Carbohydrate-Active Enzymes from Alkaliphiles

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Abstract: The market in industrial enzymes for detergents, starch, food, fabric, and so on has been estimated at more than 1600 million US dollars in 1999. The usage of detergent enzymes, such as protease, cellulase, a-amylase, and lipase, forms 35% of the world market. The enzymes now are incorporated into more than 80% of heavy-duty laundry detergents in the world. Furthermore, alkaline protease and alkaline a-amylase have been frequently used in automatic dishwashing detergents especially in the US and Europe. We have exploited large-scale production of alkaline cellulases, alkaline proteases, and an alkaline a-amylase, and the enzymes have been incorporated into heavy-duty compact detergents and/or bleaches. The problems are that traditional alkaline proteases and alkaline a-amylases are seriously inactivated by chemical oxidants and chelating reagents and are thermally unstable, especially when the enzymes are used in automatic dishwashers. Here, we describe the improvement of thermostability and oxidative stability of our alkaline liquefying a-amylase designated LAMY. Furthermore, we show a novel amylase that is extremely resistant to chemical oxidants and chelating reagents.

Key words: thermostability, oxidative stability, a-amylase, detergent enzyme, alkaliphile, Bacillus

a-Amylases (1,4-a-D-glucan glucanohydrolase [EC 3.2.1.1.]) are industrially important, especially in the starch, food, and detergent industries.1-3 Liquefying a-amylases, particularly the Bacillus licheniformis enzyme (BLA),4 are used widely in technical application fields, such as in bread making, production of glucose and fructose syrup and fuel ethanol from starch materials, and textile treatment. The demand for a-amylase for use in laundry and automatic dishwashing detergents has also been growing for several years.5 However, most of the Bacillus liquefying a-amylases, such as the enzymes from Bacillus amyloliquifaciens (BAA)6 and B. steareothermophilus (BSA),7 including BLA, have an optimal pH between 5 and 7.5; therefore, they are not practically suitable for use in laundry and dishwashing detergents with high alkalinity.

We have found an alkaline liquefying a-amylase (LAMY) from alkaliphilic Bacillus sp. strain KSM-1378.9 LAMY is highly active at alkaline pH, compared with other a-amylases reported to date, and resistant to various surfactants. However, LAMY is less thermostable than BLA; therefore, improvement of the thermostability of LAMY is desirable for use at high temperatures under alkaline conditions in automatic dishwashers. Moreover, LAMY and other Bacillus a-amylases are inactivated by chemical oxidants. We tried to improve the oxidative stability of LAMY by replacing a Met residue with non-oxidizable amino acids as in the case of alkaline proteases that acquired oxidative stability by site-directed mutagenesis.10-12 In this article, we describe properties and deduced amino acid sequence of LAMY and improvement of thermostability and oxidative stability of the enzyme by site-directed mutagenesis.

Enzymatic properties of LAMY.

LAMY was found in cultures of alkaliphilic Bacillus sp. strain KSM-1378.9 A highly purified preparation of LAMY was obtained by a simple purification procedure with high yield (35%). The specific activity was approximately 5000 U/mg protein, a value two- to five-fold greater between pH 5 and 10 than that of an industrial thermostable a-amylase (BLA) from B. licheniformis (Fig. 1A). The molecular mass of LAMY was approximately 53 kDa as judged by SDS-PAGE. The N-terminal amino acid sequence was HHNGTNGTMMQYFEW. As shown in Figs. 1A and B, the optimal pH and temperature of the liquefying activity were around pH 8.5 and 55°C. The enzyme was stable over a range between pH 6 and 10 when incubated at various pHs of different buffers at 40°C for 30 min. In the absence of CaCl2, the enzyme retained its full activity after 60 min of incubation at 45°C and pH 8.5, whereas only 32 and 3% of the original activity remained after 60 min of incubation at 50 and 60°C, respectively. However in the presence of 0.1 mM CaCl2, nearly 100 and 65% of the original activity remained at 50 and 60°C, respectively. LAMY efficiently hydrolyzed various a-glucans to yield maltotriose, maltopentaose, maltohexaose, and maltose as major end products after completion of the reaction. When it was incubated with 10 mM EDTA or EGTA, the enzyme activity decreased to 10 or 9% of the initial activity, respectively.

Deduced amino acid sequence of LAMY.

The gene for LAMY was cloned and sequenced. It contained an open reading frame (ORF) of 1545-bp encoding...
515 amino acid residues including a putative signal peptide of 31 amino acid residues (Fig. 2). The molecular mass of the mature enzyme is calculated to be 55,391 Da, a value close to the 53 kDa determined by SDS-PAGE of the purified LAMY from the culture broth of *Bacillus* sp. strain KSM-1378.9)

