Articles

Changes in Optimum pH and Thermostability of α-amylase from *Bacillus licheniformis* by Site-directed Mutagenesis of His 235 and Asp 328

Mi-Sook Kim, Sang-Kyou Lee, Han-Seung Jung, and Chul-Hak Yang

Department of Chemistry, College of Natural Science, Seoul National University, Seoul 151-742, Korea
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The α-amylase gene of *Bacillus licheniformis* has been cloned and two mutant α-amylase genes of which histidine 235 was changed to glutamine (H235Q) and aspartic acid 328 to glutamic acid (D328E) have been produced by site-directed mutagenesis. The kinetic parameters, optimum pH and thermostability of wild type(WT) and these two mutant amylases expressed in *E. coli* MC1061 have been compared after purification. The K_m values of WT, H235Q and D328E α-amylases were 0.22%, 0.73%, and 0.80%, respectively, when using starch as the substrate. The V_{max} values of wild type α-amylase and mutant α-amylases were 0.6-0.7%/minute, and did not show any significant differences among them. The optimum pH of D328E α-amylase was shifted to more acidic pH. Also, the thermostability of H235Q α-amylase was increased compared to the wild type α-amylase.

Introduction

α-Amylase (EC 3.2.1.1) hydrolyzes α-1,4-glucosidic linkage of starch in endo fashion to yield glucose, maltose, and a series of branched α-limited dextrins.[1] The α-amylases are widely distributed in plants, animals, and bacteria. Among them, α-amylase of *Bacillus* species is important in the production of corn syrups, dextrose, beer and distilled beverages industrially.[2] So increased thermostability and resistance to the chaging pH of this enzyme is desirable.

α-Amylase genes had been cloned from several *Bacillus* species.[3-7] The three-dimensional structures of two α-amylases, one from *Aspergillus Oryzae*, so-called Taka-amylase A (TAA), and the other from pig pancreas have been determined by X-ray crystallography.[8-9] However, there is little information about its reaction mechanism. In one report,[10] the active site area and substrate binding site of α-amylase were suggested by in vitro enzymatic random mutagenesis of *Bacillus stearothermophillus* α-amylase.

α-Amylase genes from *Bacillus stearothermophillus* and *Bacillus licheniformis* are very thermostable and active even at the temperature above 75℃.[11] Two mutant amylase genes with reduced thermostability have been obtained by hydroxylamine mutagenesis of enzyme gene from *Bacillus amyloliquefaciens*.[12] But it is still not clear that which amino acid residues have the critical role in their thermostability.

In this paper, α-amylase gene of *Bacillus licheniformis* has been cloned and expressed in *E. coli*. And among many amino acids which seem to be located in the substrate binding site, it was suggested that Glutamine 230 and Aspartic acid 297 are the catalytic residues, seriving as the general base and the general acid in the TAA,[8] respectively. When the sequenced DNA of the amylase gene from *B. licheniformis* was compared to the DNA sequence of the TAA gene, it was found that Aspartic acid 328 of the amylase from *B. licheniformis* corresponded to the Aspartic acid 297 of the TAA. It was reported that two Histidines among six total in the TAA were involved in maltose binding when the TAA was chemically modified[9] and one of these two Histidines was located close to residue where calcium ion binds.[8] Among amino acids which are located in homologous regions,[10] Histidine 235 and Aspartic acid 328 of *B. licheniformis* amylase were selected with the help of above information. If histidine 235 and Aspartic acid 328 are critical residues in substrate binding, even small changes in them will cause a significant change in enzyme activity. Therefore, histidine 235 has been changed to glutamine (we call it H235Q α-amylase) which is another basic amino acid, Aspartic acid 328 to glutamic acid (D328E α-amylase) which is another acidic amino acid, respectively, by site-directed mutagenesis of the gene and the mutant proteins have been expressed in *E. coli*. The kinetic parameters, the optimum pH and the thermostability of the wild type amylase (WT α-amylase) and the mutant enzymes have been compared after purification.

