The unique glycoside hydrolase family 77 amylomaltase from *Borrelia burgdorferi* with only catalytic triad conserved

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**Abstract**

Glycoside hydrolase family 77 (GH77) contains prokaryotic amylomaltases and plant-disproportionating enzymes (both possessing the 4-α-glucanotransferase activity; EC 2.4.1.25). Together with GH13 and GH70, it forms the clan GH-H, known as the α-amylase family. Bioinformatics analysis revealed that the putative GH77 amylomaltase (MalQ) from the Lyme disease spirochaete *Borrelia burgdorferi* genome (BB0166) contains several amino acid substitutions in the positions that are important and conserved in all GH77 amylomaltases. The most important mutation concerned the functionally important arginine positioned two residues before the catalytic nucleophile that is replaced by lysine in *B. burgdorferi* MalQ. Similar remarkable substitutions were found in two other putative GH77 amylomaltases from related borreliae. In order to confirm the exclusive sequence features and to verify the eventual enzymatic activity, the malQ gene from *B. burgdorferi* was amplified using PCR. A c. 1.5-kb amplified DNA fragment was sequenced, cloned and expressed in *Escherichia coli*, and the resulting recombinant protein was preliminarily characterized for its activity towards glucose (G1) and a series of malto-oligosaccharides (G2–G7). This study confirmed that the remarkable substitution of the arginine really exists and the GH77 MalQ protein from *B. burgdorferi* is a functional amylomaltase because it is able to hydrolyse the malto-oligosaccharides as well as to form their longer transglycosylation products.

**Introduction**

Amylomaltase (EC 2.4.1.25) is a member of the α-amylase family (MacGregor *et al.*, 2001) that, in the sequence-based classification of glycoside hydrolases (GH), consists of the three families GH13, GH70 and GH77 forming the clan GH-H (Coutinho & Henrissat, 1999). The members of the α-amylase family are starch hydrolyases and related enzymes (e.g. MacGregor *et al.*, 2001; Pujadas & Palau, 2001; Janecek, 2002; van der Maarrel *et al.*, 2002; Kuriki *et al.*, 2006).

Amylomaltase belongs to the family GH77 (Coutinho & Henrissat, 1999; MacGregor *et al.*, 2001). The main difference discriminating this enzyme from the members of the main GH13 α-amylase family is the lack of the domain C succeeding the catalytic (β/α)8-barrel of GH13 members (Przylas *et al.*, 2000). Amylomaltase is a 4-α-glucanotransferase – it catalyses the glucan-chain transfer from one α-1,4-glucan to another α-1,4-glucan (or to 4-hydroxyl group of glucose) or within a single linear glucan molecule to produce a cyclic-α-1,4-glucan (Takahashi *et al.*, 1993; Terada *et al.*, 1999; Kaper *et al.*, 2005). The GH77 amylomaltases have been found in microorganisms (bacteria and archaeons) and plants (including algae) to be involved in the metabolism of malto-oligosaccharides and glycogen or starch, respectively (Boos & Shuman, 1998; Lu & Sharkey, 2006). The name amylomaltase is used for the microbial GH77 4-α-glucanotransferases whereas the plant counterparts are usually called disproportionating enzymes (or shortly D-enzymes) (Takahashi *et al.*, 1993; Terada *et al.*, 1999; Kaper *et al.*, 2005).

One of the fundamental characteristics of all the α-amylase family members is the presence of conserved sequence regions (MacGregor *et al.*, 2001; Janecek, 2002) that cover the residues conserved invariantly. As the number of enzyme sequences and specificities belonging to the α-amylase family increased considerably, the number of invariant residues decreased dramatically. It has been established that there are only four residues (Janecek, 2002). These were the three catalytic residues (i.e. the catalytic triad) corresponding to the Asp206, Glu230 and Asp297 of the Taka-amylase A (MacGregor *et al.*, 2001), i.e. the...
catalytic nucleophile, proton donor and transition state stabilizer (Qian et al., 1993; Uitdehaag et al., 1999), respectively. The fourth invariant residue was the β4-strand arginine (Arg204) in position i-2 with respect to the catalytic nucleophile (β4-strand Asp206) (Janecek, 2002). It is worth mentioning that this is a functionally important arginine because in general in the α-amylase family it forms a hydrogen bond with the catalytic nucleophile and O2 of the glucosyl moiety of the substrate at subsite −1 (Uitdehaag et al., 1999; Matsuzura, 2002; Ravaud et al., 2007). It is also involved in the Cl-binding site of chloride-dependent α-amylases (Aghajari et al., 1998; D’Amico et al., 2000). The α-amylase family can thus be considered to be an enzyme family where some groups or subfamilies exhibit a high degree of sequence similarity but the overall sequence identity is extremely low (Lee et al., 2002; Osłancova & Janecek, 2002; Stam et al., 2006).

