patients. Milk IgG and sIgA fractions revealed Michaelis constants in hydrolysis of 4-nitrophenyl 4,6-O-ethylidene-α-D-maltoheptaoside in a range of 10^{-4} M. Fractions of autoantibodies from various donors revealed different mode of action in the hydrolysis of maltooligosaccharides, PNP maltooligosaccharides, and PNP-α-D-glucopyranoside.

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SESSION 4
NEW THREE-DIMENSIONAL STRUCTURES

Crystal structures of maltogenic amylase and cyclomaltodextrinase

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Maltogenic amylase, cyclomaltodextrinase (CDase), and neopullulanase share a significant sequence homology to each other. They are distinguished from small molecular weight α-amylases in that they contain an extra N-terminal domain composed of ~120 amino acids. They prefer cyclomaltodextrins (CDs) as substrates to soluble starch. Crystal structure of a maltogenic amylase from a *Thermus* species (ThMA) reveals that the enzyme is a homodimer, whose dimerization is mostly mediated by the N-terminal domain. The enzyme has a narrower and deeper active site cleft compared with those of smaller α-amylases. A modeling study shows that β-cyclomaltodextrin fits to the active site cleft snugly, indicating that helical amylose with a width wider than that of CDs would not easily fit to the cleft. We also determined the structure of a cyclomaltodextrinase which reveals a dodecameric assembly of the enzyme. The dodecameric structure can be described as a hexamer of dimer. The dimerization pattern of ThMA is conserved in the dimeric unit of the CDase. It is noted that the C-terminal domain plays a prominent role in the hexamerization of the enzyme. Solution studies also showed that the enzyme exists as a dodecamer at low salt concentration. The dodecameric structure explains why the enzyme exhibits higher efficiency in degrading CDs than the dimeric ThMA.

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References

KIM, J.-S., CHA, S.-S., KIM, H.-J., KIM, T.-J., HA, N.-C., OH, S.-T., CHO, H.-S., CHO, M.-J., KIM, M.-J., LEE, H.-S., KIM, J.-W., CHOI, K.Y., PARK, K.-H. & OH, B.-H. 1999. *J. Biol. Chem.* **274**, 26279-26286.

X-Ray crystallographic studies of *Escherichia coli* branching enzyme

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There are three reactions concerned with the synthesis of the α-1,4 and α-1,6 linkages found in plant starch and in bacterial glycogen. In the first reaction the glucosyl donor for starch synthesis, ADP-glucose, is formed by the catalytic action of ADP-glucose pyrophosphorylase. The second reaction concerns the synthesis of the α-1,4 linkages and elongation of the oligo-saccharide chains of glycogen and starch and is catalyzed in plants by starch synthases and in bacteria by glycogen synthase using ADP-glucose as the glucosyl donor. The third reaction, the synthesis of α-1,6 linkages, is catalyzed by branching enzyme (BE; 1,4-α-D-glucan: 1,4-α-D-glucan 6-α-(1,4-α-glucano) transferase; EC 2.4.1.18). BE belongs to the group of enzymes known as the α-amylase family sharing with enzymes of the group 7 conserved amino acid residues in the (α/β) barrel domain that are necessary for enzyme activity. The properties of plant and bacterial branching enzymes vary with respect to size of oligosaccharide chain transferred and with respect to their substrate preference. Maize endosperm branching enzyme I (BEI) prefers to branch amylose rather than amylopectin and mainly transfers oligosaccharide chains of 12-14 to greater than 40 glucose units long. Maize BEII prefers amylopectin and mainly transfers chains an average of 6-7 to glucose units long. Chimeric (hybrid) constructs from these isozymes have been made which have altered branching properties as compared to the normal enzymes and indicate that the C terminal end of the enzyme may be involved in catalysis and substrate preference and the N-terminal end may be involved in determining the length of oligosaccharide chain to be transferred. The BE of *E. coli* has also been studied. A truncated form having 112 amino acids deleted from the N-terminal was constructed which had a 3- to 4-fold lower catalytic efficiency than the native form. Recent results indicate that the truncated form has an altered chain transfer pattern than the wild-type enzyme. It transfers relatively fewer chains of 5-11 glucose units long and more chains longer than 12 glucose units long. This result is consistent with the view that the N-terminal is involved in size of glucan transferred. The truncated *E. coli* BE has been crystallized and high resolution data of 2.3 Å has been collected. Phasing information was obtained using isomorphous replacement and anomalous dispersion methods. The techniques of solvent flattening and four-fold non-crystallographic averaging improved the quality of the electron density map. The central (α/β) domain in BE has been established. All of the seven conserved residues necessary for activity in the α-amylase family are located in the BE (α/β) domain and the (α/β) domain and C-terminal region are structurally similar to isoamylase. Availability of BEs with altered properties can be used for transformation of plants to produce a starch with altered structure. Starch with altered structure can have properties which may be beneficial in developing new food and non-food products of economic value.

Structural basis for substrate recognition and specificity differences of barley α-amylase isozymes

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The germinating barley seed contains two major α-amylase isozyme families AMY1 and AMY2 involved in starch degradation to provide energy used by the plant embryo for growth. The three-dimensional structure of AMY2 has been solved, both in the native state (KADZIOLA et al., 1994) and in complex with acarbose (KADZIOLA et al., 1998), a pseudo-tetrasaccharide acting as a powerful inhibitor for several glycosidases. Furthermore, an endogenous protein inhibitor (BASI, 19 kDa) present in the barley seeds has been shown to be bifunctional as it inhibits both a protease from the subtilisin family and AMY2, but not AMY1. The complex AMY2-BASI has been crystallized and its structure was solved at 1.9 Å resolution (VALLÉE et al., 1998). The crystal structure of AMY1 could recently be established at high resolution (1.5 Å) (ROBERT, X. et al., in preparation), and an extended analysis of carbohydrate interactions has been performed with enzyme/substrate and enzyme/inhibitor complexes with crystals of various mutants. This now allows a direct comparison of the differences in substrate recognition and specificity on the basis of the 3D structures for both isozymes. In fact, the detailed analysis of the structural consequences of the amino acid mutations clearly shows that the organization of both isozymes is virtually identical, and that local changes are very small.

However and remarkably, these structures behave very differently when interacting with substrates and inhibitors. A subtle, but very significant conformational change occurs when sugars bind, leading to the identification of an extra sugar recognition site in AMY1, compared to AMY2. Inspection of the various complexes demonstrates the crucial role of a tyrosine and its movement in AMY1 when interacting with substrate. This reorganization is the major structural difference between AMY1 and AMY2 when acting on polysaccharides, and appears to be the consequence of a very few mutations in the direct vicinity of the above key tyrosine. Interestingly, the finding of an extra carbohydrate site in AMY1 is fully consistent with an increased affinity and hydrolytic activity already observed of that enzyme when acting on starch granules, compared to AMY2. Finally, only a few mutations, close to this new carbohydrate site suggested to contribute to starch granule binding, appear to govern the fine-tuning of these interactions and to be the major determinant for isozyme specificity.

A further analysis of the structural and isozyme-specific differences in terms of substrate recognition and specificity will be presented, with focus on the functional significance of the various mutations between the two isozymes.

References

KADZIOLA, A., ABE, J., SVENSSON, B. & HASER, R. 1994. *J. Mol. Biol*. **239**, 104-121.

KADZIOLA, A., SØGAARD, M., SVENSSON, B. & HASER, R. 1998. *J. Mol. Biol*. **278**, 205-217.

VALLÉE, F., KADZIOLA, A., JUY, M., BOURNE, Y., RODENBURG, K., SVENSSON, B. & HASER, R. 1998. *Structure* **6**, 649-659.

Three dimensional structure of *Bacillus stearothermophilus* neopullulanase

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Neopullulanase from *Bacillus stearothermophilus* hydrolyzes not only α-1,4-glucosidic linkages but also α-1,6-glicosidic linkages of several branched oligosaccharides (TAKATA et al., 1992). The reaction of the enzyme on pullulan produces panose, maltose, and glucose, the final ratio being 3:1:1. This enzyme has also been shown to hydrolyze cyclodextrin. It is one of multispecific enzymes that is classified into cyclomaltodextrinase (PARK et al., 2000). The three dimensional structures of the enzymes in this group have been determined for maltogenic amylase (KIM et al., 1999) and TVAII (KAMITORI et al., 1999). These enzymes commonly possess unique N-terminal domain, which is not present in usual α-amylase. This paper describes the three dimensional structure of neopullulanase and compares to other enzymes in the same group, focusing on the elucidation of the relative specificity of the catalytic reaction.

The crystallization was performed by the hanging drop method with protein concentration of 10 mg/ml and 18% PEG8000 as precipitant in 100mM sodium cacodylate buffer solution at pH6.5. The crystal grew to a typical size of 0.3 mm in two weeks. The intensity data were collected by using R-AXIS IV to a resolution of 2.4 Å. The structure was determined by the molecular replacement method using TVAII (PDB code: 1BVZ) as a search model. The refinement of the structure was carried out by the program X-PLOR at 2.4 Å resolution to a final R of 20.2%.

The molecule is composed of four domains: N(1-123), A(124-246, 300-506), B(247-299), and C(507-588). Domain N consists of antiparallel eight-stranded β-sheet. Domain A contains a $(\beta/\alpha)_8$ barrel structure, and B one α-helix and the other non-typical structures. Domain C also consists of antiparallel eight-stranded β-sheet. In the crystal the two molecules are associated to form a dimer with folding N and B domains with each other. The dimer formation is also confirmed in the solution state by the ultracentrifugation method. Though this enzyme has similar structural features with maltogenic amylase and TVAII, there are some significant differences as well. In the domain B near the active site, there is a consistent sequence 'FAF' or 'FGF' present among this family enzymes, however, in neopullulanase it is 'FRF'. This difference may have some significant meanings with respect to the difference in the catalytic specificities. The structure analysis of the substrate analogue complexed enzyme is underway, and will be presented in the talk.

References

KAMITORI, S., KONDO, S., OKUYAMA, K., YOKOTA, T., SHIMURA, Y., TONOZUKA, T. & SAKANO, Y. 1999. *J. Mol. Biol.* **287**, 907-921.

KIM, J.-S., CHA, S.-S., KIM, H.-J., KIM, T.-J., HA, N.-C., OH, S.-T., CHO, H.-S., CHO, M.-J., KIM, M.-J., LEE, H.-S., KIM, J.-W., CHOI, K.-Y. & PARK, K.-H. 1999. *J. Biol. Chem.* **274**, 26279-26286.

PARK, K.-H., KIM, T.-J., CHEONG, T.-K., KIM, J.-W., OH, B.-H. & SVENSSON, B. 2000. *Biochim. Biophys. Acta* **1478**, 165-185.

TAKATA, H., KURIKI, T., OKADA, S., TAKESADA, Y., IIZUKA, M., MINAMIURA, N. & IMANAKA, T. 1992. *J. Biol. Chem.* **267**, 18447-18452.

Crystal structure of an α-amylase from the hyperthermophilic archaeon *Pyrococcus woesei*

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α-Amylases (1,4-α-D-glucan glucanohydrolase; EC 3.2.1.1) belong to a widespread group of enzymes hydrolyzing the α-1,4-glucosidic bonds in glycogen, starch, and related polysaccharides. To date, several structures of α-amylases belonging to all the three domains of life, Eucarya, Eubacteria, and Archaea, have been solved and provide a useful tool for comparative studies (PUJADAS & PALAU, 2001). Hyperthermophilic archaea proliferate at temperatures around 80-100°C (HORIKOSHI & GRANT, 1998) and their proteins isolated so far show considerable thermal stability and seem to become increasingly important for the investigation of fundamental processes in structure biology such as folding and stability of proteins. To investigate the role of factors associated with elevated temperature stability, we determined the crystal structure of an α-amylase from the hyperthermophile *Pyrococcus woesei* (optimal growth at 102°C) to a resolution of 2.2 Å. This enzyme shows a very high thermostability (50% residual activity after 2.7 h incubation at 120 °C) (LINDEN et al., 2000). The orthorombic crystal belongs to the space group P212121 with unit cell parameters a = 62.9 Å, b = 78.1 Å, c = 106.3 Å, and α = β = γ = 90°, with one molecule per asymmetric unit. In addition, crystals were obtained in presence of the α-amylase inhibitor acarbose (Gift: Bayer AG, Leverkusen) and a dataset was collected to 1.7 Å. The unit cell parameters were a = 51.5 Å, b = 76.51 Å, c = 136.46 Å, and α = β = γ = 90°, with one molecule per asymmetric unit. Interestingly, this α-amylase contains five cysteine residues, with three cysteines located in the domain B of the amylase structure. Four of these cysteine residues form intramolecular disulfide bridges. Furthermore, although the primary sequence of the *P. woesei* α-amylase does not show the conservation of calcium-liganding residues as found in other family 13 amylases and the protein does not require calcium ions for activity or thermostability (JØRGENSEN et al., 1997; LINDEN et al., 2000), there is a strong indication for the presence of at least one calcium binding site in the molecule.

References

HORIKOSHI, K. & GRANT, W.D. 1998. *Extremophiles – Microbial Life in Extreme Environments.* Wiley-Liss, New York.

JØRGENSEN, S., VORGIAS, C.E. & ANTRANIKIAN, G. 1997. *J. Biol. Chem.* **272**, 16335-16342.

LINDEN, A., NIEHAUS, F. & ANTRANIKIAN, G. 2000. *J. Chromatogr. B* **737**, 253-259.

PUJADAS, G. & PALAU, J. 2001. *Mol. Biol. Evol.* **18**, 38-54.

SPECIAL BANQUET LECTURE OF THE SYMPOSIUM

Comparative genomics and the metabolism of starch

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With the growing amount of sequence and biochemical data, the number of families of glycoside hydrolases and glycoside transferases has grown over the years. As of today, there are 85 families of glycoside hydrolases and over 50 families of glycosyltransferases. Although it is likely that other families will be discovered, it is reasonable to assume that the most important families are already identified and that general inferences on the role and importance of carbohydrates in different organisms can be made. The sequence-based families of glycoside hydrolases and glycoside transferases are accessible via a regularly updated www server at URL: http://afmb.cnrs-mrs.fr/~pedro/CAZY/db.html (COUTINHO & HENRISSAT, 1999). An advantage of the sequenced-based families is that they can be readily searched in genomes, allowing a global and detailed comparison of the glycoside hydrolase/glycosyltransferase repertoire of various organisms at a genomic scale. Family assignment can constitute an effective conservative intermediate step in functional analysis that drastically limits missassignments resulting from the over- or under-prediction of enzyme properties, and that restricts enzyme functionality to specific reaction types.

