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Thermostability of three α-amylases of *Streptomyces* sp IMD 2679

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The amylolytic system of *Streptomyces* sp IMD 2679 is composed of three α-amylases, amylase I, II and III, with temperature maxima of 60, 60–65 and 65°C, respectively. Although each α-amylase displayed higher stability in the pH range 6.0–8.5 than at pH 5.0–5.5, differences in their thermostabilities were more evident as the pH increased from pH 6.0 to 8.5. There was a 14-min difference in half-lives between amylase III, the most thermostable enzyme and amylase II at pH 6.0, and a 46-min difference in the half-lives of amylase III and the least thermostable enzyme, amylase I at pH 6.5. In addition, the α-amylases underwent a pH-dependent monomer-dimer transformation. Increased thermostability of the α-amylases was reflected in the variable contents of amino acids (Arg, His, Ser) responsible for electrostatic interactions, and in the levels of aliphatic and bulky hydrophobic amino acids. There was a two-fold reduction in Cys levels in amylase III relative to amylase I and II.

Keywords: *Streptomyces* sp; α-amylase; thermostability; structure-function; dimerisation

Introduction

α-Amylases (1,4-α-d-glucan glucanohydrolase, EC 3.2.1.1) hydrolyse starch and related compounds in an endo-acting mechanism releasing maltooligosaccharides and glucose in the α-anomeric form [5]. The majority of industrial applications for α-amylases require their use at high temperatures. The upper temperature for the activity of such enzymes is, however, dependent on the limits of protein stability. Proteins have a temperature range within which structural integrity is maintained. Outside this range denaturation occurs resulting in the loss of activity [1].

The physicochemical basis of increased protein thermostability has been a subject of investigation since the late 1960s. Comparative studies on homologously-related proteins, coupled with advances in protein engineering, suggest structural trends characteristic of thermostable proteins [4,11,17]. Higher thermostability has been attributed to alterations in specific amino acid sequences resulting in enhanced conformational stability and a reduced entropy of unfolding, both of which increase the reversibility of the thermoinactivation process [17]. Conflicting results, however, have made it apparent that no global rule for increasing thermal stability is as yet available.

The main components in the amylolytic system of *Streptomyces* sp IMD 2679 are three α-amylases differing in their thermostability properties. This system provided a unique opportunity to examine and extend the knowledge of protein stability. Subtle differences in the amino acid composition of each of the enzymes are discussed in the context of implications for differential thermostability, and conformity and/or non conformity, with structural trends previously proposed to mediate thermostability.

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Materials and methods

Microorganism

Streptomyces sp IMD 2679 was isolated and maintained as previously described [10].

Enzyme production and purification

Crude enzyme was produced at 40°C as previously described [10]. The amylolytic system of *Streptomyces* sp IMD 2679 was purified by ammonium sulphate (0–60%) fractionation, followed by affinity chromatography on bacitracin-Sepharose 4B, ultrafiltration, Superose-12 gel filtration and finally preparative native polyacrylamide gel electrophoresis. The final step in the purification procedure separated the amylolytic system into three distinct α-amylase components, amylase I, II and III. Each α-amylase had a specific activity of 3042 units mg^{-1} protein.

α-Amylase assay

α-Amylase was assayed by the addition of 0.5 ml enzyme to 0.5 ml soluble starch (1%, w/v) in 0.1 M acetate buffer, pH 5.5, followed by incubation at 40°C for 30 min. The reaction was stopped and reducing sugars determined with 3,5-dinitrosalicylic acid according to the method of Bernfeld [2].

Half-lives of thermoinactivation

The half-lives of thermoinactivation were measured by incubating aqueous solutions of each enzyme at the desired pH (buffer conditions are indicated below) at 65°C. Samples were withdrawn after specific time intervals, cooled on ice for 10 min, then diluted appropriately with 0.1 M acetate buffer, pH 5.5; the residual activity was then determined. The catalytic half-lives were calculated using Enzfitter, a non-linear regression analysis programme [7].

Amino acid composition
Amino acid analysis was carried out at Alta Bioscience, School of Biochemistry, University of Birmingham, UK. The amino acid composition was determined as the number of residues per mole of protein.