It was, therefore, reasonable to anticipate whether a member of the α-amylase family exists (i.e. could be revealed) that contains conserved only the catalytic triad. The eventual loss of the invariance of the above-mentioned arginine was first demonstrated by Machovic & Janecek (2003), who found a lysine in the corresponding position in the putative GH77 amylo maltase present in the Borrelia burgdorferi genome (Fraser et al., 1997).

The main goal of this work was therefore to verify whether or not the natural Arg-to-Lys substitution does exist in the above-mentioned unique GH77 member from B. burgdorferi and, if yes, then to deliver the first experimental proof that such protein may retain its activity.

Materials and methods

Bioinformatic analysis

The amino acid sequences of real and hypothetical GH77 amylomaltases (Table 1) were retrieved from GenBank (Benson et al., 2007). The set was collected in an effort to make a comparison of all real prokaryotic amylomaltases and plant-disproportionating enzymes. All known amylomaltase-like hypothetical proteins from sequenced genomes of four borreliae (Fraser et al., 1997; Glockner et al., 2004, 2007; Pettersson et al., 2007) were also included the analysis. The set of compared enzymes was completed by selected hypothetical GH77 members – according to the CAZY database (Coutinho & Henrissat, 1999) – covering as wide as possible spectrum of bacterial, archaeal and plant producers (Table 1).

In order to obtain all the eventual examples of the studied arginine-to-lysine substitutions in these proteins and related enzymes from the α-amylase family, especially from the unfinished genome sequencing projects, the BLAST (Altschul et al., 1990) tools were used.

### Table 1. List of GH77 members used in the present study

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<th>Source</th>
<th>Accession numbers*</th>
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*The accession numbers are the GenPept Protein_id Nos. from Genbank.
†The length in amino acid residues.

The amino acid retrieved sequences were aligned using the program CLUSTALW (Thompson et al., 1994) and the selected conserved sequence regions covering the strands β2, β3, β4, β5 and β7 of the catalytic (β/α)8-barrel were cut and used for further analysis. The evolutionary tree was calculated on the European Bioinformatics Institute’s server for CLUSTALW (http://www.ebi.ac.uk/clustalw) as a PHYLP tree type (Felsenstein, 1985) using the alignment of the selected conserved sequence regions. The tree was displayed with the program TREEVIEW (Page, 1996).

Bacterial strains, media and plasmids

Two strains of Escherichia coli were used (Novagen): MC1061 for vector propagation and BL21 (DE3) for gene
expression. Competent cells of *E. coli* were prepared and transformed according to standard molecular biology protocols (Sambrook *et al*., 1989). Plasmids used were pLitmus 28i (New England Biolabs) for sequencing and pET21d(+) (Novagen) for gene expression.

**Cloning of the malQ gene**

Based on the nucleotide sequence of the putative GH77 amylomaltase (malQ) from *B. burgdorferi* genome (BB0166; GenBank Acc. No.: AE000783; protein id.: AAC66547.1) (Fraser *et al*., 1997), the primers for PCR amplification of the malQ gene were designed as follows:

- **B1** (NcoI) primer 5'-GGAGGGATCCCATATGAAAATAAAAAAACAAAAAGGATATTTAAC-3'
- **B2** (XhoI) primer 5'-CCGGCTCGAGAGCCCTGGCATAAAGGCTTGTAAAAAACT-3'

NcoI and XhoI restriction sites (bold face) were introduced in the forward (B1) and reverse (B2) primers, respectively, to allow the cloning into pLitmus 28i for sequence analysis and pET21d(+) for expression. Start codon ATG in the B1 primer sequence is underlined; the B2 primer does not contain the malQ gene stop codon.

Using these primers and the DNA from *B. burgdorferi* (a gift from Dr Manesh Bhide, University of Veterinary Medicine, Kosice, Slovakia) as a template, a c. 1.5-kb DNA fragment was amplified using PCR. The obtained amplified-PCR product was digested by restriction endonucleases NcoI and XhoI and then cloned into both pLitmus 28i for sequence analysis and pET21d(+) for gene expression. Recombinant MalQ protein contained 6xHis tag at the C-terminus.

