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Thermostability of L-phenylalanine aminotransferase from thermophilic bacteria*

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Summary. Various mesophilic and thermophilic bacteria were screened for the presence of thermostable L-phenylalanine aminotransferases. With organisms from culture collections best results were obtained with *Thermus aquaticus* and *Bacillus caldolyticus*. Cell-free extracts of these bacteria contained enzymes which did not lose activity by heat treatment at 60°C for 25 min, although they became rapidly inactivated during incubation at 70°C. *Bacillus* species able to grow at 70–75°C in mineral medium with phenylalanine as the sole carbon- and energy source were subsequently isolated in pure culture. At 70°C *Bacillus* strain IS1 grew on phenylalanine with a doubling time of 35 min and synthesized a phenylalanine aminotransferase which only slowly lost activity when incubated at 70°C and was stable at 60°C for at least 7 h.

During the purification of the phenylalanine aminotransferase from *Bacillus* IS1 only a single peak of activity was observed consistently. This enzyme showed activity with phenylalanine and tyrosine but not with aspartate. The apparent K_m values for phenylalanine and tyrosine were 0.95 and 0.77 mM, respectively. The enzyme had an optimum pH of 6.4 and a temperature optimum of 71.5°C for the deamination of phenylalanine. Similar levels of the enzyme were synthesized during growth of *Bacillus* IS1 on a variety of substrates, suggesting that it functions in phenylalanine (and tyrosine) biosynthesis rather than in phenylalanine catabolism.

Introduction

The last step in the biosynthesis of L-phenylalanine is generally catalyzed by aromatic amino acid aminotransferase (Umbarger 1980). This enzyme transaminates phenylpyruvate to L-phenylalanine using aspartate or glutamate as the amino donor. In some organisms able to grow on phenylalanine as a carbon- or nitrogen source, inducible phenylalanine aminotransferases have been detected which catalyze the initial step in phenylalanine degradation (Kishore et al. 1976; Lee and Desmazaud 1985; Lee et al. 1985; Ziehr and Kula 1985). The enzyme may be used for the amination of phenylpyruvate resulting in the stereospecific synthesis of L-phenylalanine (Ziehr and Kula 1985; Yonaha and Soda 1986). Currently there is a strong interest in biocatalysts from thermophilic organisms. In addition to the higher thermostability of these enzymes they are generally also more resistant to denaturing agents and more tolerant to higher solute concentrations (Sonnleitner 1983; Sonnleitner and Fiechter 1983; Ng and Kenealy 1986). We have studied the thermostability of phenylalanine aminotransferase in a number of (thermophilic) bacteria. In this paper we report the purification and characterization of a phenylalanine aminotransferase from *Bacillus* IS1, a thermophilic bacterium able to use phenylalanine as a growth substrate.

Materials and methods

Microorganisms and growth conditions. The strains used and their growth temperatures are listed in Table 1. *Escherichia coli* K12 was grown in mineral medium (medium A; Miller 1972) with glucose (10 mM). *Nocardia* sp. 239 (Hazeu et al. 1983), *Bacillus* C1 (Clement et al. 1986), and *Bacillus* IS1, *Bacillus* IS2 (see below) were grown at pH 7.0 in mineral medium contain-

* Dedicated to Prof. Dr. H. J. Rehm on the occasion of his 60th birthday

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Table 1. Characteristic properties of strains used

Strain	Growth and assay temperatures (°C)		Phenylalanine amino-transferase activity (nmol·min ⁻¹ ·mg ⁻¹ prot.)
<i>Escherichia coli</i> K12	37	37	146
<i>Nocardia</i> sp. 239	37	37	47
<i>Bacillus</i> C1	50	45	190
<i>Bacillus acidocaldarius</i>	55	45	131
<i>Bacillus flavothermus</i>	55	45	49
<i>Bacillus stearothermophilus</i>	65	55	180
<i>Bacillus caldolyticus</i>	70	60	85
<i>Thermus aquaticus</i>	70	60	306
<i>Bacillus</i> IS1	70	60	175
<i>Bacillus</i> IS2	75	65	139

ing (per litre): NaH₂PO₄, 1.5 g; K₂HPO₄, 4.65 g; (NH₄)₂SO₄, 1.0 g; MgSO₄, 0.2 g; trace element solution, 0.2 ml (Vishniac and Santer 1957), plus glucose (10 mM), methanol (100 mM) or L-phenylalanine (7.5 mM), respectively. *Bacillus acidocaldarius* ATCC 27009 (Darland and Brock 1971) was grown at pH 3.5 in glucose (10 mM)-yeast extract (0.5 g/l) medium. *Bacillus stearothermophilus* 1503-4R (ATCC 7954), *Bacillus flavothermus*, and *Bacillus caldolyticus* were grown at pH 7.0 in trypton (10 g/l), yeast extract (5 g/l), sodium chloride (10 g/l) broth. The latter two strains were obtained from Dr. W. Heinen, University of Nijmegen, The Netherlands, *Thermus aquaticus* YT-1 (ATCC 25104) was grown in Castenholz medium (DSM medium 86).

