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Alkaline active maltohexaose-forming α-amylase from *Bacillus halodurans* LBK 34

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Abstract

The gene encoding Amy 34, a maltohexaose-forming α-amylase from *Bacillus halodurans* LBK 34 isolated from Lake Bogoria, Kenya, was cloned and sequenced. The mature peptide consists of 958 amino acids with a theoretical molecular weight of 107.2 kDa and p*I* 4.41, respectively. The gene was expressed in *Escherichia coli* and the recombinant enzyme purified to homogeneity by a combination of metal chelate affinity and size exclusion chromatography. The pure enzyme exhibited optimum activity at 60 °C and pH 10.5–11.5. The enzyme retained over 60% activity after incubation at 55 °C for 4 h and was most stable at pH 9.0. Complete inhibition of enzyme activity was observed in presence of 5 mM Cu^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+} and 5 mM EDTA. The enzyme displayed 80% of its original activity in presence of 1% (w/v) SDS and was stable in presence of up to 5 mM DTT. Maltohexaose (G6) was the main initial product of starch hydrolysis while other products formed were G4 > G2 > G5 > G3 and G1. The main end product of the enzyme's action on amylose, amylopectin and maltodextrin is maltotetraose. Amy 34 could not hydrolyse pullulan, α and β-cyclodextrin but could hydrolyse γ-cyclodextrin to produce glucose, maltose and maltotetraose. Maltotetraose was the smallest α-(1–4) linked maltooligosaccharide that could be hydrolysed by the enzyme.

Keywords: Alkaliphile; Amylase; *B. halodurans*; Maltohexaose

1. Introduction

The demand for maltooligosaccharides with a degree of polymerisation greater than three has increased due to their properties such as sweetness, ease of digestibility and absorbability, making them useful as fillers, viscosity controlling, moisture retaining, flavour preserving and crystallisation preventing agents, for potential uses in food, beverages, cosmetics, pharmaceutical and fine chemical industries [1,2]. The production of these maltooligosaccharides is also quite tedious and expensive. Hence, microbial enzymes that predominantly form maltooligosaccharides of a specific length allow the production of larger amounts of these oligosaccharides.

Maltohexaose producing amylases from *Aerobacter aerogenes*, *Klebsiella pneumoniae* [3,4] and various *Bacillus* species are known [1,2,5–8]. A few maltohexaose producing amylases active under alkaline conditions have also been reported, including amylases from *Bacillus* sp. H-167 [9,10], *Bacillus clausii* BT-21 [11] and *Bacillus* sp. GM 8901 [12]. The latter two amylases initially produce maltohexaose on hydrolysis of starch, which is subsequently converted to maltotetraose during extended hydrolysis.

In this work, we report on the cloning and expression of the gene encoding Amy 34, an alkaline active maltohexaose-forming α-amylase from *Bacillus halodurans* LBK 34, isolated from a soda lake in Kenya, and purification and characterisation of the recombinant enzyme. *B. halodurans* is a commonly found species in alkaline

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environments [13]. The presence of an open reading frame (ORF ID: BH0413) encoding a maltohexaose-forming amylase in this species is known from the complete genome sequence of *B. halodurans* C-125 [14] (Extremobase: http://www.jamstec.go.jp/genomebase/micrHome_bha.html), although the corresponding enzyme from this species has not been studied before.

2. Materials and methods

2.1. Organism isolation and cultivation

Isolate LBK 34 was isolated from a water sample collected from hot springs around Lake Bogoria, Kenya and was found to have highest identity (over 99%) with *B. halodurans* species by 16S rDNA sequence analysis [15]. The isolate was cultivated in Horikoshi II medium [16] at pH 10.0 and 37 °C for 18 h, with orbital shaking at 200 rpm.

2.2. PCR amplification, sequencing and cloning of amy34 gene

Genomic DNA was extracted from *B. halodurans* LBK 34 cells and purified according to Sambrook et al. [17].

Primers for the amplification of the gene encoding Amy 34 were designed taking as reference, *Bh0413* open reading frame (accession no. AP001508), which encodes an alpha-amylase G-6 precursor from the *B. halodurans* C-125 database (http://www.jamstec.go.jp/genomebase/micrHome_bha.html). Sequencing of the gene was performed by primer walking using the ABI PRISM ready reaction dRhodamine terminator cycle sequencing kit version 3.0 (Applied Biosystems, Foster City, CA, USA) followed by analysis of the reaction mix in a DNA sequencer.

The mature peptide of Amy 34 was cloned into pET 30 (Novagen, Madison, WI, USA), a ligation independent vector, to produce a recombinant plasmid containing the insert fused with a hexa-histidine (His_6) tag at its N-terminal end. Single stranded complementary overhangs (underlined) were introduced by PCR using the forward primer 5′-GACGACGACAAGATGGTTGATGCCTCTCAAGGTGG-CGAG-3′, and reverse primer 5′-GAGGAGAAGC-CCGGTTTACTTAGGAGTTCCACTTAAGAT-3′ (ThermoHybaid, Ulm, Germany) with a combination of *Taq* and *Pfu* DNA polymerase (Promega Corporation, Madison, WI, USA) to ensure high fidelity [18]. The PCR amplification conditions used were: initial denaturation at 94 °C for 2 min followed by 30 cycles each consisting of 94 °C for 1 min, 60 °C for 1 min, 70 °C for 3.5 min, with a final extension step at 70 °C for 7 min. The resulting PCR products were gel purified using the Qiagen purification kits (Qiagen, GmbH, Hilden, Germany) and resuspended in TlowE buffer (10 mM Tris–HCl, pH 8.0, 0.1 mM EDTA). T4 DNA polymerase (Novagen, Madison, WI, USA) treatment of the insert for the generation of vector compatible overhangs and annealing of the insert to the ligation independent vector, pET 30 were performed according to the manufacturer's instructions.