When suitably aligned, the deduced amino acid sequence of LAMY shows moderate similarity to BAA, BSA and BLA with 66.7, 68.6 and 68.9% identity, respectively. The deduced amino acid sequence of G6-producing α-amylase from alkaliphilic *Bacillus* sp. strain 707 has the highest identity to that of LAMY (83.5%). The three-dimensional structures of some α-amylases from different origins have been determined by X-ray crystallography, and they appear very similar and contain three domains, A, B and C, as first reported by Buisson et al.12) for porcine pancreatic α-amylase. Essentially, LAMY has the (β/α)n barrel structure, as the structure elements are shown to be underlined and labeled by a1–α8 for α-helices and β1–β8 for β-sheets in Fig. 2. Four conserved regions which are necessary for the catalytic activity of α-amylase13–14) are well conserved in LAMY as Asp102 to His107, Gly232 to His240, Gly266 to Lys269 and Phe328 to Asp333 for regions I, II, III and IV, respectively. The regions are believed to form the active center, the substrate binding site, and the calcium binding site. The three BLA residues (Asn104, Asp200 and His235) involved in calcium binding conserved in LAMY. The BLA residues for chloride binding, Arg229 and Asn326, correspond to Arg234 and Asn331 in LAMY. Like other liquefying α-amylases, LAMY has a distinct internal sequence, a domain B loop (Arg171 to Tyr200), which is believed to play an important role in the liquefaction of starch.15)

For evaluation of the properties of and large-scale production of LAMY, we used a new excretion vector, pHSP 64, to develop a hyperproduction system for *Bacillus subtilis*.15,16) The structural gene for LAMY was amplified by PCR and cloned into *SalI-SmaI* site of pHSP64. The transformed *B. subtilis* hyperproduced the α-amylase activity, corresponding to approximately 1.0 g protein per liter of an optimized liquid culture.17) No significant differences in physiological or catalytic properties were observed between the recombinant enzyme and the native enzyme produced by *Bacillus* sp. strain KSM-1378.

**Improvement of thermostability of LAMY.**

LAMY is relatively less thermostable than BLA; therefore, it is desirable to improve the thermostability of LAMY for use at high temperatures under alkaline conditions, especially in automatic dishwashing machines. Suzuki et al.10) demonstrated that the thermostability of BAA was drastically improved by the deletion of Arg176-Gly177 and substitution of Lys269 for Ala, using site-directed mutagenesis. They proposed that an increase in hydrophobicity by changes in charged residues enhanced the thermostability of this enzyme. Machius et al.19) also suggested that the loop containing the Arg-Gly residues in BAA has two more amino acid residues than BLA and that this could cause increased mobility of this region and decreased thermostability of the whole protein. In fact, the amino acid sequences of LAMY, BAA and BSA, all conserved the corresponding Arg-Gly residues at the respective amino acid positions. However, substituted Ala269 in BAA corresponds to Ala274 for LAMY and Ala269 for BLA in their original aa sequences. In support of this scenario, we deleted Arg181-Gly182 residues from the LAMY molecule by site-directed mutagenesis.20) LAMY and Arg181-Gly182 deleted LAMY (∆RG) were very similar with respect to specific activity, pH-activity curve, temperature-active curve, susceptibility to inhibitors, and pattern of hydrolysis products from soluble starch and maltooligosaccharides. As expected, ∆RG acquired increased thermostability (see Fig. 5); however, it also acquired increased pH stability and resistance to sodium dodecyl sulfate and especially chelating reagents (Fig. 3).

Recently, Suvd et al.21) analyzed the 3D structure of
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Fig. 2. Amino acid sequence alignment of LAMY and other Bacillus amylases.

Each numbering starts after the respective signal peptide. The four conserved regions, I, II, III and IV, are boxed. Domain A and B are indicated by dotted box and shading, respectively. The remaining C-terminal sequence corresponds to domain C. Underlined sequences correspond to the secondary structure elements $\alpha_1-\alpha_8$ and $\beta_1-\beta_8$ of the BLA $(\alpha/\beta)_8$ barrel (domain A) according to Machius et al. 19) BLA residues involved in the catalytic site (*), calcium binding site (○), and chloride binding site (●) are indicated above the sequences. Sequence accession numbers are as follows: strain 707; enzyme, P19571; BAA, P00692; BSA, P06279; and BLA, P06278.

BSA and showed that the insertion of Ile181-Gly182, instead of Arg179-Gly180, pushes away a spatially contact- ing region containing calcium coordinating Asp207. As a result, Ca$^{2+}$ cannot coordinates Asp207. Instead, a water molecule participates in coordinating Ca$^{2+}$ According to them, this may be the reason why BSA, BAA and LAMY are less thermostable than BLA. Actually, in the case of LAMY, Thr183-Gly184 and Asp209 seem to be spatially identical to Thr181-Gly182 and Asp207 in BSA judging from a model structure of LAMY with BSA as template (Fig. 4). 22) To examine whether this prediction is actually applicable to LAMY, we deleted the Thr183-Gly184 in LAMY by site-directed mutagenesis and compared the mutant (zi TG) with the wild type and 0 RG. 22) The resistance to EDTA of both L RG and 0 TG was essentially equal, and both mutants were more thermostable than LAMY even after being heated at 70°C for 60 min in the presence of Na$^+$ and Ca$^{2+}$ (Fig. 5). Furthermore, 0 TG was clearly more thermolabile than 0 RG, regardless of whether the cations were present or not. Our results with LAMY are clearly contrary to the prediction by Suvd et al. 21) It is suggested strongly that the microenvironmental topology around the dipeptide-containing loop in LAMY (possibly also the case for BAA) may be different from that in BSA. The contribution of loop stabilization by shortening two amino acid residues to the thermostabilization of the enzyme may be marginal, because only two more hydrogen bonds are gained on the loop (data not shown). At present, we cannot explain how the deletion mutations acquire chelator resistance or thermostabilization.
Fig. 3. Effects of SDS and EDTA on the activities of LAMY and ΔRG.

