Unique features of several microbial α-amylases active on soluble and native starch
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Identification of a Novel α-Amylase from Marine Bacillus megaterium NL3 Representing a New GH13 Subfamily

Fean D. Sarian, Tjaard Pijning, Ihsanawati, Zeily Nurachman, Marc J.E.C. van der Maarel, Lubbert Dijkhuizen and Dessy Natalia
Abstract

Microbes employ a large diversity of α-amylase to degrade starch as growth substrate. Most of these α-amylase have been classified in Glycoside Hydrolase family 13 (GH13) and their three-dimensional structures and reaction mechanisms have been studied in detail. New variants however are still identified from diverse natural environments. Here we report the characterization of a novel α-amylase enzyme from Bacillus megaterium NL3, a bacterial strain isolated from Kakaban landlocked marine lake, Derawan Island, East Kalimantan, Indonesia. The mature recombinant BmaN1 consisting of 505 amino acid residues shares about 90% amino acid sequence similarity with the putative α-amylases of Bacillus flexus, Bacillus aryabhattai, B. megaterium strain DSM319, and strain WSH-002. Phylogenetic analysis clustered BmaN1 and its homologs as a separate branch in family GH13, representing a new subfamily. Sequence alignments revealed that members of this subfamily lack one of the conserved aspartate residues in their catalytic region, however BmaN1 still possessed normal α-amylase activity. Modelling of the BmaN1 active site suggested that members of this new subfamily are likely to use a histidine instead of an aspartate residue as transition state stabilizer.
Introduction

α-Amylases are ubiquitously present in nature. In humans, α-amylases are synthesized primarily in the salivary glands and the pancreas (1, 2). The digestion of starch in humans begins with the action of salivary α-amylase and is completed by the pancreatic α-amylase converting starch into glucose (3). α-Amylases are also produced by many plants and microorganisms, in the latter mostly as extracellular enzymes to degrade glycogen and starch to smaller oligosaccharides and finally into glucose that serves as an energy source.

α–Amylases act on the α(1→4) glycosidic linkages in glycogen, starch, and related α-glucans. The vast majority of α-amylases belongs to glycoside hydrolase (GH) family 13 (4), constituting 20 different reaction and product specificities including, glycoside hydrolases (EC 3.2.1.x), glucanotransferase enzymes (EC 2.4.1.x and EC 2.4.99.16), and isomerases (EC 5.4.99.11; EC 5.4.99.15 and EC 5.4.99.16), all sharing a conserved structural scaffold (4). The first experimentally determined three-dimensional (3D) structure of α-amylase was Taka α-amylase A (TAA), the amylase from Aspergillus oryzae (5). The crystal structure of TAA revealed that α-amylases have three characteristic domains: A, B, and C (6). The A domain containing the catalytic residues is the most conserved domain, with a typical (β/α)8- or TIM-barrel motif comprised of eight stranded parallel β-sheet surrounded by eight α-helices. Domain B is found inserted between the third β-strand and the third α-helix of the (β/α)8-barrel and varies in length and structure. The C domain folds into eight antiparallel β-strands, is connected to the A domain by loops and seems to be an independent domain with unknown function (6, 7). Despite low similarity between the amino acid sequences of α-amylases from animals, plants, and microbes, the GH13 enzymes share four highly conserved regions that are involved in the formation of the catalytic site. The α-amylase active site is located in an open cavity between the A and B domains, and contains the invariably carboxylic acid Asp206, Glu230 and Asp297 (TAA numbering) that are essential for catalysis, acting as nucleophile, general acid/base and transition state stabilizer, respectively (5).
Recently, several α-amylase and related enzymes composed of a \((\beta/\alpha)_7\)-barrel (an irregular TIM-barrel domain) have been classified into families GH57 and GH119 (8, 9). Both of these enzyme families are at present considerably smaller than GH13 and only few have been characterized in detail (10). The first determined 3D structure of GH57 was that of the 4-α-glucanotransferase from *Thermococcus litoralis* (TLGT). X-ray crystallography supported by site-directed mutagenesis of TLGT revealed that it has two catalytic residues, Glu123 and Asp214, acting as the catalytic nucleophile and the general acid/base, respectively (11). No 3D structure is currently available for GH119 members. In addition to the structural differences between GH13 and GH57-GH119 family members, there are also distinctive conserved regions between these families (8). The GH57 and GH119 families possess their own five conserved sequence regions (12).

We screened several microbial strains isolated from a unique land-locked marine lake located in Kakaban island for the production of α-amylases. Kakaban island is part of the Derawan Islands, East Kalimantan, Indonesia. Kakaban lake is also known as Jellyfish lake or Halimeda lake. It was originally the lagoon of an atoll, formed by corals over a period of two million years. As a result of movements in the earth’s crust the coral reef was raised above the sea level, trapping 5 km\(^2\) of seawater within a 50 meter high ridge, effectively creating a land-locked marine lake (13). It has a pH of 7.6-7.8, a salinity of 23-26% and a tidal range of 11 cm. There is only one other similar ecosystem known which is found on Rock Island-Palau in Micronesia (14).