2.3. Expression of recombinant Amy 34 in E. coli

Recombinant plasmid containing the mature peptide of Amy 34 was purified and transformed initially into *E. coli* Novablue cloning host and subsequently into *E. coli* BL21(DE3) expression host (Novagen, Madison, WI, USA). The recombinant host was grown in LB medium in shake flasks at room temperature (25 °C) with orbital shaking at 180 rpm. When the optical density at 600 nm of the cultures reached 0.6–0.8, expression was induced by the addition of IPTG to a final concentration of 1 mM.

Overnight culture (40 ml) of the induced *E. coli* BL21(DE3) cells was harvested by centrifugation (12,200 × *g*, 10 min) at 4 °C in a Sorvall RC-5B centrifuge. The cell pellet was resuspended in 2 ml of 20 mM phosphate buffer pH 8.0, and sonicated at 60 W/cm^2 (four treatments of 60 s, amplitude of 0.5 cycles, with 60 s intervals between the cycles) with a UP 400S sonicator (Dr. Hielscher, GmbH, Stahnsdorf, Germany) to release the recombinant protein. The cell debris was separated by centrifugation at 16,000 × *g* for 10 min and the resulting supernatant used as the source of soluble recombinant protein.

2.4. Purification of recombinant Amy 34

Recombinant His_6-tagged Amy 34 was purified by immobilized metal ion affinity chromatography (IMAC) on Cu^{2+}-iminodiacetic acid-Sepharose CL-6B column. The affinity adsorbent was prepared by coupling iminodiacetic acid (IDA) to epoxy-activated Sepharose CL-6B (Amersham Biosciences, Uppsala, Sweden) as described by Hermanson et al. [19], which was then mixed with five volumes of $CuSO_4$ solution (5 mg/ml) for 30 min. The resulting Cu^{2+} loaded gel was packed in a column (8.5 cm × 1.75 cm) washed with 10 bed volumes of water, followed by 10 bed volumes of 50 mM imidazole to remove loosely bound copper, and finally with 10 bed volumes of water prior to equilibration with binding buffer consisting of 20 mM phosphate buffer, pH 8.0 containing 10 mM imidazole and 0.5 M NaCl. Two millilitres of clarified Amy 34 sample were applied on the column at a flow rate of 0.5 ml/min. The column was then washed with five bed volumes of binding buffer to remove unbound proteins, followed by elution of the bound protein using 200 mM imidazole in 20 mM phosphate buffer, pH 8.0 containing 0.5 M NaCl. The absorbance of the eluate was continuously monitored at 280 nm, and amylase activity of the eluted fractions determined.

Fractions with amylase activity were pooled and concentrated 20-fold using Vivaspin columns (Vivascience AB, Hannover, Germany) with a molecular cut off of 100 kDa to enhance the recovery of full length mature peptide. The concentrated enzyme was then subjected to size exclusion chromatography on Sephacryl S-200 (Amersham Biosciences,

Uppsala, Sweden) column (13.5 cm × 0.96 cm), equilibrated with 20 mM phosphate buffer, pH 8.0 containing 0.5 M NaCl at a flow rate of 0.20 ml/min.

2.5. Amylase activity determination

Amylase activity was routinely estimated by determination of the reducing sugars liberated from starch hydrolysis. Fifty microlitres of appropriately diluted enzyme solution was added to 450 μl of 0.3% (w/v) soluble starch solution in 50 mM glycine–NaOH buffer pH 10.0 (preincubated at the appropriate temperature) and the mixture was incubated at 55 °C for 10 min. The amount of reducing sugars produced was determined by the dinitrosalicylic method [20]. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of reducing sugars per minute under the standard assay conditions.

2.6. Protein determination

Protein concentration during purification was determined using the bicinchoninic acid (BCA) method with bovine serum albumin as the standard [21].

2.7. Polyacrylamide gel electrophoresis

Purity of the enzyme at different stages of purification was analysed by SDS–PAGE as described by Laemmli [22] using 10% acrylamide gel. Protein bands were visualised by silver staining of the gel after electrophoresis.

Native PAGE of crude culture supernatant of *B. halodurans*, clarified homogenate of recombinant *E. coli* cells and purified recombinant Amy 34 was performed on 12% acrylamide gel. Amylase activity staining of the gel was performed as described by Kim et al. [12].

2.8. Analysis of hydrolysis products

End products formed by the action of Amy 34 on various substrates such as starch (0.3%, w/v) amylose, amylopectin, maltodextrin, pullulan, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose (0.5%, w/v), and α, β and γ-cyclodextrin (20 mM), respectively, were analysed by thin layer chromatography using the HPTLC Camag equipment (Camag, Muttenz, Switzerland). Defined amounts of the reaction mixtures in 30% (v/v) acetonitrile were applied as 8 mm bands on 20 cm × 10 cm precoated Kieselgel 60 F254 silica gel plates (Merck, Darmstadt, Germany) using the Camag ATS3 autosampler. The plates were developed with a solvent system of butanol:acetic acid:water (3:1:1; v/v/v), dried in the automated Camag ADC development chamber, immersed for 5 s in the Camag chromatogram immersion device containing 10% (v/v) sulphuric acid in ethanol as the detection reagent and heated for 5–10 min at 120 °C. Products were visualised as brown narrow bands on a clear background and were densitometrically evaluated at 500 nm wavelength (tungsten lamp) with a Camag TLC scanner 3 in absorbance/reflection mode with the slit dimensions set at 4.0 mm × 0.45 mm. The relative amounts of linear sugars obtained were calculated on each chromatographic plate from the integrated peak areas. Calibration curves for G1–G7 linear sugars (0.01–0.05%, w/v; Sigma St. Louis, MO, USA) in 30% (v/v) acetonitrile were attained under similar conditions.