Growth was in conical flasks filled for 25% of the volume in a shaking waterbath set at 200 rpm. Growth was followed by measuring the absorbance of the cultures at 433 nm using a Vitatron 280 colorimeter (Vitatron, Dieren, The Netherlands).

Isolation of *Bacillus* IS1 and *Bacillus* IS2. Organisms able to grow in mineral medium (see above) with L-phenylalanine as the sole carbon- and energy source were isolated from the waste water treatment system of a sugar beet factory. After repeated transfers in this medium, cells were plated out on phenylalanine (7.5 mM)-gelrite (15 g/l; Kelco, San Diego, USA) and incubated at 70 or 75 °C. Single colonies were picked and transferred several times to check purity.

Preparation of cell-free extracts. Cells from the mid-exponential phase of growth were harvested by centrifugation at 6,000 × g for 15 min at 4 °C, washed once and resuspended in 50 mM potassium phosphate buffer pH 7.5. Cells were disrupted by sonication for 3 min at 4 °C in a MSE 100W ultrasonic desintegrator. Unbroken cells and debris were removed by centrifugation at 40,000 × g for 30 min at 4 °C. The supernatants, which contained 2–3 mg/ml protein, were used as cell-free extracts for enzyme assays.

Assay of L-phenylalanine aminotransferase (EC 2.6.1.5.). In order to avoid possible instabilities of coupling enzymes at higher incubation temperatures, a discontinuous assay for the formation of phenylpyruvate was used, based on its absorbance at 318 nm in alkaline environment (Scandurra and Cannella 1969; Collier and Kohlhaw 1972). The reaction mixture (0.5 ml) was incubated in closed Eppendorf tubes in a waterbath and contained: 100 mM potassium phosphate buffer pH 6.5; 20 mM L-phenylalanine; 0.2 mM pyridoxal-5-phosphate; and limiting amounts of extract. After temperature equilibra-

tion for 5 min, the reaction was started by the addition of 20 mM neutralized 2-oxoglutarate. At various time intervals the reaction was terminated by the addition of 0.5 ml 2 M NaOH. After 5 min at room temperature the absorbance at 318 nm was determined in a Hitachi-Perkin Elmer 139 single beam spectro-photometer, against a zero time blank to which NaOH had been added before the 2-oxoglutarate. The molar absorption coefficient of phenylpyruvate at 318 nm was taken as 17,500. Enzyme activities are expressed as nmol of phenylpyruvate formed·min⁻¹·mg⁻¹ of protein. The thermostability of the various phenylalanine aminotransferases was determined by incubating 0.1 ml cell-free extract in closed Eppendorf tubes in a waterbath at various temperatures. After cooling on ice the remaining activities were measured as described above.

Assay of tyrosine aminotransferase (EC 2.6.1.5.). The assay for tyrosine aminotransferase activity was identical to that described above for phenylalanine aminotransferase, except that L-tyrosine (2 mM) was substituted for phenylalanine. After addition of NaOH the solution was incubated for 30 min at room temperature and the absorbance at 330 nm determined. The molar absorption coefficient of 4-hydroxyphenylpyruvate at 330 nm was taken as 19,500 (Collier and Kohlhaw 1972).

Assay of aspartate aminotransferase (EC 2.6.1.1.). The assay for aspartate aminotransferase activity was based on the 2-oxoglutarate dependent formation of oxaloacetate from aspartate. The reaction mixture (1.0 ml) contained: 100 mM potassium phosphate buffer pH 6.5 or 100 mM Tris. HCl buffer pH 8.0; 10 mM L-aspartate; 0.2 mM pyridoxal-5-phosphate; 10 mM neutralized 2-oxoglutarate and enzyme. The molar absorption coefficient of oxaloacetate at 265 nm was taken as 780 (Collier and Kohlhaw 1972).