The present paper reports the characterization of a novel α-amylase produced by *Bacillus megaterium* strain NL3 isolated from a sea anemone from the Kakaban land-locked marine lake. *B. megaterium* strains have been reported as producers of extracellular α-amylases (15). Analysis of the full genome sequences of *B. megaterium* strain DSM319 and strain WSH-002 has revealed that they encode several putative amylolytic enzymes (16, 17). From the *B. megaterium* NL3 isolate, a gene coding for an α-amylase, BmaN1 was identified. Of the three conserved catalytic residues in GH13 α-amylases, only two catalytic amino acids were
identified in BmaN1, being Asp203 and Glu231. In spite of this, BmaN1 was shown to be active towards soluble starch. Phylogenetic analysis clustered BmaN1 and its homologs as a separate branch in family GH13, representing a novel subfamily.
Results

Screening and characterization of bacteria producing extracellular α-amylases

Twenty bacterial isolates from Kakaban landlocked marine lake were tested for production of α-amylase by the starch hydrolysis test. Eight bacterial isolates grown on MB plates containing red-dyed amylpectin or raw starch, produced clear zones around their colonies. The isolate NL3 showed the largest clearing zone on starch agar plates, indicating a relatively high α-amylase activity and was selected for further study. A 16S rDNA sequence analysis showed that the sea anemone associated bacterial strain NL3 was most closely related to *Bacillus megaterium*. This result was in agreement with biochemical and physiological properties (data not shown) and hence the selected isolate was designated as *B. megaterium* NL3. α-Amylase activity was detected in culture supernatant of strain NL3 by measuring its activity towards soluble starch. The extracellular α-amylase enzyme was partially purified by ammonium sulphate precipitation at a 50-80% concentration, as this fraction contained most of the α-amylase activity. The 50-80% ammonium sulphate sample of the supernatant was run on SDS-PAGE gels and by activity staining, a single protein band with molecular mass of approximately 55 kDa was found (data not shown).

Molecular identification of α-amylase

Using degenerate α-amylase specific primers and inverse PCR, a DNA fragment of 2.3 kb was obtained. Analysis of the nucleotide sequence of this fragment showed that an open reading frame of 1515 bp with clear α-amylase sequence similarity, with an ATG as the initial codon and TAG as the stop codon was present. This gene was designated as *bmaN1*. According to the prediction, the putative ribosomal binding site (RBS) corresponds to the AGGAGG sequence located 12 nucleotides upstream of the start codon. A probable catabolite responsive element (CRE) was found together with possible -10 (TATAAT) and -35 (TTAAC) regions (Fig. 5.1). The CRE sequence showed only one mismatch in the last position when compared to
the consensus sequence (TGT/AAANCGNTNA/TCA) (26). The ORF downstream from *bmaN1*, had *lacI* as predicted product and is oriented in the same direction from *bmaN1*.

The sequence of *bmaN1* was then used to perform a homology search using BLASTN software against the GenBank nucleotide collection (nr/nt) database (27). On the basis of multiple sequence alignments, the *bmaN1* gene exhibits the highest identity (97%) with the putative α-amylase of *B. megaterium* strain DSM319, and 94% identity with the putative α-amylase of the *B. megaterium* strain WSH-002. The full-length DNA sequence of the putative α-amylase gene of *B. megaterium* NL3, *bmaN1*, has been deposited in the GenBank database under the accession no. AGT45938.

**Fig 5.1 Sequence analysis of the 5′ region of the B. megaterium NL3 bmaN1 gene.** The putative -10 and -35 promoter regions (*underlined*), a putative CRE (*overlined*), the ribosome-binding site (*double-underlined*), and the start codon (*boldface*) are shown.

The BmaN1 polypeptide deduced is 505 amino acid residues in length with a clear putative signal peptide sequence of 23 residues preceding the mature enzyme, as predicted by SignalP 4.0 Server (28). The molecular weight and *pI* of BmaN1 were predicted using ExPaSy server ([http://web.expasy.org/protparam](http://web.expasy.org/protparam)) as 56934 Da and 9.05, respectively. The deduced amino acid sequence of BmaN1 was subjected to BLASTP in GenBank revealing that BmaN1 is considerably similar to the glycoside hydrolase family 13 (GH13). BmaN1 shared the highest identity (39%) to α-amylase from *Geobacillus thermoleovorans* (GTA, GenBank accession number: 4E20_A) (29) of all α-amylases characterized functionally and biochemically. Furthermore, BmaN1 shared 95, 94, 94, and 92% amino acid sequence identity with the α-amylase catalytic regions, I to IV, of *B. flexus* (GenBank accession number: WP_050691052),
B. megaterium DSM319 (GenBank accession number: ADF37524), B. aryabhattai (GenBank accession number: WP_043980530), and B. megaterium WSH-002 (GenBank accession number: AEN91476), respectively. These α-amylases have not yet been characterized experimentally yet.

To identify the conserved regions and the catalytic triad of BmaN1, the amino acid sequence of BmaN1 was aligned with α-amylases from archaea and bacteria that have been classified into various GH13 subfamilies (Table 5.2). The conserved regions II, III, and IV are clearly present in BmaN1. However, the region corresponding to region I is different from any of the α-amylases. Regions I-IV are the well-known conserved sequence regions in GH13 α-amylases; region II, III, and IV contain the three carboxylic residues (Asp, Glu, and Asp, respectively) that together function as a catalytic triad. Surprisingly, two of these residues are absent in BmaN1 (Table 5.2 and supplementary data). First, at the position of the conserved nucleophilic aspartate residue, BmaN1 has a lysine residue (Lys202). Interestingly however, directly downstream of this lysine residue is an aspartate residue (Asp203). Second, the aspartic acid residue that stabilizes the covalent transition state is not present in BmaN1; it is replaced by a histidine residue (His294). Alignment with the sequences from B. flexus, B. megaterium DSM319, B. aryabhattai, and B. megaterium WSH-002 (supplementary data) showed that also in these sequences, a lysine and a histidine are present at the positions of the nucleophilic and the transition state stabilizing residues, respectively. Also the aspartate residue directly downstream of the lysine residue is conserved in these sequences.
Table 5.2 Comparison of amino acid sequences of the four conserved regions of *B. megaterium* NL3 BmaN1 and various α-amylases from selected members of GH13 subfamilies