3. Results and discussion

3.1. Extracellular amylolytic activity of B. halodurans LBK 34

Activity staining of amylases from cell-free culture supernatant of the native host, *B. halodurans* LBK 34 on native PAGE revealed five bands at pH 10.0 and 55 °C (Fig. 1). All the bands were observed from the onset of amylase activity production during cultivation of the isolate. The largest band was found to decrease in intensity while the smallest increased in intensity with time, implying that the different isoforms were formed as a result of proteolytic cleavage of the largest protein (data not shown). Cultivation of the microorganism in presence of a protease inhibitor (PMSF) at concentrations of up to 5 mM did not alter the formation and activity of the different amylase variants (data not shown). Multiple forms of amylolytic enzymes from various *Bacillus* sp. have previously been reported. The alkaliphilic *Bacillus* sp. GM 8901 maltotetraose-forming amylase is cleaved to produce five isoforms [12] by a serine protease present in the cell free culture supernatant, but the amylase activity was found to be inhibited by 1 mM PMSF. The G_6-amylase from alkaliphilic *Bacillus* sp. H-167 was proteolytically processed at the C-terminus into three isoforms; expression of the G_6-

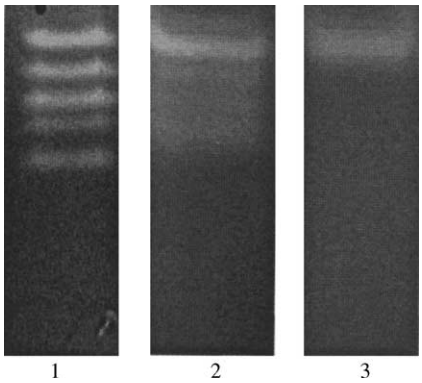

Fig. 1. Detection of amylolytic activity of *B. halodurans* LBK 34 and recombinant Amy 34 on native (12%) PAGE. The gel was incubated at 55 °C in 1% (w/v) starch solution, pH 10.0, for 2–3 h with shaking (80 rpm) and subsequently stained with iodine solution (0.5%, w/v, KI and 0.05%, w/v, I_2). Lane 1: *B. halodurans* LBK 34 cell-free culture supernatant; lane 2: crude recombinant Amy 34 (clarified homogenate); lane 3: purified recombinant Amy 34.

amylase gene in recombinant *E. coli* led to the formation of two more minor amylases [9,10,23]. Three isoforms were also detected in the extracellular fluid of *B. clausii* BT-21, whose G_6-amylase shows more than 90% identity with the *Bacillus* sp. H-167 amylase [11].

3.2. Sequence analysis of amy34 gene

Amplification of the *amy34* gene from *B. halodurans* LBK 34 using primers designed from *Bh0413* ORF (accession no. AP001508) gave a 3 kb PCR product encoding a mature peptide of 958 amino acid residues, with theoretical molecular weight of 107.2 kDa and isoelectric point of 4.41. The gene sequence has been deposited in the GenBank database and has been assigned the accession number AY528737. A signal peptide sequence of 33 aa residues was also deduced from the sequence (www.cbs.dtu.dk/services/SignalP). Sequence analysis of *amy34* gene revealed about 97% identity with the α-amylase G6 precursor gene sequence in the *B. halodurans* C-125 genome, 75% identity with the *B. clausii* BT-21 maltooligosaccharide-forming amylase [11], and 74% identity with the alkaliphilic *Bacillus* sp. H-167 G_6-amylase gene [24].

By amino acid sequence alignment of various amylolytic enzymes using Clustal W (Version 1.74), the four conserved regions commonly found within the α-amylase family [25] were identified within the Amy 34 sequence including the three proposed catalytic residues, Asp in region II, Glu in region III and Asp in region IV (Table 1).

3.3. Expression and purification of recombinant Amy 34

The gene encoding Amy 34 amylase was cloned into pET 30 vector containing the *T7 lac* promoter and transformed into *E. coli* BL21(DE3) cells. Expression of the gene after induction with 1 mM IPTG led to the intracellular production of 45-fold higher amount of soluble and active amylase activity with a specific activity of 117 U/mg, as compared to the extracellular production of the enzyme (specific activity of 0.07 U/mg) from the wild-type *B. halodurans* LBK 34. With full induction (1 mM IPTG final concentration), enzyme production was quite stable and levels of recombinant enzyme produced remained stable during prolonged (overnight) cultivation at room temperature.

The crude recombinant enzyme obtained after sonication of the *E. coli* cells showed the presence of multiple bands on native PAGE (Fig. 1), indicating that the mature peptide undergoes proteolytic cleavage as was observed with the α-amylase from the native host. Although the expression host, *E. coli* BL21(DE3) is naturally deficient of the ATP dependent proteinase, Lon and the outer membrane proteinase, OmpT [26], protease activity has been detected in this host, as a result of induction-related stress response [27], whereby the metabolic burden imposed on the cells to produce the recombinant proteins leads to induction of host cell proteases in order to release amino acids from existing proteins and also resulting in proteolysis of the recombinant proteins [28–30].

