Hyperexpression of the Gene for a Bacillus α-Amylase in Bacillus subtilis Cells: Enzymatic Properties and Crystallization of the Recombinant Enzyme*

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We have constructed a new excretion vector, pHSP64, to develop a hyperexpression system for Bacillus subtilis [Sumitomo et al., Biosci. Biotechnol. Biochem., 59, 2172–2175 (1995)]. The structural gene for a novel liquefying semi-alkaline α-amylase from the alkaliphilic Bacillus sp. KSM-1378 was amplified by PCR. It was cloned into a Sac I-Smal site of pHSP64 and the recombinant plasmid obtained was introduced into B. subtilis. The transformed B. subtilis hyperproduced the α-amylase activity extracellularly, corresponding to approximately 1.0 g (5 × 10^6 units) per liter of an optimized liquid culture. The recombinant enzyme was purified to homogeneity by a simple purification procedure with very high yield. No significant differences in physicochemical and catalytic properties were observed between the recombinant enzyme and the native enzyme produced by Bacillus sp. KSM-1378. The enzymatic properties of the recombinant enzyme were further examined with respect to the responses to various metal ions. The recombinant enzyme could easily be crystallized at room temperature within one day in a buffered solution of 10% (w/v) ammonium sulfate (pH 6.5).

Key words: alkaliphile; Bacillus; α-amylase; cloning; expression

α-Amylase (1,4-α-β-glucan glucanohydrolase; EC 3.2.1.1) and debranching pullulanase (pullulan 6-glucanohydrolase; EC 3.2.1.14) are enzymes of industrial importance, particular in food and detergent industries. We have found unique debranching enzymes, such as an alkaline pullulanase, an alkali-resistant neopullulanase, and an alkaline isoamylase (EC 3.2.1.68), in strains of alkaliphilic Bacillus. We also found an alkaline amylopullulanase in cultures of alkaliphilic Bacillus sp. KSM-1378 for the first time, which efficiently hydrolyzes the α-1,6 linkages of pullulan and the α-1,4 linkages of carbohydrates at different active sites. These amylooligosaccharides enzymes can clean difficult-to-remove starch-based stains, such as gravy, pudding, and potato, in dishwashing and laundry detergents under alkaline conditions.

There are many reports on α-amylases of various microbial origins, such as the enzymes, for example, from Aspergillus oryzae (Taka-amylase), B. licheniformis (BLA), B. amyloliquifaciens (BAA), and B. stearothermophilus (BSA). The catalytic properties and the structures of these microbial α-amylases are understood on the basis of the three-dimensional structure of porcine pancreatic enzyme, as first analyzed by Buisson et al. We have found a liquefying semi-alkaline α-amylase (LAMY) from an alkaliphilic Bacillus isolate, KSM-1378, and shown that the catalytic properties and the amino acid sequence of this enzyme are quite different from those of the enzymes reported so far.

To study the molecular properties of bacterial enzymes from various origins, we have constructed and used a new vector for B. subtilis, pHSP64, for efficient extracellular production of foreign and protein-engineered enzymes. This report describes the hyperexpression in B. subtilis cells of the gene for the 53-kDa LAMY (lamy) from Bacillus sp. KSM-1378 using pHSP64 and the enzymatic properties of the recombinant enzyme.

Materials and Methods

Bacterial strains, plasmids, and propagation. The source of the enzyme and genomic DNA examined in this study was Bacillus sp. KSM-1378. It was grown in an liquid medium, as described previously. B. subtilis ISW1214 (leuA8 metB5 hsrM1) harboring a pHSP64 that contained the lamy gene, designated pHSP-LAMY, was propagated at 30°C for 60 hr in an optimized liquid medium composed of (w/v) 9% maltose, 0.05% yeast extract (Difco), 16% corn steep liquor, 1.0% meat extract, 0.02% KH₂PO₄, 5 mM CaCl₂, and 15 μg/ml tetracycline (pH 7.4).