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Region I</th>
<th>Region II</th>
<th>Region III</th>
<th>Region IV</th>
<th>Accession numbera</th>
<th>GH13 subfamily</th>
</tr>
</thead>
<tbody>
<tr>
<td>BmaN1</td>
<td>EFPLTI</td>
<td>GYYVKDIDQ</td>
<td>GEING</td>
<td>FLDDVH</td>
<td>T1SIF2</td>
<td>new</td>
</tr>
<tr>
<td><em>B. amyloliquefaciens</em></td>
<td>DVVLNH</td>
<td>GFRIDAAKH</td>
<td>AEWYQ</td>
<td>FVENHD</td>
<td>P00692</td>
<td>5</td>
</tr>
<tr>
<td><em>Pyrococcus woesei</em></td>
<td>DVVINH</td>
<td>GWRFDYVKG</td>
<td>GEYWD</td>
<td>FVANHD</td>
<td>Q7LYT7</td>
<td>7</td>
</tr>
<tr>
<td><em>Hordeum vulgare</em></td>
<td>DIVINH</td>
<td>AWRLDFARG</td>
<td>AEWWD</td>
<td>FVDNH</td>
<td>P00693</td>
<td>6</td>
</tr>
<tr>
<td><em>Homo sapiens</em></td>
<td>DAVINH</td>
<td>GFRIDASKH</td>
<td>GEVID</td>
<td>FVDNH</td>
<td>P04746</td>
<td>24</td>
</tr>
<tr>
<td><em>D. melanogaster</em></td>
<td>DVVFNH</td>
<td>GFRVDAAHK</td>
<td>GEVID</td>
<td>FVDNH</td>
<td>P08144</td>
<td>15</td>
</tr>
<tr>
<td><em>Streptomyces limosus</em></td>
<td>DSVINH</td>
<td>GFRIDAAKH</td>
<td>GBAIH</td>
<td>FVDNH</td>
<td>P09794</td>
<td>32</td>
</tr>
<tr>
<td><em>Xanthomonas campetris</em></td>
<td>DVVFNH</td>
<td>GFRVDAAHK</td>
<td>GEVIT</td>
<td>FAVTHD</td>
<td>Q56791</td>
<td>27</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>DAVINH</td>
<td>GFRVDAH</td>
<td>GEILQ</td>
<td>WVESHD</td>
<td>P00691</td>
<td>28</td>
</tr>
<tr>
<td><em>G. thermoleovorans</em></td>
<td>DFVANH</td>
<td>GYRLDTVRH</td>
<td>GEVVS</td>
<td>FLDNH</td>
<td>G8N5V2</td>
<td></td>
</tr>
<tr>
<td><em>Aspergillus oryzae</em></td>
<td>DVVANH</td>
<td>GLRIDTVKH</td>
<td>GEVLD</td>
<td>FVENHD</td>
<td>P0C1B3</td>
<td>1</td>
</tr>
<tr>
<td><em>Vibrio fischeri</em></td>
<td>DGVFGH</td>
<td>GWRIDQAYQ</td>
<td>AEWWN</td>
<td>MLGNHD</td>
<td>B5FDL2</td>
<td>37</td>
</tr>
<tr>
<td><em>D. thermophilum</em></td>
<td>DLVVNH</td>
<td>GFRIDAAKH</td>
<td>GEVWD</td>
<td>FLRNH</td>
<td>P14899</td>
<td>36</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>DVVMNH</td>
<td>GFRVDTAKH</td>
<td>GEAWG</td>
<td>YLSSHD</td>
<td>P25718</td>
<td>19</td>
</tr>
<tr>
<td>CONSENSUS</td>
<td>D<strong>NH G<em>R</em>D</strong>H GE** F**NHD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The catalytic residues are shown in shadow and bold. Representatives of the 13 subfamilies of GH13 that showed α-amylase specificity were compared. Organisms: *B. amyloliquefaciens* = *Bacillus amyloliquefaciens*; *D. melanogaster* = *Drosophila melanogaster*; *G. thermoleovorans* = *Geobacillus thermoleovorans*; *D. thermophilum* = *Dictyoglomus thermophilum*. The available gene sequences are aligned by ClustalW. aAccession numbers from the protein sequence database.
Fig 5.2 Structural comparison of BmaN1 of B. megaterium NL3 and GTA of Geobacillus thermoleovorans. (A) The BmaN1 model structure, (B) The structure of BmaN1 (orange) superimposed on GTA (grey) structure, (C) Active-site region in a superposition of BmaN1 with GTA including the acarbose bound in subsites -2 to +2 (white carbon atoms). Active-center residues of BmaN1 (orange) and GTA (grey) are given as stick models and labeled in orange (BmaN1) and black (GTA).