Here we will present the results of a comparative analysis of all glycoside hydrolases and glycosyltransferases in a selection of 21 complete genomes covering Archaea, Bacteria and Eukaryotes (Table 1) with a special emphasis on enzymes involved in starch/glycogen biosynthesis and biodegradation.

Table 1. List of the genomes analysed for glycoside hydrolases and glycosyltransferases.

Organism	Kingdom
Aeropyrum pernix	Archaea
Methanococcus jannaschii	Archaea
Archaeoglobus fulgidus	Archaea
Halobacterium sp. NRC-1	Archaea
Methanobacterium thermoautotrophicum	Archaea
Pyrococcus horikoshii	Archaea
Thermoplasma acidophilum	Archaea
Pyrococcus abyssi	Archaea
Haemophilus influenzae	Bacteria
Aquifex aeolicus	Bacteria
Deinococcus radiodurans	Bacteria
Mycobacterium tuberculosis	Bacteria
Thermotoga maritima	Bacteria
Escherichia coli	Bacteria
Synechocystis sp.	Bacteria
Bacillus subtilis	Bacteria
Saccharomyces cerevisiae	Eukaryote
Drosophila melanogaster	Eukaryote
Caenorhabditis elegans	Eukaryote
Arabidopsis thaliana	Eukaryote
Homo sapiens	Eukaryote

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References

COUTINHO, P.M. & HENRISSAT, B. 1999. In: *Recent Advances in Carbohydrate Bioengineering* (GILBERT, H.J., DAVIES, G., HENRISSAT, B. & SVENSSON, B., eds), pp. 3-12, The Royal Society of Chemistry, Cambridge.

SESSION 5
PROTEIN ENGINEERING

Structural basis of the synthesis of large cycloamyloses by amylomaltase

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Larger cycloamyloses probably contain helix-like structures similar to a cycloamylose with 26 glucose units whose crystal structure analysis revealed that this macrocycle folds into two short left-handed V-amylose helices in an antiparallel arrangement (GESSLER et al., 1999). A hydrophobic channel of 5.0-5.5 Å diameter runs along the axis of the V-helix. This channel may form complexes with a large variety of hydrophobic guest molecules, leading to possible industrial applications. The synthesis and purification of large amounts of these compounds is currently the major obstacle.

Amylomaltase has been found to be useful for the synthesis of cycloamyloses with a ring size of 17 and more (TAKAHA et al., 1996; TERADA et al., 1999). We have recently determined structures of amylomaltase from *Thermus aquaticus* (PRZYLAS et al., 2000a) and of a complex with the maltotetraose inhibitor acarbose (PRZYLAS et al., 2000b). Amylomaltase is a member of the α-amylase superfamily of enzymes. The protein lacks the C-terminal domain C, which is present in all related α-amylases and it contains a novel, mainly α-helical, subdomain B2.

Two acarbose molecules are bound to the enzyme, one in the active site groove at subsite -3 to +1 and a second one ~14 Å away from the non-reducing end of the acarbose bound to the catalytic site. Together, these binding sites suggest a possible binding path for cycloamylose formation. A loop around residue 460 may sterically prevent the formation of smaller cycloamyloses. Unlike the situation in other enzymes of the α-amylase family, the inhibitor is not processed and the inhibitory cyclitol ring of acarbose, which mimicks the half chair conformation of the transition state, does not bind to the catalytic subsite -1.

References

GESSLER, K., USÓN, I., TAKAHA, T., KRAUSS, N., SMITH, S.M., OKADA, S., SHELDRICK, G.M. & SAENGER, W. 1999. *Proc. Natl. Acad. Sci. USA* **96**, 4246-4251.

PRZYLAS, I., TOMOO, K., TERADA, Y., TAKAHA, T., OKADA, S., SAENGER, W. & STRÄTER, N. 2000a. *J. Mol. Biol.* **296**, 873-886.

PRZYLAS, I., TERADA, Y., FUJII, K., TAKAHA, T., SAENGER, W. & STRÄTER, N. 2000b. *Eur. J. Biochem.* **267**, 6903-6913.

TAKAHA, T., YANASE, M., TAKATA, H., OKADA, S. & SMITH, S. M. 1996. *J. Biol. Chem.* **271**, 2902-2908.

TERADA, Y., FUJII, K., TAKAHA, T. & OKADA, S. 1999. *Appl. Environ. Microbiol.* **65**, 910-915.

Structure determination and engineering to explore the function of glycosyltrehalose trehalohydrolase from the hyperthermophilic archaeum *Sulfolobus solfataricus*

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Glycosyltrehalose trehalohydrolase (GTHase) is an α-amylase with unique exo-amylolytic activity for glycosyltrehalosides (KATO et al., 1996). It cleaves the α-1,4 glycosidic bond adjacent to the trehalose moiety to release trehalose and maltooligo saccharide. Unlike other α-amylases, the GTHase from the hyperthermophilic archaeum *Sulfolobus solfataricus* KM1 does not require Ca^{2+} for activity, and it contains an N-terminal extension of ~100 amino acids which is common to glycosidases that recognize branched oligosaccharides.

The crystal structure of GTHase from *Sulfolobus solfataricus* KM1 has been solved to 3.0 Å by multiple isomorphous replacement (FEESE et al., 2000). Crystallography revealed the enzyme to exist as a homodimer covalently linked by an intermolecular disulfide bond at residue Cys-298. Although the existence of the intermolecular disulfide bond was confirmed by biochemical analysis and mutagenesis Cys-298→Val, the role of this intermolecular disulfide is not known. It also revealed that this enzyme consists of three major domains (A, C, E) and two sub-domains (B, D). While domains A, B, and C are common within α-amylases, the N-terminal extension forms an independent novel domain E connected to the catalytic $(\beta/\alpha)_8$ barrel (domain A) by an extended linker. The functionally essential Ca^{2+} binding site found in the domain B of other α-amylases was found to be replaced by hydrophobic packing interactions.

To identify the role of catalytic residues, several mutations were introduced according to the structural similarities to other known α-amylases. One mutation Glu-283→Gln resulted in complete inactivation. The crystal structure of this mutant in complex with maltotriosyl trehalose was also determined at 2.7 Å resolution. The structural geometry of the bound substrate suggests that both Glu-283 and Asp-252 are involved in catalytic action of GTHase as a general acid and a nucleophile, respectively. These residues were further mutated to other nucleophilic residues to alter the catalytic mechanism as is successfully done in T4 phage lysozyme (KUROKI et al., 1995; 1999).

The enzyme also contains a very unusual excursion in the $(\beta/\alpha)_8$ barrel structure of the catalytic domain. This excursion originates from the bottom of the $(\beta/\alpha)_8$ barrel between helix 6 and strand 7, but folds upward in a distorted β-hairpin structure to form a part of the substrate binding cleft wall that is possibly critical for the enzyme's unique substrate selectivity. Participation of an α-β loop in the formation of the substrate binding cleft is a novel feature that is not observed in other known $(\beta/\alpha)_8$ enzymes.

References

KATO, M., MIURA, Y., KETTOKU, M., KOMEDA, T., IWAMATSU, A. & KOBAYASHI, K. 1996. *Biosci. Biotech. Biochem.* **60**, 925-928.

FEESE, M.D., KATO, Y., TAMADA, T., KATO, M., KOMEDA, T., MIURA, Y., HIROSE, M., KONDO, K., KOBAYASHI, K. & KUROKI, R. 2000. *J. Mol. Biol.* **301**, 451-464.

KUROKI, R., WEAVER, L.H. & MATTHEWS, B.W. 1995. *Nature Struct. Biol.* **2**, 1007-1011.

KUROKI, R., WEAVER, L.H. & MATTHEWS, B.W. 1999. *Proc. Natl. Acad. Sci. U.S.A* **96**, 8949-8954.

Characterization of the functional module responsible for low temperature optimum of a rice α-amylase (Amy 3E)

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Cultured cells of rice produce two α-amylase isozymes, AMY-I(Amy 1A) and AMY-III(Amy 3E). Using a bacterial expression system, eight chimeric genes constructed with various combination of AMY-I and AMY-III cDNA fragments were expressed, and each recombinant chimeric protein was characterized. Four of the eight recombinant enzymes, having region c (one of the four regions having unconserved base sequences between AMY-I and AMY-III cDNAs) of AMY-I showed the same enzyme characteristics as that of native AMY-I, which had high temperature optimum at 50°C. The other four chimeric proteins carrying region c of AMY-III showed AMY-III type characteristics, which were a low temperature optimum at 25°C. Site directed mutagenesis of the region c of AMY-III indicates that α-helix 5 is responsible for the characteristics of low temperature optimum.

Designing the product specificity of α-amylases

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Significant improvement have been obtained in the last years development of stable α-amylases, which have resulted in variants of the *Bacillus licheniformis* α-amylase capable of liquefy starch at high temperature (above 95°C) and acid pH (i.e. pH 5.5) and without requiring addition of calcium. These new α-amylases have simplified the process of the industrial conversion of starch to glucose/fructose syrups and improved the process robustness (BISGARD-FRANTZEN et al., 1999).

In order to simplify the starch conversion process further, we have focused on the product specificity of the α-amylase. The branched tri-saccharide, panose is an undesirable by-product in the conversion of starch to glucose/fructose syrups. The working hypothesis has been that in the starch liquefaction process, the used α-amylase hydrolyze amylopectin close to the 1,6-branching point forming panose precursors, which later in the saccharification step are converted to panose by the glucoamylase. To minimize panose formation, it is necessary today to inactivate the α-amylase prior to saccharification, thus an α-amylase with the desired product specificity wound not require inactivation and would therefore be an improvement to the process.

Despite a high sequence and structure homology, α-amylases from different *Bacillus* sources are known to give different reaction products when degrading starch (SVENSSON, 1994). To evaluate the affinity for branched substrate, we have analyzed the degradation of a synthetic branched oligosaccharide, G9' by the α-amylases from *Bacillus licheniformis* (BLA), *B. stearothermophilus* (BSA) and *B. amyloliquefaciens* (BAA), and found that only BLA is able to hydrolyze this substrate, while BAA and BSA have no or only weak activity.

These findings were used in combination with a structural alignment, to point out disparities in the substrate binding cleft. Five residues were identified and the specific residues in BLA were mutated towards BAA and BSA resulting in longer products from starch hydrolysis. By combination and optimization of the specific substitutions, we have developed a variant of BLA with reduced affinity for branched substrates, while the starch hydrolysis activity and the general stability were maintained. Evaluation of the variants under application relevant conditions has confirmed a reduced formation of panose.

References

BISGAARD-FRANTZEN, H., SVENDSEN, A., NORMAN, B., PEDERSEN, S., KJÆRULFF, S., OUTTRUP, H. & BORCHERT, T.V. 1999. *J. Appl. Glycosci.* **46**, 199-206.

SVENSSON, B. 1994. *Plant Mol. Biol.* **25**, 141-157.

Site-directed mutagenesis of key amino acids in the active site of amylosucrase from *Neisseria polysaccharea*

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Amylosucrase (AS) from *Neisseria polysaccharea* is a remarkable member of the family 13 of glycoside hydrolases. Opposed to most of the enzymes from family 13, AS shows an unusual specificity for sucrose and an ability to catalyse from this high-energy substrate the synthesis of an amylose-like polymer and the release of fructose. Polymer synthesis, the main reaction, is also accompanied by the formation of oligosaccharide side-products (POTOCKI DE MONTALK et al., 2000a). In addition, when glycogen is added to the reaction mixture, this enzyme transfers glucose from the sucrose donor onto the polymer branches (POTOCKI DE MONTALK, 2000b). Finally, we have recently shown that AS also catalyses the disproportionation of maltooligosaccharides (ALBENNE et al., 2001). This property enlarges the substrate specificity of AS.

Like in all α-amylases structures, the three-dimensional AS structure reveals a catalytic $(\beta/\alpha)_8$-barrel A domain, a B domain and a C-terminal β-sheet domain. However, two additional domains can be distinguished in the structure: a helical N-terminal domain and an extended loop inserted between strand β7 and helix α7 called domain B' (SKOV et al., 2001). The catalytic residues D286, E328, D393, H187 and H392, always conserved in the α-amylase family, interact with the substrate and their roles have been confirmed by site-directed mutagenesis (SARCABAL et al., 2000).

A structural analysis of the E328Q mutant complexed with sucrose led to a detailed description of the active site and of the interaction between sucrose and AS at the subsites –1 and +1 (MIRZA et al., 2001). Besides the five residues previously identified, several other conserved residues such as Y147, F250, R284 were found to occupy a key position. In addition, unconserved residues found at the subsites –1 and +1 are thought to be involved in the specificity for sucrose. Each of theses positions constitutes a target for site-directed mutagenesis. Mutant characterisation is actually under progress. The substrate specificity for sucrose and/or maltooligosaccharides, the analysis of products synthesised and the ability for acceptor glucosylation are currently examined from pure mutants.

This approach enables to acquire further insight into structure/function of amylosucrase and into the structural features that are involved in its specificity.

References

ALBENNE, C., REMAUD-SIMEON, M., POTOCKI DE MONTALK, G., WILLEMOT, R.M. & MONSAN, P. 2001. *Submitted.*

MIRZA, O., SKOV, L.K., REMAUD-SIMEON, M., POTOCKI DE MONTALK, G., ALBENNE, C., MONSAN, P. & GAJHEDE, M. 2001. *Submitted.*

POTOCKI DE MONTALK, G., REMAUD-SIMEON, M., WILLEMOT, R.M., SARCABAL, P., PLANCHOT, V. & MONSAN, P. 2000a. *FEBS Lett.* **471**, 219-223.

POTOCKI DE MONTALK, G., REMAUD-SIMEON, M., WILLEMOT, R.M. & MONSAN, P. 2000b. *FEMS Microbiol. Lett.* **186**, 103-108.

SARCABAL, P., REMAUD-SIMEON, M., WILLEMOT, R.M., POTOCKI DE MONTALK, G., SVENSSON, B. & MONSAN, P. 2000. *FEBS Lett.* **474**, 33-37.

SKOV, L.K., MIRZA, O., HENRIKSEN, A., POTOCKI DE MONTALK, G., REMAUD-SIMEON, M., SARCABAL, P., WILLEMOT, R.M., MONSAN, P. & GAJHEDE, M. 2001. *Submitted.*

Glucan binding domain of streptococcal glucosyltransferases

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Cariogenic Mutans Streptococci contribute to tooth decay in the presence of sucrose due to their acidogenicity and ability to synthesise extracellular polymers that enable the formation of stable bacterial biofilms and act as an extracellular carbon store. The bacteria secrete glucosyltransferases (GTFs) that catalyse the conversion of sucrose to glucan and are closely related to other glucansucrases produced by species of *Leuconostoc* and *Lactobacillus*. These enzymes are all of high molecular weight (c. 160,000 Da) and have an N-terminal variable domain, followed by a catalytic domain and a C-terminal glucan-binding domain (GBD). The central catalytic core contains alternating α-helices and β-sheets. Multiple alignment with family 13 glycosidases indicated that GTF have a circularly-permuted $(\alpha/\beta)_8$ barrel structure and catalytic residues corresponding to those in amylases. These predictions have been confirmed experimentally by site-directed mutagenesis studies, which have also revealed that residues outside the barrel region are essential for function (MONCHOIS et al., 2000).