The determined nucleotide sequence of the gene coding for the recombinant amylomaltase-like GH77 protein was deposited with the GenBank (Benson *et al*., 2007) under the Acc. No. EF634455.

**MalQ production and purification**

*Escherichia coli* BL21 (DE3) cells containing the recombinant malQ gene in pET21d(+) expression vector, designated as pET21Bor, were grown in Luria–Bertani medium supplemented with 100 μg mL⁻¹ ampicillin at 37°C. Gene expression was induced by adding the isopropyl-β-D-thiogalactoside (IPTG) to a final concentration of 0.5 mM when the absorbance of the culture medium measured at 600 nm reached 0.3. The cultivation was then continued for 15 h at 30°C. The cells from 2-L culture were harvested by centrifugation (7000 g, 15 min, 4°C) and the pellet was frozen at −20°C.

Before use, the frozen *E. coli* cells were suspended in sterile water and then disrupted by sonication; the crude extract was centrifuged (12 500 g, 20 min, 4°C) to remove the cell debris. The supernatant was filtered through a bacteriological filter (0.22 μm pore size, Millipore) and harvested for subsequent purification. Solid ammonium sulphate was added slowly to the resulting supernatant to 70% saturation. The precipitate formed was removed by centrifugation at 15 000 g for 20 min and dissolved in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl and 10 mM imidazole, pH 8). Ammonium sulphate was removed by dialysis against the lysis buffer. Protein solution was loaded onto an Ni-NTA (NTA, nitriolate acid) Superflow column and the 6xHis-tagged MalQ was purified by two successive Ni-NTA affinity purification steps under native conditions according to the Qiagen protocol. The eluates were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the positive ones were pooled, dialysed against buffer A (10 mM KH₂PO₄–Na₂HPO₄, pH 7), filtered through a bacteriological filter (0.22 μm pore size, Millipore) and stored at −20°C. For further characterization of the enzyme activity, 20 μL of the enzyme solution was used.

**Partial biochemical characterization of the GH77 enzyme**

The amylomaltase-like activity of the recombinant protein (MalQ) was determined as glucose released from the maltotriose substrate by the glucose oxidase method (Miwa *et al*., 1972). One unit of enzyme activity was defined as the amount of enzyme, that produced 1 μmol glucose min⁻¹ under the assay conditions. The activity was assayed in a 30-μL reaction mixture containing 1% substrate (w/v) in 20 mM sodium acetate buffer (pH 5.5) and the enzyme solution. The mixture was incubated at 37°C for 15 h. The temperature and pH optima of the enzyme were determined by incubating the reaction mixtures at 20, 30, 37 and 45°C and pH 3.5, 4.0, 5.0, 5.5, 6.0, 7.0, 8.0, 8.5 and 9.5, respectively. In order to gain an insight into the eventual specificity of the MalQ, the reaction with glucose (G1; Sigma) and a series of malto-oligosaccharides (maltose, maltotriose, maltotetraose, maltohexaose and maltoheptaose; G2–G7; Sigma) was investigated with thin-layer chromatography (TLC) (Terada *et al*., 1999) using different enzyme concentrations (0, 5.35, 10.7 and 21.4 μM mL⁻¹). Three-microlitre aliquots of reaction mixtures were spotted onto a TLC plate (Silica Gel 60, Merck) that was developed three times with 1-butanol–ethanol–water (5 : 5 : 3, v/v). The carbohydrates were detected by spraying the plate with sulphuric acid–methanol (1 : 1, v/v) and then baked at 130°C for 5 min.