A topological alignment of BmaN1 and the putative α-amylase of B. megaterium DSM319, Bacillus sp. 278922, B. flexus, B. aryabhattai, B. megaterium WSH-002, and GTA was made (supplementary data). Almost all β-strands and α-helices of the TIM
barrel in domain A, and the Greek key motif in domain C, are conserved in these α-amylases. A model of the three-dimensional structure of BmaN1 was generated by the PHYRE server (25) and visualized by the MacPymol software (30). The BmaN1 protein displayed 40% homology (100% confidence, 85% sequence coverage) with the X-ray crystal structure of *Geobacillus thermoleovorans* α-amylase (GTA, PDB code: 4E20) (29) which was used as a template for the modelling. The comparison between the model and the GTA crystal structure revealed that the global topology is almost the same (Fig. 5.2B). The BmaN1 protein model folds into three distinct domains: a central A domain of 366 residues harboring a (β/α)₈ barrel, with an irregular loop domain of 37 residues (domain B) connecting the third β-sheets strand and the third α-helix of the barrel. The C domain of 79 residues has an eight-stranded anti-parallel β-sandwich-like fold (Fig. 5.2A).

Superposition of acarbose-bound GTA with the BmaN1 model demonstrates that of three catalytic residues found in GH13 α-amylases, only residue Glu231 of BmaN1 superimposes with the corresponding residue in GTA (Glu246), and presumably is the general acid/base in BmaN1. As already concluded from sequence alignments, two of the three catalytic residues are not conserved in the BmaN1 model. Lys202 replaces the catalytic aspartate (Asp217 of GTA); however, Asp203 directly downstream of the lysine is positioned nearby and has its carboxylic acid side chain pointing into the presumed substrate binding groove. Furthermore, at the position corresponding to the nucleophile, His294 replaces the transition-state stabilizing aspartate residue (Asp314) found in α-amylases.

To elucidate the evolutionary status of BmaN1, a phylogenetic tree of α-amylases was constructed using representatives from each of the GH13 subfamilies as well as the *B. megaterium* NL3 α-amylase, BmaN1 and the putative amylase of *B. megaterium* DSM319, *Bacillus* sp. 278922, *B. flexus*, *B. aryabhattai*, *B. megaterium* WSH-002. To date, a total of 40 subfamilies of GH13 can be found in the Carbohydrate-Active enzyme (CAZy) database (http://www.cazy.org/), with the α-amylase specificity present in the subfamilies 1, 5, 6, 7, 15, 19, 24, 27, 28, 32, 36,
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and 37 (31). The unrooted distance tree was based on the alignment of the sequences of the conserved regions, I to VII, of α-amylases. BmaN1 does not cluster with any of the previously defined GH13 subfamilies (Fig. 5.3). This characterized BmaN1 forms a cluster together with the uncharacterized α-amylases from *B. flexus, B. megaterium* DSM319, *B. aryabhattachi*, and *B. megaterium* WSH-002, and this cluster is most closely related to the GH13_α group comprised of several *Bacillus* α-amylases (32) (Fig. 5.3). The amino acid identity of the sequences in the uncharacterized group with BmaN1 was in the range of 90-95%, while similarity of these sequences with the nearest neighbor (GTA group) was below 30%.

At present, a few amylolytic enzymes with no sequence similarity to family GH13 have been classified in families GH57 and GH119. Some GH57 members are known to lack one or even both catalytic residues (12). Since only two corresponding residues to the conserved catalytic site of α-amylase are identified in BmaN1, a comparative analysis of BmaN1 to GH57 and GH119 families was done. In phylogenetic analysis, BmaN1, 8 amylases of GH57 and 2 amylases of GH 119 formed three separate clusters (data not shown).

**Fig 5.3 Phylogenetic relationship of BmaN1 with α-amylases of GH13 subfamilies.** The tree was constructed using the neighbor-joining method based on the sequence alignment of α-amylases conserved regions (I-VII) of a selection of GH13 α-amylases performed using Clustal X embedded within the MEGA4 software. The GH13 α-amylases are divided into subfamilies. Protein database numbers are shown following the sources of the α-amylases. Bootstrap values, expressed as a percentage of 1000 replications, are given at the branching nodes. The scale bar at the lower left corner refers to the substitution rate (substitution/site).
Characterization of BmaN1

The gene encoding BmaN1 was cloned in vector pMM1525 and this recombinant plasmid was transformed to *B. megaterium* MS941. A transformant with clear α-amylase activity, as detected on starch plates by iodine staining, was selected and grown in liquid medium. The culture medium was saturated with 50-80% concentrations of ammonium sulphate to purify the BmaN1 α-amylase enzyme. The molecular weight of the partially purified BmaN1 was estimated to be 55 kDa as judged from activity staining after protein renaturing on SDS-PAGE gels (Fig. 5.4, lane 2). In contrast, no band was observed in the culture supernatant of *B. megaterium* MS941 carrying pMM1525 without any insert (Fig. 5.4, lane 1).

![Fig 5.4 SDS-PAGE(left) and activity staining after renaturation (right image) of BmaN1 purified from *B. megaterium* MS941. M, molecular weight marker; lane 1, pMM1525 empty vector; lane 2, BmaN1 protein. Activity staining was performed by soaking the starch-containing gel in 50 mM maleate buffer pH 6.0 and incubating at 55 °C for 4 h. The clear bands after iodine solution treatment indicate amylolytic activity.](image)

Amylolytic activity of BmaN1 was measured spectrophotometrically by incubating it with soluble starch and measuring the increase in the amount of reducing sugars released over a period of 40 min (Fig. 5.5). A clear increase in reducing ends was
observed, resulting in an activity of 8.4 U/mg of protein. BmaN1 was found to be most active at 55 °C and pH 6.0. The main end products formed from soluble starch were glucose and maltose. Minor amounts of longer maltooligosaccharide were also found (Fig. 5.6).

