Role of the C-Terminal Region of $\beta$-Amylase from Barley

Naohiro Yoshigi, Hirohisa Sahara, and Shohi Koshino

Brewing Research Laboratories, Sapporo Breweries Ltd., Yaizu, Shizuoka 425

Received for publication, July 19, 1994

To investigate the role of the C-terminal region of barley $\beta$-amylase, plasmid $\Delta 54$ was constructed with an expression vector (pBETA92) of barley $\beta$-amylase by site-directed mutagenesis. *Escherichia coli* JM109 harboring plasmid $\Delta 54$ was expected to express $\Delta 54$ $\beta$-amylase in which 54 amino acid residues were deleted from the C-terminus. The enzyme production started in the logarithmic phase, increased linearly, and reached a maximum after 12 h. $\Delta 54$ $\beta$-amylase gave a single activity band on isoelectric focusing (pI 6.85). $\Delta 54$ $\beta$-amylase was purified from the cells by consecutive $\alpha$-cyclodextrin/Sepharose 6B column chromatography. A comparison of the properties of the mutant enzyme with those of the original recombinant $\beta$-amylase [Biosci. Biotech. Biochem. (1994) 58, 1080-1086] revealed two major differences. First, the original recombinant $\beta$-amylase showed heterogeneity on isoelectric focusing, but $\Delta 54$ $\beta$-amylase gave a single main band of protein (pI 6.85). Therefore, the isoelectrophoretic heterogeneity of the original recombinant $\beta$-amylase was apparently due to its C-terminal region. Secondly, $\Delta 54$ $\beta$-amylase lacked thermostability. Therefore, it was concluded that the C-terminal region was significantly involved in the thermostability of $\beta$-amylase.

Key words: $\beta$-amylase, barley, C-terminal region, thermostability.

MATERIALS AND METHODS

Site-Directed Mutagenesis—The construction of an expression vector (plasmid $\Delta 54$) for $\Delta 54$ $\beta$-amylase was done by site-directed mutagenesis (7). The oligonucleotides used for generating the base substitutions were as follows; 5'-CCCTTCCAGTAGCACACCG as a mutation primer and 5'-GGTTGAGTATTCACCAGTC as a selection primer. A single base substitution for encoding $\beta$-amylase was confirmed by sequencing. DNA sequencing was done using the dideoxy method (8) with 5'-GGTTCCATTACATGGTGGCAGGGAGCTCCC as a primer and dye-terminators with a DNA sequencer (Model 373A, Applied Biosystems).

E. coli JM109 (recA1 endA1 gyrA96 thi-1 supE44 relA1 [F' traD36 proAB lacIqZM15]) harboring plasmid $\Delta 54$ was expected to express $\Delta 54$ $\beta$-amylase in which 54 amino acid residues were deleted from the C-terminus.

Course of $\beta$-Amylase Production and Assay of $\beta$-Amylase Activity—These methods were described previously (4).

Preparation of a Starting Material for Purification of $\Delta 54$ $\beta$-Amylase—*E. coli* JM109 carrying plasmid $\Delta 54$ was cultivated in an L-broth (100 ml×4) containing 50 $\mu$g/ml ampicillin and 0.1 mM isopropyl $\beta$-D-thiogalactoside in four 500-ml Erlenmeyer flasks. Cultivation was done for 15 h at 37°C with shaking at 180 strokes/min. After centrifugation at 5,000× g for 10 min to remove the culture medium, packed cells were suspended in 40 ml of 0.25% lysozyme solution [20 mM Tris-HCl, 30 mM NaCl (pH 7.5)], left on ice for 30 min, and disrupted by ultrasonication (Model 450 SONIFIER, Branson Ultrasoneics) at 50 W for 30 s followed by centrifugation at 27,300× g for 20
min. The supernatant (38.5 ml), added to the same volume of 20 mM Tris-HCl buffer (pH 7.5) containing 30 mM NaCl, was used as a starting material for the purification of \( \Delta 54 \) \( \beta \)-amylase.

