The Role of Highly Conserved Tryptophan in the Sixth Conserved Region at Substrate Specificity of α-amylase

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ABSTRACT

Early in this study, an α-Amylase from Bacillus megaterium WHO (BMW) was isolated from hot springs of Ramsar (North of Iran), and its gene was cloned in E.coli. Based on its conserved sequence regions and substrate specificity, it was classified as intermediary group enzymes with the specificity of oligo-1,6-glucosidase and neopullulanase subfamilies. In the sixth conserved region (83-QVNGIWMMP), like oligo-1,6-glucosidase subfamily, there is a highly conserved Trp, instead of Tyr for neopullulanase subfamily. In this study, through Trp88Tyr mutation the role of this amino acid in the substrate specificity of enzyme was investigated. The specificity of enzyme against starch, pullulan, amylose and amylopectin was determined. Compared to the wild type, thermal stability and the catalytic efficiency of the mutant increased while product pattern of enzyme didn’t change by mutation. As expected, the neopullulanase activity of enzyme increased.

1 INTRODUCTION

Glycoside hydrolases and transglycosylases have been grouped into more than 135 families based on their amino acid sequences rather than specificity (Coutinho et al., 1999). Enzymes of one family have three-dimensional structure, mechanism and some sequence similarities. The α-amylase belongs to a large clan of three families 13, 70 and 77 of glycoside hydrolases (i.e. clan GH-H) covering hydrolases, transferases, and isomerases (Horvathova et al., 2001). Enzymes of these families mainly act on α-1,4 or α-1,6-glucosidic linkages; however, some of them can hydrolyze α-1,1, α-1,2, α-1,3 and α-1,5-glucosidic bonds. α-amylase, α-glucosidase, oligo-1, 6-glucosidase, pullulanase, isoamylase, CGTase, the branching enzymes, amylopullulanase, and neopullulanase belong to α-amylase family (Macgregor et al., 2001). These multidomain proteins share a common (α/β)₈ barrel as a catalytic domain (Stam et al., 2006). Four conserved sequence regions (I, II, III and IV) covering the C-termini strands of β3, β4, β5, and β7 of the catalytic (α/β)₈ barrel domain were previously identified to play

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important functional roles in α-amylases [Nakajima et al., 1986]. Three additional conserved sequence regions (V, VI and VII) were proposed by comparing the amino acid sequences of α-amylases and the related enzymes. The fifth region (V) is located between β3 strand and helix α3 near the calcium-binding aspartate 175(Taka-Amylase A) while regions VI and VII cover the β2 and β8 strands, respectively. It seems that these regions play a role in determining the enzyme specificity (Janecek et al., 1992). Region VI seems to be important for the (α/β)8 barrel folding and is useful in discriminating α-amylase subfamilies. In the oligo-1,6-glucosidase subfamily, this region contains a highly conserved Trp instead of Tyr in neopullulanase subfamily (Janecek et al. 2002). Oligo-1,6-glucosidase subfamily consists of oligo-1,6-glucosidase, α-glucosidase, dextran glucosidase, trehalose-6-phosphate hydrolase, amyllosucrase, sucrose phosphorylase, isomaltulose synthase and trehalose synthase. Neopullulanase subfamily is formed by three specificities: cyclomaltodextrinase, maltogenic amylase and neopullulanase (Majzlova, et al., 2013). Early in the study, an α-Amylase from Bacillus megaterium WHO (BMW) was isolated from hot springs of Ramsar (North of Iran), and its gene was cloned in E.coli (Yazdani et al., 2009; Ghollasi et al., 2010). Nasrollahi et al., (2011) reported the best refolding condition for the enzyme. The enzyme contains 531 amino acids with a molecular weight of about 60 kDa. Amino acid sequence of the sixth conserved region is 83-QVNGIWMMP. Based on its conserved sequence regions, it was suggested that this α-amylase belongs to the intermediary group enzymes. In this study, the role of very conserved amino acid (Trp 88) in the sixth conserved regions was investigated. Therefore, Trp 88 was substituted with Tyr by site direct mutagenesis to switch the sixth region of the wild type enzyme more similar to neopullulanase subfamily. The neopullulanase activity is defined by the rate of pullulan degradation relative to starch degradation. If this constant increases, enzyme neopullulanase activity will be increased. The enzyme specificity was determined in each case and the kinetic parameters were calculated against starch, amyllose, amyllopectin and pullulan. Both TLC and HPLC were carried out to analyze the end products of the wild type enzyme and its variant.

2 MATERIALS AMD METHODS
2.1 Chemicals
All chemicals were purchased from commercial sources: dinitrosalicylic acid (DNS), starch, Glucose (GO) Assay Kit (Sigma Chemical Co., St. Louis, Mo, USA); dNTP, MgCl2, and Taq polymerase (Fermentas, Vilnius, Lithuania); restriction enzymes (Boehringer Mannheim); Ninitrilotriacetic acid (NTA) column (Qiagen, Munich, Germany); Tris (liofilchem, Italy); CaCl2 (Merck, Germany). All other chemicals were of analytical grade.

