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The highly stereospecific enzyme catalysed transamination of 4-fluorophenylglyoxylic acid to 4-(S)-fluorophenylglycine

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Abstract

Cell free extracts from a number of bacterial strains belonging to the *Pseudomonas* and *Bacillus* genera were found to catalyse the conversion of 4-fluorophenylglyoxylic acid to 4-(S)-fluorophenylglycine in high optical purity (99% ee).
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1. Introduction

Arylglycines are an important class of non-proteinogenic amino acids that are found in a number of important biologically active compounds [1]. For example, ρ-hydroxyphenylglycine is a side-chain constituent of the antibiotic amoxicillin [2], and more functionalised arylglycines are found in numerous peptides and glycopeptides, such as the vancomycins [3]. The asymmetric synthesis of arylglycines is complicated by the lability of the α-methine proton and consequently renders the asymmetric syntheses of these compounds a challenging task. Various approaches, each with its own limitations, have been developed including: enzymatic resolution of racemic Strecker-derived amides and esters [4–6], Friedal-crafts additions to chiral cationic glycine equivalents [7,8], asymmetric Strecker reactions [9–11], electrophilic amination of chiral enolates [12–14], nucleophilic ring opening of aryl epoxy alcohols [15] and photolysis of active (β-hydroxyamino) carbene–chromium complexes [16]. Our interest in non-proteinogenic amino acids required the synthesis of 4-(S)-fluorophenylglycine. Previously, reported synthesis of 4-(S)-fluorophenylglycine required either an elaborate chemical synthesis employing highly toxic reagents [16] or the enzymatic resolution of the Strecker-derived amide [17]. A more direct approach that has become increasingly recognised [18,19] as a valuable tool in the synthesis of optically pure amino acids involves the use of transaminases. Transaminase mediated by a co-factor, such as pyridoxal 5-phosphate catalyses, the transfer of an amino group to an α-keto-acid in a stereospecific manner. Therefore, in our study, we were interested in identifying a transaminase that would convert the readily accessible keto-acid, 4-fluorophenyglyoxylic acid (1); (Fig. 1) to the amino acid, 4-fluorophenylglycine (2); in a

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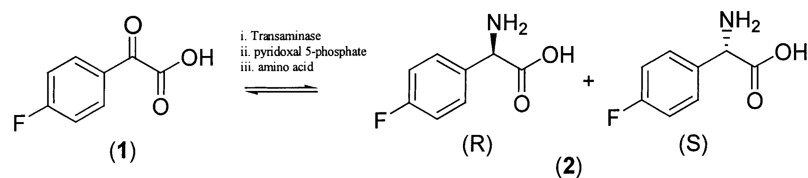

Fig. 1. Reaction scheme for the transamination of 4-fluorophenylglyoxylic acid.

stereospecific manner. We now wish to report our results in identifying bacterial strains belonging to the *Pseudomonas* and *Bacillus* genera in their ability to transaminate 4-fluorophenylglyoxylic acid to 4-fluorophenylglycine in high optical purity.

2. Experimental

2.1. Materials

Chemicals used were of reagent grade and were purchased from either Fisher Scientific (Springfield, NJ), Sigma Chemical Co. (St. Louis, MO) or Aldrich Chemical Co. (Milwaukee, WI), and pre-formulated cultivation media were purchased from Difco (Detroit, MI). The keto-acids were either prepared from the reaction between the appropriate Grignard reagent and diethyl oxolate or purchased from Aldrich Chemical Co. ^{1}H NMR data were obtained on a Bruker 250 DPX (250.13 MHz) and samples were prepared in CD_3OD. ^{13}C NMR data were obtained on a Bruker 250 DPX (62.9 MHz) and samples were prepared in CD_3OD. Mass spectra were recorded on a TSQ-70 triple quadrupole mass spectrometer fitted with a TSP2 thermospray source operating in the positive ion mode.

2.2. Analytical

The HPLC assay for the determination of 4-fluorophenylglycine concentration was performed with a Zorbax RX-C8 (250 mm × 4.6 mm) column at 22°C, and compounds were detected at 210 nm. Separation was achieved employing a gradient elution of two mobile phases at a flow rate of 1 ml min^{-1}. Phase A consisted of 0.1% phosphoric acid in acetonitrile and phase B consisted of 0.1% phosphoric acid. The amount of phase A delivered linearly increased from 10 to 60% over 20 min, and under these conditions 4-fluorophenylglyoxylic acid and 4-fluorophenylglycine eluted after 11.2 and 11.7 min, respectively. HPLC assay for the determination of the optical purity of 4-fluorophenylglycine was performed with a Crownpack CR+ (150 mm × 4.0 mm) column at 22°C, and UV detection at 254 nm. Separation was achieved employing an isocratic mobile phase consisting of 10% methanol in water (acidified to pH 2.0 with perchloric acid) at flow rate of 1 ml min^{-1}. Under these conditions (R) and (S) 4-fluorophenylglycine eluted after 3.0 and 7.0 min, respectively. Spectroscopic confirmation of product identity was obtained from the analysis of material isolated from the enzyme reaction mixture.