Because of the importance of insoluble α1,3-linked glucans in adherence of bacteria in dental plaque, we are studying GTF-I of *Streptococcus downei* that produces glucans with α(1-3) linkages, and investigating the structure and function of the glucan-binding domain and its interaction with the core containing the $(\alpha/\beta)_8$ barrel. The GBD of GTF-I is predicted to be mainly β-sheet and contains a series of related but non-identical repeats, each of c. 40 amino acids, in which a number of aromatic residues and glycines are universally conserved. Repeats showing the same pattern of conservation are also found in the non-enzymatic GbpA glucan-binding protein of *Streptococcus mutans* and in the glucansucrases of *Leuconostoc mesenteroides* (JANECEK et al., 2000). Similar repeat regions are found in the choline-binding proteins of *Streptococcus pneumoniae*, pneumococcal bacteriophages, toxins of *Clostridium difficile* and surface proteins of several other Gram-positive bacteria. Analysis of the genome sequence of *S. mutans* has recently revealed the existence of another protein of unknown function containing repeats.

The repeat regions contain clusters of aromatic residues (Y, W, F) that may interact with the sugar units of glucan, and polar and acidic residues (K, D) that may allow hydrogen bonding with the hydroxyl residues on the sugar. The glucan-binding domain of GTF-I was cloned and expressed as a His-tagged fusion protein in *E. coli*. The His-tag allowed development of a rapid method for immobilising recombinant protein and measurement of the capacity to bind biotin-labelled dextran. Serial truncations of the binding domain showed that removal of 1 or 2 repeat units resulted in reduced binding while removal of 3 abolished all binding activity. Site-directed mutagenesis of specific residues and error-prone PCR of the last 2 repeats has allowed construction of a library of mutants that were screened for binding activity in order to determine the contribution of individual amino acid residues.

References

MONCHOIS, V., VIGNON, M., ESCALIER, P.C., SVENSSON, B. & RUSSELL, R.R.B. 2000. *Eur. J. Biochem*. **267**, 4127-4136.
JANECEK, S., SVENSSON, B. & RUSSELL, R.R.B. 2000. *FEMS Microbiol. Lett.* **192**, 53-57.

SESSION 6
RAW STARCH BINDING AND DEGRADING

Functional studies on the barley α-amylase raw starch-binding domain

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The mature form of barley seed low-pI α-amylase (BAA1) is a polypeptide 414 amino acids in length and possesses a raw starch-binding site in addition to the catalytic site. A truncated cDNA encoding the C-terminal region (134 aa), the proposed raw starch-binding domain (SBD), was synthesized via PCR and expressed in *E. coli*. To determine binding, rSBD was purified by affinity chromatography with cyclohepta-amylose (CHA) as ligand cross-linked to Sepharose. This work demonstrates that a SBD is located in the C-terminal region and retains function in the absence of the N-terminal and catalytic regions. This property is analogous to that of *Aspergillus niger* glucoamylase (GAMY). In other work, deletion and mutagenesis experiments reveal that the conserved amino acids at the C-terminal end of rBAA1 expressed in the yeast *Pichia pastoris* are required for enzymatic activity. This region most likely plays a role in structural stability, unlike GAMY, which retains activity on soluble starch in the absence of its SBD. Details and recent updates on this work will be presented.

C-Terminal fusion of barley α-amylase isozyme 1 with the starch binding domain of glucoamylase from *Aspergillus niger*

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Certain members of the glycoside hydrolase families 13, α-amylases, 14, β-amylase and 15, glucoamylase, have high affinity for starch granules due to the presence of a starch binding domain (SBD) (JANEČEK & ŠEVČÍK, 1999). Most fungal glucoamylases (GAs) contain a SBD and glucoamylase GA-I from *Aspergillus niger* has a SBD (approx. 100 aa) connected to the catalytic domain via a 70 aa long *O*-glycosylated linker (SVENSSON et al., 1983). SBD increases the activity towards starch granules of this GA by around 100 fold (SVENSSON et al., 1982). In contrast to the well-defined separate SBD module of GA, barley α-amylase possesses on the surface of the catalytic domain a site involved in binding onto starch granules (KADZIOLA et al., 1998).

Construction of a gene encoding a C-terminal fusion protein of *A. niger* SBD (aa 471-616) with barley α-amylase 1 was conducted to enhance the performance of barley α-amylase on starch granules. Directed by the AMY1 signal sequence *A. niger* transformants secreted correctly N-terminally processed AMY1-SBD and AMY1 (JUGE et al., 1998). The K_d for barley starch granules for AMY1-SBD was 0.13 mg × mL^{-1} compared to K_d = 0.8 mg × mL^{-1} for AMY1, showing that increased affinity was achieved by the SBD-addition. Compared to AMY1 the specific activity of AMY1-SBD improved almost two-fold towards barley starch granules and towards soluble potato starch, but was essentially unaltered towards a maltooligossacharide substrate. Moreover the degree of multiple attack on amylose DP440 increased significantly. In conclusion, the fusion of SBD to AMY1 led to the production of an active and conformationally stable multidomain enzyme, which reacted with enhanced enzymatic activity towards starch substrates. The implications of these findings are discussed.

References

JANEČEK, Š. & ŠEVČÍK, J. 1999. *FEBS Lett.* **456**, 119-125.
JUGE, N., SVENSSON, B. & WILLIAMSON, G. 1998. *Appl. Microbiol. Biotechnol.* **49**, 385-392.
KADZIOLA, A., SØGAARD, M., SVENSSON, B. & HASER, R. 1998. *J. Mol. Biol.* **279**, 205-217.
SVENSSON, B., LARSEN, K., SVENDSEN, I. & BOEL, E. 1983. *Carlsberg Res. Commun.* **48**, 529-544.
SVENSSON, B., PEDERSEN, T. G., SVENDSEN, I., SAKAI, T. & OTTESEN, M. 1982. *Carlsberg. Res. Commun.* **47**, 55-69.

Molecular cloning and functional expression of non-extra-SBD-domain type of raw starch degrading glucoamylase from *Saccharomycopsis fibuligera* IFO 0111

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Glucoamylase from dimorphous yeast *Saccharomycopsis fibuligera* IFO 0111 (GluSf0111) displays ability to digest raw starch. Here we report the isolation of the full length cDNA for this glucoamylase, expression and characterisation of the gene product. cDNA encodes 489 amino-acid residues (aa) corresponding to the mature protein, 26 aa to the putative signal peptide and three potential N-glycosylation sites. The calculated molecular mass of the putative mature protein is 54 590 Da. The recombinant protein isolated from *Saccharomyces cerevisiae* AH22 is fully active with characteristics similar to the original glucoamylase. Amino acid alignment of the entire sequence of GluSf0111 revealed 60% identity and 77% similarity with 492 aa long polypeptide chains, i.e. catalytic $(\alpha/\alpha)_6$–barrel domains of *S. fibuligera* glucoamylases Glu (ITOH et al., 1987; ŠEVČÍK et al., 1998) and Gla (HOSTINOVÁ et al., 1991), both unable of raw starch digestion. Protein similarity search further showed that amino acid sequence of GluSf0111 had no similarity to any known starch-binding domain found in glucoamylases and other amylolytic enzymes.

References

ITOH, T., OHTSUKI, I., ZAMASHITA, I. & FUKUI, S. 1987. *J. Bacteriol.* **169**, 4171-4176.

HOSTINOVÁ, E., BALANOVÁ, J. & GAŠPERÍK, J. 1991. *FEMS Microbiol. Lett.* **83**, 103-108.

ŠEVČÍK, J., SOLOVICOVÁ, A., HOSTINOVÁ, E., GAŠPERÍK, J., WILSON, K.I. & DAUTER, Z. 1998. *ACTA CRYST.* **D54**, 854-866.

SESSION 7
STABILITY AND STABILISATION

Maltosyltransferase of the hyperthermophilic bacterium *Thermotoga maritima*: crystal structure and its implications for the novel transfer specificity and thermostability of the enzyme

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Maltosyltransferase (MTase) is an extremely thermostable enzyme which belongs to glycosyl hydrolase family 13 (the α-amylase family). The enzyme is a non-hydrolytic maltodextrin glycosyltransferase which catalyses the transfer of maltosyl units from α-1,4-linked glucans or malto-oligosaccharides to other α-1,4-linked glucans, malto-oligosaccharides or glucose (MEISSNER & LIEBL, 1998). To date, the only organism known to produce a starch-converting enzyme with this unique reaction chemistry is the hyperthermophilic bacterium *Thermotoga maritima*, a heterotroph with a maximum growth temperature of 90 °C. In addition to MTase, *T. maritima* possesses a second maltodextrin glycosyltransferase, 4-α-glucanotransferase (GTase), also a member of the α-amylase family which displays a broad transfer specificity (HEINRICH et al., 1994). MTase is extremely insensitive to thermoinactivation with half-lives of about 2.5 h, 17 h and 21 d recorded at 90, 85 and 70 °C (pH 6.5), respectively. The enzyme is a homodimer. Crystals of recombinant MTase have been obtained by the hanging-drop vapor-diffusion method (BURKE et al., 2000) The crystal structures of MTase and its complex with maltose have been determined at 2.4 and 2.1 Å resolution, respectively. As had been predicted from the enzyme's primary structure relatedness with family 13 of glycosyl hydrolases, MTase contains a $(\beta/\alpha)_8$ barrel supersecondary structure with the active site cleft including the three proposed catalytic residues, Asp385, Glu414 and Asp468, at the C-terminal end of the barrel. Two distinct maltose binding sites were identified. The X-ray analysis of the structure of MTase provides an explanation for the strict transfer specificity of the enzyme. Also, conclusions can be drawn about features at the molecular level contributing to the resistance of MTase against thermoinactivation.

References

BURKE, J., ROUJEINIKOVA, A., BAKER, P.J., SEDELNIKOVA, S., RAASCH, C., LIEBL, W. & RICE, D.W. 2000. *Acta Crystallogr.* **D56**, 1049-1050.

HEINRICH, P., HUBER, W. & LIEBL, W. 1994. *System. Appl. Microbiol.* **17**, 297-305.

MEISSNER, H. & LIEBL, W. 1998. *Eur. J. Biochem.* **250**, 1050-1058.

Engineering the thermostability of *B. licheniformis* α-amylase

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Bacillus licheniformis α-amylase (BLA) is a highly thermostable enzyme which is widely used in biotechnological processes. Although it is produced by a non-thermophilic bacteria, it remains active for several hours at temperatures over 90°C under conditions of industrial starch hydrolysis. It is also far more thermostable than the α-amylases from *B. stearothermophilus* and *B. amyloliquefaciens* despite the strong sequence similarities between these three proteins. BLA provides therefore an interesting model for protein engineers investigating on enzyme thermostability and thermostabilization. Over the last decade, we have performed an extensive mutational and structural analysis on BLA in order to elucidate the origin of its unusual thermal properties and, if possible, increase its thermostability even further. Before the three-dimensional structure was known, we had used "blind" mutagenesis and identified two critical positions where amino acid substitutions could either increase or decrease significantly the rate of irreversible thermoinactivation (DECLERCK et al., 1990; JOYET et al., 1992). The structural effect of the stabilizing mutations have first been predicted from molecular modeling (DECLERCK et al., 1995), then interpreted from the wild-type BLA crystal structure (DECLERCK et al., 1997) and finally visualized in the crystal structure of a hyperthermostable variant (MACHIUS, M. et al., submitted). Once a detailed three-dimensional structure of BLA was solved, structure-based mutagenesis was used to probe the role of protein regions and residues possibly important for the enzyme thermostability. Residues involved in salt-bridges, calcium-binding or potential deamidation processes have been replaced with various amino acids and the effect of the mutations has been tested *in vitro*. Our results confirmed the importance of domain B in determining the overall thermostability of BLA and revealed the key role of the electrostatic interaction network entrapping the Ca-Na-Ca metal triad observed at the interface between domain A and B. Moreover, we identified several new stabilizing substitutions, one of them (Asn190Phe) leading to a 6-fold increase of the enzyme's half-life at 80°C (DECLERCK et al., 2000). Combined with other thermostabilizing mutations previously identified, this mutation lead to hyperthermosatble variants several hundred times more thermostable than the wild-type enzyme. The crystal structure of this variant was recently determined and enabled to get insights into the structural features leading to BLA thermostabilization (MACHIUS, M. et al., submitted). Finally, we have added two stabilizing mutations identified by others (BISGAARD-FRANTZEN et al., 1999) and observed a global increase, compared to wild-type, of about 20°C in the temperature of half-inactivation. In the course of this mutational study we have constructed over 500 BLA variants bearing single or multiple mutations, among which many were found to be either highly detrimental or slightly beneficial to the stability. The cumulative effect of the mutations enabled to modulate the enzyme's stability over a range of 50°C without perturbing significantly the amylolytic function. Although a full understanding of the origin of BLA natural thermoresistance has not yet been reached, our study demonstrated that it is not optimized and that it can be increased or decreased artificially by several means.

References

BISGAARD-FRANTZEN, H., SVENDSEN, A., NORMAN, B., PEDERSEN, S., KJÆRULFF, S., OUTTRUP, H., BORCHERT, T.V. 1999. *J. Appl. Glycosci*. **46**, 199-206.

DECLERCK, N., JOYET, P., GAILLARDIN, C. & MASSON, J.M. 1990. *J. Biol. Chem.* **265**, 15481-15488.

DECLERCK, N., JOYET, P., TROSSET, J.Y., GARNIER, J. & GAILLARDIN, C. 1995. *Protein Eng.* **8**, 1029-1037.

DECLERCK, N., MACHIUS, M., CHAMBERT, R., WIEGAND, G., HUBER, R. & GAILLARDIN, C. 1997. *Protein Eng.* **10**, 541-549.

DECLERCK, N., MACHIUS, M., WIEGAND, G., HUBER, R., & GAILLARDIN, C. 2000. *J. Mol. Biol.* **301**, 1041-1057.

JOYET, P., DECLERCK, N. & GAILLARDIN, C. 1992. *Biotechnology (N Y)* **10**, 1579-1583.