![Graph showing reducing ends vs. Incubation period](image1)

**Fig 5.5 Incubation of BmaN1 (open squares) with soluble starch; open circles: control (empty vector).** 1% (w/v) soluble starch and 12.5 µg/mL of the BmaN1 protein were incubated for various time at 55 °C. Each data point represents the means of triplicate experiments.

![HPLC analysis](image2)

**Fig 5.6 HPLC analysis for hydrolyzed products of soluble starch by BmaN1.** A reaction mixture containing 1% (w/v) soluble starch, 12.5 µg/mL of BmaN1, 50 mM maleate buffer pH 6.0 in a total volume of 0.1 mL was incubated at 55 °C. G1, glucose; G2, maltose; ≥G3, maltooligosaccharides.
Discussion

In the present study, *Bacillus megaterium* NL3 was found to produce an extracellular α-amylase, BmaN1. Several novel structural properties of the BmaN1 protein were found using bioinformatics tools and homology modelling. The secondary structure prediction of BmaN1 showed a typical \((\beta/\alpha)_8\) barrel structure, which is a common feature of α-amylase glycoside hydrolase family 13. The overall structure of BmaN1 can be divided into three domains (see Fig. 5.3): the first domain is composed of 339 residues (1-139 and 177-377) corresponding to domain A in the α-amylase family enzymes, containing an eight-stranded parallel β-barrel and eight α-helices surrounding the β-barrel (33). This barrel is quite similar to the \((\beta/\alpha)_8\)-barrel found in maltogenic amylase from *Pseudomonas saccharophila* and *Bacillus stearothermophilus* (34), that both have an additional helix between Aα6 and Aβ7, which is a three-turn helix lying nearly parallel with the Aα6 strand. The B-domain with residues 140-176 is formed by a single α-helix interrupting the \((\beta/\alpha)_8\)-barrel between the third β-sheet and the helix, and the C-terminal domain with residues 378-505. The members of GH13 possess related \((\beta/\alpha)_8\) barrels with a small irregular loop B domain, while GH57 members contain the catalytic \((\beta/\alpha)_7\) barrel and the GH119 have a yet uncharacterized motif. On the basis of this information we conclude that BmaN1 belongs to family GH13. In sequence alignments, the BmaN1 protein showed more than 90% similarity to the putative *B. flexus*, *B. megaterium* DSM319, *B. aryabhattai*, and *B. megaterium* WSH-002 α-amylases. Phylogenetic analysis of BmaN1 together with these five homologous putative α-amylases against other GH13 family enzymes from the NCBI database indicated the uniqueness of these 6 proteins. They have their own separate branch that constitutes a new GH13 subfamily (Fig. 5.3).

GH13 α-amylases have four conserved regions that are important for catalytic activity or substrate binding (6). Sequence alignments revealed that the conserved GH13 regions I-IV of BmaN1 are remarkably different. The sequence of region II (\(^{199}\text{GYKYDD}^{205}\) in BmaN1) suggests that Asp203 may function as the nucleophile, although it is one position downstream of the expected position, where in BmaN1 a
lysine residue is found. In region III (\textsuperscript{229}GEING\textsuperscript{233}), the general acid/base residue, Glu230, is conserved. In region IV (\textsuperscript{289}FLDDVH\textsuperscript{294}), a histidine (His294) replaces the canonical aspartate that functions as a transition-state stabilizer (Fig. 5.2). Sequentially similar motifs have also been recognized in the 5 BmaN1 homologs representing unclassified putative \(\alpha\)-amylases, of various \textit{Bacillus} species. They have been added to GH13 because part of their sequence exhibits similarity to \(\alpha\)-amylase (http://www.cazy.org).

A three-dimensional structure of BmaN1 was built by homology modelling using Quick Phyre. The secondary and tertiary structures of the BmaN1 model are similar to that of the template enzyme (GTA, PDB code: 4E2O). Superimposition of the active site of BmaN1 on GTA revealed several differences as well as similarities in the active site structure. In particular, there is an insertion of a lysine residue in the catalytic region around Asp203 (BmaN1 numbering). As a consequence, Asp203 seems to be shifted in position, although still positioned proximate to the substrate. In the absence of an experimental 3D structure, it is unclear whether or not it can perform the nucleophilic attack. In addition, residue Asp314 in GTA, providing hydrogen bonds to the sugar moiety in subsite -1, is substituted by His294 in BmaN1. Although different from aspartate, a histidine side chain is also able to form hydrogen bonds; this residue thus may act as alternative transition state stabilizer.

\(\alpha\)-Amylase activity was observed following expression of BmaN1 in \textit{B. megaterium} expression host. The specific activity of the BmaN1 (8.4 U/mg from a DNS assay) is in the moderate range of \(\alpha\)-amylase activity (www.brenda-enzymes.info). Glucose and maltose were the dominant products of BmaN1 incubated with soluble starch (Fig 5.6). This mode of action is very similar to that of the amylase from \textit{Bacillus} sp. IMD 435 that releases glucose and maltose as the major products on hydrolysis of both soluble starch and raw corn starch (37). Interestingly, with regards to the amylolytic activity, our results show that despite the substitution of a negatively charged aspartate residue by a positively charged histidine (His294), BmaN1 still has an \(\alpha\)-amylase activity. The reaction mechanism of BmaN1 thus is essentially
similar to the general catalytic reaction mechanism of α-amylases. Further experiments are needed to validate the role of the proposed catalytic residues, i.e. by constructing and characterizing mutant enzymes with different residues at this position, and/or determining the 3D structure, preferably in complex with a substrate.