2.2 Bacterial growth and enzyme extraction
E. coli BL-21, harboring a recombinant plasmid, was grown overnight at 37 °C in 25 mL culture medium containing ampicillin (100 mg/mL). 1 ml of an overnight culture was added into 100 ml of fresh TB culture containing Amp at 37°C until A600 reached 1.5. Protein expression was induced by adding 10 mM lactose. Cultures were incubated at 22°C for 6 hours. Subsequently, the culture was centrifuged at 5000 g for 15 min at 4°C. Pellet was resuspended in 5 ml buffer (20 mM Tris-HCl, 20 mM CaCl2 pH 7.2). Cells were
lysed by sonication (20 sec on, 1 min off for ten times) and centrifuged at 12000 x g for 15 min at 4°C. Pellets (containing inclusion body proteins) were washed twice by the washing buffer (Tris 20 mM, CaCl₂ 20 mM, Urea 2 M pH 7.2), then, they were centrifuged and kept frozen.

2.3 Purification and refolding of the enzyme

Pellets were resuspended in binding buffer (tris (20 mM), CaCl₂ (30 mM), imidazole (20 mM) and Urea (8 M) pH 7.2) and applied on Ni-NTA Agarose column that was equilibrated by the same buffer. Unbounded proteins were washed by a binding buffer containing 20 mM imidazole. Protein elution was done using a concentration of 300 mM imidazole in binding buffer. The fractions exhibiting enzyme activity were pooled separately and analyzed by SDS-PAGE. To refold the enzyme after the chromatographic step, pure fractions were combined and diluted to a final concentration of 20 mM tris, 30 mM CaCl₂, 0.3 mgr/ml protein and 2 M urea.

2.4 Enzyme assay

α-Amylase activity was measured by Bernfeld method (Bernfeld, 1995). The reaction system contained 0.3 mL of 1% (W/V) soluble starch in 20 mM Tris-HCl, 20 mM CaCl₂ (pH 7.2) and 0.1 mL of the enzyme solution. The reaction was stopped by adding 1.0 mL of DNS (3, 5-Dinitrosalicylic acid 3,5-Dinitrosalicylic acid) and heated in boiling water for 5 min. One unit of enzyme activity was defined as the amount of enzyme required to generate 1 μmol of reducing ends per minute under the catalysis conditions described above (Olesen et al., 2000).

2.5 Thermal stability

To determine the thermal stability of the enzyme, the enzyme was incubated for several periods of time at 60°C in 20 mM Tris-HCl containing 20 mM CaCl₂ (pH 7.2). Residual activity was determined under the standard assay conditions.

2.6 Site-directed mutagenesis

E. coli DH5α and E. coli BL-21 were used as the hosts for cloning and expression, respectively. Plasmid pET-21a containing α-Amylase gene from B. megaterium WHO served as the vector for mutation and expression. The term wild type in this study refers to E. coli BL-21 that had been transformed with this plasmid previously. Site directed mutagenesis was carried out using QuikChange method. The following mutagenic oligonucleotide primers were designed individually: F: 5´-GGATTTATATGATGCCGTAAACCCTTCTCC-3´and R: 5´-GGCATATATAAATCCCGTTTACTTGAAAGATC-3´ for W88Y in the sixth conserved region (83-QVNGIWMMP). The pcr reaction mixture contained 0.1 μg template plasmid, 10x PCR buffer containing MgCl₂, 0.1 mM mixed dNTp, Long DNA polymerase (1U) and 15 μM of forward or revers primer in two different 25 μL volumes. The mixture was heated at 95 °C for 5 min and then subjected to thermal cycling (6 cycles of 95°C for 1 min, 58 °C for 1 min, and 68 °C for 6 min). Then, the two vials were combined and 1U long DNA polymerase was added and subjected to the same thermal cycling condition for 24 cycles. The product was treated by dpnI and then transformed into E. coli DH5α. Some colonies were selected and sequenced to confirm the mutation. Appropriate plasmids were then transformed to E. coli BL-21.

2.7 Thin-layer chromatography (TLC)
The end products formed as a result of starch and pullulan hydrolyzes were analyzed by Thin-layer chromatography (TLC). TLC is developed with solvent system containing 1-butanol/acetone/water (5:3:1). TLC Aluminum Sheets Si 60 F254 5 x 7.5cm was used. Sugars were detected with sulfuric acid/methanol (1:1).

2.8 HPLC analysis

Knauer hplc system was used to determine the products of pullulan and starch degradation by amylase. The products were separated by Eurokat Ag (300 x 8 mm) column. Water was used as eluent at 75 °C by the Flow rate of 0.5 ml/min. Sugars were detected by Refractive index detector (RID).

