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Identification of essential histidine residues in a recombinant α-amylase of thermophilic and alkaliphilic *Bacillus* sp. strain TS-23

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Abstract To understand the structure-function relationships of a truncated *Bacillus* sp. strain TS-23 α-amylase, each of His-137, His-191, His-239, His-269, His-305, His-323, His-361, His-436, and His-475 was replaced with leucine. The molecular masses of the purified wild-type and mutant enzymes were approximately 54 kDa. The specific activity of His323Leu and His436Leu was decreased by more than 52%, while His239Leu, His305Leu, and His475Leu showed activity similar to that of the wild-type enzyme. As compared with the wild-type enzyme, His323Leu and His436Leu exhibited a 62% decrease in the value of k_{cat}/K_m. Alterations in His-191, His-239, His-305, and His-475 did not cause a significant change in the K_m or k_{cat} values. At 70°C, a decreased half-life was observed in His436Leu. These results indicate that His-137, His-269, and His-361 of *Bacillus* sp. strain TS-23 α-amylase are important for proper catalytic activity and that His-436 may contribute to the thermostability of the enzyme.

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α-Amylases (α-1,4-glucan-4-glucanohydrolases, EC 3.2.1.1) catalyze the hydrolysis of α-1,4-glucosidic linkages in starch and related polysaccharides. α-Amylases are produced by various microorganisms and are industrially important, especially in the starch-processing industry (Vihinen and Mäntsälä 1989). Besides their use in starch hydrolysis, α-amylases are also used in a number of other industrial applications, such as laundry detergents (Ito et al. 1998) and as anti-staling agents in baking (Sahlström and Bräthen 1997). Based on the amino acid sequence similarities, most of the starch-hydrolyzing enzymes belong to one family, family 13 glycosyl hydrolases (Henrissat 1991). The crystal structures of α-amylases have shown that these enzymes consist of domains A [which is formed of eight (α/β) barrels], B, and C [e.g. *Bacillus licheniformis* α-amylase (Machius et al. 1995)]. Four highly conserved regions (designated regions I, II, III, and IV) are usually present in the enzymes of family 13. Amino acids in these conserved regions play a role in positioning the substrate into the correct orientation in the active site, proper orientation of the nucleophile, transition state stabilization, and polarization of the electronic structure of the substrate (Lawson et al. 1994; Strokopytov et al. 1996; Utidehaag et al. 1999). Earlier, a *Bacillus* sp. strain TS-23 α-amylase gene was cloned and expressed in recombinant *Escherichia coli* (Lin et al. 1997). In this work, we replaced the nine histidine residues at positions 137, 191, 239, 269, 305, 323, 361, 436, and 446 in the primary amino acid sequence of the truncated *Bacillus* sp. TS-23 α-amylase [His_6-tagged N- and C-terminal mutants (ΔNC)] by leucine, using site-directed mutagenesis in an attempt to probe the residues essential for proper catalytic activity.

To investigate the essential histidine residues of *Bacillus* sp. strain TS-23 α-amylase, we compared the amino acid sequence of the enzyme with those of various amylolytic enzymes. As shown in Fig. 1, His-137, His-

Enzyme	Region I	Region II	Region IV
	•	•	•
Bacamy	DVFNH	GFRLDAVKH	LVDNHD
Bksamy	DVMNH	GYRLDAIKH	FVDNHD
Bstamy	DVFDH	GFRLDGLKH	FVDNHD
Humamy	DAINH	GFRLDASKH	FVDNHD
Baramy	DIINH	DGRLDWGPH	FVDNHD
Baccgt	DFPNH	GIRVDAVKH	FIDNHD
Kaepul	DVYNH	GFRFDLNGY	YVSKHD
Bstnep	DAFNH	GWRLDVANE	LLGSHD
Pamiso	DVYNH	GFRFDLASV	FIDDHD
Bceglu	DLVNH	GFRMDVINF	LYNNHD