Relative molecular mass
The relative molecular mass (M_r) was determined by gel filtration on a prepacked Pharmacia (Uppsala, Sweden) HR 10/30 FPLC Superose-12 column. The standards used were: cytochrome c, M_r 12 400; chymotrypsin, M_r 22 500; ovalbumin, M_r 45 000; bovine serum albumin, M_r 66 000 and γ-globulin, M_r 160 000. The column was equilibrated with buffer (as indicated below). Aliquots (200 μl) of each protein sample in the appropriate buffer were applied to the column by means of a valve injection system (Pharmacia). A flow rate of 0.5 ml min^{-1} was used and column eluents were detected using a UV monitor and recorded with a Spectra Physics (Manchester, UK) SP 4270 integrator.

Results

Effect of temperature on activity of amylase I, II and III
Amylase I, II and III had temperature maxima of 60, 60–65 and 65°C, respectively (Figure 1). At 70°C, amylase III displayed higher % relative activity (greater than 70% of the maximum) than amylase I (15%) or II (49%).

Effect of pH on the rate of irreversible thermoinactivation of amylase I, II and III
The relative strengths of protein interactions including electrostatic interactions are pH-dependent, with the pH of a solution influencing the overall charge of the protein. To evaluate differences in thermostabilities of amylase I, II and III from *Streptomyces* sp IMD 2679, the half-lives of inactivation were determined over the pH range 5.0–8.5 (Figure 2a). With pH, there were major differences in the thermostability properties of the three α-amylases. Differences in half-life values of the most stable α-amylase (amylase III) and the other two amylases were examined relative to pH (Figure 2b). The variation in half-lives between the enzymes was minimal at pH 5.0 and 5.5; however, as the pH increased, differences in the thermostabilities became evident. The highest variation in stability

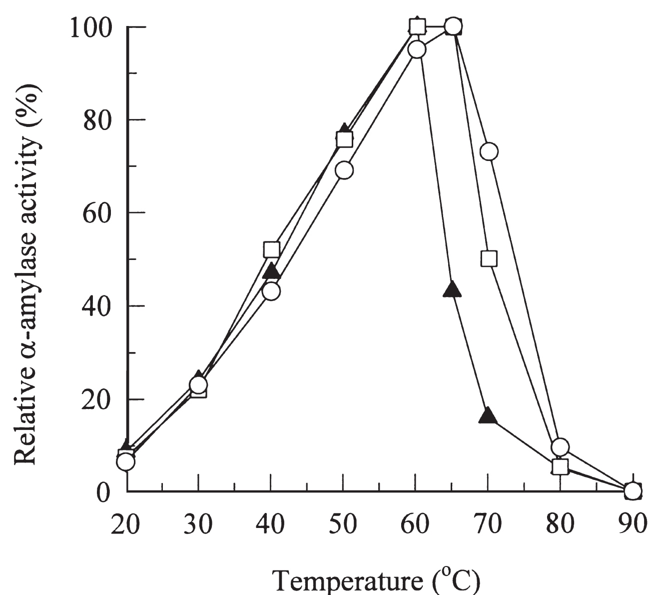

Figure 1 Determination of the temperature profiles of amylase I, II and III of *Streptomyces* sp IMD 2679. α-Amylase activity was assayed using starch (final assay concentration 0.5%, w/v), in 0.1 M acetate buffer, pH 5.5, as substrate over a period of 30 min at the temperatures indicated. Relative α-amylase activity (%) at each temperature is calculated in relation to activity at maximum temperature (100%): ▲ amylase I; □ amylase II; ○ amylase III.