The recombinant Amy 34 bearing the His_6 tag was purified to homogeneity from the clarified *E. coli* homogenate by a combination of IMAC, ultrafiltration and size exclusion chromatography with a 10-fold purification and 47% yield (Table 2). The molecular weight was calculated to be about 119 kDa by SDS–PAGE (Fig. 2), which was in the range (53–159 kDa) of molecular weights of other alkaline active maltooligosaccharide-forming α-amylases from various *Bacillus* species [11,12,24,31–35].

Table 1
The four conserved regions found in the amino acid sequences of enzymes within the α-amylase family

Enzyme	Region I	Region II	Region III	Region IV
Amy 34	DVVMNH	GIDGFRVD	EALW	FNVLSYLSQHD
G6 amylase (C-125)	DVVMNH	GIDGFRVD	EALW	FNVLSYLSQHD
G6 amylase (*K. pneumoniae*)	DVVMNH	GIDGFRVD	EWKK	FNVLSYLSSHD
α-amylase	DAVINH	GIDGFRID	EAWG	FNPVSYMSSHD
TAKA amylase	DVVANH	SIDGLRID	EVLD	FVE-----NHD
CGTase	DFAPNH	GVDGIRVD	EWFL	FID-----NHD
Neopullulanase	DAVFNH	DIDGWRLD	EIWH	FNLLG---SHD
Amylopullulanase	DVVLNH	TIDYFRVD	EAWG	FLG-----SHD
	*	* *	*	**
		!	!	!

Regions I, II, III and IV are the four well-known conserved sequence regions within the α-amylase family of enzymes [25]. Conserved amino acid residues involved in catalysis are indicated with (*). The three proposed catalytic residues are indicated with (!). The amino acid sequences of the following enzymes were used for the alignment: Amy 34 [AY528737]; *B. halodurans* C-125 maltohexaose-forming amylase [Q9KFR4]; *K. pneumoniae* maltohexaose-forming amylase [Q9RHR1]; *V. cholerae* α-amylase [Q9KL86]; *A. oryzae* Taka amylase [Q96TH4]; *B. circulans* CGTase [P30920]; *B. stearothermophilus* neopullulanase [Q9AIV2] and *Bacillus* sp. alkaline amylopullulanase [P70983].

Table 2
Purification of recombinant Amy 34 from clarified *E. coli* cell homogenate

	Total units (U)	Total protein (mg)	Specific activity (U/mg)	Purification factor	Yield (%)
Crude	3042	26.0	117	1.0	100.0
IMAC	2698	3.0	899	7.7	88.7
Gel filtration	1440	1.2	1200	10.3	47.3

3.4. Effect of temperature and pH on Amy 34 amylolytic activity

The pure recombinant Amy 34 was found to display optimum activity at 60 °C when assayed with 0.3% (w/v) soluble starch at pH 10.0. A rapid decrease in activity was seen with further increase in temperature, reaching 50% of the optimum at 70 °C and 15% at 90 °C. At the lower end of the temperature scale, 35% of the optimum activity was observed at 25 °C.

Determination of thermal stability of the enzyme, by incubating the enzyme solution at a particular temperature followed by measurement of residual activity at 55 °C, revealed that after 4 h incubation, Amy 34 retained complete activity at temperatures up to 35 °C, and 60% activity at 55 °C (Fig. 3). After 30 min incubation at the optimal temperature of activity (60 °C), 50% activity was lost, suggesting that the enzyme was more sensitive to denaturation in the absence of substrate. The presence of substrate during activity measurements confers some protection on the enzyme against thermal inactivation, allowing it to be active at higher environmental temperatures [33,36].

Amy 34 displayed optimal activity at pH 10.5–11.5 at 55 °C, but when stored for 30 min (55 °C) at pH 10.5 and pH 11.0 prior to activity measurements, it lost over 30 and 60% of its original activity, respectively (Fig. 4). Most of the alkaline active amylases from alkaliphilic *Bacillus* species also exhibit similar properties, with the optimum pH and temperature for their activities ranging between pH 8.0–12.0 and 50–60 °C, respectively [11,12,23,31–33].

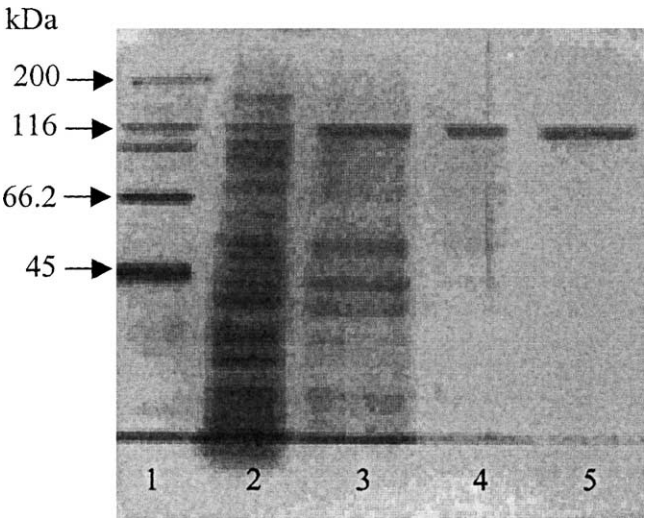

Fig. 2. SDS–PAGE analysis of recombinant Amy 34 during purification from *E. coli*. Lane 1: high range molecular weight marker (Bio-Rad Laboratories, CA, USA); lane 2: clarified cell homogenate; lane 3: eluate from metal chelate affinity column; lane 4: retentate after concentration by ultrafiltration; lane 5: eluate from gel filtration.