Isolation of DNA and transformation. Genomic DNA from Bacillus sp. KSM-1378 was prepared as described by Saito and Miura, and plasmid DNA was isolated by the alkaline extraction procedure of Birnboim and Doly. B. subtilis cells were transformed with plas-
mids by the method of Chang and Cohen.\textsuperscript{29}

\textbf{Amplification and sequencing of DNA.} Primer DNAs were designed for the amplification of the \textit{lamy} gene in the genomic DNA. Since pHSP64 contained part of the signal peptide sequence, MMLRRKTKQLGR, of a \textit{Bacillus} endoglucanase,\textsuperscript{15} we inserted PAQA between the incomplete signal peptide and the LAMY structural amino acid sequences. The incomplete signal sequence has been proved to be very important for the hyperexpression of foreign genes in our host-vector system\textsuperscript{10} and the AQA in the PAQA functions as the recognition site of the \textit{Bacillus} signal peptide.\textsuperscript{29} The primer sequences used were primer A, 5'-GAGTCGACCAGCACAAGCCCCATCATAATGG-3', and primer B, 5'-AAGGTTTCAATTTATGGGTGTAT-3'.\textsuperscript{13} They were prepared with a DNA synthesizer (model 392A; Applied Biosystems) and were purified with a DNA Refinement System (model Dnastec-1000; Astec). PCR was done in a DNA thermal cycler (model 480; Perkin-Elmer), using each primer (0.2 \mu g) plus genomic DNA (1.0 \mu g) from \textit{Bacillus} sp. KSM-1378. The reaction conditions were as follows: 5 min at 94°C followed by 20 cycles of for 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, and a final 15 min at 72°C. The reaction mixture contained 200 \mu M dNTPs, 25 mM KCl, 5 mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, 2 mM MgSO\textsubscript{4}, 2.5 units \textit{Pwo} DNA polymerase, and 10 mM Tris-HCl buffer (pH 8.85) (Boehringer Mannheim) in a reaction volume of 100 \mu l. Products of PCR were purified with a Geneclean II kit (Bio 101 Inc.) and they were used for sequencing or for subcloning.

Sequencing was done by the dyeoxy chain-termination method of Smith et al.,\textsuperscript{23} using fluorescent terminators and an automated DNA sequencer (model 377A; Applied Biosystems). Both strands of the DNA were sequenced and computer analysis was done using a GENE- \textit{TYX} program (SDC Software Development). When necessary, DNA fragments that had been amplified by PCR were sequenced directly after purification with a Geneclean II kit.

\textbf{Assays of enzymatic activity.} \textit{\textalpha}-Amylase activity was routinely measured at 40°C in a 1.0-ml reaction mixture that contained 0.5 ml of a 1.0\% (w/v) solution of soluble starch (from potato; Sigma) in 50 mM Tris-HCl buffer (pH 8.5) and 0.1 ml of a suitably diluted solution of enzyme. The reducing sugar formed was measured by the dinitrosalicylic acid procedure.\textsuperscript{26} One unit of enzymatic activity was defined as the amount of protein that produced 1 \textmu mol of reducing sugar as glucose per min under the conditions of the assay. Protein was measured by the method of Bradford,\textsuperscript{27} with a protein assay kit (Bio-Rad) and bovine plasma albumin as the standard.

\textbf{Purification of enzyme.} The recombinant LAMY was purified such that it yielded a single band of protein on SDS-PAGE after precipitation by ammonium sulfate and chromatographies on DEAE-Toyopearl 650M (Tosoh) and CM-Toyopearl 650S (Tosoh), as reported previously for the native enzyme from the alkalophilic \textit{Bacillus} sp. KSM-1378.\textsuperscript{13} The absorbance at 280 nm was used to monitor protein in column effluents.

\textbf{Electrophoresis.} SDS-PAGE was done essentially as described by Laemmli\textsuperscript{20} on slab gels (90 mm × 90 mm, 2.0 mm thickness). Molecular masses were estimated by SDS-PAGE [10\% (w/v) acrylamide] with low-range molecular-mass standards (Bio-Rad), which included phosphorylase \textit{b} (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). Proteins were stained with a Rapid CBB KANTO dye (Kanto Chemical).