Materials and Methods

Preparation of mutant α-Amylase genes. α-Amylase gene of *Bacillus licheniformis* was cloned in *E. coli* by using pBR322 as a cloning vector. The chromosomal DNA from *B. licheniformis*[13] (ATCC 27811) was isolated as described by Palva.[14] Standard DNA manipulations were performed as described by Maniatis *et al.*.[15] Transformation of *E. coli* MC 1061 was performed by the method of Mandel *et al.*[16] and the colonies bearing α-amylase gene were screened as suggested by Mielenz.[6] The restriction map of this recombinant DNA is shown in Figure 1. The DNA sequence of α-amylase gene was determined by M13-dideoxy chain termination method.[17] Site-directed mutagenesis was performed according to the method of Eckstein[18] by using the synthetic oligonucleotides as shown in Table 1, and the mutations were confirmed by nucleotide sequencing. Also the strategy used in

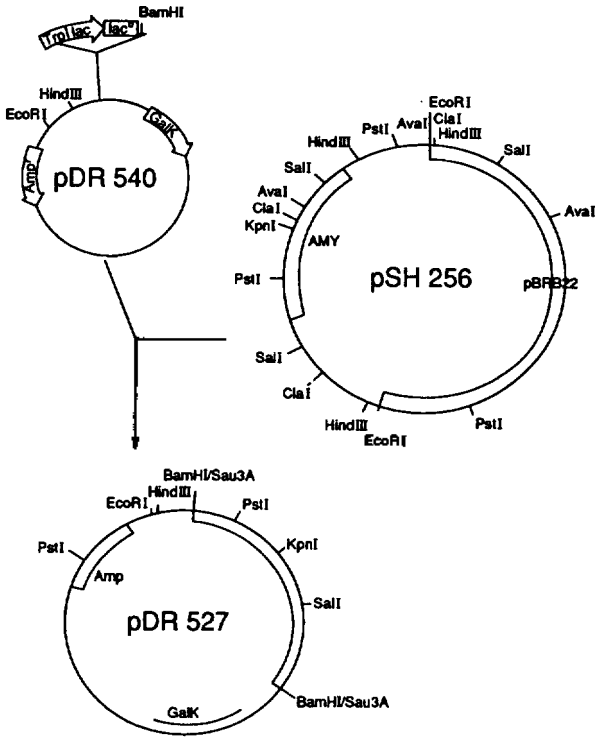

Figure 1. Restriction map of α-amylase gene bearing plasmid, pSH256. The 4.3 Kb of right hand side represent the pBR322 and about 3.8 Kb of left hand side represent the insert. The region 'AMY' represents about 1.5 Kb α-amylase coding region.

Table 1. Oligonucleotides used for site-directed mutagenesis

Mutant	Sequence of DNA(upper) and oligonucleotide (lower)
His235Gln	3' AG TTT GAG TAA TTT AAA 5'
	5' TC AAA CA ATT AAA TTT 3'
	A
	Lys Gln Ile Lys Phe
Asp328Glu	3' TG GTA CTA TGT GTC GGC 5'
	5' AC CAT GA ACA CAG CCG 3'
	G
	His Glu Thr Gln Pro

Amino acid numbering is based on the sequence of *Bacillus licheniformis* α-amylase.

this mutagenesis is shown in Figure 2.

Assay of α-amylase. The modified DNS [2-OH-3,5-dinitrobenzoic acid] method of Bernfelt[19] was used for the assay of purified α-amylase. To 0.5 m*l* of 1% soluble starch solution, 0.4 m*l* of 0.02 M Tris acetate (pH 7.5) and 0.1 m*l* of enzyme solution were added and incubated for 10 minutes at 37 ℃. After reaction, 0.3 m*l* of DNS reagent was added to 0.1 ml of the reaction mixture, and in boiling water bath after 10 minutes color was appeared. The colored mixture was cooled in tap water and 2.1 m*l* of distilled water was added and the optical density of the solution was measured with spectrophotometer at the wavelength 525 nm.