**Purification of \( \Delta 54 \) \( \beta \)-Amylase—Immobilization of \( \alpha \)-cyclodextrin (\( \alpha \)-CD) on epoxy-activated Sepharose 6B (Pharmacia Biotechnology) was done by the method of Vretblad (10). To the starting material (78 ml, 430 units), solid (NH\(_4\))\(_2\)SO\(_4\), was added up to 30% saturation. The suspension was left for 1 h and centrifuged at 14,000 \( \times \) g for 20 min to remove the precipitated proteins. The supernatant (82 ml) was put on an \( \alpha \)-CD/Sepharose 6B column (2.5 \( \times \) 8.2 cm) preequilibrated with 50 mM acetate buffer (pH 5.5) containing 30% saturated (NH\(_4\))\(_2\)SO\(_4\). Elution was successively done with 600 ml of 50 mM acetate buffer (pH 5.5) containing 30% saturated (NH\(_4\))\(_2\)SO\(_4\), and 200 ml of 50 mM acetate buffer (pH 5.5); the flow rate was maintained at 5 ml/min. \( \Delta 54 \) \( \beta \)-amylase was eluted with 50 mM acetate buffer (pH 5.5). Fractions containing \( \beta \)-amylase were combined (50 ml) and solid (NH\(_4\))\(_2\)SO\(_4\) was added to make 30% saturation. After a 20-min centrifugation at 27,300 \( \times \) g, the supernatant was placed on an \( \alpha \)-CD/Sepharose 6B column once again. Elution was successively done with 200 ml of 50 mM acetate buffer (pH 5.5) containing 30% saturated (NH\(_4\))\(_2\)SO\(_4\), and 200 ml of 50 mM acetate buffer (pH 5.5). \( \Delta 54 \) \( \beta \)-amylase was eluted with 50 mM acetate buffer (pH 5.5). Fractions containing \( \beta \)-amylase were pooled (50 ml) and used in the following experiments as the purified \( \Delta 54 \) \( \beta \)-amylase preparation.

**Electrophoresis—SDS-PAGE, PAGE, and isoelectric focusing (IEF)** were done with the Phast System (Pharmacia Biotechnology) using ready-made gels (PhastGel Gradient 8-25 for SDS-PAGE and PAGE, and PhastGel IEF 3-9 for IEF) according to the protocol. The protein was stained using a silver staining kit (Pharmacia Biotech). In the cases of PAGE and IEF, the activity staining was done as follows. The plate was incubated in 3% soluble starch in 50 mM acetate buffer (pH 5.5) at 37°C for 30 min. The supernatant was heated at 100°C for 5 min. After cooling, 20 \( \mu \)l-samples were subjected to HPLC to identify each maltooligosaccharide produced with a slight modification of the method described elsewhere (12). The column was eluted with acetonitrile-deionized water (70:30, v/v). The amount of each maltooligosaccharide on HPLC was determined by using the peak area method (13). The relative initial velocity, the \( K_m \) and \( V_{max} \) values, and the hydrolysis limit for each polysaccharide were calculated from the amount of the maltose produced, while for each maltooligosaccharide, the relative initial velocity, and the \( K_m \) and \( V_{max} \) values were obtained from its decrease.

**RESULTS**

**DNA Sequencing**—The original recombinant \( \beta \)-amylase lacked four amino acids at positions 2-5 (Glu-Val-Asn-Val) when compared with the presumed amino acid sequence of barley \( \beta \)-amylase (4), but the numbering of the amino acids was based on the amino acid sequence of barley \( \beta \)-amylase to avoid confusion.

The plasmid \( \Delta 54 \) exhibited a G-T transversion leading to Glu-482 by a stop codon (GAG-TAG, data not shown).

**Course of \( \beta \)-Amylase Production by E. coli JM109 Harboring Plasmid \( \Delta 54 \)**—The enzyme production started in the logarithmic phase, increased linearly, and reached a maximum after 12 h (Fig. 1A). \( \Delta 54 \) \( \beta \)-amylase gave a single activity band on isoelectric focusing (pI 6.85), and its pI did not change throughout the incubation (Fig. 1B).

**Purification of \( \Delta 54 \) \( \beta \)-Amylase and Electrophoresis**—A typical elution profile of \( \Delta 54 \) \( \beta \)-amylase on 2nd \( \alpha \)-CD/Sepharose 6B column chromatography is shown in Fig. 2. The final purified \( \Delta 54 \) \( \beta \)-amylase preparation had a specific activity of 365 units per mg protein with a final activity yield of 37.4%.

On SDS-PAGE, the purified \( \Delta 54 \) \( \beta \)-amylase gave a main band of protein at an apparent molecular weight of 54,000 (Fig. 3A, lane 1). Its migration was larger than that of the original recombinant \( \beta \)-amylase (Fig. 3A, lane 2). Its molecular weight was calculated to be 54,129 from its amino acid sequence.

The final \( \Delta 54 \) \( \beta \)-amylase preparation gave a single main band of protein on PAGE and IEF (Fig. 3B and D), and this band exhibited amylolytic activities (Fig. 3, C and E).