Fig. 1 Sequence alignments in the conserved regions I, II, and IV of various amylolytic enzymes. *Bacamy Bacillus* sp. strain TS-23 α-amylase (Lin et al. 1997); *Bksamy Bacillus* sp. strain KSM-K38 α-amylase (Hagihara et al. 2001); *Bstamy Bacillus stearothermophilus* α-amylase (Ihara et al. 1985); *Humamy* human pancreatic α-amylase (Nishide et al. 1984); *Baramy* barley α-amylase (Rogers and Millman 1983); *Baccgt Bacillus* sp. number 1011 cyclodextrin glucanotransferase (Kimura et al. 1987); *Kaepul Klebsiella pneumoniae* pullulanase (Katsuragi et al. 1987); *Bstnep B. stearothermophilus* neopullulanase (Kuriki and Imanaka 1989); *Pamiso Pseudomonas amyloderamosa* isoamylase (Amemura et al. 1988); *Bceglu B. cereus* oligo-1,6-glucosidase (Watanabe et al. 1990); *solid circles* essential histidine residues of *Bacillus* sp. strain TS-23 α-amylase

Table 1 Primers used for site-directed mutagenesis of the *Bacillus* sp. strain TS-23 α-amylase gene

Enzyme	Nucleotide sequence (5′-3′)[a]
His137Leu	GTCTTTAAT**CTT**AAGGCGGG
His191Leu	CGTTGGTAT**CTT**TTGACGGT
His239Leu	GATATGGAT**CTC**CCTGAGGT
His269Leu	GTAAAA**CTT**ATTAAATACAG
His305Leu	AATAAGCTG**CTT**AATTAC
His323Leu	GCACCTTTG**CTT**AACAACTT
His361Leu	GTCGATAAC**CTC**GACACGCA
His436Leu	TACATTGAC**CTT**CAAGACAT
His475Leu	GGTAAAAAA**CTT**GCTGGAAA

[a]Nucleotides in **bold** represent the mutations that introduce the desired amino acid substitutions

269, and His-361 of *Bacillus* sp. strain TS-23 α-amylase are highly conserved in α-amylases, and are located in regions I, II, and IV, respectively. His-323 is present in the α6 helix of domain A, while His-239 and His-305 are situated at the loop regions of the same domain. His-191, His-436 and His-475 are located in domains B and C, respectively. Although His-436 is conserved in the aligned microbial α-amylases, this residue is located outside the highly conserved regions, implying that it is not directly involved in the catalytic activity.

For replacement of histidine residues in His_6-tagged ΔNC, pQE-AMYΔNC (Lo et al. 2001) was subjected to restriction digestion with *Bam*HI and *Hin*dIII, and a 1.6-kb *Bam*HI-*Hin*dIII fragment was subcloned into the corresponding sites of M13mp19 to generate pM19-AMY. Oligonucleotide-directed mutagenesis was performed on pM19-AMY according to the procedures described by Kunkel (1987) with the Bio-Rad Muta-Gene version 2 mutagenesis kit (Bio-Rad Laboratories, Richmond, Calif., USA). The primers used (from Quality Systems, Taipei, Taiwan) are listed in Table 1. The mutagenized 1.6-kb *Bam*HI-*Hin*dIII fragments were subcloned into pQE-30 to generate pQE-AMYΔNC137/191/239/269/305/323/361/436/475, respectively. After verification of the altered sequence, the recombinant plasmids were transformed into *E. coli* M15 (Qiagen, Valencia, Calif., USA) for gene expression induced by isopropyl-beta-D-thiogalactopyranoside (IPTG). Starch-plate assay showed that *E. coli* M15 (pQE-AMYΔNC191/239/305/475) could hydrolyze the starch in the medium to an extent comparable to that of the control (data not shown). However, negligible halos were observed from *E. coli* M15 harboring pQE-AMYΔNC137/269/361. The remaining two transformants produced halos smaller than *E. coli* M15 (pQE-AMYΔNC).