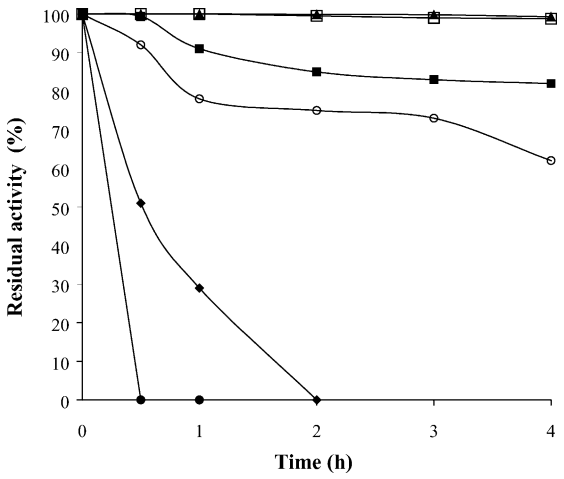

Fig. 3. Effect of temperature on the stability of recombinant *B. halodurans* Amy 34. The enzyme was incubated at 25 °C (▲), 35 °C (□), 45 °C (■), 55 °C (◯), 60 °C (♦) and 65 °C (●) in 50 mM glycine–NaOH buffer pH 9.0 and samples were taken at different time intervals for determination of residual activity under standard assay conditions. Enzyme activity corresponding to 100% was 1.30 U/ml.

3.5. Effect of metal ions and additives on the activity of Amy 34

Amy 34 activity was tested in the presence of various metal ions. The activity was not significantly influenced at 1 mM

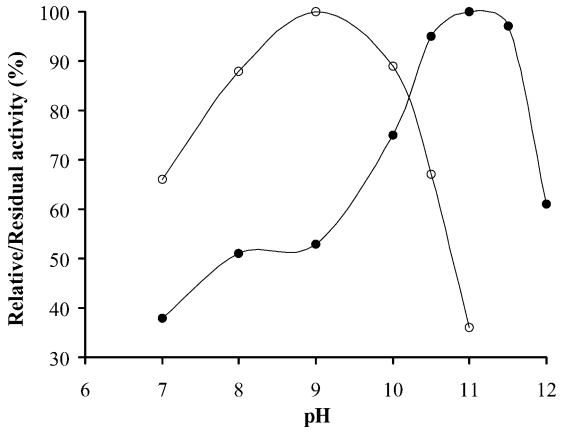

Fig. 4. Effect of pH on activity (●) and stability (◯) of recombinant *B. halodurans* Amy 34. The buffers (20 mM) used were acetate–sodium acetate (pH 4.0–5.5), phosphate (pH 5.5–8.0), Tris–HCl buffer (pH 7.5–9.0) and glycine–NaOH (pH 8.5–12.5). For determination of stability, the enzyme was incubated with the mentioned buffers at 55 °C for 30 min prior to measurement of residual activity under standard assay conditions. Enzyme activity corresponding to 100% activity was 1.30 U/ml.

concentration of most of the metal ions, but no activity was detected at 5 mM Cu^{2+}, Fe^{2+}, Fe^{3+} and Mn^{2+} ions, and less than 60% activity with Ca^{2+} ions (Table 3). Stimulation of activity was observed in presence of 1 mM Co^{2+} and 5 mM Ni^{2+} ions, respectively. Calcium ions play an important role in the maintenance of enzymatic activity and structural integrity of amylases, including those from various alkaliphilic *Bacillus* species [11,12,31–33]. Inhibition of amylase activity at higher Ca^{2+} ion concentration, as seen in this study, has earlier been reported also for *Pyrococcus woesei* α-amylase [37]. Inhibition of catalytic activity of certain amylases at relatively high concentrations of Ca^{2+} ions (2–10 mM) has been ascribed to interference caused by the binding of the metal ion at the secondary binding site within the substrate binding cleft that involves the catalytic residues [38,39].

The Amy 34 enzyme was also sensitive to the presence of EDTA, exhibiting 40% of its original activity and no activity, respectively, at 1 and 5 mM concentration of the metal chelator (Table 3). Effect of EDTA on α-amylases from alkaliphilic *Bacillus* species varies considerably, with some being unaffected in presence of EDTA at concentrations as high as 100 mM [34], while others are completely inhibited by 1 mM EDTA [12]. Enzyme activity was not affected by dithiothreitol, suggesting the absence of any critical disulphide groups. Furthermore, it displayed 80 and 40% activity in the presence of 1 and 5% SDS, respectively (Table 3). *Bacillus* sp. TS-23 amylase has earlier been shown to be resistant to SDS [33].

3.6. Mode of action of Amy 34

Investigation of the products formed by the action of Amy 34 on hydrolysis of soluble starch showed maltohexaose (G6) to be the predominant initial product, the yields of which decreased with time to 40% after 2 h of reaction time, while lower maltooligosaccharides were formed in the order G4 > G2 > G5 > G3 > G1 (Fig. 5). Similar hydrolysis patterns of the amylases from *Bacillus* sp. H-167 and *B. clausii* BT-21 on starch, have also been reported [10,11]. The smallest oligosaccharide that the enzyme could accept as a substrate was maltotetraose to yield G1, G2 and G3 (Fig. 6). The products of hydrolysis of maltopentaose were predominantly G4 and G1 that of maltohexaose were G4, G5, G2, G1 and traces of G3, while G6 was liberated as the main product with maltoheptaose along with G4, G5, G2, G1 and G3.