**Results and discussion**

**Bioinformatics analysis**

The well-established fact that there are at least four residues conserved invariantly throughout the entire α-amylase family is no longer sustainable because the sequences of the three (*B. burgdorferi, Borrelia garinii* and *Borrelia afzelii*) of...
Fig. 1. (a) Selected conserved sequence regions in representative GH77 amylomaltases and putative GH77 proteins. The regions correspond to the strands $b_2$, $b_3$, $b_4$, $b_5$ and $b_7$ of the catalytic ($b_4/a_8$)-barrel domain. The individual taxonomic groups are distinguished from each other by colours, the borreliae also being differentiated from the rest of the bacteria. Colour code for the amino acid residues: catalytic triad, blue; invariant arginine, yellow; functional histidine, violet; additional conserved residues, black; substituted residues and exclusive positions in borrelial GH77 proteins, red and grey, respectively; three strange positions in *Borrelia turicatae* enzyme, green. The invariant catalytic triad of the entire clan GH-H is indicated by asterisks. The position with the most interesting mutation found in the three borrelial GH77 proteins, that is, the $b_4$-stand arginine-to-lysine exchange, is emphasized by an arrow. (b) Evolutionary tree of compared GH77 amylomaltases and putative amylomaltase-like proteins. The tree is based on the alignment of the above conserved sequence regions.
the four borrelial GH77 amylomaltase-like proteins have the β4-strand arginine substituted by a lysine (Fig. 1a). This observation supports the possibility that the Arg/Lys substitution actually exists naturally, i.e. the eventuality of a sequencing error is minimized. Moreover, all the three above-mentioned GH77 amylomaltase-like members exhibit several additional remarkable sequence features that distinguish them from the rest of the GH77 enzymes. These are both the exclusive residues and the specific substitutions (Fig. 1a). The former features are represented by: (1) serine (Ser49 in the amino acid sequence of B. burgdorferi protein) and phenylalanine (Phe56) in the region covering the strand β2 of the (β/α)8-barrel; (2) glutamate (Glu223) and methionine (Met226) in the region β3; and (3) tryptophan (Trp352) and valine (Val353) in β5, whereas the latter are formed by: (1) alanine (Ala57) instead of proline in the region covering the strand β2; (2) asparagine (Asn227) instead of aspartate in the region β3; (3) phenylalanine (Phe356) and glutamine (Gln357) (or glutamate) replacing the otherwise invariant leucine and glycine in β5; and (4) glycine (Gly406) (or serine) instead of histidine in β7. The arginine-to-lysine (Lys305) substitution in the β4-strand region (Fig. 1a) is the most important change that deserves special attention from the point of view of the entire α-amylase family, i.e. the clan GH-H, because the arginine (Arg204 in Taka-amylase A) is known to be conserved in more than several thousands of sequenced members of the clan GH-H and was thus considered to be one of the indispensable α-amylase family residues (Coutinho & Henrissat, 1999; MacGregor et al., 2001; Janecek, 2002; Kuriki et al., 2006).

All these sequence features can also be reflected in the evolutionary tree of the GH77 real amylomaltases and putative amylomaltase-like proteins (Fig. 1b) where the three borreliae proteins are positioned on a separate branch on a long distance from the rest of the bacterial, archaeal and plant counterparts. It is worth mentioning, however, that the β4-strand arginine is conserved (i.e. not mutated) in the fourth representative of available borrelial GH77 proteins from the recently released genome sequence of *Borrelia turicatae* (Pettersson et al., 2007). As can be seen from the conserved sequence regions (Fig. 1a), this GH77 amylomaltase-like protein contains only a few exclusive features characteristic of the three remaining borreliae and, on the other hand, possesses those that are typical for ordinary GH77 amylomaltases. This intermediary nature of its sequence also resulted in a position in the evolutionary tree (Fig. 1b) placing the GH77 *B. turicatae* member between the three exclusive borrelial amylomaltase-like proteins and the representatives of the rest of the family GH77.

With regard to the eventual function of the three proteins with arginine-to-lysine mutation, the conserved catalytic triad (Fig. 1a) supports the possibility that their protein function has been maintained (i.e. either the 4-α-glucanotransferase function or, in a wider sense, a function of an enzyme that is a member of the α-amylase family). This, interestingly, might not be true for the intermediary amylomaltase-like protein from *B. turicatae* that contains the third
catalytic residue (the β7-strand aspartate), i.e. the transition state stabilizer, substituted by a glutamate (Fig. 1a).

It should be pointed out that the arginine exchange by lysine is really unique. According to the best of our knowledge, it was possible to detect the same mutation by BLAST (Altschul et al., 1990) only in one protein in addition to the three examples from borreliae. This 825-residue-long protein (GenPept Protein_id. No. EAL49704.1) is a fragment of putative GH77 amylomaltase from the genome of protist parasite Entamoeba histolytica (Loftus et al., 2005) that exhibits the segment 493_IIKFDSISF (c.f. with Fig. 1a).

The three putative GH77 amylomaltase-like enzymes from B. burgdorferi, B. garinii and B. afzelii (Fig. 1) are thus attractive models for experimental studies within the entire α-amylase clan GH-H focused on verifying the existence of the mutation and its eventual effects.