Single colonies of the recombinant *E. coli* cells were transferred to 10 ml Luria-Bertani medium supplemented with 100 μg/ml ampicillin and 25 μg/ml kanamycin and grown at 37°C for 12 h. Then 100 μl of this pre-culture was inoculated into 10 ml of the same medium and incubated at 28°C with shaking until the absorbance at 600 nm reached 0.6. IPTG at a final concentration of 0.5 mM was added and the cultivation continued for another 3 h. As shown in Fig. 2A, a major protein of approximately 54 kDa was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) when the recombinant *E. coli* cells were induced with IPTG. Activity staining showed that His191Leu, His239Leu, His305Leu, His323Leu, and His475Leu have amylolytic activity (Fig. 2B), suggesting that histidine residues at these positions are not important for catalytic activity of the enzyme. As expected, no amylolytic activity was observed in His137Leu, His269Leu, and His361Leu. Interestingly, a complete loss of amylolytic activity was observed in His436Leu (Fig. 2B). Because the cells were heated at 100°C for 5 min before activity staining, it is possible that this residue plays an important role in the thermostability of the enzyme.

To purify the expressed proteins, the recombinant cells were collected from 100 ml cultured medium and homogenized as described previously (Lo et al. 2001). After removal of the cell debris by centrifugation at 12,000 *g* at 4°C for 20 min, the supernatants were immediately mixed with nickel nitrilotriacetate resin pre-equilibrated with binding buffer (5 mM imidazole, 0.5 mM NaCl, and 20 mM Tris-HCl; pH 7.9). Then the wild-type and mutant proteins were eluted from the resin with a buffer containing 0.5 M imidazole, 0.5 M NaCl, and 20 mM Tris-HCl (pH 7.9). As shown in Fig. 2C, the recombinant enzymes were purified until nearly homogeneous.

To determine the specific activity of each mutant His_6-tagged ΔNC, amylase activity of the purified enzymes was assayed in accordance with the procedure described by Lin et al. (1994) and concentrations of protein were determined with a Bio-Rad protein assay kit using bovine serum albumin as the standard. As shown in Table 2, no enzymatic activity was detected in His137Leu, His269Leu, and His391Leu. The generally accepted catalytic mechanism of the α-amylase family is