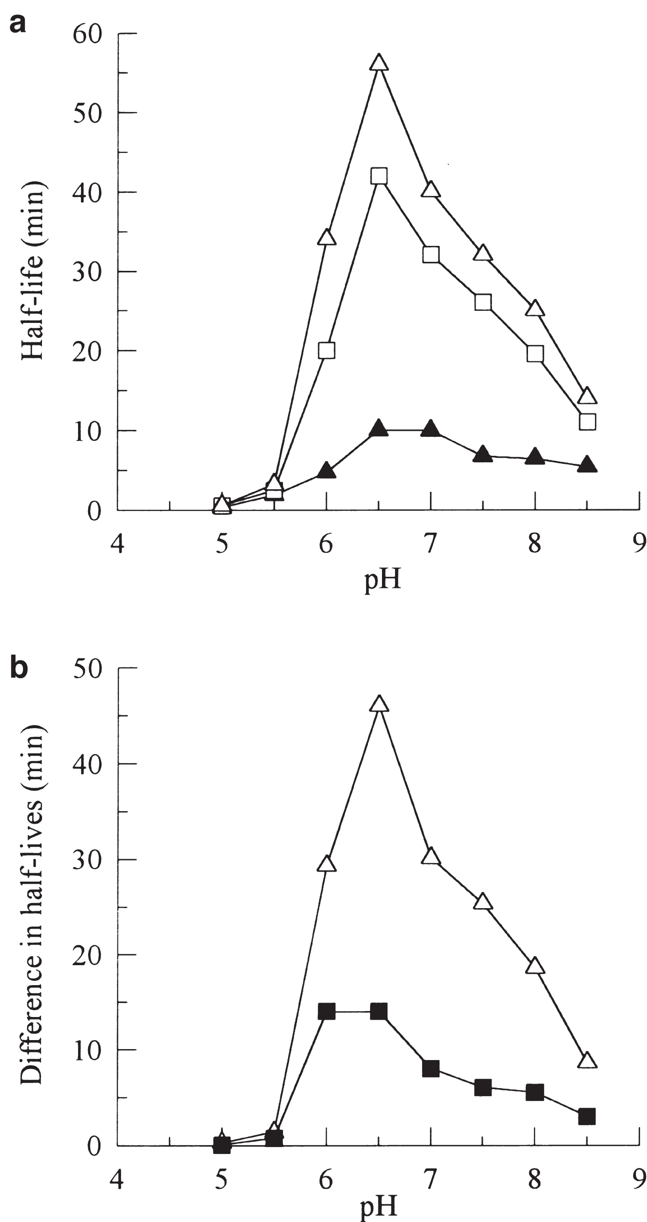

Figure 2 The effect of pH on the respective half-lives of irreversible thermoinactivation of amylase I, II and III from *Streptomyces* sp IMD 2679. Enzyme was incubated at 65°C, at pH 5.0 and pH 5.5 (0.05 M acetate buffer) and at pH 6.0–8.5 (0.05 M Tris/maleate buffer). (a) Effect of pH on thermostability: ▲ amylase I; □ amylase II; △ amylase III. (b) Differences in half-life values between: △ amylase III and I; ■ amylase III and II.

Table 1 The effect of pH on the relative molecular mass of amylase I, II and III of *Streptomyces* sp IMD 2679

Superose-12, gel filtration (Eluent)	Relative molecular mass (M_r)		
	Amylase I	Amylase II	Amylase III
0.05 M acetate buffer, pH 5.5, containing 6 M guanidine hydrochloride	47 900	48 400	48 400
0.05 M Tris/HCl buffer, pH 8.5, containing 6 M guanidine hydrochloride	84 140	84 140	84 140

between amylase III and I occurred at pH 6.5, yielding a 46-min difference in half-lives, whilst with amylase III and II this occurred between pH 6.0–6.5 with a 14-min difference.

Effect of pH on the relative molecular mass of amylase I, II and III

The relative molecular mass (M_r) of each α-amylase was determined by gel filtration in the presence of 6 M guanidine hydrochloride to preclude non-specific interactions between the enzymes and the gel matrix. At pH 5.5, a value in the range 47 900–48 400 was obtained for each enzyme (Table 1). By increasing the pH to 8.5, a value (84 140) almost twice that obtained at pH 5.5, was observed.

The amino acid composition of amylase I, II and III

Comparison of the amino acid composition of amylase III and II, revealed lower levels of His, Trp and Arg in amylase II (Table 2). The ratios of the His, Trp and Arg contents in amylase III relative to those in amylase II were 1: 0.7, 1:0.9 and 1:0.9, respectively (Table 2). Conversely, higher levels of Glu and Cys were detected in amylase II.

Greater variations in amino acid composition were observed on comparing the amino acid content of amylase III to that of amylase I. Lower levels of Asp, Glu, Pro, Phe, Trp, and Arg as well as higher levels of Thr, Ser, Cys, Val, Ile and His were observed in amylase I. The largest difference between amylase I and II and the most thermostable enzyme, amylase III was observed with respect to the Cys content (Table 2). Two-fold lower levels of this amino acid were observed in amylase III compared with amylase I and II.