\textbf{Sequencing of N-terminal regions.} To analyze the N-terminal sequence of the purified recombinant LAMY, a polyvinylidene difluoride membrane (Prosurb; Perkin-Elmer) had been wetted with methanol. Protein samples were blotted on the membrane and their amino acid sequences were directly identified in an amino acid sequencer (model 476A; Perkin-Eimer).

\textbf{Chromatographic analysis of the products of hydrolysis of polysaccharides.} Soluble starch was dissolved in 4.5 ml of 10 mM phosphate buffer (pH 8.0), at a final concentration of 0.2\% (w/v). A portion (0.5 ml) of the enzyme preparation was added to the reaction mixture, which was then incubated at 30°C for an appropriate time. At intervals, samples were taken and the reaction in them was stopped by boiling for 5 min. The boiled samples were chromatographed on thin-layer plates of silica gel 60 (10 cm × 20 cm; Merck) (TLC) in a solvent system composed of butanol, pyridine, and water (6:4:3, v/v). Chromatograms were developed by spraying with the aniline-diphenylamine reagent, as reported elsewhere.\textsuperscript{6} The products from soluble starch were measured by HPLC on a column of High Performance Carbohydrate (4.6 mm × 250 mm) equipped with a pump (model LC Module 1 plus) and a refractive index detector (model 430) (Waters). Twenty \mu l of the boiled reaction mixture were injected into the column and products were eluted with 65\% (v/v) acetonitrile at 35°C and at 1.4 ml/min. Each product eluted was measured using data analysis software, 805 Data Station (Waters).

\textbf{Results and Discussion}

\textbf{Hyperexpression in \textit{B. subtilis} of the PCR product in pHSP64} By PCR, using the \textit{Bacillus} sp. KSM-1378 genomic DNA and primers A and B, we amplified a DNA fragment, the size of which was estimated to be 1.5 kbp by electrophoresis along with 2 DNA cleaved with \textit{HindIII} in 1% (w/v) agarose gel. The 1.5-kbp PCR product was digested with \textit{SalI} and the digest was inserted into the \textit{SalI-SmaI} site of pHSP64 (5.5 kbp), as shown in Fig. 1. The resultant plasmid, designated pHSPLAMY (7.1 kbp), was introduced into \textit{B. subtilis} ISW1214 cells and one of the transformants obtained was grown at 30°C for 70 hr, with shaking, in an optimized liquid medium.
**Bacillus sp. KSM-1378**

**primer A, primer B**

**PCR**

1.5 kbp

**Sall**

**l amy**

**SalI, SmaI**

**Ligase**

**Amp**

**pHSP64**

5.5 kbp

**pHSLAMY**

7.1 kbp

**Fig. 1.** Schematic Representation of Construction of the L amy Gene in the Plasmid, pHSLAMY. pHSLAMY was constructed from pHSP64 and the amplified 1.5-kbp fragment, as described in Materials and Methods. The arrows inside plasmids indicate the positions and the orientations of the sequences encoding resistance to tetracycline (Tet) and ampicillin (Amp). The PCR product that encodes the L amy gene and the possible promoter region from the Bacillus sp. KSM-64 endoglucanase gene, inserted in pHSLAMY, are indicated by hatched and thick arrow bars, respectively. Immediately downstream of the nucleotide sequence of the promoter region that includes the potential -35 and -10 regions and the putative Shine-Dalgarno (SD) sequence, part of the nucleotide sequence and its deduced amino acid sequence of the L amy gene product (stippled by dotted arrow) are shown, starting immediately downstream of the inserted signal sequence AQA indicated by dotted line. The 13 amino acid residues from M to P, upstream of the AQA, may function as the signal peptide in this case. The N-terminal amino acid sequence of the mature extracellular enzyme produced by transformed B. subtilis is double-underlined. The convergent arrows shown in the sequence are primers A and B that were used to amplify the 1.5-kbp gene by PCR.