Purification of phenylalanine aminotransferase. Enzyme purification was performed with a fast protein liquid chromatography (FPLC) system which consisted of a Model GP-250 gradient programmer, Model P-500 high precision pumps, a Model V-7 valve, a Single Path Monitor UV-1 and a Frac-100 fraction collector (Pharmacia, Uppsala, Sweden). The enzyme was purified via a two step anion exchange chromatography procedure on a Mono Q HR 5/5 column. The flow rate was set at 1.0 ml/min and 0.5 ml fractions were collected. After preparation of cell-free extracts (6 mg protein per ml) of phenylalanine-grown cells of *Bacillus* IS1, samples (0.5 ml) were injected and eluted with a linear sodium chloride gradient (0–1 M) in

20 mM Bis-Trispropane buffer pH 7.0, containing 0.05 mM pyridoxal-5-phosphate. Enzyme activities were measured as described above and peak fractions were pooled for further purification. After desalting on a Sephadex G25 (fine) column (1 × 5 cm) as described by de Koning et al. (1987), a sample (2.5 ml) containing 0.3 mg protein was again applied to a Mono Q column and eluted with a non-linear sodium chloride gradient (0–1 M; see Fig. 5). Fractions were analyzed for phenylalanine aminotransferase activity.

Molecular weight determinations. Molecular weight of the purified phenylalanine aminotransferase preparation was estimated by gel filtration on a Superose 6 column. Samples (0.2 ml) containing 50 µg protein were injected and eluted with a 50 mM potassium phosphate/150 mM sodium chloride buffer pH 7.0, with a flow rate of 0.4 ml/min at room temperature. Thyroglobulin, mw 670,000; gamma globulin, mw 150,000; ovalbumin, mw 44,000; myoglobin, mw 17,000; and vitamin B12, mw 1,350, were used as markers (gel filtration standards of Bio-Rad, Richmond, USA).

SDS-PAA gel electrophoresis. Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed according to Laemmli and Favre (1973) with the calibration proteins Combithek 104558 from Boehringer (Mannheim, FRG) as references. After electrophoresis the gels were stained with the silver staining technique as described by Wray et al. (1981).

Protein determination. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Results

Using crude cell-free extracts, the activity of phenylalanine aminotransferase(s) at pH 6.5 was determined in various organisms (Table 1). For each of the organisms an assay temperature was selected identical to or slightly below the growth temperature. At these assay temperatures no loss of enzyme activity occurred during an incubation period of at least 30 min. In all assays the observed rate was linear for at least 20 min and proportional to the amount of extract added (Fig. 1). Phenylpyruvate is a relatively unstable compound which is known to decompose spontaneously in, amongst others, phenylacetaldehyde (Cavallini and Stirpe 1957). However, in control experiments run at the various assay temperatures, no significant loss of phenylpyruvate was evident over the time period used.

The thermostability of the enzyme(s) synthesized by the various organisms was tested over a range of temperatures (Fig. 2). The enzymes present in the mesophilic and thermotolerant bacteria *Escherichia coli*, *Nocardia* sp. 239 and *Bacillus* C1, were stable when cell-free extracts were incubated at the respective growth temperatures but rapidly lost activities at higher temperatures (Fig. 2A). In

Fig. 1. Phenylpyruvate formation from phenylalanine and 2-oxoglutarate by cell-free extracts of *Bacillus* IS1 incubated at 60°C. Enzyme activity: 168 nmol·min⁻¹·mg⁻¹ of protein. ● 7.5 µg of protein; ○ 15.0 µg of protein

Fig. 2. A, B. Thermostability of phenylalanine aminotransferase in cell-free extracts of various bacteria. After heat treatment the enzyme solution was cooled on ice and the remaining activity assayed. The assay temperatures used for the various bacteria and the initial activities are indicated in Table 1. The maximum incubation temperature tested at which 100% activity was maintained is given between brackets. A. ●, *Escherichia coli*, 55°C (37°C); ○, *Nocardia* sp. 239, 50°C (37°C); ▼, *Bacillus* C1, 65°C (45°C); ▽, *Bacillus flavothermus* 60°C (45°C). B. ●, *Bacillus acidocaldarius*, 65°C (55°C); ○, *Bacillus caldolyticus*, 70°C (60°C); ▼, *Bacillus stearothermophilus*, 70°C (55°C); ▽, *Thermus aquaticus*, 70°C (60°C); ■, *Bacillus* IS1, 70°C (60°C); □, *Bacillus* IS2, 75°C (70°C); ×, *Bacillus* IS2, 70°C

comparison the enzymes present in thermophilic bacteria displayed a considerably enhanced thermostability (Fig. 2B). With bacteria from culture collections best results were obtained with *Thermus aquaticus* and *Bacillus caldolyticus*. These or-

ganisms possess enzymes which were stable at 60°C for at least 30 min although they became inactivated rapidly when cell-free extracts were incubated at 70°C, their growth temperature.