**Conclusion**

An extracellular α-amylase of the new bacterial isolate *B. megaterium* NL3, BmaN1, has been identified and produced in a *B. megaterium* expression host. Based on amino acid sequence similarity and phylogenetic analysis, the BmaN1 and five unclassified homologous α-amylases belong to a new GH13 subfamily. Interestingly, the highly conserved and catalytic aspartic acid residue in conserved region IV is replaced by a histidine in BmaN1. Taken together, this paper provides evidence that BmaN1 is a novel α-amylase with an unusual catalytic triad.

**Acknowledgements**

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Material and Methods

Materials

All chemicals used were reagent grade and were obtained from either Fermentas (Maryland, USA) or Difco Laboratories (New Jersey, USA).

Bacterial strains, plasmids, and growth conditions

Twenty microbial strains (gift of Prof. Ocky Karna Radjasa of Diponegoro University, Indonesia) that had been isolated from Kakaban landlocked marine lake (Derawan Islands, East Kalimantan, Indonesia) were screened for extracellular α-amylase activity. The isolates were cultured in marine broth (MB) medium containing 0.25% (w/v) yeast extract, and 0.5% (w/v) peptone in filtered sea water (Seaworld, Ancol, Jakarta, Indonesia) at 30 °C. *B. megaterium* MS941 (MoBiTec, Germany) and *Escherichia coli* TOP10 were grown at 37 °C in LB medium (1% (w/v) Bacto-tryptone, 1% (w/v) NaCl and 0.5% (w/v) yeast extract). Ampicillin and tetracyclin were used at concentrations of 100 µg/ml and 12 µg/ml, respectively. The medium was autoclaved at 120 °C for 30 min prior to adding the antibiotics. Plasmid pGEM-T (Promega, USA) was used for PCR product cloning, whereas pMM1525 (MoBiTec, Germany) was used as expression vector.

Screening of α-amylase producing bacteria

Bacterial isolates were inoculated on MB agar plates supplemented with 1.0% (v/v) red-dyed amylopectin (18) and then incubated at 30 °C for 24 hrs. The appearance of a clear zone against a red background was indicative for the production of α-amylase activity. The positive isolates were then subjected to a second screening round using MB agar plates containing 1.0% (w/v) potato or wheat starch granules. A clearing zone around the bacterial colony indicated that the starch granules was hydrolyzed and thus that the isolate produced extracellular α-amylase activity.
Bacterial identification

Isolate NL3 showing the largest clearing zone on starch-agar plates was identified by 16S rDNA sequencing. Chromosomal DNA of strain NL3 was isolated using Wizard Genomic DNA Purification (Promega). The 16S rDNA gene was amplified by PCR using universal primers UniB1 and BactF1 (Table 5.1). The resulting 1.4 kb fragment was sequenced using the dideoxy-chain termination method (Macrogen, South Korea). Bacterial isolates were identified by aligning their 16S rDNA sequence with those of known bacteria using NCBI BLASTn (http://www.ncbi.nlm.nih.gov). The 16S rDNA gene sequence was submitted to GenBank (accession number KU862862).

Cloning of the α-amylase-encoding gene and plasmid construction

Two degenerate primers (Table 5.1) were designed based on the amino acid sequences of the well-conserved regions (region VI-VII) of α-amylases from several Bacilli. The first α-amylase gene fragment was amplified by polymerase chain reactions (PCR), using chromosomal DNA from B. megaterium NL3 as a template and the two degenerate primers. The PCR products were inserted into pGEM-T vector (Promega) and transformed into E. coli TOP10. Plasmid DNA of the transformed E. coli TOP10 was isolated and the nucleotide sequence of the inserted DNA was determined using the dideoxy-chain termination method (Macrogen). The resulting nucleotide sequence was used to design a set of primers, NL3_SP8-invF1 and NL3_SP8-invR1 (Table 5.1), to amplify parts of α-amylase gene beyond the conserved region. The chromosomal DNA was partially digested with EcoRV and then self-ligated using T4 DNA ligase (Fermentas). Inverse PCR (iPCR) (Ochman et al. 1988) was performed with Dream Taq polymerase (Fermentas) and the primers listed in Table 5.1 using the self-ligated DNA fragment as a template. Analysis of sequence data and sequence similarity searches was performed using the BLAST program of the National Center for Biotechnology Information (NCBI). Primers pMM-NL3-F and pET/MM-NL3-R were used to amplify the complete open reading frame of the α-amylase gene which was designated as bmaN1.
Table 5.1 Primers used in amplification by PCR