### Table: Purification of Recombinant LAMY

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Total protein (mg)</th>
<th>Total Activity (units)</th>
<th>Specific activity (units/mg)</th>
<th>Yield (%)</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture broth</td>
<td>131</td>
<td>158867</td>
<td>1214</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>102</td>
<td>134753</td>
<td>1317</td>
<td>85</td>
<td>1.1</td>
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<tr>
<td>(60% saturation)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEAE-Toyopearl</td>
<td>32</td>
<td>132275</td>
<td>4049</td>
<td>83</td>
<td>3.3</td>
</tr>
<tr>
<td>(unadsorbed)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CM-Toyopearl</td>
<td>24</td>
<td>117442</td>
<td>4873</td>
<td>74</td>
<td>4.0</td>
</tr>
</tbody>
</table>

plus tetracycline (15 µg/ml). The product of the L amy gene was excreted extracellularly at a level of 5×10⁸ units/l, which corresponds to approximately 1.0 g of LAMY protein/l. When the "incomplete" signal sequence MMLRKKTQKLGR in pHSLAMY replaced the original complete signal sequence, having 30 amino acid residues from Bacillus sp. KSM-64, the productivity of the recombinant enzyme was unexpectedly decreased to a level of 10³ units/l. Excretion of α-amylase activity by B. subtilis harboring pHSP64 was negligible.

**Purification of the recombinant enzyme**

The recombinant LAMY excreted in the culture was purified to homogeneity very simply by ammonium sulfate precipitation and chromatographies on columns of DEAE-Toyopearl 650M and then CM-Toyopearl 650S, as described elsewhere. A typical overall purification of the enzyme is summarized in the Table. The overall yield was very high (74%) and the specific activity toward soluble starch was approximately 4,900 units/mg protein, a value very close to the 5,000 units/mg protein for the purified preparation of the native enzyme.
from Bacillus sp. KSM-1378. Therefore, there may be no significant difference in the folded conformation around the active site between the native and recombinant enzymes.

Properties of the purified enzyme

The molecular mass of the recombinant LAMY was estimated to be approximately 53 kDa by SDS-PAGE, a value identical to the 53 kDa measured for the native enzyme produced by Bacillus sp. KSM-1378 (Fig. 2A). The isoelectric points were also similar between the two enzymes, both at around pH 9. Optimal temperature and pH for activity of the recombinant LAMY were observed at 55–60°C and at pH 8. The N-terminal amino acid sequence was HHNGTNGTMMQYFELWH, which was identical to the sequence of the extracellular mature enzyme produced by the original strain. This indicates that the AQA inserted, in frame, immediately downstream of the incomplete signal sequence functioned well as the recognition site of the signal peptidase of the host B. subtilis ISW1214, as described by Perlman and Halvorson29 and Simonen and Palva.30 If this inserted signal peptide were cleaved on the C-terminal side of A (alanine), the molecular mass of the entire LAMY would be 55,391 Da, which is close to the 53 kDa found for the recombinant LAMY.

The purified recombinant enzyme could easily be crystallized. It was dissolved in 10 mM MOPS buffer (pH 6.5) plus 1 mM CaCl₂ to a concentration of 3.5–5 mg/mL. To the solution was added ammonium sulfate at a final concentration of 10% (w/v). Plane-like crystals of LAMY were readily grown at room temperature within one day, as shown in Fig. 2B.

Substrate specificity of the recombinant LAMY

The recombinant LAMY hydrolyzed soluble starch (from potato), glycogen (from oyster), amyllopectin (from potato), amyllose (from potato; degree of polymerization 17), and dextrin (from corn) at a relative rate of 100:94:133:39:12, a value similar to the relative rate (100:83:114:37:7) for the native enzyme produced by Bacillus sp. KSM-1378. Pullulan (from Aureobacterium pullulans), dextran (from potato), and α-, β- and γ-cyclodextrins were practically inert as substrates for the native and recombinant enzymes.