Stabilization of *Bacillus licheniformis* α-amylase through introduction of hydrophobic residues at the surface

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Based on a broad mutagenesis study involving more than 175 mutants, we have identified variants of the highly thermostable and irreversibly unfolding α-amylase from *Bacillus licheniformis* (BLA) that unfold at higher temperatures than the wild type and that exhibit kinetic hyperthermostability, *i.e.* they unfold more slowly. When incubated at 85°C under representative conditions, the wild-type enzyme shows a half-life of about 14 min, whereas the half-life of the most hyperthermostable variant combining five mutations is 32 times as long. Four out of the five amino acid changes result in the replacement of small or hydrophilic residues at the surface by large and hydrophobic residues. Crystal structure analysis at 1.71 Å resolution indicates that the stabilization is due to a) extension of the concept of increased hydrophobic packing, usually applied to cavities, to surface indentations, b) introduction of favorable aromatic-aromatic interactions on the surface, c) specific stabilization of intrinsic metal binding sites, and d) stabilization of a β-sheet by introducing, at a strategic position, a residue with high β-sheet forming propensity.

Increasing the hydrophobicity of protein surfaces is considered thermodynamically unfavorable as it decreases solubility. With respect to stability, there is recent evidence, although controversial, that incorporation of hydrophobic residues at the surface might not necessarily be destabilizing. Our study is an example that incorporation of hydrophobic residues does not only not reduce stability, but it in fact increases stability drastically. However, not all investigated hydrophobic surface substitutions act this way. We find that the most stabilizing mutations are at sites that participate in cooperatively formed substructures. The degree of stabilization that we observe for mutations at these sites does not seem to be directly proportional to the underlying forces when compared with other surface substitutions. Although at first puzzling, such an „over-stabilization“ can be rationalized by the classical theory of helix-coil transitions, which explains how a small stabilization of initiation sites for unfolding can result in a drastically increased overall stability. Solvent exposed cooperatively formed substructures represent „weak spots“ in a protein as they likely act as initiation sites for unfolding. Therefore, as a general way to improve the stability of proteins, we suggest that special attention be paid to stabilizing these sites at which even mutations that appear to be thermodynamically unfavorable should be explored.

Stability, activity and binding properties study of α-amylase upon interaction with Ca^{2+} and Co^{2+}

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The interaction of α-amylase from *Bacillus amyloliquefaciens* (BAA) with divalent calcium and cobalt cations was studied by equilibrium dialysis, isothermal titration microcalorimetry, UV spectrophotometry and temperature scanning spectrophotometry methods at 27 ^{o}C in Tris buffer solution at pH=7.5. There is a set of 17 binding sites for calcium binding on the enzyme with weak positive co-operativity in binding. The binding of calcium is exothermic ($\Delta H = -16.0$ kJ.mol^{-1}) with mean dissociation binding constant of 0.55 mM. The binding of calcium caused the more stability of the enzyme against surfactant and thermal denaturation. Moreover, the binding of calcium prevents from the spontaneous decrease in biological activity of α-amylase. There is a set of 25 non-cooperative binding sites for cobalt binding on the enzyme. The binding of cobalt is exothermic ($\Delta H = -18.5$ kJ.mol^{-1}) with mean dissociation binding constant of 0.12 mM. The enzyme activity increased significantly with an increasing concentration of cobalt; however, the temperature of denaturation of the enzyme decreased. So, divalent calcium and cobalt cations are stabilizer and activator, respectively, for BAA.

Structural determinants of cold adaptation and stability in a psychrophilic α-amylase

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The psychrophilic α-amylase from *Pseudoalteromonas haloplanktis* (AHA) belongs to the family of chloride-dependent α-amylases. This group includes all known animal α-amylases as well as those from some Gram-negative bacteria, covering a large range of living temperatures (D'AMICO et al., 2000). The cold-adapted AHA is the largest known protein that unfolds reversibly according to a two-state transition as shown by Differential Scanning Calorimetry (FELLER et al., 1999). Mutants of this enzyme carrying additional weak interactions found in thermostable α-amylases were produced (D'AMICO et al., 2001). It is shown that single amino acid side chain substitutions can significantly modify the melting point T_m, the calorimetric enthalpy ΔH_{cal}, the cooperativity and reversibility of unfolding and the kinetic parameters k_{cat} and K_m. We also devised a new method using Isothermal Titration Calorimetry to provide a direct and continuous monitoring of activity thus allowing determination of inactivation rate constants at a denaturing temperature. The correlation between thermal inactivation and unfolding reversibility displayed by the mutants shows that stabilizing interactions increase the frequency of side reactions during refolding, leading to intramolecular mismatches or aggregation typical of large proteins. Although all mutations were located far from the active site, their overall trend is to decrease both k_{cat} and K_m by rigidifying the molecule and to protect mutants against thermal inactivation. The effects of these mutations indicate that the cold-adapted α-amylase has lost a large number of weak interactions during evolution in order to reach the required conformational plasticity for catalysis at low temperatures, therefore producing an enzyme close to the lowest stability allowing maintenance of the native conformation.

References

D'AMICO, S., GERDAY, C. & FELLER, G. 2001. *J. Biol. Chem.* (in press).
D'AMICO, S., GERDAY, C. & FELLER, G. 2000. *Gene* **253**, 95-105.
FELLER, G., D'AMICO, D. & GERDAY C. 1999. *Biochemistry* **38**, 4613-4619.

POSTER PRESENTATIONS ABSTRATCS

Session 1: New primary structures

G.R. Bignell, J. Pandya & I.H. Evans: Alpha-amylases from the soil yeast *Lipomyces starkeyi*. (P 1.1)

A. Chagolla-López, L. Herrera-Estrella & A. Blanco-Labra: Functional characterization and expression in *Escherichia coli* of an alpha-amylase inhibitor gene from amaranth (*Amaranthus hypochondriacus*). (P 1.2)

K.A. Cheong, T.K. Cheong, T.J. Kim, C. Park, J.H. Park, S.B. Lee, M.H. Lee, J.H. Choi & K.H. Park: Cloning of thermostable maltogenic amylase gene from *Bacillus thermoalkalophilus* ET2 and the enzymatic characteristics of its gene product. (P 1.3)

R.F. Martins & R. Hatti-Kaul: Purification and characterisation of a cyclodextrin glycosyltransferase from an alkaliphilic *B. agaradhaerens* strain. (P 1.4)

M. Nishimoto, H. Mori, A. Kimura & S. Chiba: Production of honeybee alpha-glucosidase isozymes in *Pichia pastoris* and their properties. (P 1.5)

M. Shahhoseini, A.A. Ziaee, N. Ghaemi & A.A. Pourbabaii: An unusual DNA sequence encoding a hyperthermostable alpha-amylase isolated from a native strain of *B. licheniformis*. (P 1.6)

Session 2: Specificity and evolution

Š. Janeček, B. Svensson & E.A. MacGregor: Evolution of the alpha-amylase family members containing the C-terminal raw starch-binding domain. (P 2.1)

O. Markovič & P. Biely: A simple differentiation of alpha-amylase and amyloglucosidase in gels. (P 2.2)

R. Mikkelsen, L. Baunsgaard & A. Blennow: The effects of starch branching enzyme and R1 on the phosphorylation of starch. (P 2.3)

C. Park, M.J. Kim, J.S. Baek, J.S. Cho, H.K. Bae, S.B. Lee, S.J. Lee, J.W. Yoon, Y.K. Kim & K.H. Park: Application of transglycosylation activity of *Bacillus stearothermophilus* maltogenic amylase (BSMA). (P 2.4)

Session 3: Catalytic and binding mechanism

F. Chang-Pi-Hin, P. Debeire, F. Duchiron & M.J. O'Donohue: Mode of action of the recombinant type II pullulanase from *Thermococcus hydrothermalis* and hyperthermophilic archaebacteria. (P 3.1)

L. Kandra, G. Gyémánt & A. Lipták: Action patterns of alpha-amylases on modified maltooligosaccharides. (P 3.2)

M.J. Kim, J.S. Cho, J.S. Baek, H.S. Lee, S.B. Lee, J.W. Kim, K.S. Lee, J.H. Shim & K.H. Park: Comparative inhibition study of novel amylase inhibitors against the amylolytic enzymes. (P 3.3)

B. Kramhøft, M. Tovborg Jensen, K. Sass Bak-Jensen, H. Mori, J. Nohr & B. Svensson: Multiple attack and transglycosylation reactions catalyzed by alpha-amylase and limit dextrinase from barley. (P 3.4)

L. Lo Leggio, I. Hilden, H. A. Ernst & S. Larsen: Structural and sequence analyses of glycogen branching enzymes. (P 3.5)

N. Ouldjeriouat, V. Desseaux, R. Koukiekolo, M. Santimone, B. Svensson & G. Marchis-Mouren: Mechanism of barley malt alpha-amylase (AMY): inhibition of amylose and maltoheptaose hydrolysis by acarbose and cyclodextrins. (P 3.6)

Session 4: New three-dimensional structures

Ľ. Pribišová, L. Janda, Ľ. Urbániková & J. Ševčík: Crystallization and preliminary X-ray study of alpha-1,4-glucosidase from *Thermomonospora curvata*. (P 4.1)

Session 5: Protein engineering

T.J. Kim, P.K. Nielsen & B. Svensson: Enzymatic properties of chimeric enzymes of barley alpha-amylase isozymes. (P 5.1)

M.H. Rivera, X. Soberón, A. López-Munguía & G. Saab: Role of amino acid residues conserved near to

catalytic site in *Bacillus licheniformis* alpha-amylase in transglycosylation reaction. (P 5.2)

A. Shaw, R. Bott & A.G. Day: Protein engineering of *Bacillus licheniformis* α-amylase for low pH performance. (P 5.3)

M.J.E.C. van der Maarel, B. van der Veen, J.C.M. Uitdehaag, B.W. Dijkstra & L. Dijkhuizen: Key role of hydrophobic residues in determining the transglycosylating activity of cyclodextrin glycosyltransferase. (P 5.4)

Session 6: Raw starch binding and degrading

A.L. Hansen & B. Wischmann: Enzymatic modification of raw potato starch. (P 6.1)

V. Horváthová, M. Navrátil & E. Šturdík: Hydrolysis of starch substrates by amylases before fermentation. (P 6.2)

A. Kováčová & Š. Janeček: Hunting in the molecular-biology databases for the sequences related to the raw starch-binding domain. (P 6.3)

R. Rodriguez Sanoja, J. Morlon-Guyot, J. Jore, J. Pintado, J.P. Guyot & N. Juge: Identification of a new type of starch binding domain: the tandem repeat units in the C-terminal region of *Lactobacillus amylovorus* alpha-amylase. (P 6.4)

A. Vos, W. Eeuwema, L. Dijkhuizen & M.J.E.C. van der Maarel: Genome mining for alpha-amylases of *Microbacterium aurum* involved in the degradation of native potato starch granules. (P 6.5)

Session 7: Stability and stabilisation

S. D'Amico, C. Gerday & G. Feller: Calorimetric characterization of mutants from a cold-active alpha-amylase. (P 7.1)

W.T. Ismaya, T. Setiana, B. Mulyana, D. Natalia & S. Soemitro: Stabilization and protein engineering of alpha-amylase from *Saccharomycopsis fibuligera*. (P 7.2)

K. Khajeh & M. Nemat-Gorgani: Chemical modification of bacterial alpha-amylases: changes in tertiary structures and the effect of additional calcium. (P 7.3)

M. Nemat-Gorgani & K. Khajeh: Proteolysis of bacterial alpha-amylases: segmental mobility and the effect of additives. (P 7.4)

P.K. Turnert, O.H. Fridjonsson, E. Nordberg-Karlsson, G.O. Hreggvidsson, O. Holst, P. Schönheit & J.K. Kristjansson: Exploring diversity of thermostable starch-modifying enzymes from Icelandic hot springs. (P 7.5)

POSTER SESSION 1
NEW PRIMARY STRUCTURES

α-Amylases from the soil yeast *Lipomyces starkeyi*

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The soil yeast *Lipomyces starkeyi* is a member of an ascomycetous genus with an interesting range of hydrolytic and degradative activities, including the ability to assimilate starch; some *Lipomyces* species, like *L. kononenkoae* and *L. starkeyi*, are particularly effective in this respect. We have investigated the amylolytic system of one strain of *L. starkeyi* - NCYC 1436 - via enzyme purification and characterisation studies (BIGNELL et al., 2000; J. PANDYA, Thesis in preparation). Aerobic cultures were grown at 30 °C on a rich medium, with starch as sole carbon source, for various time periods up to 15 days. Proteins were precipitated from culture supernatants using ammonium sulphate, and then fractionated by various combinations of hydrophobic interaction chromotography and ion exchange chromatography. Chromatography using Q-Sepharose achieved useful separation of α-glucosidase and α-amylase activities, both of which showed complex elution behaviour. Gel electrophoresis of a fraction enriched in α-glucosidase activity, followed by activity-staining, showed the presence of a single α-glucosidase enzyme species; non-denaturing electrophoretic analysis indicated that this enzyme had a molecular mass of approximately 170,000. Chromatographic fractions enriched in α-amylase activity - as assayed using the Phadebas reagent (a cross-linked covalently dyed starch substrate) – were also analysed electrophoretically: a starch-iodine activity stain revealed three distinct amylase species. The molecular masses of the three enzymes were approximately 50,000, 70,000 and 80,000. The 70,000 species, on extraction from a gel, did not show activity with the Phadebas substrate, and so may not be a true α-amylase. A purified α-amylase fraction (80kDa) was found to have a pH optimum between 4 and 5, and showed moderate thermostability. Circular dichroism studies were also carried out on the same sample, using a Jasco J600 spectropolarimeter, with the kind assistance of Dr. A. DRAKE (Birkbeck College). The CD spectrum (260-185) was determined over a wide pH range, allowing Δε (CD) at 208nm to be plotted again pH. A large Δε change between pH 2.6 and 3.6 was observed, indicative of a change in protonation states of aspartate residues involved in the conformation of the protein. Possible inferences are that one or more aspartate residues, probably surface-located, have a key role in conformational stability; pH denaturation occurs in two distinct phases, with optimal catalytic activity occurring at the first inflection, approximately pH 5.0.

References

BIGNELL, G.R., BRUCE, I.J. & EVANS, I.H. 2000. *Biotechnol. Lett.* **22**, 1713-1718.