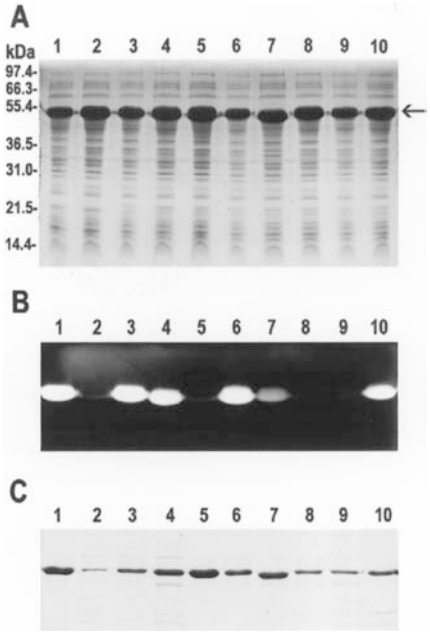

Fig. 2A–C Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. **A** Total cell proteins from *Escherichia coli* M15 transformants visualized by Coomassie blue staining. **B** Total cell proteins from *E. coli* M15 transformants visualized by activity staining. **C** Purified wild-type and mutant proteins. Protein markers were phosphorylase b (97.4 kDa), bovine serum albumin (66.3 kDa), glutamic dehydrogenase (55.4 kDa), lactate dehydrogenase (36.5 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). For activity staining, cells induced by isopropyl-beta-D-thiogalactopyranoside were collected from 1 ml culture broth, mixed with 100 μl of 1×SDS-sample buffer, heated at 100°C for 5 min, and centrifuged at 12,000 *g* for 20 min; 3 μl of the resulting materials was subjected to SDS-PAGE (10% acrylamide gel). The gels were immediately immersed into 1% soluble starch in 50 mM Tris-HCl buffer (pH 8.5). The amylolytic band was visualized by soaking the gels in a solution of 0.01N–0.1 N KI. Lanes are as follows: *1 E. coli* M15 (pQE-AMYΔNC); *2 E. coli* M15 (pQE-AMYΔNC137); *3 E. coli* M15 (pQE-AMYΔNC191); *4 E. coli* M15 (pQE-AMYΔNC239); *5 E. coli* M15 (pQE-AMYΔNC269); *6 E. coli* M15 (pQE-AMYΔNC305); *7 E. coli* M15 (pQE-AMYΔNC323); *8 E. coli* M15 (pQE-AMYΔNC361); *9 E. coli* M15 (pQE-AMYΔNC436); *10 E. coli* M15 (pQE-AMYΔNC475). *Arrow* indicates the position of His_6-tagged ΔNCs. Lanes in **C** are as follows: *1* wild-type enzyme; *2* His137Leu; *3* His191Leu; *4* His239Leu; *5* His269Leu; *6* His305Leu; *7* His323Leu; *8* His361Leu; *9* His436Leu; *10* His475Leu

that of the α-retaining double displacement (Kuriki and Imanaka 1999). The mechanism involves two catalytic residues in the active sites: a glutamic acid as acid/base catalyst and an aspartate as the nucleophile. In the catalytic process, the critical histidine residues of amylolytic enzymes have been proposed to be involved in the binding of substrate (Ishikawa et al. 1992, 1993; Nakamura et al. 1993; Takase 1994; Tseng et al. 1999; Watanabe et al. 2001). Histidine residues implicated in substrate binding have generally been localized to high-similarity regions (Matsuura et al. 1984; Buisson et al. 1987; Igarashi et al. 1998). In *Bacillus* sp. strain TS-23 His_6-tagged ΔNC, these include the highly conserved residues His-137 (region I), His-269 (region II), and His-361 (region IV). It is likely that these residues in

Table 2 Specific activities and kinetic parameters of wild-type and mutant His_6-tagged ΔNCs

Enzyme	Specific activity (U mg^{-1})[a]	K_m (mg ml^{-1})[b]	k_{cat} (s^{-1})[b]	k_{cat}/K_m (ml mg^{-1} s^{-1})
Wild-type	187.3	2.9	168.5	58.1
His191Leu	201.8	3.2	181.6	56.8
His239Leu	209.1	2.6	186.1	71.6
His305Leu	179.3	3.7	161.3	43.6
His323Leu	89.1	2.7	80.2	29.7
His436Leu	79.2	3.4	71.5	21.0
His475Leu	215.5	2.8	193.9	69.3
His137Leu	ND[c]	ND[c]	ND[c]	ND[c]
His269Leu	ND[c]	ND[c]	ND[c]	ND[c]
His361Leu	ND[c]	ND[c]	ND[c]	ND[c]

[a]One unit of amylase activity is defined as the amount of the enzyme that releases an amount of reducing sugar equivalent to 1 μmol glucose min^{-1} under the assay conditions. Specific activity is expressed as units mg $protein^{-1}$

[b]Values of k_{cat} and K_m were calculated by fitting the initial rates as a function of soluble starch concentration to the Michaelis-Menten equation

[c]*ND* not detected

His_6-tagged ΔNC play a role in binding the substrate during the catalytic process. The specific activity was significantly decreased by the mutations introduced at positions 323 and 436 of the enzyme, while substitution of other histidines with leucine showed no apparent change in enzymatic activity. The kinetic parameters for His191Leu, His239Leu, His305Leu, and His475Leu are similar to that for the wild-type enzyme with small changes in both K_m and k_{cat} values (Table 2). His323Leu and His436Leu were severely compromised catalytically with more than a 60% decrease in the value of k_{cat}/K_m, indicating the importance of these two residues in catalytic activity. Since these changes are not as dramatic as those involved in His137Leu, His269Leu and His361Leu, it may be that these residues play a minor or indirect role in the catalytic mechanism. Such effects might be due to long-range interactions or local structural rearrangements of more critical residues in the active site as a consequence of the mutations.