The product pattern of the recombinant LAMY with soluble starch (0.2%, w/v) as substrate was examined by both TLC and HPLC. As far as the hydrolysis products were analyzed by TLC, the major products were both maltotriose (G3) and maltopentaose (G5) with intermediate products, maltotetraose (G6), maltoheptaose (G7), and larger malto-oligosaccharides, as shown in Fig. 3A. Apparently, maltose (G2) and maltotetraose (G4) were generated very slightly and glucose (G1) could not be detected on TLC plates during the digestion up to 20 hr. Quantitative analysis by HPLC gave a pattern of hydrolysis similar to that obtained by TLC, except for unnegligible production of G1 and G2, as shown in Fig. 3B. After completion of the reaction (20 hr of digestion), the major end products were G5, G3, and, in addition, G2. G1 was also formed significantly after 20 hr of incubation. A typical product composition at equilibrium was as follows; G1, 0.7 mM; G2, 1.1 mM; G3, 1.2 mM; G4, 0.2 mM; G5, 1.3 mM; G6, 0.3 mM, and G7, 0.0 mM. The apparent discrepancy of product ratio observed with the two methods may simply be due to weak reactions of the aniline-diphenylamine reagent with G1 and G2 on TLC plates.

Protective effects of some cations on the thermal stability of the recombinant LAMY

The thermal stability of the enzyme was assessed in 50 mM Tris-HCl buffer (pH 8.5) after heating for 20 min at various temperatures. The enzyme was stable up to 45°C. Complete loss of enzymatic activity was observed after heating to 60°C. When Ca²⁺ was present, the enzyme was stable up to 55°C and the inactivation temperature shifted from 60°C to 70°C (data not shown). Ca²⁺ and Na⁺ ions were found to protect the thermostability at 60°C of LAMY completely, at concentrations of approximately 0.5 mM and 12.5 mM, respectively. Ca²⁺ ions are known to involve in catalysis and stabilization of amylase molecules.12,23 The striking protective effect of Na⁺ is very unusual and cannot readily be explained, but it could arise from an unidentified interaction of this cation with free carboxyl groups of some amino acid residues in the LAMY molecule, or from changes in the gross conformation of the enzyme. The following cations (1 mM each) were practically without effect on the thermal stability; K⁺ (added up to 50 mM), Ba²⁺, Be²⁺, Mg²⁺, Mn²⁺, Co²⁺, Cu²⁺, Fe²⁺, Sr²⁺, Ag²⁺, Pd²⁺, Fe³⁺, and Al³⁺ ions.

Reversal inactivation of LAMY by EDTA

When the purified LAMY was dialyzed without Ca²⁺ ions, it retained almost full activity, suggesting that catalytic and/or structure-stabilizing Ca²⁺ ions are tightly

Fig. 2. SDS-PAGE (A) and Crystallization (B) of the Purified Recombinant Enzyme. A. SDS-PAGE: lane a, the molecular mass markers (calibration in kDa); lane b, a purified recombinant LAMY; lane c, a purified LAMY produced by Bacillus sp. KSM-1378. The arrow indicates the position of the protein band of the recombinant enzyme. Detailed methods for electrophoretic analysis are described in Materials and Methods. B. A photograph of crystal of LAMY. The scale bar indicates 10 μm.
bound to the enzyme molecule. EDTA had a significant negative effect on the activity of LAMY, and this effect was apparent at a level as low as 0.2 mM. When LAMY was incubated with 0.2 mM EDTA at 30°C for 60 min in 50 mM Tris-HCl buffer (pH 8.5), it lost its activity almost completely, as shown in Fig. 4. Upon addition of 0.5 mM Ca²⁺ to the reaction mixture, LAMY recovered its activity immediately, demonstrating a reversibility of the inhibition of the enzyme by EDTA. The ability of Ca²⁺ to neutralize the inhibitory action of EDTA is support for the involvement of this divalent cation in the catalytic process of LAMY.

We are now analyzing the tertiary structure of the recombinant LAMY by a molecular replacement method and by reference to the deduced amino acid sequence of this enzyme and to the crystal and/or modeling structures of other α-amylases, such as Taka-amylase, BLA, BAA, and BSA.

A number of genes for BAA,32) BLA,33) and BSA34,35) have been cloned and expressed in B. subtilis cells with low yields. By using our hyperproduction system with pHSP64 and B. subtilis, together with the simple purification procedure described, we can easily and readily study structure-function relationships of homogeneous preparations of wild-type and protein-engineered α-amylases. Analysis of the structure of LAMY may explain its unique physicochemical and catalytic properties.

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References
Recombinant Bacillus α-Amylase 1725