Table 2 Amino acid composition of amylase I, II and III of *Streptomyces* sp IMD 2679

Amino acid	Number of residues per mole Amylase			Ratio Amylase	
	I	II	III	III:I	III:II
Asp	45.9	56.8	56.2	1:0.8	1:1.0
Thr	32.9	29.5	29.5	1:1.1	1:1.0
Ser	21.0	17.3	16.9	1:1.2	1:1.0
Glu	11.1	16.0	12.4	1:0.9	1:1.3
Pro	12.2	14.3	13.9	1:0.9	1:1.0
Gly	ND	ND	ND	ND	ND
Ala	ND	ND	ND	ND	ND
Cys[a]	3.7	3.9	1.5	1:2.5	1:2.6
Val	28.2	25.0	25.3	1:1.1	1:1.0
Met	10.0	10.5	10.7	1:0.9	1:1.0
Ile	20.1	17.7	18.2	1:1.1	1:1.0
Leu	22.3	21.2	22.5	1:1.0	1:0.9
Tyr	21.7	22.5	22.5	1:1.0	1:1.0
Phe	19.1	20.7	21.1	1:0.9	1:1.0
His	36.6	19.1	26.7	1:1.4	1:0.7
Trp	4.8	6.6	7.4	1:0.6	1:0.9
Lys	23.9	22.7	23.9	1:1.0	1:0.9
Arg	14.8	15.9	16.9	1:0.9	1:0.9

ND = not determined (due to buffer interference).
[a]Determined as cysteic acid.

Discussion

Streptomyces sp IMD 2679 produced three α-amylases differing in their thermostability properties. The existence in nature of related multiple forms of enzymes presents valuable opportunities, which cannot be engineered in the laboratory, to investigate and expand the knowledge of the structure-function relationships of enzymes.

A number of highly thermostable enzymes occur as stable oligomeric structures [6,15]; the oligomeric nature of multisubunit enzymes is thought to confer increased stability. Each of the α-amylases underwent a pH-dependent monomer-dimer transformation. As each form possessed higher stability at pH 8.5 than at pH 5.5, the transformation evident at pH 8.5 may provide an adaptive mechanism to increase stability under adverse conditions.

The influence of pH on the susceptibility of the α-amylases to thermoinactivation also reflects a difference in the ionisation of internal amino acid side chains and could indicate subtle differences in the primary structure of the α-amylases manifested in their thermostability. An increase in the Arg content correlated with increasing thermal stability from amylase 1 (14.8 Arg residues) to amylase II (15.9) and amylase III (16.9). Others have noted an increase in the Arg: Lys ratio in thermophilic proteins [11,12]. This would allow for formation of stronger salt linkages as Arg possesses a higher pK_a (12.0) than Lys (9.5) and, unlike Lys, Arg remains positively charged at highly alkaline pH values and at higher temperatures [17]. With the amylolytic components of *Streptomyces* sp IMD 2679, the increase in Arg content was not, however, accompanied by an equivalent decrease in Lys content. However, His levels were greatly reduced in amylase II (19.1) and amylase III (26.7) in contrast to amylase I (36.6). Examination of the individual amino acid contents of each amylase indicated that the most pronounced reduction lay in the His content between amylase III and II. As His possesses a pK_a in the region of pH 6.0 [13], its replacement by Arg would allow for the formation of stronger salt linkages in amylase II and III. In addition, as the Arg and His levels are higher in the most stable α-amylase (amylase III) than in amylase II, this strengthens the argument for the involvement of salt linkages in stabilising the structure of amylase III. Such a proposal can be substantiated by examining the pH dependency of thermoinactivation, as the greatest variation in half-lives between amylase II and III occurred at pH 6.0.

Polar amino acids such as Ser and Thr are less common in thermophilic proteins [11]. With the amylolytic system

of *Streptomyces* sp IMD 2679 the highest level of Thr was detected in amylase I and a decrease in Ser accompanied increased thermostability. In addition, higher levels of the bulky hydrophobic amino acids Trp, Phe and Pro and reduced levels of the aliphatic non-polar amino acids Ile, Val and Thr were detected in amylase II and III over amylase I. Such replacements would enhance hydrophobicity, whilst reducing the entropy of unfolding of the protein backbone. A number of studies have demonstrated a relationship between stability and hydrophobic content [1,4].

Increasing the proline content of a protein has been proposed to have a stabilising effect [14,16]. Although a higher proline content was detected in amylase II and III relative to amylase I, a direct relationship between proline content and thermostability was not observed.

The greatest difference in amino acid composition between the most thermostable α-amylase, amylase III, and the less thermostable α-amylases II and I, was noted in the Cys content, with a greater than two-fold reduction in the content of this amino acid in amylase III. Lepock *et al* [8] proposed that an important step towards increasing enzyme stability involved removal of free cysteine residues. Such residues are susceptible to oxidation, and thermal inactivation of a number of α-amylases has been attributed to such a modification [3]. The presence of free thiols within a protein containing disulphides also leads to oligomerisation through thiol–disulphide interchange [9]. The latter may have contributed to dimerisation observed with the α-amylases of *Streptomyces* sp IMD 2679. Thus, a lower Cys content (as seen with amylase III) could imply fewer free thiol groups available for nucleophilic attack (thiol–disulphide interchange) increasing the stability of existing disulphide bridges and enhancing protein stability.

