The purification and some properties of a stereospecific D-asparaginase from an extremely thermophilic bacterium, *Thermus aquaticus*

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A specific D-asparaginase was isolated and crystallized from *Thermus aquaticus* strain T351. It is present in larger amounts than the L-asparaginase. The enzyme has a molecular weight of 60000, an isoelectric point of 4.8 and a K_m of 2 mM. It has 6 disulphide bonds/molecule, and a histidine residue at the active site. It is inhibited by keto acids and by high salt concentrations.

The occurrence of L-asparaginase in various bacteria, fungi and plants has been reported by many workers, and its presence in Gram-negative bacteria is particularly well documented (Wriston & Yellin, 1973). These L-asparaginases are generally not completely stereospecific, and a specific D-asparaginase enzyme has been reported only once, in *Brucella abortus* (Altenbern & Housewright, 1954).

There has been speculation over a number of years over the incorporation of D-amino acids into proteins, or as substrates for enzymes. Some bacteria are known to use D-amino acids as substrates and to incorporate them into cell walls, e.g. *Bacillus anthracis* (Hanby & Rydon, 1946) and *Bacillus licheniformis* (Bodansky & Perlman, 1969). At the high growth temperatures, above 70°C, of extremely thermophilic bacteria thermal racemization of amino acids will occur much more rapidly than at room temperature, and it may be that the ability to utilize D-amino acids will be an advantage.

We have discovered the presence of substantial amounts of an active and completely stereospecific D-asparaginase in an extremely thermophilic bacterium, *Thermus aquaticus* strain T351. In the present paper we report on the purification and some properties of this enzyme.

Methods

Bacterial growth

Thermus aquaticus strain T351 was maintained, grown and harvested as described previously (Hickey & Daniel, 1979).

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Asparaginase assay

The Nessler method of Bergmeyer (1974) was used for the assay of cell-free D-asparaginase except that 0.2 M- $\text{Na}_2\text{B}_4\text{O}_7$ /NaOH buffer, pH 8.5, was used instead of Tris buffer, whereas the method of Peterson & Ciegler (1969) was used to determine activity in whole cells. A chromatographic assay method was also used in which the enzyme extract was incubated with 40 mM-D-asparagine for 15 min and spotted on Whatman no. 1 chromatography paper together with a control sample taken immediately after mixing of the two components. The chromatograph was run with a phenol/water (4:1, w/v) mixture and the amino acids were located by spraying with ninhydrin.

Enzyme purification

All purification steps were performed at room temperature (approx. 20°C) in 50 mM-Tris/HCl buffer, pH 8.2, unless otherwise stated. Bacteria were ruptured in a French Pressure Cell (Amicon Corp.) at 70 MPa. The 240 000 g supernatant from this suspension was dialysed against distilled water. The non-diffusible material was concentrated by rotary evaporation to approx. 1 ml, and loaded on to a QAE (quaternary ammonium ethyl)-Sephadex A-50 column (15 cm × 2.5 cm) and eluted with increasing concentrations of NaCl (0.05–0.5 M). The fractions with D-asparaginase activity were pooled (an L-asparaginase was separated also at this point) and applied to a Bio-Gel HTP hydroxyapatite column (15 cm × 2.5 cm) after being desalted by ultrafiltration on a PM 10 (Amicon) ultrafilter. The fractions were eluted with five separate step concentrations of 50 mM to 0.5 M-sodium phosphate buffer, pH 7.2.

Peak fractions containing D-asparaginase were desalted and concentrated by using a PM 10

ultrafilter, and applied in a 1.0–1.5 ml amount to a Sephadex G-200 column (65 cm $\times$ 2.5 cm) that had been calibrated with proteins of known molecular weight. Fractions containing D-asparaginase were applied to a second QAE-Sephadex A-50 column, as above, but eluted with a linear 0.1–0.2 M-NaCl gradient in 0.1 M-Na₂B₄O₇/NaOH buffer, pH 8.2. Fractions containing D-asparaginase were then applied to a second hydroxyapatite column and eluted with a 50 mM–0.1 M-sodium phosphate buffer, pH 7.2, gradient containing 0.1 mM-(NH₄)₂SO₄ (Skarstedt & Bascombe, 1974). Fractions containing D-asparaginase were freeze-dried and then dissolved in 200 μ l of 50 mM-phosphate buffer, pH 7.2. This solution was then applied to centre wells on a 2 cm $\times$ 2 cm cellulose acetate gel plate. A potential of 450 V with 6 mA was applied for 60 min. The major protein band was separated by cutting the plate into thin sections with a razor blade after locating it with Ponceau S dye. After the gel had been stripped off the backing and macerated with 0.1 M-sodium borate buffer, pH 9.2, the activity was confirmed by the chromatographic assay, and the D-asparaginase was centrifuged at 27 000 g (r_{av} , 4.25 cm) for 30 min. The supernatant was then dialysed against distilled water for 6 h. Crystallization was then achieved by the method of Roberts *et al.* (1972).