Functional characterization and expression in *Escherichia coli* of an α-amylase inhibitor gene from amaranth (*Amaranthus hypochondriacus*)

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α-Amylase inhibitors are widespread in nature. Although their physiological role in plants remains unclear, most of them are active against heterologous enzymes and they could play an important protective role against the attack of animal predators. In amaranth, an ancestral Mexican crop, we have isolated and characterized an α-amylase inhibitor (AAI). This AAI represents a small fraction of the total proteins present in amaranth seeds. It consists of 32 amino acid residues with three disulfide bridges, adopting a common structure designed as the inhibitor cystein knot motif (ICK) (CHAGOLLA-LÓPEZ et al., 1994). AAI showed strong inhibition of amylases of insects which represents important pests of stored grains (*Tribolium castaneum, Prostephanus truncatus, Callosobruchus maculatus,* among others), but it does not inhibit mammalian α-amylases. Presently, we are interested in the functional characterization and modification of AAI. The first step toward this goal is the cloning and expression of our inhibitor in bacterial system (*E. coli*). In this work, a synthetic gene coding for AAI was cloned and expressed in *E. coli* using pINIII-ompA expression system (GHRAYEB et al., 1984). The recombinant protein (rAAI), containing four additional amino acids at its N-terminal end, was secreted into the medium. After purification by ion exchange chromatography, the protein was found to be fully active and to strongly react with polyclonal antibodies raised against the AAI purified from seeds.

References

CHAGOLLA-LÓPEZ, A., BLANCO-LABRA, A., PATHY, A., SÁNCHEZ, R. & PONGOR, S. 1994. *J. Biol. Chem.* **269**, 23675-23680.

GHRAYEB, J., KIMURA, H., TAKAHARA, M., HSIUNG, H., MASUI, Y. & INOUYE, M. 1984. *EMBO J.* **3**, 2437-2442.

Cloning of thermostable maltogenic amylase gene from *Bacillus thermoalkalophilus* ET2 and the enzymatic characteristics of its gene product

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A thermostable maltogenic amylase gene of *Bacillus thermoalkalophilus* ET2 (*btma*) has been cloned in *Escherichia coli* using PCR. It was found that the cloned *btma* gene (open reading frame of 1,767 bp) encoded 588 amino acid residues with a molecular mass of 68,774 Da. Deduced amino acid sequence analysis showed that BTMA had 78.2% identity with *Bacillus stearothermophilus* maltogenic amylase (BSMA). BTMA is a thermostable and alkalophilic maltogenic amylase with the optimal reaction temperature and pH at 70 ^{o}C and pH 8.0, respectively. Analytical ultracentrifugation and gel permeation chromatographic analyses revealed that BTMA existed in a monomer-dimer equilibrium with a molar ratio of 1:1 in 50mM Glycine-NaOH buffer at pH 8.0. The monomer-dimer equilibrium is shifted toward monomer at high ionic strength and low enzyme concentration.

Purification and characterisation of a cyclodextrin glycosyltransferase from an alkaliphilic *Bacillus agaradhaerens* strain

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The enzyme, cyclodextrin glycosyltransferase (CGTase) from an alkaliphilic microorganism, identified as *Bacillus agaradhaerens*, has been purified and characterized. CGTase converts starch and related sugars into cyclic oligosaccharides called cyclodextrins (CDs) comprising six or more glucose units attached by α-1,4 glucosidic bounds. CDs have the ability to form inclusion complexes with a variety of compounds, which can be of interest to pharmaceutical, cosmetic and food industry, as well as in organic synthesis, separation processes and bioconversions.

The *B. agaradhaerens* strain LS-3C was isolated from a sample from an Ethiopian soda lake. The organism grown at pH 10 and 37 ^{o}C produced CGTase into the extracellular medium at a level of 0.31 U/ml. The enzyme was purified by adsorption to corn starch and elution by 1 mM β-CD. The molecular weight of the pure protein was estimated to be 91 kDa by SDS-PAGE, and its isoelectric point was 6.9. The pure enzyme was stable over a broad range of pH, between pH 5.0 and 11.4, and was most active at pH 9. It could be stored in a buffer at pH 8 at 4 ^{o}C for over 1 month without losing its activity. The CGTase was optimally active at 55 ^{o}C, however, it maintained its stability only up to a temperature of 30 ^{o}C at pH 8. The thermostability of the enzyme was enhanced in the presence of its substrate, product and $CaCl_2$, respectively. With 5 % maltodextrin as substrate, a maximum conversion of about 10% to CDs was obtained after 4 h of reaction with 2 U enzyme per gram substrate. Mainly β-CD was produced. The N-terminal sequence of the CGTase has also been determined.

This is the first report of a CGTase from a *B. agaradhaerens*.

Production of honeybee α-glucosidase isozymes in *Pichia pastoris* and their properties

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In adult honeybees, three α-glucosidase isozymes (HBG-I, -II, and -III) are localized in ventriculus corresponding to stomach and intestine in mammal, ventriculus and hemolymph, and hypopharyngeal gland, respectively. The enzymes have high hydrolyzing activity toward sucrose (TAKEWAKI et al., 1980) with different kinetic features. HBG-I is an allosteric enzyme showing the negative cooperative reaction on sucrose (KIMURA et al., 1990), whereas HBG-II shows the positive cooporativity (TAKEWAKI et al., 1993). HBG-III catalyzes the typical Michaelis-Menten type reaction. α-Glucosidase found in honey had the same properties as those of HBG-III, suggesting that HBG-III is secreted from hypopharyngeal gland into nectar and hydrolyzes sucrose during the formation of honey.

α-Glucosidase is divided into two families, α-glucosidase family-I and -II, according to amino acid sequences (CHIBA, 1997). Family-I enzymes share four conservative regions found in α-amylase family. Based on the primary structures of HBG-I, -II, and -III deduced from the cloned cDNAs, the three honeybee enzymes belong to family-I. They display approximately 40% identical residues in each pair of the sequences.

Recombinant α-glucosidases were produced in *Pichia pastoris* under the control of AOX1 promoter. HBG-II and -III were secreted into the media by their own signal sequences and accumulated up to 30 and 13 mg per 1 L of medium, respectively, after 200 hour induction, whereas HBG-I was not produced. Although the purified HBG-II was highly glycosylated (21% as mannose) much more than the native enzyme, the kinetic properties for maltose, maltotriose, and phenyl α-glucoside were similar to those of the native enzyme. The recombinant HBG-III also showed the similar enzymatic properties to native HBG-III.

To identify the Glu residue in region-3, Glu259 and Glu269 in HBG-III were replaced by Gln by site-directed mutagenesis. The E259Q and E269Q HBG-III purified from the culture media had 50% and 0.04% of specific activities of the wild type enzyme, respectively. Combined with the results that the specific activities of D206N and D331N HBG-III were 0.004% and 0.002% of the native enzyme, respectively, three essential catalytic residues were identified to be Asp206, Glu269, and Asp331.

References

CHIBA, S. 1997. *Biosci. Biotechnol. Biochem.* **61**, 1233-1239.
KIMURA, A., TAKEWAKI, S., MATSUI, H., KUBOTA, M. & CHIBA, S. 1990. *J. Biochem.* **107**, 762-768.
TAKEWAKI, S., CHIBA, S., KIMURA, A., MATSUI, H. & KOIKE, Y. 1980. *Agric. Biol. Chem.* **44**, 731-740.
TAKEWAKI, S., KIMURA, A., KUBOTA, M. & CHIBA, S. 1993. *Biosci. Biotechnol. Biochem.* **57**, 1508-1513.

An unusual DNA sequence encoding a hyperthermostable α-amylase isolated from a native strain of *B. licheniformis*

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The gene encoding a thermostable α-amylase enzyme, belonging to a native strain of *B. licheniformis* isolated from wheat grinder factory waste water, around Kashan city, has been cloned in *E. coli* following the design of modified primers relevant to its promoter and terminal sites. The nucleotide sequence of the cloned gene has been determined by automated DNA sequencing method. The data show that the gene is 1539 base pairs long and its deduced protein consists of 482 amino acids, similar to what has been so far reported for this gene. Comparison of the inferred nucleotide sequence with those from other strains of *B. licheniformis* reported in databanks revealed about 6.5% of differences in the base sequence. Although most of these changes are at the third position of the codons resulting in no changes in the amino acid component, there are 20 different amino acids including those in the positions of 133 and 209. These two positions have been reported to have a critical role on thermostability of the enzyme. In our study, these are Glu and Thr respectively, which predict an elevated effect on thermostability.

POSTER SESSION 2
SPECIFICITY AND EVOLUTION

Evolution of the α-amylase family members containing the C-terminal raw starch-binding domain

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Approximately 10% of amylolytic enzymes belonging to the three families 13, 14 and 15 of α-amylase, β-amylase and glucoamylase, respectively (HENRISSAT, 1991), contain at the C-terminal end of their sequence the raw starch-binding domain (SBD) (SVENSSON et al., 1989; JANEČEK & ŠEVČÍK, 1999). In the α-amylase family, this domain has been well-known as domain E of cyclodextrin glucanotransferase (CGTase) (KLEIN & SCHULZ, 1991; PENNINGA et al., 1995). In the present study we focused on the analysis of amino acid sequence similarities and differences among the members of the α-amylase family that contain this SBD module in order to contribute to the evolutionary picture of the family. The amylases that may contain a new type of SBD (RODRIGUEZ SANOJA et al., 2000; SUMITANI et al., 2000; TIBBOT et al., 2000) are outside the scope of this work.

The analysed set of sequences from the α-amylase family consisted of 38 enzymes possessing 6 different specificities: 23 CGTases, 1 acarviose transferase, 1 maltogenic amylase, 10 α-amylases, 2 maltotetraose-producing amylases and 1 maltopentaose-producing amylase. The CGTases (except for the CGTase from *Klebsiella*), acarviose transferase and maltogenic amylase are five-domain enzymes, whereas the α-amylases together with maltotetrao- and maltopentao-hydrolases contain only four domains. Thus all the α-amylase family members studied have in common the catalytic $(\beta/\alpha)_8$-barrel domain (domain A), domain B protruding out of the barrel as the β3-α3 connection, domain C (just after the catalytic barrel), and the SBD (domain E). The evolutionary trees based on the alignment of the individual domain sequences reveal that, in fact, each of the four domains (A, B, C and E) may have its own history, although the differences found for the first three domains are not large. The trees for the first three domains, and especially for A and B, exhibit clustering and branching close to each other, with two main detectable groups: four-domain and five-domain enzymes. The maltogenic amylase, acarviose transferase and two CGTases (the only CGTase from archaebacteria and the only four-domain CGTase from *Klebsiella*) seem to lie between the typical bacterial five-domain-containing CGTases and the four-domain amylases. The SBD tree confirmed that this domain may reflect rather the evolution of species than evolution of the individual amylolytic specificities (JANEČEK & ŠEVČÍK, 1999). The most striking example is the clustering of the two *Pseudomonas* maltotetraohydrolases with the *Klebsiella* CGTase which is far away from the rest of CGTases. In the tree based on the alignment of all the four domains (A, B, C and E) the features seen in the SBD tree are not visible so that the influence of the long sequence of the catalytic $(\beta/\alpha)_8$-barrel domain predominates.

References

HENRISSAT, B. 1991. *Biochem. J.* **280**, 309-316.
JANEČEK, Š. & ŠEVČÍK, J. 1999. *FEBS Lett.* **456**, 119-125.
KLEIN, C. & SCHULZ, G.E. 1991. *J. Mol. Biol.* **217**, 737-750.
PENNINGA, D., VAN DER VEEN, B.A., KNEGTEL, R.M., VAN HIJUM, S.A., ROZEBOOM, H.J., KALK, K.H., DIJKSTRA, B.W. & DIJKHUIZEN, L. 1996. *J. Biol. Chem.* **271**, 32777-32784.
RODRIGUEZ SANOJA, R., MORLON-GUYOT, J., JORE, J., PINTADO, J., JUGE, N. & GUYOT, J.P. 2000. *Appl. Environ. Microbiol.* **66**, 3350-3356.
SUMITANI, J., TOTTORI, T., KAWAGUCHI, T. & ARAI, M. 2000. *Biochem. J.* **350**, 477-484.
SVENSSON, B., JESPERSEN, H., SIERKS, M.R. & MACGREGOR, E.A. 1989. *Biochem. J.* **264**, 309-311.
TIBBOT, B.K., WONG, D.W. & ROBERTSON, G.H. 2000. *J. Protein Chem.* **19**, 663-669.

A simple differentiation of α-amylase and amyloglucosidase in gels

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Endo-acting α-1,4-glucanases can be specifically detected in gels using a soluble starch-aromatic dye conjugate, such as Ostazine Brilliant Red-starch introduced by us earlier (BIELY et al., 1988). The principle of the detection is the liberation of dyed low-molecular mass fragments which show higher rate of diffusion in gels and which can easily be washed out of the gels in contrast to the non-hydrolyzed polymer. The exo-acting enzymes such as amyloglucosidase or β-amylase do not liberate dyed fragments from OBR-starch because their action ceases on the first dye molecule linked to the polysaccharide chain from the non-reducing end as a steric barrier. However, both types of enzymes, α-amylase and amyloglucosidase can be detected using the classical method based on the iodine-starch complex formation. The drawback of this detection in the case of amyloglucosidase is the requirement for more or less complete starch degradation that may take a relatively long time in the case of low amyloglucosidase activities. The way to solve this problem is to use in the detection gel the lowest possible concentration of starch stainable with iodine. Then the lack of activity on OBR-starch and positive activity in the iodine/starch assay serves as experimental evidence for the absence of α-amylase and for the presence of amyloglucosidase.

References

BIELY, P., MISLOVIČOVÁ, D., MARKOVIČ, O. & KALÁČ, V. 1988. *Anal. Biochem.* **172**, 176-179.

The effects of starch branching enzyme and R1 on the phosphorylation of starch

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Amylopectin and amylose are the two major α-D-glucan polymers of starch granules in higher plants. A fundamental property of amylopectins from most plant sources is the small amount of glucose moieties (0.1-1%) with phosphate covalently linked at the C-2, C-3 or C-6 of the glycosyl unit. Modulation of starch branching enzyme, a member of the amylolytic enzyme family, has been suggested to be a main determinant affecting starch bound phosphate in many natural starches as indicated by the strong correlation between the chain length distribution of natural starches and the degree of phosphorylation (BLENNOW et al., 2000) and by employment of the antisense technique in potato showing that suppression of SBE I and II resulted in a 3-4 fold increase in starch bound phosphate (SCHWALL et al., 2000).

Another protein, which has been reported to be of importance to starch phosphorylation in potato, is the R1 protein (LORBERTH et al., 1998). In our laboratory we are studying various R1 isoforms of *Arabidopsis thaliana* by reverse genetics in order delineate the metabolic and starch structural effects of starch phosphorylation.

References

BLENNOW, A., ENGELSEN, S.B. MUNCK, L. & MØLLER, B.L. 2000. *Carbohydr. Polym*. **41**, 163-174.

LORBERTH, R., RITTE, G., WILLMITZER, L. & KOSSMANN, J. 1998. *Nature Biotechnol*. **16**, 473-477.

SCHWALL, G.P. SAFFORD, R., WESTCOTT, R.J., JEFFCOAT, B., TAYAL, A., SHI, Y.-C., GIDLEY, M.J. & JOBLING, S.A. 2000. *Nature Biotechnol*. **18**, 551-554.