Experimental evidences

The sequencing of the cloned B. burgdorferi GH77 amylomaltase-like gene revealed that it is six nucleotides longer (1527 vs. 1521 including the stop codon) than the putative malQ gene (BB0166) from the complete sequence of the B. burgdorferi genome (Fraser et al., 1997). At the amino acid sequence level, it means that the recombinant real GH77 protein from B. burgdorferi contains three inserted residues in comparison with the BB0166 one. These are the Ala2 succeeding the initiation methionine at the N-terminus and Leu508-Glu509 at the very C-terminus. In addition, there are five single conservative substitutions between the two amino acid sequences (Fig. 2). These eight subtle changes are, however, negligible with regard to the exclusive sequence features (Fig. 1a) that are all present in the real unique GH member, which also cover the most important arginine-to-lysine natural mutation (Fig. 2).

SDS-PAGE revealed that the molecular mass of the expressed recombinant protein is c. 59 kDa (Fig. 3) as a sufficiently clear single protein band was detected after the second purification on an Ni-NTA column. This corresponds well with the molecular weight of 59.7 kDa calculated on the Expasy server (Gasteiger et al., 2005) from the amino acid sequence determined.

The preliminary TLC enzyme activity tests with maltotriose as a substrate (Miwa et al., 1972) indicated that the recombinant protein is able to release glucose from maltotriose. The temperature and pH optima were determined to be around 37°C and 5.5, respectively (data not shown), when maltotriose was used as a substrate. The enzyme for these biochemical tests was used at a concentration of 21.4 mU mL⁻¹.

The most important findings of this study are shown in Fig. 4. There are no doubts that the B. burgdorferi MalQ, which possesses the natural arginine-to-lysine substitution, was able to perform both reactions typical for a GH77 amylomaltase (Terada et al., 1999; Kaper et al., 2005; Park et al., 2007), i.e. to hydrolyse the malto-oligosaccharides (G₂–G₇) and to form their transglycosylation products. This was especially clear when a higher enzyme concentration (21.4 mU mL⁻¹) was used (Fig. 4, lane ‘3’ for each malto-oligosaccharide). With glucose as a substrate, expectedly, no transglycosylation products were observed (data not shown). When maltose was used, only maltotriose and...
maltotetraose were produced by transglycosylation; however, all the other malto-oligosaccharides tested (G3–G7) were comparable effective substrates for the enzyme under these conditions because similar longer transglycosylation products were produced (Fig. 4). It is therefore possible to conclude that our protein does possess the exact amylomaltase activity.

Conclusions

This study confirmed that the remarkable substitution of extremely well-conserved and functional arginine (Arg291 in *Thermus aquaticus* GH77 amylomaltase) exists naturally in the GH77 protein member from *B. burgdorferi* that obviously exhibits the amylomaltase activity. Based on the previous structure/function studies with GH77 amylomaltases ([Przylas et al., 2000; Fujii et al. 2007]), it was not possible to speculate on the effect of the arginine-to-lysine mutation discussed here because most work was focused on the role of two aromatic residues (Tyr54 and Tyr101 in the *T. aquaticus* enzyme) involved in the second glucan-binding site. There have also been very few examples of the arginine-to-lysine mutants prepared artificially within the entire α-amylase family. For example, such a mutant of *Bacillus steaothermophilus* α-amylase had 12% of the specific activity of the parental enzyme ([Vihinen et al., 1990]) and the same mutant of the maize branching enzyme also retained only some residual activity ([Libessart & Preiss, 1998]).

In *T. aquaticus* GH77 amylomaltase, the Arg291 forms a hydrogen bond just with the catalytic nucleophile β4-strand Asp293 ([Przylas et al., 2000]). Because in our enzyme the arginine is replaced by lysine (Lys305) which could eventually take on the role of the original arginine, it is not possible to conclude whether or not the catalytic triad alone (i.e. the three catalytic residues without the above-mentioned arginine) is enough for a GH-H protein to be a real functional member of the α-amylase family. It would be necessary to make a mutant *B. burgdorferi* GH77 amylomaltase with lysine at the β4-strand replaced with a neutral amino acid residue (e.g. alanine) and to measure its activity. Moreover, our enzyme contains not only the lysine (Lys305) instead of the otherwise invariant arginine, but it also lacks the aspartate (in β3; Asn227) and histidine (in β7; Gly406) that are both important functional residues throughout the α-amylase family ([Uitdehaag et al., 1999; MacGregor et al., 2001; Janecek, 2002; Kuriki et al., 2006]). It would thus be of interest to also find out how these two additional mutated positions influence the enzyme activity. Our future efforts will therefore be focused on elucidating all those eventualities that may lead to novel goals in protein engineering and design of the α-amylase family enzymes.

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References


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