Electrophoresis

Analytical polyacrylamide-gel electrophoresis was performed as described by Davis (1964). Gels were stained for protein with Coomassie Brilliant Blue (0.2% in methanol containing 9.2% acetic acid). D-Asparaginase activity was located by the method of Padjack & Padjack (1972). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed by incubating proteins at 37°C for 12 h in 0.1 M-phosphate buffer, pH 7.6, containing 1% (w/v) sodium dodecyl sulphate and 1% (v/v) 2-mercaptoethanol. Sucrose (30%, w/v) was added just before application of the mixtures to the sample gels. Gel tubes were 10–12 cm long, and separating gels were of composition 7.5% acrylamide and contained 0.1 M-K₂HPO₄/NaH₂PO₄ buffer, pH 7.1, and 0.1% sodium dodecyl sulphate. The sample contained 10–20 μ g of protein determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Electrophoresis was performed at 8 mA/tube for 2 h. Identification of the sodium dodecyl sulphate front, staining procedures and determination of relative migration were as described by Shapiro *et al.* (1967).

Other methods

The Universal buffer of Roberts (Rowley & Wriston, 1967) was used to determine the pH optimum, the exact pH being measured at 75°C.

Disulphide analysis was done by the method of Anderson & Wetlaufer (1975). Free thiol groups were determined by the methods of Robyt *et al.* (1971) and Riddles *et al.* (1979).

Carbohydrate was estimated by the orcinol method (Winzler, 1955).

Results and discussion

The purification results are summarized in Table 1. A complete separation of the D- and L-asparaginases was effected on the first QAE-Sephadex A-50 column. The D-asparaginase was pure by the criteria of a single band on polyacrylamide-gel electrophoresis and on cellulose acetate electrophoresis. The molecular weight of D-asparaginase, determined by gel-filtration chromatography on Sephadex G-200, was about 60 000. The results of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis suggest the enzyme may be made up of two 30 000-mol.wt. subunits. The isoelectric point of the enzyme was 4.8.

The enzyme contained less than 0.01 mol of carbohydrate/mol of enzyme. The determination of free thiol groups yielded a ratio of 0.06 mol of 3-carboxylato-4-nitrothiophenolate formed/mol of enzyme, and it was concluded that no free thiol groups are present. The disulphide analysis gave 6.65, 5.58 and 5.89 mol of 3-carboxylato-4-nitrothiophenolate formed/mol of enzyme in three determinations, indicating the presence of 6 disulphide bonds/molecule. Control analyses on egg-white lysozyme, which has 4 disulphide bonds/molecule (Canfield & Liu, 1965), gave values of 3.73, 4.14 and 4.23 mol/mol.

The activity of D-asparaginase was unaffected by phenylglyoxal (Takahashi, 1968), by salicylaldehyde (Ajitai *et al.*, 1970) or by phenylmethanesulphonyl fluoride (1.6 mM), suggesting that none of arginine, lysine or serine is involved at the active site.

Ethoxyformic anhydride (3 mM) strongly inhibited the enzyme, but this inhibition did not occur in the presence of 19 mM-hydroxylamine hydrochloride, suggesting that an active-site histidine residue had been acylated (see, e.g., Berger, 1975). When the reaction of the enzyme with ethoxyformic anhydride was monitored spectrophotometrically, an increase in absorbance was seen at 242 nm, but not at 278 nm. The increase was reversible with hydroxylamine hydrochloride. These results tend to confirm that histidine is involved at the active site of the enzyme (Muhlrad *et al.*, 1967).