3 RESULTS AND DISCUSSION

Although million years of evolutionary pressure may have gone into optimizing an enzyme’s pattern of substrate specificity, it is now becoming clear that the overall structure of an enzyme can be compatible with alternative specificities. The divergence of glycosyl hydrolases to acquire new specificities is not unexpected given the stereochemical resemblance between some of their substrates. By comparing the amino acid sequences of α-amylases, it has been revealed that there are seven conserved sequence regions. Regions I, II, III and IV that contained the important functional residues were recognized in 1986. Regions VI, VII (that cover β2 and β3 strands respectively) and region V (near the C terminus of domain B) were established in 1990. These regions seemed to have a role in the enzyme substrate specificity determination. In recent years, it has been suggested that the oligo-1,6-glucosidase and neopullulanase subfamilies of α-amylase, can be classified based on the amino acid sequence of fifth and sixth conserved regions (Nakajima et al., 1997; Janček et al., 2002). Oligo-1,6-glucosidase subfamily with the highly conserved tryptophan in sixth conserved sequence contains oligo-1,6-glucosidase, α-glucosidase, dextran glucosidase, trehalose-6-phosphate hydrolase, amyllosucrase, sucrose phosphorylase, isomaltulose synthase and trehalose synthase (Oslancova et al., 2002). There is a well-conserved Trp instead of a highly conserved Tyr in neopullulanase subfamily. Conserved region VI was assumed to be important in substrate specificity and the folding of (α/β)8 barrel. The sequence of the VI region in BMW-amylase is 83-QVNGIWMMP. Although some studies have been carried out to change the enzyme characteristics of one subfamily into another, there is no experimental data that shows the relationship between this amino acid and the substrate specificity of the enzyme. BMW-amylase-that had been isolated from the hot springs of Ramsar-was previously classified as intermediary. BMW-amylase can act on starch, pullulan, amylose, amylopectin and α-cyclodextrin. In all cases, glucose is produced as the end product. In this study, we investigated the role of Trp 88 in the substrate specificity of BMW-amylase. Therefore, mutant W88Y was constructed as explained in the materials and methods section. Enzymatic characteristics were investigated after the purification of the protein. Michaelis-Menten diagrams of the wild type enzyme and its variant were depicted for starch, pullulan, amylopectin and amylose (Fig. 1). K_m and V_max values were determined using prism software and the enzyme concentration was calculated by Bradford method (Bradford, 1976).

Fig. 1. Michaelis-Menten diagram of wild type BMW-amylase and its mutant for substrates; starch, pullulan, amyllopectin and amylose (Wild type ♦. W88Y ●).

As shown in Table 1, mutant W88Y was more active than the wild type with lower Km values. Catalytic efficiency of this mutant increased about 150% for all the substrates. Thermal stability analysis of the mutant was also investigated. W88Y revealed 40% of the initial activity after incubation at 60°C for 120 minutes while the activity of the wild type enzyme decreased to 15% (Fig. 2). The wild type enzyme can degrade starch, pullulan, amylose and amyllopectin to glucose as the end product. To investigate the effect of mutation in product specificity of enzyme, both TLC (Thin layer chromatography) and hplc analysis were done.

Table 1
Kinetic parameters of BMW-amylase wild type and mutants D203E, W88Y and W88Y, D203E toward starch, pullulan, amyllopectin and amylose.

<table>
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<tr>
<th></th>
<th>STARCH</th>
<th>PULLULAN</th>
<th>AMYLOPECTIN</th>
<th>AMYLOSE</th>
</tr>
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<tr>
<td></td>
<td>K&lt;sub&gt;f&lt;/sub&gt;</td>
<td>K&lt;sub&gt;c&lt;/sub&gt;</td>
<td>K&lt;sub&gt;c&lt;/sub&gt;/K&lt;sub&gt;f&lt;/sub&gt;</td>
<td>K&lt;sub&gt;f&lt;/sub&gt;</td>
</tr>
<tr>
<td>Wild type</td>
<td>1.05</td>
<td>6.12</td>
<td>5.8</td>
<td>0.8</td>
</tr>
<tr>
<td>W88Y</td>
<td>0.9</td>
<td>7.2</td>
<td>8.0</td>
<td>0.7</td>
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Fig. 2. Thermal stability of the wild type enzyme and the mutants at 60°C in 20 mM Tris-HCl containing 20 mM CaCl2 (pH 7.2). (Wild type ♦. W88Y ●).
As shown in Fig. 3, mutated enzyme can hydrolyze pullulan and starch to glucose. Production of glucose as the end product of mutant W88Y was confirmed by hplc (Fig. 4). Thus, mutations didn’t affect product formation and the product pattern of enzyme didn’t change. Mutation in amino acid 88 (W88Y) improved enzyme both in thermal stability and enzyme catalytic efficiency. Therefore, it can be important in enzyme activity. The ratio of catalytic efficiency of the enzyme for starch to pullulan, was calculated about 5.52 for wild type while it was 3.74 in the case of mutant. So, pullulan degradation specificity of W88Y mutant enzyme increased about 1.48 fold compared to the wild type.
Thus, as it was expected, the neopullulanase future of enzyme increased by replacing tryptophan 88 with tyrosine.

Although some experiments have been done to investigate the role of conserved regions in amylase specificity, activity, and calcium binding (Zareian, et al., 2010; Horinouchi, et al., 1988; Sadeghi, et al., 2013; Majzlova, et al., 2013), many of the reports about the conserved sequences are made by bioinformatic analysis using sequence alignment tools. This study is the first experimental report on the role of a highly-conserved amino acid in the sixth conserved region of α-amylase.

REFERENCES
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