Iodine method proposed by Fuwa[20] and modified by Takahashi *et al.*[21] were also used for α-amylase which was not purified.

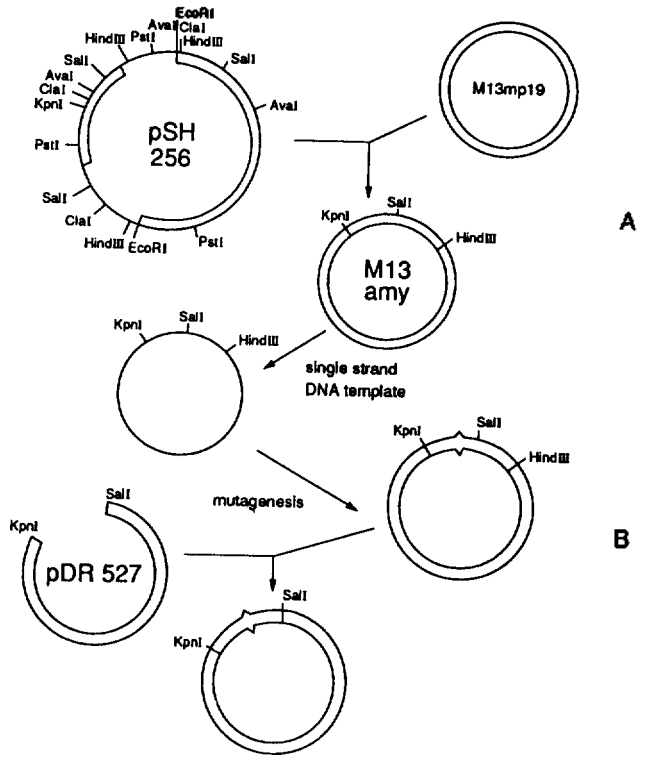

Figure 2. (A) Subcloning of α-amylase gene using pDR540, tac promoter vector. The pDR 540 was completely digested with BamHI and pSH256 bearing α-amylase coding gene was partially digested with Sau 3A and colony that had -amylase activity was selected after transformation. The recombinant vector with the smallest insert was selected and named as pDR527. Also the restriction map of pDR527 is presented. (B) The strategy of site-directed mutagenesis. The KpnI-HindIII fragment of pSH256 was recombined with the M13mp19 and named as M13amy. After isolation of single strand DNA template from the M13amy, site-directed mutagenesis reaction was carried out and the clone was transformed to *E. coli*. Then the mutant DNA was selected by sequencing and the mutagenized DNA was isolated and double digested with KpnI, SalI. Then the small KpnI-SalI fragment (about 0.5 Kb) of mutagenized α-amylase gene was ligated with the large KpnI-SalI fragment (about 7.0 Kb) of pDR527. So, the mutagenized whole α-amylase gene was obtained.

Purification and characterization of α-amylase. The wild type and two mutant α-amylases expressed in *E. coli* were purified by ammonium sulfate fractionations, DEAE-cellulose chromatography, CM-cellulose chromatography, and Sephadex G-100 gel filtration as performed by Saito.[13] The Kinetic parameters were obtained by Hofstee plots with soluble starch as substrate. In order to determine the optimum pH, Britton-Robinson buffer was used for pH 3.0-10.0. To 0.5 m*l* of 1% soluble starch in distilled water, 0.2 m*l* of two times concentrated pH buffer, 0.2 m*l* of distilled water, and 0.1 m*l* of enzyme diluted with distilled water were added, and the enzyme activity of the solution was measured after incubation for 10 minutes at 37 ℃. To compare the thermostability of wild-type α-amylase and mutated

Table 2. Purification table of WT α-amylase

	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Ammonium sulfate fractionation	185.3	595.8	3.2	100
DEAE-cellulose	40.9	508.0	12.4	85.2
CM-cellulose	4.3	457.6	107.6	76.8
Sephadex G-100	0.9	223.9	246.3	37.6