Application of transglycosylation activity of *Bacillus stearothermophilus* maltogenic amylase (BSMA)

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Maltogenic amylases (MAases) are distinctively different from other amylolytic enzymes. They are able to not only hydrolyze cyclodextrins (CDs), poor substrates for amylolytic enzymes, but also catalyze the transglycosylation reaction in the presence of various acceptor molecules, consequently accumulating the transglycosylation products. The transglycosylation activity of *Bacillus stearothermophilus* MAases (BSMA) has been employed to modify the acceptor molecules such as ascorbic acid, neohesperidin, and naringin, thus creating the transglycosylated molecules for their improved characteristics and application. The structures of transfer products were determined by ^{13}NMR, MALDI-TOF MS, and FAB MS analyses. The results revealed that the maltosyl-transfer products with α-(1,6)-glycosidic linkage were major transglycosylated compounds when maltotriose was used as a donor molecule. Biochemical characteristics of the resulting transglycosylated products showed that the MAases could be applied to improve the properties of various compounds using their transglycosylation activities.

POSTER SESSION 3
CATALYTIC AND BINDING MECHANISM

Mode of action of the recombinant type II pullulanase from *Thermococcus hydrothermalis* and hyperthermophilic archaebacteria

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Pullulanases are debranching enzymes that hydrolyse α(1,6) bonds in pullulan. According to their additional ability or inability to degrade the α(1,4) glucosidic bonds in other substrates such as amylose or starch, they can be classed as pullulanase type II or pullulanase type I, respectively. In recent years, many pullulanases have been isolated from a wide variety of thermophilic microorganisms. Among these microorganisms, the Thermococcales family, is particularly well represented. Indeed, the genes encoding pullulanases type II from *Pyrococcus woesei* (RÜDIGER et al., 1995), *P. furiosus* (DONG et al., 1997) and *Thermococcus hydrothermalis* (GANTELET & DUCHIRON, 1998; ERRA-PUJADA et al., 1999), have been successfully cloned and expressed in *E. coli*. Although the *T. hydrothermalis* pullulanase (Th-Apu) is much bigger than those of *P. woesei* (Pw-Apu) or *P. furiosus* (Pf-Apu), these three enzymes appear to be highly homologous. Sequence analysis has shown that the *P. furiosus* pullulanase displays over 80% similarity with a N-terminal region of the pullulanase from *T. hydrothermalis* and biochemical characterization has shown that all three pullulanases share similar properties. However, despite a good degree of consensus in the available data concerning the properties of these enzymes, subtle differences in substrate specificity have been detected. Unlike Pw-Apu, Th-Apu does not hydrolyse the α(1,6) linkage in panose, but can hydrolyse β- and γ-CDs. Th-Apu also possesses hydrolytic activity towards DP_6 oligosaccharides while Pf-Apu does not (BROWN & KELLY, 1993). Moreover, the inhibitory effect of α-CD is different depending on the pullulanase tested. Indeed, the amylolytic activities of both Th-Apu and Pf-Apu are inhibited by the presence of α-CD, whereas Pw-Apu is unaffected.

Therefore, in order to better define the substrate specificity of Th-Apu and to investigate its mode of action we have performed a detailed study of this enzyme. In a first instance, we have sought to provide unambiguous data concerning the Th-Apu-mediated pullulan degradation. Using ^{1}H NMR analysis of the reaction products, we have shown that the unique product of this reaction is maltotriose. This indicates that Th-Apu displays true pullulanase activity in the presence of pullulan. In support of this conclusion, we have also shown that, as one would expect of a pullulanase, other α(1,6)-linked polysaccharides are not degraded by Th-Apu.

With regards to amylolytic activity, Th-Apu degrades amylose and produces small oligosaccharides (mainly DP_1 to DP_7). Using smaller malto-oligosaccharides (DP_4, DP_7 and pNP-DP_3) as substrates we have also shown that Th-Apu appears to act in an exo-like manner, releasing glucose from the reducing end of the substrate.

Finally, in order to further investigate the mode of action of Th-Apu, we have studied the course of CD degradation and have monitored amylose and pullulan hydrolysis using rheological analysis. In this way, we have been able to clearly distinguish between the exo and endo-like modes of action of Th-Apu.

References

RÜDIGER, A., JORGENSEN, P.L. & ANTRANIKIAN, G. 1995. *Appl. Environ. Microbiol.* **61**, 567-575.
DONG, G., VIEILLE, C. & ZEIKUS, J.G. 1997. *Appl. Environ. Microbiol.* **63**, 3577-3584.
GANTELET, H. & DUCHIRON, F. 1998. *Appl. Microbiol. Biotechnol.* **49**, 770-777.
ERRA-PUJADA, M., DEBEIRE, P., DUCHIRON, F. & O'DONOHUE, M.J. 1999. *J. Bacteriol.* **181**, 3284-3287.
BROWN, S.H. & KELLY, R.M. 1993. *Appl. Environ. Microbiol.* **59**, 2614-2621.

Action patterns of α-amylases on modified maltooligosaccharides

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In the course of our studies of convenient substrates for α-amylases, 2-chloro-4-nitrophenyl (CNP) and 4,6-O-benzylidene-modified 4-nitrophenyl (Bnl-NP) β-maltooligosaccharides (dp 4-8) were synthesised from cyclodextrins using a chemical procedure (FARKAS et al., 1997). For the preparation of CNP-maltooligosides of longer chain length, in the range of dp 8-11, a new chemoenzymatic procedure was developed using rabbit skeletal muscle glycogen phosphorylase b. These substrates were used for further studies of the action pattern of porcine pancreatic α-amylase (PPA), human salivary α-amylase (HSA) and *Bacillus licheniformis* α-amylase (BLA). The hydrolysis products and the remaining substrates were separated and quantified by HPLC, and the columns and elution conditions were selected accordingly. Separation of CNP- and NP-glucosides and their products, produced by amylase, was very effective on an APS column, since the separated hydrolysis products were the members of the same substrate series. However, the benzylidene-modified substrate representatives of the three different maltooligosaccharide series (Bnl-NP- and NP-glucosides, and 4,6-O-benzylidene-oligosaccharides) could be separated successfully on RP18 column. Our results revealed that the binding region in HSA is longer than the five subsites usually considered in the literature and suggested the presence of at least six subsites; four glycone binding sites (-4, -3, -2, -1) and two aglycon binding sites (+1, +2). In the ideal arrangement, the six subsites are filled by a glucopyranosyl unit and the release of maltotetraose (G_4) from the nonreducing end is dominant. The binding modes of the benzylidene derivatives indicated a favourable interaction between the Bnl group and subsite (-3) and an unfavourable one with subsite (-4). As compared to the action of PPA on the same substrates, the results showed differences in the structure of active sites of HSA and PPA (KANDRA & GYÉMÁNT, 2000). PPA exhibited a unique pattern of action on CNP-maltooligosaccharides by cleaving the maltotriose units from the nonreducing ends and leaving CNP-glycosides, or by cleaving CNP-G_2 units from the reducing ends to leave maltooligosaccharides. The product pattern of the 4,6-O-benzylidene oligomers was very interesting and markedly different from that of the unmodified substrates. Modification of the nonreducing end of NP glycosides to give a 4,6-O-benzylidene-D-glucopyranosyl group indicated a favourable interaction between the Bnl group and the subsites (-3) and (-5) but an unfavourable one with subsite (-4), which resulted in a clear shift in the product pattern. The results obtained by the digestion of the benzylidene-protected substrates confirm a multiple attack mechanism for PPA and HPA (KANDRA et al., 1997). The binding region is longer in BLA than in human amylases. Our results suggested the presence of at least eight subsites; five glycone binding sites and three aglycone ones. The binding modes of substrates will be discussed on the basis of the known features of the structures of α-amylases.

References

FARKAS, E., JÁNOSSY, L., HARANGI, J., KANDRA, L. & LIPTÁK, A. 1997. *Carbohydr. Res.* **303**, 407-415.
KANDRA, L., GYÉMÁNT, G., FARKAS, E. & LIPTÁK, A. 1997. *Carbohydr. Res.* **298**, 237-242.
KANDRA, L. & GYÉMÁNT, G. 2000. *Carbohydr. Res.* **329**, 579-585.

Comparative inhibition study of novel amylase inhibitors against the amylolytic enzymes

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The inhibition properties of various amylase inhibitors including acarbose, acarviosine-glucose, isoacarbose, and G1-PTS on the amylolytic enzymes were investigated. Inhibition kinetic parameters of inhibitors on maltogenic amylase, α-amylase, α-glucosidase, and Maltogenase® were compared with those of acarbose. It was found that acarviosine-glucose and isoacarbose were potent inhibitors for baker's yeast α-glucosidase and α-amylase, respectively. Interestingly, G1-PTS was the most potent inhibitor against the maltose-producing amylases such as maltogenic amylase and Maltogenase®. Furthermore, the determination of kinetic constants indicated that the inhibition of G1-PTS against the maltogenic amylase followed the slow-binding inhibition mechanism. This result implies that G1-PTS and acarbose bind to the enzyme subsites in a different manner each other.

Multiple attack and transglycosylation reactions catalyzed by α-amylase and limit dextrinase from barley

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Barley α-amylase (AMY, EC 3.2.1.1) and limit dextrinase (LD, EC 3.2.1.41) both belong to glycoside hydrolase family 13 and contain sequence motifs characteristic of the catalytic $(\beta/\alpha)_8$-barrel domain (MACGREGOR et al., 2001). LD is larger than AMY and has additional domains. AMY exclusively cleaves α-1,4-glucoside linkages, whereas LD cleaves only α-1,6-linkages.

AMY has an active site comprising 10 subsites. Mutant enzymes were obtained by site directed mutagenesis at subsites –6 through +4 and are compared with respect to their catalytic properties and to products formed during the initial stage of amylose hydrolysis. Both the wild-type and the mutant enzymes have 2-3 fold higher k_{cat}/K_m on amylose of DP 440 than on a very short amylose of DP 17. The degree of multiple attack (DMA) is defined as the number of cleavages per enzyme-amylose encounter subsequent to the first hydrolytic attack (ROBYT & FRENCH, 1967). AMY exerts a low but significant DMA of 2 (KRAMHFØT & SVENSSON, 1998). A mutant at subsite –6 has a DMA of 3, whereas one at subsite +1/+2 has a DMA of 1. Thus, altered DMA can be demonstrated following amino acid substitutions involving substrate binding sites. The results are discussed in terms of structural changes of the enzymes as predicted from the mutations. Several amylolytic enzymes possess a separate starch binding domain (SBD). For a genetically produced fusion protein, AMY-SBD, consisting of barley AMY and the starch binding domain of a glucoamylase from *Aspergillus niger* (JUGE, N. et al., manuscript submitted), DMA was increased to give a value of 3 compared to the DMA of 2 for AMY. Thus, the presence of a SBD apparently enhanced the lifetime of the active enzyme-substrate complex resulting in increased DMA.

A common feature of family 13 enzymes is their capacity to catalyze transglycosylation. While AMY typically quickly degrades its own transglycosylation products, LD has the special advantage of producing transglycosylation products, which are not necessarily good substrates for the enzyme. LD from barley malt (KRISTENSEN et al., 1998) thus catalyzed significant transglycosylation using maltosylfluoride as donor and a range of linear maltooligosaccharides (G_2 through G_4), branched oligosaccharides (panose and isopanose), and the cyclic β-cyclodextrin as acceptors. Products of transglycosylation were identified after purification by anion exchange chromatography. In all cases transglycosylation was clearly demonstrated. The transglycosylation yield (transfer of donor relative to hydrolysis of donor) depended on the acceptor and varied from 100% (isopanose) to ~7% (β-cyclodextrin). The other acceptors gave transglycosylation yields varying from ~25% (G_4) to ~70% (panose). Hydrolysis by LD of the α-1,6 bonds formed during transglycosylation occurred to different extent, only transglycosylated panose and β-cyclodextrin being completely hydrolyzed.

In conclusion, it is demonstrated that degradation of high DP amylose by variants of AMY involves multiple attack, which can be manipulated by rational protein engineering. Furthermore, transglycosylation efficiency by LD varies with acceptor structures and reveals potential for production of novel oligosaccharides.

References

KRAMHØFT, B. & SVENSSON, B. 1998. *Progr. Biotechnol*. **15**, 343-347.
KRISTENSEN, M., PLANCHOT, V., ABE, J.-I. & SVENSSON, B. 1998. *Cereal Chem*. **75**, 473-479.
MACGREGOR, E.A., JANECEK, S. & SVENSSON, B. 2001. *Biochim. Biophys. Acta* **1546**, 1-20.
ROBYT, J.F. & FRENCH, D. 1967. *Arch. Biochem. Biophys*. **122**, 8-16.

Structural and sequence analyses of glycogen branching enzymes

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An N-terminally truncated form of *E. coli* glycogen branching enzyme (GBE) missing the first 107 residues has been produced (HILDEN et al., 2000) and found to retain half of the activity of full length GBE. Similar findings have been independently reported by others (BINDERUP et al., 2000). Both groups pursued in parallel crystallization of truncated GBE, but the maximum resolution obtained by our crystals was 3.6 Å. The crystal structure of GBE has now been determined (see the contribution by J. PREISS to these conference proceedings), however to our knowledge nobody has succeeded in obtaining crystals of the intact GBE suitable for structure determination. The structure and function of the N-terminal domain is thus still unclear.

We have previously divided branching enzymes as having a long (group 1) or short (group 2) N-terminus, with *E. coli* GBE belonging to group 1. A PSI-BLAST (ALTSCHUL et al., 1997) search using the N-terminal domain of *E. coli* GBE hit several branching enzyme sequences. Many group 1 sequences, including the *E. coli* GBE sequence itself, were hit twice, at the N-terminus itself and in the portion from residue 107 to 208, suggesting a domain duplication. The 107-208 region has previously been reported (BINDERUP et al., 2000) to share homology with the N-domain (see recent review on family 13, MACGREGOR et al., 2001) found in isoamylase from *P. amyloderamosa* (KATSUYA et al, 1998) and α-amylase II from *T. vulgaris* R-47 (KAMITORI et al., 1999). Deletion studies on the latter suggest the involvement of this domain in substrate binding (YOKOTA et al., 2001). A 3-D model is presented based on threading results and secondary structure prediction.

A preliminary homology model of human branching enzyme is also presented and used to aid the molecular interpretation of mutations reported for two related inherited human diseases, polyglucosan body disease and type IV glycogen storage disease (BAO et al., 1996).

References

ALTSCHUL, S.F., MADDEN, T.L., SCHÄFFER, A.A., ZHANG, J., ZHANG, Z., MILLER, W. & LIPMAN, D.J. 1997. *Nucleic Acids Res.* **25**, 3389-3402.