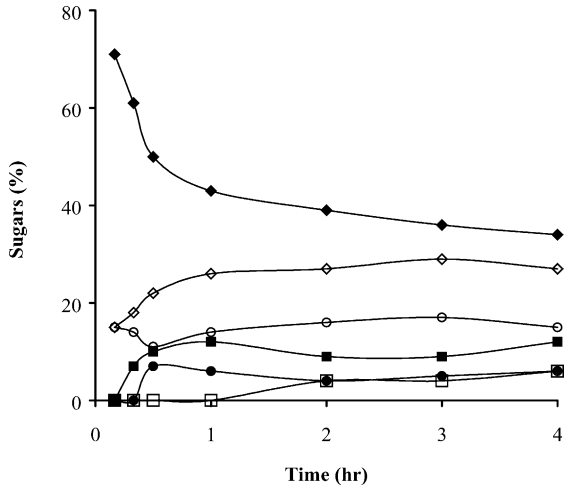

Fig. 5. Analysis of the end products formed by the action of recombinant *B. halodurans* Amy 34 (1.30 U/ml) on starch at 55 °C in 50 mM glycine buffer pH 10.0 at various time intervals. Symbols denote: (□) glucose; (◯) maltose; (●) maltotriose; (◊) maltotetraose; (■) maltopentaose and (⧫) maltohexaose. The reducing sugars were quantified using the Camag TLC scanner.

Table 3
Effect of metal ions and additives on Amy 34 activity

	Relative activity (%)	
	1 mM	5 mM
Ca^{2+}	98.6	59.2
Cu^{2+}	98.3	–
Co^{2+}	105.9	95.0
Ni^{2+}	98.0	113.4
Fe^{2+}	98.9	–
Fe^{3+}	93.9	–
K^{+}	90.3	88.6
Mg^{2+}	84.5	76.7
Mn^{2+}	92.0	–
Na^{+}	98.6	98.1
Zn^{2+}	95.3	85.2
DTT	99.9	98.6
EDTA	58.5	–

SDS concentration (%)	Relative activity (%)
0.1	93.7
0.5	86.7
1	81.5
5	42.7

Hydrolysis of amylose, amylopectin and maltodextrin yielded predominantly maltotetraose (Fig. 6). Pullulan was not hydrolysed by Amy 34 indicating its inability to attack the α-1,6-linkage. Incubation of the enzyme with α, β and γ-cyclodextrin revealed that the enzyme could only hydrolyse γ-cyclodextrin with G4, G2, G1 and traces of G3 as the end products (Fig. 6) although the rate of hydrolysis of γ-CD by Amy 34 was much lower as compared to that of starch. Such a catalytic behaviour has earlier been reported for a raw starch degrading α-amylase from *Bacillus* sp. IMD 340 [40]. Few other amylases are reported to have the ability to hydrolyse cyclodextrins with varying rates of hydrolysis [41–43]. In contrast, other maltooligosaccharide-forming alkaline active amylases from various *Bacillus* species are reported to have no hydrolytic action on cyclodextrins [11,12,32–34].

The product profile of the Amy 34 activity suggests the enzyme to belong to the category of exo-amylases, such as maltotetraose (EC 3.2.1.60) and maltohexaose (EC 3.2.1.98)-forming enzymes, and cyclodextrin glycosyltransferases, CGTases, (EC 2.4.1.19), although the latter have low

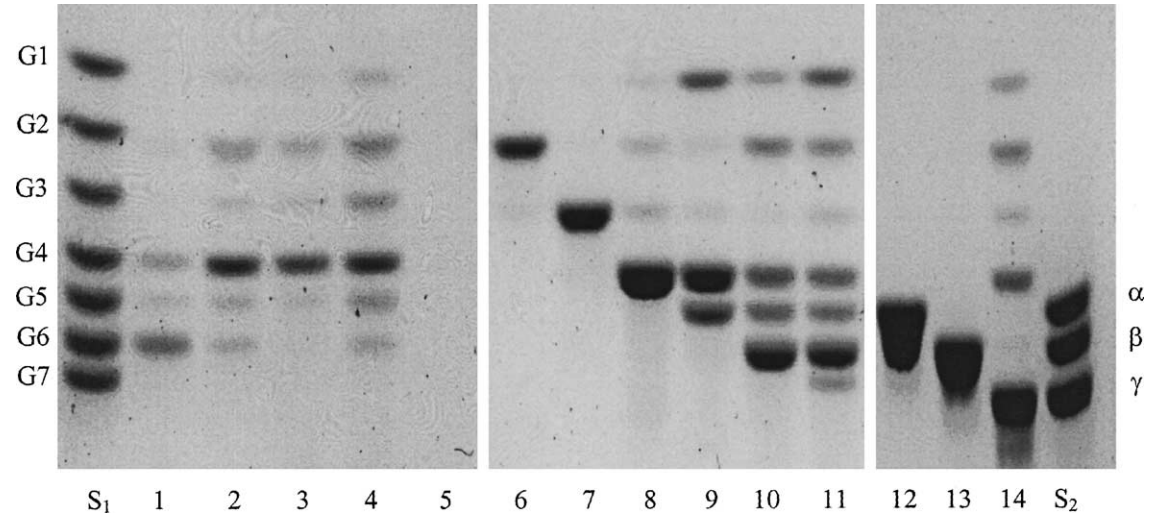

Fig. 6. HPTLC analysis of the end products formed by the action of Amy 34 (1.0 U/ml) after 1 h incubation at 55 °C, in 50 mM glycine buffer pH 10 with 0.3% (w/v) starch (1), 0.5% (w/v) amylose (2), 0.5% (w/v) amylopectin (3), 0.5% (w/v) maltodextrin (4), 0.5% (w/v) pullulan (5) and after 4 h incubation at 55 °C with maltose (6), maltotriose (7), maltotetraose (8), maltopentaose (9), maltohexaose (10), maltoheptaose (11), 20 mM α-cyclodextrin (12), 20 mM β-cyclodextrin (13) and 20 mM γ-cyclodextrin (14). S_1 and S_2 are linear oligosaccharide and cyclodextrin standards, respectively. Maltotriose band in lane 1 appears faint but was detectable with the Camag TLC scanner.

hydrolytic activity and also exhibit transglycosylation activity [44].