Table 3. Purification table of D328E α-amylase

	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Ammonium sulfate fractionation	217.4	557.1	2.6	100
DEAE-cellulose	107.2	443.7	4.1	79.6
CM-cellulose	5.8	537.6	92.9	96.5
Sephadex G-100	0.8	100.4	129.1	18.0

Table 4. Purification table of H2350Q α-amylase

	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Ammonium sulfate fractionation	214.7	95.7	0.4	100
DEAE-cellulose	156.0	77.3	0.5	80.7
CM-cellulose	0.7	27.1	36.36	35.1
Sephadex G-100	0.5	21.1	42.2	27.3

α-amylases, enzyme solutions were incubated at different temperatures for 15 minutes. The 0.1 m*l* enzyme solution was taken and the 0.4 m*l* of buffer solution and 0.5 m*l* of 1% starch solution were added and the residual activity was measured after incubation for 10 minutes at 37 ℃. This thermostability tests of the enzymes were done at pH 8.0 buffer solutions with and without 1 mM calcium ion.

Results and Discussion

The structural gene of α-amylase was sequenced after cloning and the result is well agreed with the data of Yuuki *et al.*.[22] *E. coli* bearing pSH 256 secret about 24% of total α-amylase activity to the culture medium. However, it cannot be said that secretion system of *Bacillus* α-amylase is the same as that of *E. coli*, for not all of α-amylase activity is found in culture medium.

The result of overall purification procedures of the wild type and two mutant α-amylases are presented in Table 2, 3,and 4. After purification, SDS polyacrylamide gel electrophoresis of the purified α-amylase showed two protein bands, the major of which is α-amylase. The measured kinetic parameters are shown in Table 5. The K_m values of D328E and H235Q α-amylases are 3-4 times larger than that of WT amylase. This increase in K_m value means that the mutant enzymes are not able to bind substrate as well as WT amylase . In other words, the aspartic acid 328 and histidine 235 residues are involved in the substrate binding. But the little differences in V_{max} values between wild type and mutant enzymes means that the maximum velocity of them is not affected by the mutation of these residues as long as the substrate is bound.

Table 5. The values of kinetic parameters of WT, D328E and H2350 and α-amylases

Enzyme	K_m (%)	V_{max} (%/min)
WT α-amylase	0.22	0.60
D328E α-amylase	0.80	0.76
H235Q α-amylase	0.73	0.67

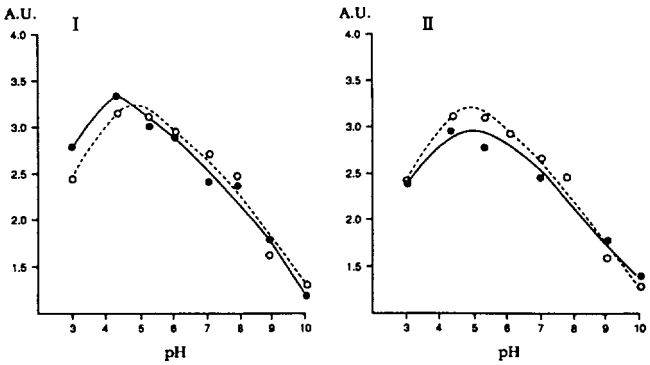

Figure 3. Activity of α-amylase according to the change of pH. Activity units (A.U.) were mesured from pH 3.0 to pH 10.0 according to the DNS assay method. Nearly all the same units of purified enzymes were used for each α-amylases. ○ reperesent for WT α-amylase in both graph and ● represent for D328E α-amylase (I) or for H235Q α-amylase (II).