**Effects of pH and Temperature on the Activity and Stability**—\( \Delta 54 \) \( \beta \)-amylase had almost the same pH profile for the activity as the original recombinant \( \beta \)-amylase (Fig. 4A), but the effect of pH on the stability was different in the alkaline region (Fig. 4B). The original recombinant \( \beta \)-
Role of C-Terminal Region of $\beta$-Amylase

**Fig. 1.** Course of $\Delta54$ $\beta$-amylase production and IEF analysis. A, course of $\Delta54$ $\beta$-amylase production: ●, intracellular $\beta$-amylase; ○, extracellular $\beta$-amylase; □, cell growth. B, IEF analysis. Intracellular fractions (0.27–3.7 mU) were put on a plate. The numbers 6, 12, 24, 30, and 48 denote incubation time (h). Standard proteins were as follows: amyloglucosidase (pI 3.50), soybean trypsin inhibitor (pI 4.55), $\beta$-lactoglobulin (pI 5.20), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase B (pI 6.55), horse myoglobin (pI 6.85 and 7.35), lentil lectin (pI 8.15, 8.45, and 8.65).

**Fig. 2.** A typical elution profile of $\Delta54$ $\beta$-amylase on 2nd $\alpha$-CD/Sepharose 6B column chromatography. Fractions of 10 ml each were collected and assayed for protein (●) by the method of Lowry et al. with BSA as the standard (14) and for $\beta$-amylase activity (○). The arrow indicates the position at which the elution buffer was changed.

Amylase was found to be stable at pHs up to 9.5, while $\Delta54$ $\beta$-amylase was unstable at pH 9.5.

The original recombinant $\beta$-amylase and $\Delta54$ $\beta$-amylase had maximum activity at 55 and 50°C, respectively (Fig. 4C). When incubated at 55°C for 30 min, $\Delta54$ $\beta$-amylase almost completely lost its enzyme activity, but the original recombinant $\beta$-amylase did not (Fig. 4D).

**Effects of Metal Ions and Other Reagents, and Substrate Specificities**—Cu$^{2+}$, Hg$^{2+}$, Zn$^{2+}$, Ag$^+$, and Cd$^{2+}$ inhibited $\Delta54$ $\beta$-amylase activities almost completely at a final concentration of 1 mM. p-Chloromercuribenzoate (pCMB) inhibited $\Delta54$ $\beta$-amylase activity completely at a final concentration of 0.1 mM, but EDTA (1 mM) did not. These results were the same as those with the original recombinant $\beta$-amylase (4).

Table I summarizes the substrate specificities of $\Delta54$ $\beta$-amylase.

**Table I.** Substrate specificity of $\Delta54$ $\beta$-amylase.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Initial velocity (%)</th>
<th>$K_m$ (mg/ml)</th>
<th>$V_{max}$ (\mu mol/min)</th>
<th>Hydrolysis limit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maltotriose</td>
<td>0.07</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Maltotetrose</td>
<td>80</td>
<td>1.45</td>
<td>2.18</td>
<td>114</td>
</tr>
<tr>
<td>Maltopentaose</td>
<td>146</td>
<td>1.04</td>
<td>1.26</td>
<td>195</td>
</tr>
<tr>
<td>Maltohexaose</td>
<td>153</td>
<td>0.549</td>
<td>0.555</td>
<td>192</td>
</tr>
<tr>
<td>Maltolentaose</td>
<td>109</td>
<td>0.429</td>
<td>0.372</td>
<td>165</td>
</tr>
<tr>
<td>Amylose (DF=17)$^a$</td>
<td>101</td>
<td>0.794</td>
<td>0.286</td>
<td>170</td>
</tr>
<tr>
<td>Soluble starch</td>
<td>100</td>
<td>0.917</td>
<td>—</td>
<td>166</td>
</tr>
<tr>
<td>Amylopectin</td>
<td>116</td>
<td>1.69</td>
<td>—</td>
<td>215</td>
</tr>
<tr>
<td>Oyster glycogen</td>
<td>37</td>
<td>—</td>
<td>—</td>
<td>47.0</td>
</tr>
<tr>
<td>Liver glycogen</td>
<td>27</td>
<td>—</td>
<td>—</td>
<td>34.8</td>
</tr>
</tbody>
</table>

$^a$DF, average degree of polymerization.

**DISCUSSION**

Production of the original recombinant enzyme started in the logarithmic phase and increased linearly up to about 12
The original recombinant $\alpha$-amylase gave two major (pI 5.43 and 5.63) and four minor (5.20, 5.36, 5.80, and 6.13) activity bands on IEF (4). However, the $\Delta 54$ $\alpha$-amylase gave a single activity band (pI 6.85), as shown in Fig. 1B. The results obtained suggest that the heterogeneity of the original recombinant $\alpha$-amylase on IEF is due to amino acids in the C-terminal region.