BAO, Y., KISHNANI, P., WU, J.Y. & CHEN, Y.T. 1996. *J. Clinic. Invest.* **97**, 941-948.

BINDERUP, K., MIKKELSEN, R. & PREISS, J. 2000. *Arch. Biochem. Biophys.* **377**, 366-371.

HILDEN, I., LO LEGGIO, L., LARSEN, S. & POULSEN, P. 2000. *Eur. J. Biochem.* **267**, 2150-2155.

KAMITORI, S., KONDO, S., OKUYAMA, K., YOKOTA, T., SHIMURA, Y., TONOZUKA, T. & SAKANO, Y. 1999. *J. Mol. Biol.* **287**, 907-921.

KATSUYA, Y., MEZAKI, Y., KUBOTA, M. & MATSUURA, Y. 1998. *J. Mol. Biol.* **281**, 885-897.

MACGREGOR, E.A., JANECEK, S. & SVENSSON, B. 2001. *Biochim. Biophys. Acta* **1546**, 1-20.

YOKOTA, T., TONOZUKA, T., KAMITORI, S. & SAKANO, Y. 2001. *Biosci. Biotech. Biochem.* **65**, 401-408.

Mechanism of barley malt α-amylase (AMY): inhibition of amylose and maltoheptaose hydrolysis by acarbose and cyclodextrins

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Acarbose is an AMY1 inhibitor (SØGAARD et al., 1993). In the present work inhibition by acarbose of amylose and maltoheptaose AMY-1 and AMY2 catalysed hydrolysis was studied to understand further the inhibition mechanism. Graphical analysis of the kinetic data shows that with both substrates the inhibition is of the uncompetitive type (one binding site). The same result was obtained with AMY1 and AMY2. The corresponding model indicates that under these conditions acarbose does not bind to the active site, however a secondary site is postulated. X-ray data of the acarbose-AMY2 complex show two binding sites: the active site cleft and the surface secondary site; the latter involving stacking of one molecule of acarbose with Trp 276 and Trp 277 (KADZIOLA et al., 1998). This additional site very likely corresponds to the site inferred from the kinetic experiment. The non-observed occupancy of the active site by acarbose in our experiment might result from the different conformation of acarbose in solution (BOCK & PEDERSEN, 1984; our unpublished results) and in the crystal (KADZIOLA et al., 1998). Change of conformation needed for binding at the active site in the crystal may result from different conditions, especially higher acarbose concentration (10 mM) and time (20 h) in the soaking of the crystal than used in the kinetics studies.The secondary site is referred to as the starch granule binding site.

α- and β-cyclodextrin although to a lower extent inhibit the AMY1 catalysed hydrolysis of amylose. Statistical analysis shows that the inhibition is of the non-competitive type. This is consistent with the binding of β-cyclodextrin at the starch binding site (SØGAARD et al., 1993). The inhibitory efficiency of acarbose and cyclodextrins as compared to that of porcine pancreatic α-amylase (PPA) and indeed reflects the respective specificities of the active site of PPA and AMY.

References

BOCK, K. & PEDERSEN, H. 1984. *Carbohydr. Res.* **132**, 142-149.
KADZIOLA, A., SØGAARD, M., SVENSSON, B. & HASER, R. 1998. *J. Mol. Biol.* **278**, 205-217.
SØGAARD, M., KADZIOLA, A., HASER, R. & SVENSSON B. 1993. *J. Biol. Chem.* **268**, 22480-22484.

POSTER SESSION 4
NEW THREE-DIMENSIONAL STRUCTURES

Crystallization and preliminary X-ray study of α-1,4-glucosidase from *Thermomonospora curvata*

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The α-1,4-glucosidase (EC 3.2.1.20) from *Thermomonospora curvata* is a thermotolerant intracellular enzyme degrading small maltooligosacharides to glucose. The enzyme hydrolyses *p*-nitrophenol-α-D–glucopyranoside, sucrose, maltose, and maltodextrins from maltotriose up to maltoheptaose with decreasing efficiency. The α-1,4-glucosidase exhibits optimum activity at pH 7.0-7.5 and 54 ^{o}C. The activity of the enzyme is inhibited by heavy metals and completely inactive in temperature 65 ^{o}C. The sequence of α-1,4-glucosidase consists of 544 amino acids with deduced MW of 60 885 Da and with experimentally determined isoelectric point 4.1. Based on the HENRISSAT classification the α-1,4-glucosidase belongs to the family 13 of glycoside hydrolases. Secondary structure elements of α-1,4-glucosidase were predicted by computer-assisted analysis and show similar topology as α-amylase family of enzymes with $(\alpha/\beta)_8$ barrel structure. Proximate enzyme to α-1,4–glucosidase, with known structure, is oligo-1,6-glucosidase from *Bacillus cereus* (30% identity in primary structure).

For crystallization trials hanging-drop vapor diffusion method at room temperature was used. Protein solution was prepared by dissolving lyophilized non-recombinant α-1,4-glucosidase in 0.05 M phosphate buffer at pH 7.2 to a concentration of 30 mg.ml^{-1}. 1.2 M ammonium sulfate and 0.025 M natrium chloride in 0.1 M phosphate buffer at pH 7.0 was used as the well solution. Crystallization drops of 4 μl volume were prepared by mixing equal volumes of protein and the well solution. After three month the maximum dimension of the crystals was about 0.06 mm. The crystal diffracted to 2.2 Å resolution at 100K using synchrotron radiation at EMBL-Hamburg beam line X 31. The crystal died after several exposures. The space group is trigonal R3 with unit cell parameters a=b=108.6 Å, c=154.3 Å, α=β=90^{o}, γ=120^{o}.

POSTER SESSION 5
PROTEIN ENGINEERING

Enzymatic properties of chimeric enzymes of barley α-amylase isozymes

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Barley malt contains two α-amylase isozyme families readily distinguished by their different isoelectric points, AMY1 (pI=4.7~4.9) and AMY2 (pI=5.9~6.1). Although these isozymes share about 80% sequence identity at the amino acid level, their enzymatic properties are quite different. AMY1 has higher affinity for calcium ions than AMY2 and only AMY2 is strongly inhibited by the endogenous barley α-amylase/subtilisin inhibitor (BASI). In addition, the dominating AMY2 in barley malt is poorly expressed in *Saccharomyces cerevisiae* and *Pichia pastoris* in comparison with AMY1. In order to identify regions responsible for these two isozyme-specific properties, six chimeric enzymes were constructed and expressed in *Pichia pastoris* and their enzymatic properties and expression levels were investigated. The three sequence parts used for the constructions of isozyme chimeras are specified as follows; (I) N-terminus to α3, this includes domain B containing all except one of the calcium binding ligands (aa 1~160), (II) α3 to α7b, containing the catalytic residues (aa 161~318), and (III) β8 to the C-terminus (aa 319~414 in AMY1/403 in AMY2). As a result, only chimeric AMY112 showed the same trend in calcium ion dependency of activity as that of AMY1, but the chimeric AMY122 and AMY221 were similar to AMY2. The rest of the chimeras were categorized to a group with intermediate properties, but closely resembling AMY2. The binding of BASI with the different chimeric enzymes will also be presented.

Role of amino acid residues conserved near to catalytic site in *Bacillus licheniformis* α-amylase in transglycosylation reaction

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Industrial starch processing is one of the most important agroindustries due to the low cost and availability of the raw material that can be modified by a variety of enzymes giving rise to a large number of products. α-Amylases are the most common enzymes involved in depolymerize starch by two main mechanisms industrially defined as liquefying or saccharifying. The *Bacillus licheniformis* α-amylase is a liquefying α-amylase widely used industrially because of its thermostability. However, its efficiency in starch depolymerization is limited, requiring the complementary activity of saccharifying enzymes with the disadvantage of having to modify the reactor conditions. In the present work we describe the analysis of the primary and 3-D structure of homologous proteins to try to identify the residues involved in processing the reaction specificity in order to transform the thermostable *B. licheniformis* α-amylase towards a more saccharifying enzyme. So far, we have identified positions V286 (MATSUI et al., 1994) and Y56 (SAAB-RINCÓN et al., 1999) as targets. Mutants in these positions have been produced and purified. Their main kinetic properties are described in this presentation.

References

MATSUI, I., YONEDA, S., ISHIKAWA, K., MIYAIRI, S., FUKUI, S., UMEYAMA, H. & HONDA, K. 1994. *Biochemistry* **33**, 451-458.

SAAB-RINCÓN, G., DEL-RÍO, G., SANTAMARÍA, R.I., LÓPEZ-MUNGUÍA, A. & SOBERÓN, X. 1999. *FEBS Lett.* **453**, 100-106.

Protein engineering of *Bacillus licheniformis* α-Amylase for low pH performance

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Currently, industrial scale starch liquefaction is constrained to operating at pH 6.0, and above, as the enzyme used in the process, *Bacillus licheniformis* α-amylase (BLA) is unstable, at the lower pH under conditions used. Using sequence and structure analysis to rational known improved variants, an information based strategy was used to design additional mutations. Of the six mutant proteins designed, all six were more stable.

Key role of hydrophobic residues in determining the transglycosylating activity of cyclodextrin glycosyltransferase

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Although members of the α-amylase family use the α-retaining mechanisms in their conserved catalytic site, it is striking to find that they vary widely in their substrate and product specificity. Besides the presence of different domains attached to the catalytic core, these differences can also be attributed to differences in the architecture of the active site. A major subdivision within the α-amylase family can be made on the basis of the preferred acceptor: (i) water leading to hydrolysis of the donor substrate, as is found for α-amylase or isoamylase, or (ii) another saccharide resulting in a transglycoslyation reaction, as is found for cyclodextrin glycosyltransferase (CGTase) or amylomaltase (AMase). The transferase activity of CGTase is almost 350 times higher than the rate of hydrolysis; for AMase virtually no hydrolysis can be measured. Studies on neopullulanase and α-amylase have suggested that the presence of hydrophobic residues around the catalytic site disfavours the use of water as an acceptor. We have studied the structure-function relationships of CGTase and AMase in detail using protein crystallography, 3D structure elucidation and site-directed mutagenesis. We found that phenylalanine residues in the vicinity of the catalytic triade play an important role in determining the exclusive transferase activity of CGTase.

POSTER SESSION 6
RAW STARCH BINDING AND DEGRADING

Enzymatic modification of raw potato starch

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Native starch is modified genetically, physically and chemically to obtain a wide range of useful functional properties. Mostly the hydrolysis results in soluble dextrins. However hydrolysing intact raw starch at temperatures below their gelatinisation temperature yields insoluble granules. The success of such enzyme hydrolysis depends on both botanical origin of the starch and choice of enzyme. Potato starch is in general more difficult to hydrolyse than other starches.

In this study native potato starch is treated with a limited and controlled enzyme hydrolysis at a temperature below the gelatinisation temperature. The enzyme used has been glucoamylase (*Aspergillus niger*). The incubation conditions have been: 45°C, pH 4.5, time (0h – 48h), and glucoamylase concentrations (10 U/ml, 50 U/ml and 100 U/ml). The degree of hydrolysis has been calculated as hydrolysed starch in % of total. Maximal hydrolysis was 40%, which was obtained by incubation for 48 h with 100 U/ml.

The melting characteristics of the enzyme hydrolysed starch samples were studied by differential scanning calorimetry (DSC). The expected effect of enzyme hydrolysis was masked by a concomitant annealing effect. Further analysis of the gel forming ability was analysed by a controlled stress rheometer (stress tech), a viscometer (rapid visco analyzer, RVA) and a texture analyzer (TA). From these preliminary data it has been shown that the enzyme hydrolysis yield potato starches with altered gelatinisation temperature, and pasting profiles as well as stronger and more elastic gels.

Hydrolysis of starch substrates by amylases before fermentation

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Starch and starch-containing substrates are widespread in nature and also in industrial praxis. They can predominantly find their application in a process of alcoholic fermentation, which is carried out in fermentors allowing simultaneous saccharification and fermentation of suitable substrates. The main aim of this work was to contribute to enzymatic hydrolysis of starch substrates usable for ethanol fermentation using immobilized cells performed in an industrial scale.

Hydrolysis of starch substrates and their consecutive use for ethanol fermentation is a question of optimization when using various enzymatic products under various conditions of hydrolysis. We tried to maximize the saccharification degree in order to achieve an easily and quickly fermentable form with minimal operating expenses.

The aim of this work was to test various enzymatic products of two world-known companies providing amylolytic enzymes (Novozyme, Denmark; Gamma Chemie, GmbH, Germany) for hydrolysis of selected starch substrates.

The course of hydrolysis was followed in a suspension of pure starch in distilled water using various amounts of the products at various pH values of the substrate. Termamyl (thermostable α-amylase) by Novozymes was used for liquefaction and the AMG (amyloglucosidase) or San Super 240 L (amyloglucosidase with additional α-amylase and proteinase activity) were used for saccharification. The results showed that Termamyl with AMG is the most appropriate combination. When using the products of Gamma Chemie, Gamalpha HT (thermostable α-amylase) was used for liquefaction and Gamylo 300 L (amyloglucosidase), Gamylo Super 300 L (amyloglucosidase), Gamaltase 240 L (amyloglucosidase) were used for the consecutive saccharification.

The enzyme products of Novozymes were used due to their higher performance of hydrolysis of starch to glucose for the enzymatic hydrolysis of starch substrates. The spectrum of substrates included flours, i.e. whole-meal wheat flour, scrap wheat flour, corn flour and pure starch. The highest yield of hydrolysis of starch to glucose was achieved in case of the whole-meal wheat flour.

Hunting in the molecular-biology databases for sequences related to the raw starch-binding domain

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The ability to bind and degrade raw starch in the α-amylase family enzymes resides in most cases in the C-terminal domain, called the starch-binding domain (SBD) (SVENSSON et al., 1989). Until now this SBD was thought to exist exclusively in microbial producers of amylolytic enzymes. Recently it has been recognised that this property may be connected to other sequence-structural elements, such as some C-terminal repeats (RODRIGUEZ-SANOJA et al., 2000; SUMITANI et al., 2000), or a part of the catalytic $(\beta/\alpha)_8$-barrel domain (TIBBOT et al., 2000), or even as a starch granule binding site (SØGAARD et al., 1993). The present work focuses on the C-terminal SBD. It used the wealth of sequence information stored in the molecular-biology databases (especially from the sequencing of complete genomes) in an effort to search for sequences similar to that of SBD. The main goal was to show as wide a spectrum of proteins (both experimentally confirmed and putative ones) and organisms as possible that may contain the sequence features related to the SBD.