The activity changes of α-amylases at the pH from 3.0 to 10.0 is shown in Figure 3. It seems that no change occurs in the optimum pH in the case of H235Q α-amylase. On the other hand, D328E α-amylase has large differences between acitivities at pH 4.0 and at pH 8.0, and the optimum pH of this α-amylase has been shifted to more acidic pH range. If aspartic acid 328 is a catalitic residue, the change in the optimum pH can be explained by the difference between pKa's of aspartic acid (3.8) and glutamic acid (4.3). Therefore, it can be said that negative charge at aspartic 328 had an effect on the optimum pH.

The activity changes of WT and two mutant α-amylases in the solution without and with 1 mM calcium ion in range from the temperature 40 to 90℃ are shown in Figure 4. and Figure 5. respectively. From these results, it can be said that all of three enzymes with calcium ions are much more stable after incubation at 80℃ than without calcium ion. This confirms the fact that calcium ion stabilize tertiary structure of α-amylase. Also, H235Q α-amylase is much more stable at high temperatures compare to the other two enzymes in the reaction of both conditions (with and without

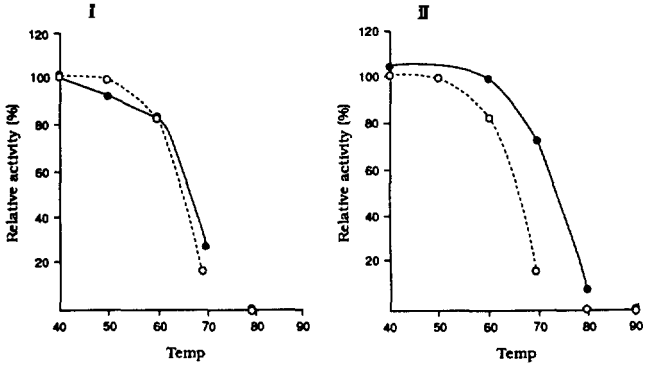

Figure 4. Thermostability of WT, D328E and H235Q α-amylases in the absence of calcium ion. The relative activity was obtained for each temperature from 40 ℃ to 90 ℃ by the DNS assay method. The enzyme reaction buffer contains no calcium ion. The enzymes incubated at 4 ℃ instead of at each measuring temperatures were used as the control enzyme. ○ represents for the WT α-amylase and ● represents for the D328E α-amylase (I) or for the H235Q α-amylase (II).

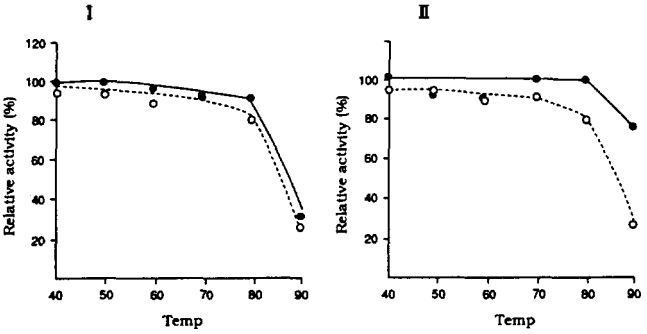

Figure 5. Thermostability of WT, D328E and H235Q α-amylases in the presence of 1 mM calcium ion. The relative activity was obtained for each temperature from 40 ℃ to 90 ℃ by the DNS assay method. The enzyme reaction buffer contains 1 mM calcium ion. The enzymes incubated at 4 ℃ instead of at each measuring temperatures were used as the control enzyme. ○ represents for the WT α-amylase and ● represents for the D328E α-amylase (I) or for the H235Q α-amylase (II).

calcium ions). When calcium ion has been added to enzyme, H235Q α-amylase had 75% activity, D328E α-amylase 30%, and WT α-amylase 25% of its activities after 15 minutes of incubation at 90 ℃. The H235Q α-amylase has a improved thermal stability.

Consequently, it have been shown that aspartic acid 328 and histidine 235 in α-amylase are not only involved in substrate binding, but also α-amylase with changed optimum pH and with increased thermostability were obtained by site-directed mutagenesis of its gene.

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