4. Concluding remarks

Maltooligosaccharide-forming amylases are thought to be the evolutionary link between α-amylases and cyclodextrin glycosyltransferases (CGTases) and are proposed to be the "intermediary enzymes", exhibiting features from both groups of enzymes [45].

B. halodurans Amy 34 amylase is yet another addition to the unique group of enzymes, which produced maltohexaose as the main product with starch as the substrate and maltotetraose as the main product with other substrates. A blast search of the putative protein sequence deduced from the translation of the gene sequence of the Amy 34 enzyme against the structural protein database (PDB), further revealed highest structural identities with various CGTases whose three-dimensional structures have been solved. This makes it an interesting candidate for further investigations regarding its activity features resembling the CGTases. Such studies are currently in progress.

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References

[1] Bealin-Kelly F, Kelly CT, Fogarty WM. The α-amylase of the caldoactive bacterium *Bacillus caldovelox*. Biochem Enzymol 1990;1:149–59.
[2] Ben Ali M, Mhiri S, Mezghani M, Bejar S. Purification and sequence analysis of the atypical maltohexaose forming α-amylase of the *B. stearothermophilus* US100. Enzyme Microbial Technol 2001;28:537–42.
[3] Kainuma K, Kobayashi S, Ito T, Suzuki S. Isolation and action pattern of maltohexaose producing amylase from *Aerobacter aerogenes*. FEBS Lett 1972;6:281–5.
[4] Monma N, Nakakuki T, Kainuma K. Formation and hydrolysis of maltohexaose by an extracellular exo-maltohexaohydrolase. Agric Biol Chem 1983;46:1121–9.
[5] Ben Ali M, Mezghani M, Bejar S. A thermostable α-amylase producing maltohexaose from a newly isolated *Bacillus* sp. US100: study of activity and molecular cloning of the corresponding gene. Enzyme Microbial Technol 1999;24:584–9.
[6] Fogarty WM, Bealin-Kelly F, Kelly CT, Doyle EM. A novel maltohexaose forming α-amylase from *Bacillus caldovelox*: patterns and mechanisms of action. Appl Microbiol Biotechnol 1991;36:484–9.
[7] Takasaki Y. Production of maltohexaose by α-amylase from *Bacillus circulans* G-6. Agric Biol Chem 1982;46:1539–47.
[8] Taniguchi H, Jae CM, Yoshigi N, Maruyama Y. Purification of *Bacillus circulans* F-2 amylase and its general properties. Agric Biol Chem 1983;47:511–9.
[9] Hayashi T, Akiba T, Horikoshi K. Production and purification of new maltohexaose forming amylase from alkalophilic *Bacillus* sp. H-167. Agric Biol Chem 1988;52:443–8.
[10] Hayashi T, Akiba T, Horikoshi K. Properties of new alkaline maltohexaose forming amylase. Appl Microbiol Biotechnol 1988;28:281–5.