A study comparing the SBD sequences from the three amylolytic families 13, 14 and 15 has revealed the interesting evolution of this domain (JANEČEK & ŠEVČÍK, 1999). It reflects the evolution of species rather than evolution of the individual amylases. Moreover, it has been pointed out (MINASSIAN et al., 2000) that laforin, a protein related to epilepsy, contains a few significant sequence-structural features of SBD. All these observations evoke the interest to find out the proteins closely and distantly related to SBD.

BLAST (ALTSCHUL et al., 1990) together with ProDom (CORPET et al., 2000) tools were used for performing the searches in the molecular biology databases. As query sequence the entire sequence of SBD or its parts were applied. Several proteins of microbial, plant and animal origins were identified as having sequence segments eventually related to the SBD sequence. Examples are human genethonin, mouse lamins B-2 and B-3, degreening-enhanced protein from *Chlorella protothecoides*, fructose-6-phosphate 2-kinase/fructose-2,6-bisphosphatases from *Arabidopsis thaliana*, neuroglian precursors from *Drosophila melanogaster*, two putative proteins from *Arabidopsis thaliana* and one from *Mesorhizobium loti*. Among these, the N-terminal part of human genethonin (BOUJU et al., 1998) was found as the most striking example exhibiting unambiguous sequence similarity with the entire length of the SBD sequence.

References

ALTSCHUL, S.F., GISH, W., MILLER, W., MYERS, E.W. & LIPMAN, D.J. 1990. *J. Mol. Biol.* **215**, 403-410.

BOUJU, S., LIGNON, M.F., PIETU, G., LE CUNFF, M., LEGER, J.J., AUFFRAY, C. & DECHESNE, C.A. 1998. *Biochem. J.* **335**, 549-556.

CORPET, F., SERVANT, F., GOUZY, J. & KAHN, D. 2000. *Nucleic Acids Res.* **28**, 267-269.

JANEČEK, Š. & ŠEVČÍK, J. 1999. *FEBS Lett.* **456**, 119-125.

MINASSIAN, B.A., IANZANO, L., MELOCHE, M., ANDERMANN, E., ROULEAU, G.A., DELGADO-ESCUETA, A.V. & SCHERER, S.W. 2000. *Neurology* **55**, 341-346.

RODRIGUEZ SANOJA, R., MORLON-GUYOT, J., JORE, J., PINTADO, J., JUGE, N. & GUYOT, J.P. 2000. *Appl. Environ. Microbiol.* **66**, 3350-3356.

SØGAARD, M., KADZIOLA, A., HASER, R. & SVENSSON, B. 1993. *J. Biol. Chem.* **268**, 22480-22484.

SUMITANI, J., TOTTORI, T., KAWAGUCHI, T. & ARAI, M. 2000. *Biochem. J.* **350**, 477-484.

SVENSSON, B., JESPERSEN, H., SIERKS, M.R. & MACGREGOR, E.A. 1989. *Biochem. J.* **264**, 309-311.

TIBBOT, B.K., WONG, D.W. & ROBERTSON, G.H. 2000. *J. Protein Chem.* **19**, 663-669.

Identification of a new type of starch binding domain: the tandem repeat units in the C-terminal region of *Lactobacillus amylovorus* α-amylase

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Raw starch degrading amylases are commercially important enzymes of the beverage, food and textile industry. Amylolytic lactic acid bacteria are attractive biotechnological organisms since they can directly produce lactic acid from starch. The α-amylase gene of *Lactobacillus amylovorus* has been isolated and consists of two distinct regions (GIRAUD & CUNY, 1997). The first 410 amino acids of *L. amylovorus* α-amylase are sufficient to transfer an amylolytic activity to a non-amylolytic-strain of *L. plantarum* (JORE & DE PARASIS, 1993), suggesting that the N-terminal part of this enzyme contains the active site. The 3'-terminal half of the gene exhibits a special tandem repeated units structure (GIRAUD & CUNY, 1997; MORLON-GUYOT, J., MUCCIOLO-ROUX, F., RODRIGUEZ SANOJA, R. & GUYOT, J.P., unpublished results).

In this study, we investigated the functional role of the carboxyl terminal repeat sequences of the *L. amylovorus* amylase (AmyA) by using clones encoding either the entire AmyA or the truncated amylase AmyAΔ, which is deleted of the repeated sequences. The two constructs were expressed into *Lactobacillus plantarum*, and their expression products purified, characterized and compared. AmyA and AmyAΔ exhibit similar amylase activity towards a range of soluble substrates (amylose, amylopectin and α-cyclodextrin, and soluble starch). The specific activities of both enzymes towards soluble starch are similar, but the K_M and V_{max} values of AmyAΔ were slightly higher than those of AmyA, whereas the thermal stability of AmyAΔ was lower than that of AmyA. In contrast to AmyA, AmyAΔ is unable to bind to β-cyclodextrin and presents low activity towards glycogen. More striking is that AmyAΔ cannot bind nor hydrolyse raw starch, demonstrating that the carboxyl terminal repeating units domain of AmyA is involved in the ability of this enzyme to bind to raw starch.

This is the first time that these tandem repeat units have been identified as a new type of starch binding domain, which is moreover believed to be of general occurrence among various amylolytic lactic acid bacteria α-amylases.

References

GIRAUD, E. & CUNNY, G. 1997. *Gene* **198**, 149-157.
JORE, J.P.M. & DE PARASIS, J. 1993. *FEMS Microbiol. Rev*. **12**, 26.

Genome mining for α-amylases of *Microbacterium aurum* involved in the degradation of native potato starch granules

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In a study on the enzymatic degradation of potato starch granules, a *Microbacterium aurum* strain was isolated that degraded the granules efficiently by the drilling of small pores. To study the granular starch degrading enzymes of this strain, an expression gene bank was constructed in *Escherichia coli.* Transformants were screened on agar plates containing Cibacron-red dyed amylopectin; those transformants that expressed enzymes degrading amylopectin showed a clearing zone around the colony (JØRGENSEN et al., 1997). From a total of 70,000 transformants, 13 were found to have an activity towards amylopectin. One of these positive transformants, containing an 11 kb genomic insert, was selected for further characterization.

To find out which extracellular enzyme(s) were capable of degrading amylopectin, culture fluid was subjected to SDS-PAGE containing amylopectin. After renaturation it appeared that the clone secreted relatively large amylopectin-degrading enzymes of up to 140 kDa. Electron microscopy of native potato starch granules treated with culture fluid of the *E. coli* transformant showed the characteristic drilling pattern as was found for the *Microbacterium aurum* strain.

DNA sequencing of the genomic insert resulted in the identification of at least two open reading frames. These genes show high homology to α-amylases of *Streptomyces* and *Bacillus* species. One of the two genes contains C-terminal repeats with high homology to repeats known to be involved in raw starch degradation (SUMITANI et al., 2000). Also C-terminal repeats with similarity to repeats found in chitinases were found. Future studies have to reveal the exact organisation of these genes.

References

JØRGENSEN, S., VORGIAS, C.E. & ANTRANIKIAN, G. 1997. *J. Biol. Chem*. **272**, 16335-16342.
SUMITANI, J., TOTTORI, T., KAWAGUCHI, T. & ARAI, M. 2000. *Biochem. J.* **350**, 477-484.

POSTER SESSION 7
STABILITY AND STABILISATION

Calorimetric characterization of mutants from a cold-active α-amylase

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Small globular proteins that unfold according to a pure two-state process, i.e. reversibly and without stable intermediate between the native and the unfolded states, have allowed to establish the thermodynamic stability function accounting for the Gibbs energy change associated with their denaturation over physiological and non-physiological temperature ranges. The current availability of this thermodynamic function, gained by differential scanning calorimetry, offers a powerful tool for investigating the driving force allowing a polypeptide to fold into a determined native and biologically active conformation.

The cold-active and heat labile α-amylase from the Antarctic bacterium *Pseudoalteromonas haloplanktis* is an exceptional case as it is the largest known protein undergoing a reversible unfolding according to a two-state reaction pathway. It has been hypothesized that this unusual behavior is dictated by the requirement for an improved flexibility or plasticity of the protein molecule in order to perform catalysis at near-zero temperatures, therefore producing an enzyme close to the lowest possible stability of its native state (FELLER et al., 1999). Accordingly, one can expect that any mutation designed to modify the stability of this fragile edifice will induce significant perturbations of the unfolding parameters, therefore giving evidence for the structural features linked to unfolding cooperativity, enthalpic-entropic compensation or reversibility.

We report here the calorimetric characterization of mutants from the cold-active α-amylase constructed on the basis of its strong homology with heat-stable mesophilic α-amylases (D'AMICO et al., 2001). These mutants reveal the unsuspected contribution of single amino acid substitution to the thermodynamic parameters characteristic of large, heat-stable mesophilic proteins and provide new insight into the molecular adaptations of enzymes from psychrophilic organisms.

References

D'AMICO, S., GERDAY, C. & FELLER, G. 2001. *J. Biol. Chem.* (in press).
FELLER, G., D'AMICO, D. & GERDAY, C. 1999. *Biochemistry* **38**, 4613-4619.

Stabilization and protein engineering of α-amylase from *Saccharomycopsis fibuligera*

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In previous study, we have succeeded to optimize both up and down stream processes using *Saccharomycopsis fibuligera* strain R64 which was selected among 136 isolates collected from different regions in Indonesia. Although this strain was able to produce high activity and large amount of amylolytic enzymes, their stability were not suitable for industrial applications. Here we describe a part of our work in order to obtain stabilized enzymes. First, we discuss the separation of amylolytic enzymes, then the effect of polyol addition on the activity and thermal stability of both α-amylase and glucoamylase; finally we will describe ongoing work.

Extracellular amylolytic enzymes produced by *S. fibuligera* grown on media of sago starch and yeast extract, α-amylase and glucoamylase, have almost similar molecular weight and charge causing their separation is problematic. Based on hydrophobic interaction principle, we have separated and characterized both enzymes. After centrifugation at 4200 g for 15 min at 4°C, separation was conducted through 25% ammonium sulphate precipitation of the supernatant followed by column chromatography of butyl-Toyopearl 650M with stepwise gradient of 20, 15, 10, 5, 0% ammonium sulphate in 50 mM Tris/HCl buffer pH 8.0. Purification of the enzymes was performed by applying fraction of 15% or of 5% ammonium sulphate, containing of α-amylase or of glucoamylase, to a column of DEAE-Toyopearl 650M equilibrated with 25 mM Tris/HCl buffer pH 8.0, linear gradient between 0 and 1.0 M NaCl was used. The relative MW of α-amylase is 54 kD, glucoamylase is 63 kD, maximal activity is reached by both enzymes at pH 5.0 at a temperature of 50°C. Addition of Mg(II) or Ca(II) ion increased both enzymes activities. They have different both in N-terminal amino acids and in peptide pattern resulted from a chymotrypsin-cleavage.

The polyols used were glycerol, ethylenglycol, and sorbitol with various concentrations (0–4 M). Sorbitol at 4 M gave the highest stabilizing effect, while addition of glycerol or ethyleneglycol was almost no effect on both enzymes. There was a correlation between increasing amylases thermal stability and increasing both concentration and MW of polyol added. The stabilized enzymes in 4 M sorbitol were still active up to 85°C, while untreated enzymes were only active up to 60°C. There were no changes in optimum pH, but the optimum temperatures were increased from 50°C in untreated enzymes to 54°C in stabilized enzymes. Eventhough there were no significant changes in K_M values, V_{max} were decreased significantly in treated enzymes. Presumably, polyol made a layer that wrapped the enzyme molecules that might cause the substrates need time and effort to reach the enzyme active sites.

In the ongoing study, we have been working to obtain stable enzymes by chemical modification and protein engineering. Modifications with dimethyladipimidate as bifunctional reagent (crosslinking of surface functional groups), and with glyoxylic acid as hydrophilization agent of enzyme surface are in progress. We also have been conducting structure and functional relationship study by cutting the enzymes (with and without denaturation) by various proteases. Then, we will perform site-directed mutagenesis by cloning amylase genes into *Saccharomyces cerevisiae*. Condition of mutagenesis will be adjusted the structural information derived from chemical modifications and also from structure and functional relationship studies. We will then try to over-produce amylases in *Saccharomyces cerevisiae* as host.

Chemical modification of bacterial α-amylases: changes in tertiary structures and the effect of additional calcium

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Chemical modification of lysine residues in two bacterial α-amylases, a mesophilic enzyme from *Bacillus amyloliquefaciens* (BAA) and a thermophilic enzyme from *Bacillus licheniformis* (BLA) was carried out using citraconic anhydride. Modification resulted in activity and stability enhancements including a dramatic increase in stability of BAA at 80 ^{o}C, a 15-fold enhancement of activity of BLA at 15 ^{o}C with a 3-fold increase in kcat/Km at 37 ^{o}C. CD and irreversible thermoinactivation studies of these metalloproteins in native and modified forms were also carried out in the absence and presence of additional Ca^{2+} (10 mM). CD experiments indicated changes in the tertiary structures of the proteins upon modification, influenced by the presence of additional Ca^{2+}. Sorbitol was found very effective in affording protection against irreversible thermoinactivation of both forms of the enzymes in the absence and presence of Ca^{2+}. Results are discussed in terms of the usefulness of this approach involving a combination for protein stabilization and activity enhancement.

Proteolysis of bacterial α-amylases: segmental mobility and the effect of additives

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A comparative study was performed on limited and extensive proteolysis of mesophilic (from *Bacillus amyloliquefaciens*, BAA) and thermophilic (from *Bacillus lichenformis*, BLA) α-amylases using trypsin. As expected, the thermophilic enzyme showed greater resistance to digestion by the protease. While the catalytic potential of BLA was enhanced upon proteolysis, that of BAA was diminished due to this process. Combined with greater catalytic activity, a lower thermal stability was observed for BLA on proteolytic treatment. Also by CD measurements, it was found that the T_m of the native enzyme was diminished from 89 to 86 ^oC , a further indication of enhanced flexibility owing to this process. For both enzymes, the extent of proteolytic cleavage was reduced in the presence of various stabilizing agents. A lower degree of proteolysis obtained in the presence of the additives tested is suggested to occur as a result of the stabilization (or "rigidifying") effect of these cosolvents. This is presumably an outcome of preferential hydration of the proteins.

Exploring diversity of thermostable starch-modifying enzymes from Icelandic hot springs

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Conserved regions in protein families are used to design consensus primers for amplification of a spectrum of genes encoding starch-modifying enzymes from cultured as well as noncultured microorganisms. Genomic template DNA is obtained both from enrichment cultures and directly from biomass in Icelandic hot springs. The full-length genes are thereafter inserted in an expression vector and produced in *Escherichia coli*. Production and properties of proteins obtained using this strategy will be presented. The possibility to retrieve a wide diversity of genetic material using this screening technique will be discussed.

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