In an attempt to select microorganisms possessing phenylalanine aminotransferases with a higher thermostability, a mineral salts medium with L-phenylalanine as the sole carbon- and energy source was inoculated with samples from various sources and incubated over a range of temperatures. Especially with samples taken from a waste water treatment system from a sugar beet factory, isolates able to grow at temperatures between 60–75°C were readily obtained in pure culture. Invariably these organisms were Gram-positive, spore-forming, aerobic organisms. No growth occurred in samples incubated at 80°C or higher. Isolate *Bacillus* IS1 displayed doubling times in phenylalanine medium of 35 min (at 70°C), 45 min (at 75°C) and 100 and 185 min at 65°C and 60°C, respectively. With *Bacillus* IS2 a doubling time of 85 min was observed on phenylalanine at the optimum growth temperature of 75°C. Further studies showed that both organisms, in addition to L-phenylalanine only could use a limited number of other substrates. At 70°C *Bacillus* IS1 grew with glutamate ($t_d=70$ min), glucose ($t_d=240$ min), acetate ($t_d=95$ min), ethanol ($t_d=80$ min) and trypton/yeast extract ($t_d=50$ min). No growth was observed with tyrosine, tryptophan, glutamine, asparagine, betaine, glycine, valine, threonine, arginine, erythritol or xylulose. In cell-free extracts the phenylalanine aminotransferase from *Bacillus* IS1 remained stable at 60°C but slowly lost activity when incubated at 70°C for 25 min. The activity present in *Bacillus* IS2 on the other hand remained stable at 70°C but slowly lost activity when incubated at 75°C (Fig. 2B). From these data inactivation rate constants at a number of temperatures were determined. By interpolation of Arrhenius plots the inactivation temperatures (50% loss of activity in 10 min: rate constant = 0.069 min^{-1}) for the enzymes from *B. stearothermophilus*, *Thermus aquaticus*, *Bacillus* IS1, and *Bacillus* IS2 were subsequently estimated to be 71.8, 76.5, 77.5, and 84.5°C, respectively (Fig. 3). Judging from its stability at 70°C (for at least 25 min) and its inactivation temperature, the enzyme from *Bacillus* IS2 is most stable. Prolonged incubation of extracts at 60°C, however, revealed that the *Bacillus* IS2 enzyme lost about 40% of its activity within 6 h (Fig. 4). The *Bacillus* IS1 enzyme on the other hand remained stable for at least 7 h and even increased in activity in the first few hours. Based on these

Fig. 3. Arrhenius plots for the inactivation of phenylalanine aminotransferases in cell-free extracts of: *Bacillus stearothermophilus*, ●; *Bacillus* IS1, ○; *Thermus aquaticus*, □; *Bacillus* IS2

Fig. 4. Thermostability of phenylalanine aminotransferase in cell-free extracts of *Bacillus* IS1 (●) and *Bacillus* IS2 (○) during incubation at 60°C for prolonged periods of time

observations we decided to purify and characterize the enzyme from *Bacillus* IS1 in more detail.

During Mono Q anion exchange chromatography a single peak of phenylalanine aminotransferase activity was consistently observed at approximately 230 mM sodium chloride. The elution profile obtained in a second run, with a non-linear sodium chloride gradient is shown in Fig. 5. The peak fraction possessed a specific activity of

Fig. 5. Mono Q FPLC elution profile of the pooled peak fractions obtained during anion exchange chromatography of cell-free extracts of *Bacillus* IS1. —, absorbance at 280 nm; ---, sodium chloride gradient; O, phenylalanine aminotransferase activity ($\text{nmol} \cdot \text{min}^{-1} \cdot \text{ml}^{-1}$)

$2.0 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein (overall purification 12-fold). Several runs were performed and the fractions containing the highest phenylalanine aminotransferase activities were pooled and used for further studies. No loss of activity occurred during these steps and the enzyme was stable at room temperature and at 4°C for prolonged periods of time.