[11] Duedahl-Olesen L, Kragh K, Zimmerman W. Purification and characterisation of a malto-oligosaccharide forming amylase active at high pH from *Bacillus clausii* BT-21. Carbohyd Res 2000;329:99–107.
[12] Kim TU, Gu BG, Jeong JY, Byun SM, Shin YC. Purification and characterisation of a maltotetraose-forming alkaline α-amylase from an alkaliphilic *Bacillus* strain GM8901. Appl Environ Microbiol 1995;61:3105–12.
[13] Duckworth AW, Grant WD, Jones BE, Steenbergen R. Phylogenetic diversity of soda lake alkaliphiles. FEMS Microbiol Ecol 1996;19:181–91.
[14] Takami H, Nakasone K, Takaki Y, Maeno G, Sasaki R, Masui N, et al. Complete genome sequence of the alkaliphilic bacterium *Bacillus halodurans* and genomic sequence comparison with *Bacillus subtilis*. Nucleic Acids Res 2000;28:4317–31.
[15] Hashim SO, Delgado O, Hatti-Kaul R, Mulaa FJ, Mattiasson B. Starch hydrolysing *Bacillus halodurans* isolates from a Kenyan soda lake. Biotechnol Lett 2004;26:823–8.
[16] Horikoshi K. Production of alkaline amylases by alkalophilic microorganisms. II. Alkaline amylase produced by *Bacillus* no. A-40-2. Agric Biol Chem 1971;35:1783–91.
[17] Sambrook J, Fritsch E, Maniatis T. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory; 1989.
[18] Cheng S, Fockler C, Barnes W, Higuchi R. Effective amplification of long targets from cloned inserts and human genomic DNA. Proc Natl Acad Sci USA 1994;91:5695–9.
[19] Hermanson G, Mallia A, Smith P. Activation methods. In: Immobilised affinity ligand techniques. San Diego: Academic Press Inc.; 1992.
[20] Miller GL. Use of dinitrosalisylic acid reagent for determination of reducing sugars. Anal Chem 1959;31:426–8.
[21] Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, et al. Measurement of protein using bicinchoninic acid. Anal Biochem 1985;150:76–85.
[22] Laemmli U. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–5.
[23] Shirokizawa O, Akiba T, Horikoshi K. Cloning and expression of the maltohexaose-forming amylase gene from alkalophilic *Bacillus* sp. H-167 in *Escherichia coli*. Agric Biol Chem 1989;53:491–5.
[24] Shirokizawa O, Akiba T, Horikoshi K. Nucleotide sequence of the G6-amylase gene from alkalophilic *Bacillus* sp. H-167. FEMS Microbiol Lett 1990;70:131–6.
[25] Nakajima R, Umanaka T, Aiba S. Comparison of amino acid sequences of eleven different α-amylase. Appl Microbiol Biotechnol 1986;23:355–60.
[26] Gottesmann S. Proteases and their targets in *Escherichia coli*. Annu Rev Genet 1996;30:465–506.
[27] Ramchuran SO, Nordberg Karlsson E, Velut S, de Mare L, Hagander P, Holst O. Production of heterologous thermostable glycosyl hydrolases and the presence of host-cell proteases in substrate limited fed-batch cultures of *E. coli* BL21 (DE3). Appl Microbiol Biotechnol 2002;60:408–16.
[28] Harcum SW, Bentley WE. Response dynamics of 26-, 34-, 39-, 54-, and 80-kDa protease in induced cultures of recombinant *Escherichia coli*. Biotechnol Bioeng 1993;42:675–85.
[29] Harcum SW, Bentley WE. Detection, quantification and characterization of proteases in *Escherichia coli*. Biotechnol Tech 1993;7:441–7.
[30] Ramirez DM, Bentley WE. Fed-batch feeding and induction policies that improve foreign protein synthesis and stability by avoiding stress response. Biotechnol Bioeng 1995;47:596–608.
[31] Mc Tigue MA, Kelly C, Doyle EM, Fogarty WM. The alkaline amylase of the alkalophilic *Bacillus* sp. IMD 370. Enzyme Microbial Technol 1995;17:570–3.
[32] Igarashi K, Hatada Y, Hagihara H, Katsuhisa S, Takaiwa M, Uemura T, et al. Enzymatic properties of a novel liquefying α-amylasefrom an alkaliphilc *Bacillus* isolate and entire nucleotide and amino acid sequence. Appl Environ Microbiol 1998;64:3282–9.
[33] Lo HF, Lin LL, Chen HK, Hsu WH, Chang CT. Enzymatic properties of a SDS-resistant *Bacillus* sp. TS-23 α-amylase produced by recombinant *Escherichia coli*. Process Biochem 2001;36:743–50.
[34] Hagihara H, Kazuaki I, Yasuhiro H, Endo K, Ikawa-Kitayama K, Ozaki K, et al. Novel α-amylase that is highly resistant to chelating reagents and chemical oxidants from the alkaliphilic *Bacillus* isolate KSM-K38. Appl Environ Microbiol 2001;67:1744–50.
[35] Burhan A, Nisa U, Gökhan C, Ömer C, Ashabil A, Osman G. Enzymatic properties of a novel thermostable, thermophilic, alkaline and chelator resistant amylase from an alkaliphilic *Bacillus* sp. isolate ANT-6. Process Biochem 2003;38:1397–403.
[36] Ratanakhanokchai K, Kaneko J, Kamio Y, Izaki K. Purification and properties of a maltotetraose- and maltotriose-producing amylase from *Chloroflexus aurantiacus*. Appl Environ Microbiol 1992;58:2490–4.
[37] Koch R, Spreinat A, Lemke K, Antranikian G. Purification and properties of a hyperthermoactive α-amylase from archaeobacterium *Pyrococcus woesei*. Arch Microbiol 1991;155:572–8.
[38] Boel E, Brady L, Brzozowski AM, Derewenda Z, Dodson GG, Jensen VJ, et al. Calcium binding in α-amylases: an X-ray diffraction study at 2.1-Å resolution of two enzymes from *Aspergillus*. Biochemistry 1990;29:6244–9.
[39] Tanaka A, Hoshino EJ. Secondary calcium-binding parameter of *Bacillus amyloliquefaciens* α-amylase obtained from inhibition kinetics. Biosci Bioeng 2003;96:262–7.
[40] Hamilton L, Kelly C, Fogarty W. Purification and properties of the raw starch degrading α-amylase of *Bacillus* sp. IMD 434. Biotech Lett 1999;21:111–5.
[41] Lin LL, Chay CC, Hsu WH. Production and properties of a raw starch degrading amylase from the thermophilic and alkaliphilic *Bacillus* sp. TS-23. Biotechnol Appl Biochem 1998;28:61–8.
[42] Saha B, Zeikus J. Cyclodextrin degrading enzymes. Starch 1992;44:312–5.
[43] Brumm P, Hebeda R, Teague W. Purification and characterisation of the commercialised, cloned *Bacillus megaterium* α-amylase. Part I: Purification and hydrolytic properties. Starch 1991;43:315–9.
[44] van der Maarel MJEC, van der Veen B, Uitdehaag J, Leemhuis H, Dijkhuizen L. Properties and applications of starch-converting enzymes of the α-amylase family. J Biotechnol 2002;94:137–55.
[45] Janecek S. Tracing the evolutionary lineages among α-amylase and cyclodextrin glycosyltransferases: the question of the so-called "intermediary enzymes". Biologia (Bratislava) 1995;50:515–22.