(A) The stability of LAMY (open symbols) and ΔRG (solid symbols) against 0.05% SDS (○, □) was compared with each control (without SDS: ◦, ●) after incubation at 45°C and at pH 8.5 in 50 mM Tris-HCl buffer. Timed aliquots (0.1 mL) were removed from the incubations, and the enzyme activities were measured at the same temperature and pH. (B) LAMY (○) and ΔRG (●) (2.0 U/mL each) were incubated at 40°C and at pH 8.5 in 50 mM Tris-HCl buffer in the presence of EDTA at various concentrations. Samples were taken after a 30-min incubation and aliquots (0.1 mL) were used to determine the residual activity in them. The values in A and B are all shown as percentage of the respective original activity, which are each taken as 100%.

Fig. 4. Structure of LAMY.

Coordinates for BSA used as the template were obtained from the PDB using the accession number 1hvx. (A) The overall structure of LAMY. The domains A, B, and C of LAMY are colored in black, green, and cyan, respectively. The loop (Phe180-Asn195) and Arg181-Gly184 are shown in red and yellow, respectively. The Cal-Na-Cal metal triad and CalIII are also indicated as spheres in the figure. (B) A close-up view showing the region around the loop and metal triad. The metal triad, Asp209, and a water molecule are shown in different colors in the CPK representation. The amino acid residues on the loop are shown as a stick model, with oxygen atoms in red, nitrogen atoms in blue, and main chains in green.

We have shown that LAMY can also be thermostabilized by replacing proline for Arg124.29 According to the homology alignment of LAMY with BLA, Arg124 is presumed to be located on a loop between a two-stranded antiparallel β-sheet involving residues at amino acid from 113 to 121 and from 135 to 142 in domain B. Half lives at 50°C of the wild-type enzyme and Arg124Pro mutant enzyme were 4.4 and 11.3 min, respectively. Furthermore, the thermostabilization due to double mutation with Arg124Pro and ΔRG is cumulative and remarkable even in the absence of any exogenous additives. The temperature at which the enzyme is inactivated in 10 min is 47.4°C for the wild-type enzyme and 62.5°C for the double mutant enzyme. Although no information was given about the positions of β-turn in the BLA structure, the thermostabilization of Arg124Pro mutant occurs as a re-
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Fig. 5. Thermostabilization of LAMY by deletion mutations.

The thermal stability of wild-type LAMY (A), ΔRG (B) and ΔTG (C) was assessed by incubation at 70°C and at pH 8.5 in 50 mM Tris-HCl buffer. The enzymes were heated without cations (○), in the presence of 10 mM Na⁺ (■), in the presence of 0.1 mM Ca²⁺ (▲), and in the presence of both additives (●). Samples (0.1 mL) were withdrawn at timed intervals, and the residual activities were measured. The respective original activities before heating are taken as 100%.

Fig. 6. Improved oxidative stability of LAMY by substitution of a Met residue for Thr.

The mutant enzyme Met202Thr was constructed by site-directed mutagenesis. The oxidative stability against 2% H₂O₂ of the mutant (■) was examined at 30°C and at pH 8.5 in 50 mM Tris-HCl buffer plus 2 mM CaCl₂ with the wild type enzyme as control (●). Similar results obtained for Met202Ala, Met202Ser, Met202Leu and Met202Ile are not shown.

A novel calcium-free alkaline α-amylase (AmyK38).

Recently, a novel alkaline α-amylase (AmyK38) was found in a culture of an alkaliphilic Bacillus sp. strain KSM-K38. The enzyme had an optimal pH of 8.0 to 9.5 and displayed maximum catalytic activity at 55 to 60°C. The molecular mass was approximately 55 kDa, as determined by SDS-PAGE. AmyK38 was highly resistant to chelators and chemical oxidation. The enzyme contains no Ca²⁺ and requires Na⁺ (or monovalent cations) for manifestation of activity. However, thermostabilization of AmyK38 is essential if it is to have industrial applications. Several chimeric enzymes between AmyK38 and ΔRG were constructed and evaluated for thermostability. As a result, AmyK38 was successfully thermostabilized by the single substitution of Tyr11 by Phe without any changes in the kinetic features.

We have found and characterized some unique other alkaline amylolytic enzymes, such as a high-alkaline pullulanase, an alkaline resistant neopullulanase, an alkaline isoamylase, and a biheaded alkaline amylopullulanase from alkaliphilic strains of Bacillus. These enzymes can be used as effective additives in dishwashing and laundry detergents, especially together with the engineered alkaline α-amylases as mentioned in this paper.

REFERENCES