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>BactF1</td>
<td>AGAGTTTGATC(A/C)TGGCTCAG</td>
<td>16S rDNA</td>
</tr>
<tr>
<td>UniB1</td>
<td>GGTTAC(G/C)TTTGTACGACTT</td>
<td>16S rDNA</td>
</tr>
<tr>
<td>bmD_reg2F</td>
<td>GACGGATATTACGTTAAAGATATC</td>
<td>PCR of region VI-VII of bmaN1</td>
</tr>
<tr>
<td>bmD_reg6F</td>
<td>GGATTACCGCTTTTTAATCC</td>
<td>PCR of region VI-VII of bmaN1</td>
</tr>
<tr>
<td>bmD_reg3R</td>
<td>ATTTCTCTTTATTATAGAAAAAGAAGG</td>
<td>PCR of region VI-VII of bmaN1</td>
</tr>
<tr>
<td>bmD_reg7R</td>
<td>ATAAAAACGATTGCTACTCG</td>
<td>PCR of region VI-VII of bmaN1</td>
</tr>
<tr>
<td>NL3_SP8-invF1</td>
<td>ACTGCTGCTACRTTTAGC</td>
<td>iPCR of bmaN1</td>
</tr>
<tr>
<td>NL3_SP8-invR1</td>
<td>TATATKRCGCTCATGTGC</td>
<td>iPCR of bmaN1</td>
</tr>
<tr>
<td>pMM-NL3-F</td>
<td>GGATTCAGCAGATCATAAAAGATATACG</td>
<td>PCR of bmaN1</td>
</tr>
<tr>
<td>pET/MM-NL3-R</td>
<td>CGGCACGCTAGTGATGGTGGTGCGACGCCTG</td>
<td>PCR of bmaN1</td>
</tr>
</tbody>
</table>

Transformation of B. megaterium

The recombinant plasmid containing the α-amylase gene, pMM1525-bmaN1, was transformed into the expression host, B. megaterium MS941. The transformation procedure was essentially conducted as described by Puyet et al. (1987) with some modifications (19). A 0.5 ml protoplast suspension was added to a tube containing 5.0 μg DNA and 1.5 ml PEG-P (40% (w/v) PEG6000 in 1x SMMP) for each transformation and incubated for 2 min at room temperature. SMMP medium contains 3.5% (w/v) AB3 (Antibiotic medium no. 3, Difco), 1 M sucrose, 40 mM disodium maleic acid and 40 mM MgCl₂ (pH adjusted to 6.5 before autoclaving for 12
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(min) and prepared freshly before use. To the mixture, 5.0 ml SMMP was added and mixed by rolling the tube carefully. Cells were harvested by centrifugation at 2,700 x *g* for 10 min at room temperature and the supernatant was poured off immediately. The pellets were resuspended with 0.5 ml SMMP and incubated at 37 °C for 90 min with gentle shaking or rolling of tubes (max. 100 rpm). Then, 50 to 200 µl of cells were added into top agar and mixed gently by rolling the tube. The mixture was poured on a pre-warmed plate of LB containing 12 µg/ml of tetracycline and incubated at 37 °C overnight.

**Expression and partial purification of recombinant α-amylase BmaN1**

α-Amylase BmaN1 was produced by growing the *B. megaterium* MS941 harboring recombinant plasmids in 20 ml of LB medium supplemented with 12 µg/ml tetracycline at 37 °C with shaking. The overnight culture was transferred into fresh media and incubated until the 546-nm absorbance reached 0.8-1.0. Subsequently, expression was induced by adding 1% (v/v) xylose, and the culture was incubated at 18 °C with constant shaking at 150 rpm for 24 hrs. Cells were removed by centrifugation (6000 x *g*, 10 min) and the resulting supernatant was subjected to ammonium sulphate precipitation at a saturation value up to 80%. The precipitate was dissolved and dialyzed against 50 mM maleate buffer pH 6.0 at 4 °C. This partially purified α-amylase was used for further studies.

**Gel electrophoresis and activity staining**

SDS-PAGE was carried out as described by Laemmli (1970) and proteins in gels were then stained with Coomassie Brilliant Blue (Bio-Rad). For α-amylase activity test, the protein samples were separated by SDS-PAGE (20) containing 1% soluble starch. After electrophoresis, SDS was removed by washing the gels with water followed by 10 min incubation at room temperature. This was repeated twice. The gels were then immersed in the enzyme reaction buffer (50 mM maleate buffer pH 6.0) for 4.0 h at 55 °C and then stained with KI/I₂ solution for 10 min, followed by
rinsing with water. The α-amylase activity was detected as a clear zone against a purple background.

**Enzyme assay**

**DNS assay.** The α-amylase activity assay was conducted using the 3,5-dinitrosalicylic acid (DNS) method described by Miller (1955) with a slight modification (21). Briefly, the assay was performed by adding 25 µl (≈12.5 µg/mL) of enzyme sample into 25 µl of 1% (w/v) soluble starch (Fermentas, USA) in 50 mM of the appropriate buffer and then incubated at 55 °C for 20 min. To the reaction mixture, 50 µl of DNS reagent was added. The absorbance at 500 nm was measured and then the amount of reducing sugar-end was calculated using a glucose standard curve (1-2.5 mM). One unit of α-amylase activity was defined as the amount of enzyme that releases 1 µmol of reducing sugars per min under the assay conditions. The protein concentration was determined using the Lowry method (Lowry, et al. 1951) and bovine serum albumin as the standard.