The thermostabilities of wild-type His_6-tagged ΔNC and His436Leu were compared. As shown in Fig. 3, His_6-tagged ΔNC exhibited a time-dependent decrease in activity at 70°C. The half-life of wild-type enzyme was significantly longer than that of His436Leu at this temperature. This result is consistent with the findings of activity staining (Fig. 2B). The thermostability of a protein is determined by many factors, for example, packing efficiency, hydrophobic interactions, loop stabilization, reduced entropy of unfolding, and electrostatic interaction (Vieille and Zeikus 1996). While most natural proteins seem to achieve their individual stability by accumulating a large number of weakly stabilizing interactions that result in a large net effect, some have acquired specialized structural features that cannot easily be transferred in a general way into other proteins (Demirjian et al. 2001). Nevertheless, several hundreds of *B. licheniformis* α-amylase variants have been

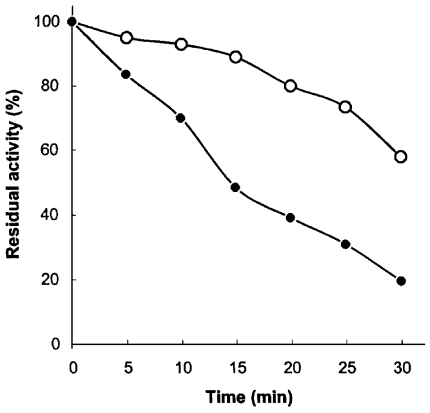

Fig. 3 Thermostability of wild-type His_6-tagged ΔNC (*open circles*) and His436Leu (*solid circles*) at 70°C. The purified enzymes were adjusted to a protein concentration of 100 μg/ml with 50 mM Tris-HCl buffer (pH 8.5). The enzyme solution was incubated at 70°C for designated time periods. After incubation, 100 μl enzyme solution was added to 0.9 ml of 1% soluble starch in 50 mM Tris-HCl buffer (pH 8.5). The reaction was then allowed to proceed at 60°C for 10 min and was stopped by the addition of 1 ml 3,5-dinitrosalicylic reagent. Subsequently, the residual activity was determined by measuring absorbance at 540 nm

acquired in various laboratories and the characterization of these constructs have led to the identification of protein regions and residues that are important for thermostability. In the study of Declerck et al. (2000), three asparagine residues (Asn172, Asn188, and Asn190) were replaced with amino acid residues and the substitution of phenylalanine in place of asparagine at position 190 leads to a sixfold increase of the enzyme's half-life at 80°C. Additionally, amino acid replacements at six histidine residues of *B. licheniformis* α-amylase reveal that His-133 is critical for the thermostability of the enzyme and replacement of this residue by leucine will increase its thermostability (Declerck et al. 1990). However, no equivalent histidine residue is present at His_6-taggedΔNC and a contrary result was observed when His-436 was replaced. For the present work, we have focused on substitution of this residue by other amino acid residues and compared the kinetic stability of the respective proteins with those of wild-type enzyme. The resulting information is expected to be valuable for our understanding of the correlation between this residue and protein stability.

In conclusion, the purpose of this investigation was to identify histidine residues of *Bacillus* sp. strain TS-23 α-amylase that are essential for catalytic activity and/or substrate binding. Toward this goal, we conducted site-directed mutagenesis on nine histidine residues and compared wild-type enzyme and selected mutant proteins with respective to their enzyme properties. Our present results suggest that His-137, His-269, and His-361 of *Bacillus* sp. strain TS-23 α-amylase are the essential catalytic residues in the amylolytic reaction.

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