A major area of interest in protein evolution is the means by which variations in amino acid composition of proteins can result in changes in enzyme stability. Increased thermostability is attributed to alterations in specific amino acid sequences resulting in enhanced conformational stability. However, from a comparison of the α-amylases of *Streptomyces* sp IMD 2679, thermostability cannot be attributed to a single amino acid variation. A combination of factors including variations in the number of hydrophobic and electrostatic interactions as well as potential protein oligomerisation are more likely to determine thermostability.

References

1. Argos P, MG Rossmann, UM Grau, H Zuber, G Frank and JD Tratschin. 1979. Thermal stability and protein structure. Biochemistry 18: 5698–5703.
2. Bernfeld P. 1955. Amylases, α- and β-. Meth Enzymol 1: 149–158.
3. Brosnan MP, CT Kelly and WM Fogarty. 1992. Investigation of the mechanisms of irreversible thermoinactivation of *Bacillus stearothermophilus* α-amylase. Eur J Biochem 203: 225–231.
4. Declerck N, P Joyet, J Trosset, JY Garnier and C Gaillardin. 1995. Hyperthermostable mutants of *Bacillus licheniformis* α-amylase: multiple amino acid replacements and molecular modelling. Protein Eng 8: 1029–1037.
5. Fogarty WM. 1983. Microbial amylases. In: Microbial Enzymes and Biotechnology (Fogarty WM, ed), pp 1–92, Elsevier Applied Science, London.
6. Laderman KA, BR Davis, HC Krutzsch, MS Lewis, YV Griko, PL Privalov and CB Anfinsen. 1993. The purification and characterisation of an extremely thermostable α-amylase from the hyperthermophilic archaebacterium *Pyrococcus furiosus*. J Biol Chem 268: 24394–24401.
7. Leatherbarrow RJ. 1987. Enzfitter, a non-linear regression data analysis program for the IBM PC. Elsevier-Biosoft, Amsterdam.
8. Lepock JR, HE Frey and RA Hallewell. 1990. Contribution of conformational stability and reversibility of unfolding to the increased thermostability of human and bovine superoxide dismutase mutated free cysteines. J Biol Chem 265: 21612–21618.
9. Matsumura M, G Signor and BW Mattews. 1989. Substantial increase of protein stability by multiple disulphide bonds. Nature 342: 291–293.
10. McMahon HEM, CT Kelly and WM Fogarty. 1997. Effect of growth rate on α-amylase production by *Streptomyces* sp IMD 2679. Appl Microbiol Biotechnol 48: 504–509.
11. Mozhaev VV and K Martinek. 1984. Structure-stability relationships in proteins: new approaches to stabilizing enzymes. Enzyme Microb Tech 6: 50–59.
12. Querol E and A Parrilla. 1987. Tentative rules for increasing the thermostability of enzymes by protein engineering. Enzyme Microb Tech 9: 238–244.
13. Schulz GE and RH Schirmer. 1979. Amino acids. In: Principles of Protein Structure (Cantor CR, ed), pp 1–16, Springer-Verlag, New York.
14. Suzuki Y, K Oishi, H Nakano and T Nagayama. 1987. A strong correlation between the increase in number of proline residues and the rise in thermostability of five *Bacillus* oligo-1,6-glucosidases. Appl Microbiol Biotechnol 26: 546–551.
15. Suzuki Y, M Nobiki, M Matsuda and T Sawai. 1997. *Bacillus thermoamyloliquefaciens* KP1071 α-glucosidase II is a thermostable M_r 540 000 homohexameric α-glucosidase with both exo-α-1,4-glucosidase and oligo-1,6-glucosidase activities. Eur J Biochem 245: 129–136.
16. Suzuki Y, N Sugita and T Kishimoto. 1997. Purification and characterization of an oligo-1,6-glucosidase from the caldoactive thermophile *Bacillus caldotenax*. Starch 49: 148–154.
17. Vieille C and JG Zeikus. 1996. Thermozymes: identifying molecular determinants of protein structural and functional stability. Trends Biotechnol 14: 183–190.