**Iodine assay.** The activity of α-amylase was also assayed by the iodine method (22) using soluble starch as substrate. A 50 µl of enzyme was mixed with 50 µl of 0.125% (w/v) soluble starch (Fermentas, USA) dissolved in 50 mM maleate buffer pH 6.0. After 20 min of incubation at 55 °C, 20 µl of 1 M HCl was added to stop the enzymatic reaction, followed by the addition of 100 µl of iodine solution (0.5% (w/v) KI and 0.15% (w/v) I₂). The blue color was read at 600 nm. One unit of the α-amylase activity was defined as the amount of enzyme that produced 10% reduction in starch-iodine staining after 20 min of incubation under the assay conditions. All enzyme assays were conducted in triplicate.

**Analysis of sugars**

The starch hydrolysis products were analyzed by high-performance liquid chromatography (HPLC). Aliquots of 100 µl of enzyme solution (12.5 µg/mL) were
incubated at 55 °C in the presence of 1% (w/v) soluble starch, maleate buffer 50 mM pH 6.0. After specific time intervals, samples were withdrawn and hydrolysis was stopped by incubation at 90 °C for 10 min. After centrifugation at 12000 x g for 10 min at 4 °C, the hydrolysates were then analyzed by HPLC (Aminex® HPX-87H system). H$_2$SO$_4$ 0.008 N served as mobile phase, the flow rate being 1 mL/min at 35 °C. The injection volume of either hydrolysate was 10 µL. Calibration of the peak area was performed based on standard glucose, maltose, and purified maltooligosaccharide (Sigma). The separated hydrolysis products were identified and calculated by comparison with the standards.

**Bioinformatics**

A phylogenetic tree of family GH13 α-amylases and BmaN1 was constructed using the conserved regions I-VII of representatives of the following α-amylase subfamilies (GH13_1, GH13_5, GH13_6, GH13_7, GH_15, GH13_19, GH13_24, GH13_27, GH13_28, GH13_32, GH13_36, and GH13_37). This set covers all GH13 subfamilies with well-defined (GH13 subfamilies 1, 5, 6, 7, 15, 19, 24, 27, 28, 32, and 37) and putative (GH13_36) α-amylase activities. The sequence alignment was performed using the Clustal W program (23) embedded within MEGA4 (24). The three-dimensional structure of the BmaN1 protein was predicted by using the Quickphyre structure program server (http://www.sbg.bio.ic.ac.uk/phyre) (25). Structural analysis of the BmaN1 protein was performed based on superimposition with the crystal structure of the α-amylase from *Geobacillus thermoleovorans* [PDB access code: 4E20]. The generated BmaN1 structures were displayed and drawn by MacPymol (http://pymol.org/educational).
References


19386.


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<table>
<thead>
<tr>
<th>Bac</th>
<th>B_megWSH00</th>
<th>B_flexus</th>
<th>B_megDSM31</th>
<th>B_aryabhata</th>
<th>EmaN1</th>
<th>GTA</th>
</tr>
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<tbody>
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<td>SKQTIDGYVRKDIDCPSAFISSFSGQLKSMDPSFL</td>
<td>RQADIDGYVRKDIDCPSAFISSFSGQLKSMDPSFL</td>
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<td>GRQADIDGYVRKDIDCPSAFISSFSGQLKSMDPSFL</td>
<td>IRETIDGYVRKDIDCPSAFISSFSGQLKSMDPSFL</td>
</tr>
</tbody>
</table>

Beta strands:

- **β4**: Bac: 1, B_megWSH00: 1, B_flexus: 1, B_megDSM31: 1, B_aryabhata: 1, EmaN1: 1, GTA: 1
- **β5**: Bac: 1, B_megWSH00: 1, B_flexus: 1, B_megDSM31: 1, B_aryabhata: 1, EmaN1: 1, GTA: 1
- **β6**: Bac: 1, B_megWSH00: 1, B_flexus: 1, B_megDSM31: 1, B_aryabhata: 1, EmaN1: 1, GTA: 1
- **β7**: Bac: 1, B_megWSH00: 1, B_flexus: 1, B_megDSM31: 1, B_aryabhata: 1, EmaN1: 1, GTA: 1
- **β8**: Bac: 1, B_megWSH00: 1, B_flexus: 1, B_megDSM31: 1, B_aryabhata: 1, EmaN1: 1, GTA: 1

Alpha helices:

- **α4**: Bac: 1, B_megWSH00: 1, B_flexus: 1, B_megDSM31: 1, B_aryabhata: 1, EmaN1: 1, GTA: 1
- **α5**: Bac: 1, B_megWSH00: 1, B_flexus: 1, B_megDSM31: 1, B_aryabhata: 1, EmaN1: 1, GTA: 1
- **α6**: Bac: 1, B_megWSH00: 1, B_flexus: 1, B_megDSM31: 1, B_aryabhata: 1, EmaN1: 1, GTA: 1
- **α7**: Bac: 1, B_megWSH00: 1, B_flexus: 1, B_megDSM31: 1, B_aryabhata: 1, EmaN1: 1, GTA: 1
- **α8**: Bac: 1, B_megWSH00: 1, B_flexus: 1, B_megDSM31: 1, B_aryabhata: 1, EmaN1: 1, GTA: 1

Alignment:

- Bac: 1, B_megWSH00: 1, B_flexus: 1, B_megDSM31: 1, B_aryabhata: 1, EmaN1: 1, GTA: 1
Supplementary data. Topological alignment of the BmaN1 primary and secondary structure with GTA (PDB 4E20) and other putative amylases. Alignment was prepared based on the super positioned models. Secondary structure: α-helix ( ), TM-helix ( ), and β-sheet ( ); residues of the catalytic site (▲). Conserved secondary structures are named.
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