When using the FPLC gel filtration technique (see Methods) a single peak of activity was detected and a molecular weight of $45,000 (\pm 2000)$ was determined for the native enzyme. When the preparations were subjected to SDS-polyacrylamide gel electrophoresis one major band in the 43,000 dalton region was observed, indicating that the enzyme is monomeric in nature. Enzyme activities were determined at 60°C over a range of pH values. A clear pH optimum was observed at 6.4 (Fig. 6A). The phenylalanine aminotransferase activity gradually increased with temperature and reached a maximum at 71.5°C (Fig. 6B). A rapid decrease in activity occurred at higher temperatures.

In addition to phenylalanine the enzyme also showed activity with tyrosine but not with aspartate. Apparent K_m values for phenylalanine and tyrosine, estimated from Lineweaver-Burk plots, were 0.95 and 0.77 mM, respectively. The enzyme displayed similar specific activities in glucose-, acetate- or ethanol-grown cells of *Bacillus* IS1

(180, 177 and $162 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein, respectively).

Discussion

While assaying for the phenylalanine aminotransferase activities in a variety of bacteria no attempts were made to optimize reaction conditions for the individual organisms. The data in Table 1 therefore may be an underestimation of the enzyme activities present. This approach nevertheless allowed a rapid screening of the thermostability of the major enzyme(s) synthesized by these organisms. For a comparison, and in view of the fact that the majority of industrial thermostable enzymes are currently produced by mesophiles (Ng and Kenealy 1986), both mesophilic and thermophilic strains were included in this study. The data obtained clearly indicate that with these strains there is a good correlation between the growth temperature of a particular organism and the thermostability of its phenylalanine aminotransferase(s) (Fig. 2). Similar observations have been made for instance for extracellular proteases (Cowan et al. 1985). No further experiments were performed to exclude a possible enzyme inactivation by proteases in cell-free extracts. However, with every organism studied we observed that the enzyme retained 100% of its activity when cell-

Fig. 6. pH (A) and temperature (B) optimum of phenylalanine aminotransferase purified from *Bacillus* IS1. A. Assayed at 60°C for 10 min. The activity obtained at pH 6.4 ($686 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein) was taken as the 100% value. B. Assayed at pH 6.4 for 10 min. The activity obtained at 71.5°C ($1150 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ protein) was taken as the 100% value

free extracts were incubated at the growth temperature, or 5 or 10°C below that value (compare Table 1 and Fig. 2). It is therefore considered unlikely that proteolysis was a major cause for enzyme inactivation.

To screen for microorganisms possessing enzymes with enhanced thermostability, enrichment cultures with phenylalanine mineral salts medium were subsequently incubated over a range of temperatures. Since in a number of organisms phenylalanine catabolism is initiated by an inducible aromatic amino acid aminotransferase, an enzyme which compared to the biosynthetic enzyme is synthesized up to relatively high levels (Jensen and Calhoun 1981), this amino acid was chosen as the carbon substrate. Pure cultures of *Bacillus* species able to grow at 70 and 75°C were readily obtained. The two strains which have been further characterized in the present study, *Bacillus* IS1 and *Bacillus* IS2, are unusual in that they are able to grow rapidly in both complex and mineral salts media without requiring further growth factors (Sundaram 1986). Their taxonomic position is still unknown. During short incubation periods (25 min) at various temperatures the phenylalanine aminotransferase activity present in *Bacillus* IS2 appeared to be most thermostable, displaying an inactivation temperature (half-life 10 min) of 84.5°C (Figs. 2 and 3). However, during prolonged incubation at 60°C the enzyme present in *Bacillus* IS1 remained stable for at least 7 h, whereas the activities present in *Bacillus* IS2 became inactivated to a considerable extent (Fig. 4). The subsequent partial purification of the phenylalanine aminotransferase from *Bacillus* IS1 and its characterization revealed that it is an aromatic amino acid aminotransferase, active with both phenylalanine and tyrosine, but not with aspartate. A similar substrate specificity has been reported for the aromatic amino acid aminotransferases in *Bacillus subtilis* (Weigent and Nester 1976; Mavrides and Comerton 1978) whereas the *E. coli* enzyme shows activity with aspartate as well (Powell and Morrison 1978). The observation that the enzyme present in *Bacillus* IS1 is synthesized constitutively suggests that it functions in aromatic amino acid biosynthesis rather than in phenylalanine catabolism. The presence of other phenylalanine degrading enzymes in *Bacillus* IS1 has not been investigated. In view of its high stability the phenylalanine aminotransferase from *Bacillus* IS1 appears to be a suitable enzyme for the stereospecific production of L-phenylalanine.

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