The pI of $\Delta 54$ $\alpha$-amylase calculated by Genetyx Version 9 (Software Development) based on the presumed amino acid sequence was 6.02, but the real pI was 6.85. Therefore, some post-translational reaction(s) might occur after protein synthesis in $E$. coli cells, but the details remain to be established.

The final $\Delta 54$ $\alpha$-amylase preparation gave a single main band of protein on PAGE and IEF (Fig. 3, B and D). The original recombinant $\alpha$-amylase showed heterogeneity on both PAGE and IEF (4). Therefore, it was confirmed that the heterogeneity of the original recombinant $\alpha$-amylase on PAGE and IEF was due to its C-terminal region.

When incubated at 55°C for 30 min, $\Delta 54$ $\alpha$-amylase almost completely lost its enzyme activity, but the original recombinant $\alpha$-amylase did not (Fig. 4D). Effects of metal ions and other reagents were almost the same as those in the case of the original recombinant $\alpha$-amylase (4). As can be seen from Table I, the values of the relative initial velocities for the substrates ranged from 80 to 153%, except for maltotriose (0.07%), oyster glycogen (37%), and liver glycogen (27%). Maltotriose was unfavorable as a substrate. The values of the hydrolysis limit for polysaccharides containing $\alpha$-1,6-bonds ranged from 34.8 to 55.9%. These results were almost the same as those of the original recombinant $\alpha$-amylase (4). Furthermore, the $K_m$ and $V_{max}$ values for various substrates were nearly the same, except for the $K_m$ value for soluble starch, as those of the original recombinant $\alpha$-amylase (4). $\Delta 54$ $\alpha$-amylase had a slightly smaller $K_m$ value for soluble starch. Therefore, barley $\alpha$-amylase, in which 54 amino acid residues are deleted from the C-terminus, loses its thermostability, but its function as a $\alpha$-amylase remains unchanged.

IEF analysis indicated that, during germination, $\alpha$-amylases in ungerminated seeds disappeared concomitantly with the production of new $\alpha$-amylase components that had higher apparent pI values, and SDS-PAGE followed by Western blotting analysis showed that during germination, the molecular weight of barley $\alpha$-amylase changed from 56,000 to 52,000 through 54,000 (15). The $\alpha$-amylase with a molecular weight of 52,000 in seeds germinated for 9 days was stable up to 52.5°C when incubated at pH 7.0 for 30 min (data not shown), and had the same thermostability as the enzyme with a molecular weight of 56,000 in unger-

**Fig. 4. Effects of pH and temperature on the activity and stability.** A, effect of pH on the activity; B, effect of pH on the stability; C, effect of temperature on the activity; D, effect of temperature on the stability. ◆, original recombinant $\alpha$-amylase; □, $\Delta 54$ $\alpha$-amylase.
minated seeds (4). Lundgard and Svensson have purified four major forms of barley $\beta$-amylase and reported that $\beta$-amylase 4 with the smallest molecular weight of 54,000 consisted of different components terminating between Gly-493 and Gln-497 in the amino acid sequence (16). It was thought that $\beta$-amylase 4 corresponded to the $\beta$-amylase in seeds germinated for 9 days, because it had the smallest molecular weight among the four major forms. $\Delta 54$ $\beta$-amylase terminating at Gln-481 lacked 12 to 16 amino acid residues from its C-terminus compared with the $\beta$-amylase 4 terminating between Gly-493 and Gln-497 or the $\beta$-amylase with a molecular weight of 52,000 in seeds germinated for 9 days. Therefore, the region from Glu-482 to Gly-493-Glu-497 might be significantly involved in the thermostability of barley $\beta$-amylase.

The soybean and barley $\beta$-amylases consisted of 495 and 535 amino acid residues, respectively, with a sequence homology of 66.7% (6). In the case of soybean $\beta$-amylase, the region composed of 12 to 16 amino acid residues corresponded to about a quarter of the tail loop consisting of about 50 amino acid residues after the $\alpha$-helix ($\alpha 8$). The amino acid sequence of this C-terminal loop showed no conserved residues between plant and bacterial $\beta$-amylases, suggesting the structural variability of this region (5), but our data suggest that the tail loop after the last helix ($\alpha 8$) of $(\alpha/\beta)_8$ structure, consisting of about 50 amino acid residues, is very important for thermostability.

REFERENCES