D-Asparaginase displayed zero activity towards 40 mM concentrations of all other substrates tested, including L-asparagine and D- and L-glutamine. Because of the thermal decomposition of glutamine at 75°C (Ratcliffe & Drozd, 1978), these results

Table 1. *Purification of D-asparaginase from Thermus aquaticus*

For full experimental details see the text. One unit of activity is defined as the amount of enzyme giving 1 mmol of NH_4^+ in 30 min under the conditions of the assay. Results are the average for four experiments.

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Relative purification	Yield (%)
1. High-speed supernatant (crude extract)	5000	12000	2.4	1	100
2. QAE-Sephadex A-50 step gradient	450	6360	14	5.9	53
3. Hydroxyapatite step gradient	105	4200	40	16.7	35
4. Sephadex G-200	21	3600	170	71	30
5. Hydroxyapatite linear gradient	10	2520	240	100	21
6. QAE-Sephadex A-50 linear gradient	6.9	1920	280	117	16
7. Cellulose acetate electrophoresis	3.9	1200	310	129	10
8. Crystallization	1.9	720	380	153	6

were confirmed by assays run at 50°C with the use of a more-sensitive but non-quantitative, chromatographic assay method for end products.

The pH optimum of the enzyme was pH 9.5, with 90% activity at pH 8.5 and 10.5.

At 75°C over 90% of the enzyme activity is retained after 10 h. At 85°C the half-life is 25 min. Fig. 1 shows the effect of temperature on the activity of the enzyme. The change in the activation energy of the enzyme at 60°C may be the result of a conformational change, such as has been observed in other enzymes from thermophilic bacteria (see, e.g. Amelunxen & Murdock, 1978). The K_m of the enzyme is the same at 75°C and at 48°C, namely 2 mM. This is of the same order as that for the L-asparaginase for the same organism (M. P. Curran, G. R. Guy & R. M. Daniel, unpublished results) and that for the D-asparaginase from *Brucella abortus* (Altenbern & Housewright, 1954).

D-Asparaginase is strongly inhibited by the keto acids 2-oxoglutarate, pyruvate and oxaloacetate. No inhibition is shown in the presence of any of the amino acids that might be expected to be biosynthetic end products of D-asparaginase action, such as cysteine (Jayaram *et al.*, 1968). This may indicate that the major role of the enzyme is in providing energy through the supply of 2-oxoglutarate into the tricarboxylic acid cycle or in the synthesis of L-amino acids via α -oxo acids, rather than in the biosynthesis of D-amino acids.

Activity is considerably diminished if the assay is performed in the presence of high concentrations of NaCl (Table 2), and a similar effect is found with KCl. The L-asparaginase of *Escherichia coli* retains 65% of its activity in 1 M-NaCl (results not shown) but is known to lose its quaternary structure in 5 M-NaCl (Wriston & Yellin, 1973), so this is a possible reason for activity loss here. However, high salt concentrations have also been shown to cause

Fig. 1. *Effect of temperature on the activity of Thermus aquaticus D-asparaginase*
For full experimental details see the text.

Table 2. *Inhibitors and activators of Thermus aquaticus D-asparaginase*

For full experimental details see the text. The following had no effect at 20 mM: L-asparagine, and neither isomer of aspartic acid, alanine, cysteine, isoleucine, leucine, methionine, valine and threonine.

Modifier	Concentration (mM)	Activity (%)
Sodium oxaloacetate	5	8
Sodium 2-oxoglutarate	5	3
Sodium pyruvate	5	5
MgSO ₄	0.1	115
MgSO ₄	10	124
EDTA	0.1	100
NaCl	100	80
NaCl	1000	18
NaCl	2000	5
p-Chloromercuribenzoate	0.2	98

loss of activity in other proteins from thermophiles (Perutz & Raidt, 1975; Chell & Sundaram, 1978), an effect proposed to be due to destabilization of external salt bridges. Mg²⁺ raises activity somewhat, but 0.1 mM-EDTA does not affect activity.

D-Asparaginase was produced by the organism in somewhat larger amounts than the L-asparaginase. By using the scoring method of Peterson & Ciegler (1969) the ranges of activities of the D- and L-asparaginases were 45–65 and 25–35 respectively. Growth on different media, e.g. with substitution of L-asparagine, DL-asparagine, L-glutamine or L-glutamic acid for the (NH₄)₂SO₄ in the normal medium, did not significantly affect the ratio of D- to L-asparaginase; nor was the ratio different at different points during growth.

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