2.3. Microbial screening

Bacterial strains were obtained from the Merck Microbial Culture Collection (Merck and Co., Rahway, NJ) or were directly isolated from soil samples obtained in NJ. All microbial cultures were preserved in 25% glycerol at −65°C. Each sample was thawed at room temperature and used to inoculate a medium of tryptic soy broth (TSB) (50 ml) supplemented with β-alanine (4 mg ml^{-1}) and incubated at 30°C for 24 h. The cells were then harvested by centrifugation (8000 rpm for 8 min at 4°C) and the cell pellet suspended in 100 mM KH_2PO_4 (30 ml) and burst under mechanical pressure (French press). The lysate (cell free extract) was separated from cell debris by centrifugation (15,000 rpm, 4°C) and used without purification for the conversion of 4-fluorophenylglyoxylic acid to 4-fluorophenylglycine as follows. A mixture of the cell free extract (3 ml), pyridoxal phosphate (10 mM in 100 mM KH_2PO_4, 1.0 ml), 4-fluorophenylglyoxylic acid (10 mg ml^{-1} in 100 mM KH_2PO_4, 2 ml), amine donor (L-alanine or L-glutamic acid, 1 M in 100 mM KH_2PO_4, 2 ml) in

Table 1
Optical purity and concentration of 4-fluorophenylglycine obtained by each strain

Strain	Amine donor	4-Fluorophenylglycine ($mg\,l^{-1}$)	Optical purity (%S)
Soil isolate 1	Alanine	309	92.8
	Glutamate	209	63.5
Soil isolate 40	Alanine	641	97.2
	Glutamate	209	94.3
Soil isolate 45	Alanine	393	99.9
	Glutamate	127	99.7
Soil isolate 26	Alanine	396	99.9
	Glutamate	183	100.0
Soil isolate 38	Alanine	287	98.0
	Glutamate	897	99.4
Soil isolate 17	Alanine	984	93.6
	Glutamate	237	94.3
Soil isolate 11	Alanine	38	98.9
	Glutamate	164	100.0
Soil isolate 51	Alanine	37	93.9
	Glutamate	146	95.8
Soil isolate 35	Alanine	53	99.9
	Glutamate	2324	99.2
Soil isolate 36	Alanine	209	98.1
	Glutamate	998	99.6
MB 4661	Alanine	1373	86.5
	Glutamate	443	97.3

100 mM KH_2PO_4 (12 ml) was incubated at 37°C with shaking (130 rpm) for 48 h. A sample was then taken and assayed for 4-fluorophenylglycine (Table 1).

2.4. Reaction of cell free extract from soil isolate 35 with 4-fluorophenylglyoxylic acid

Soil isolate 35 was cultivated at 30°C for 24 h in TSB (50 ml), supplemented with β-alanine ($4\,mg\,ml^{-1}$). The cells were harvested by centrifugation (8000 rpm for 8 min at 4°C). The supernate was then decanted off and the cell pellet suspended in 100 mM KH_2PO_4 (30 ml). After a second centrifugation, performed under the same conditions as the first one, the cell pellet was re-suspended in 100 mM KH_2PO_4 (30 ml) and burst under mechanical pressure. The lysate was separated from cell debris by centrifugation (15,000 rpm, 4°C), assayed for protein content (BCA protein assay reagent, and used without further purification for transaminase reaction. A mixture, of 4-fluorophenylglyoxylic acid (20 mg), sodium glutamate (60 mg), pyridoxal 5-phosphate (10 mM, 0.5 ml), cell free extract ($7\,mg\,ml^{-1}$ protein content, 2 ml) in 100mM Tris buffer (7.5 ml) was incubated at 25°C with shaking (150 rpm). Samples were taken periodically and assayed for 4-fluorophenylglycine.

3. Results and discussion

Bacterial strains belonging to the *Pseudomonas* and *Bacillus* genera, obtained from the Merck Microbial Culture Collection (Merck and Co., Rahway, NJ) or directly isolated from soil samples obtained in New Jersey, were evaluated for their ability to transaminate 4-fluorophenylglyoxylic acid to 4-fluorophenylglycine. Each strain (Table 1) was used to cultivate a medium of TSB supplemented with β-alanine. After an incubation period of 24 h the cells were harvested from the culture by centrifugation and burst under mechanical

pressure. The lysate from each was separated from cell debris by centrifugation and used without further purification for the amination of 4-fluorophenylglyoxylic acid to 4-fluorophenylglycine. The transaminase catalysed conversion of keto-acids to amino acids requires pyridoxal phosphate as a cofactor and an amino acid, generally either glutamate or aspartate, to act as the amine donor. The reaction is an equilibrium one that for most transaminases is near unity [20]. Therefore, a large excess of the amine donor was employed to push this equilibrium in favour of the desired amino acid. Evidence for the formation of the desired amino acid was obtained by HPLC analysis and its identity confirmed by NMR and mass spectrometry. The ^{1}H NMR spectrum showed the amino acid present, as well as, buffer. The aromatic region of the spectrum exhibited AA′ BB′X pattern, where $J_{AB} \cong J_{BX}$, $H_{a'}$ and $H_{a'}$, doublet of doublet, 7.54 ppm, $J = 5$ and 10 Hz. $H_{b'}$ and H_b, triplet, 7.16 ppm, $J = 10$ Hz, and the methine proton, broad singlet, 4.64 ppm. For the ^{13}C NMR, because of the low concentration of amino acid in the sample, the signals for the quaternary carbons were not observed. However, signals for both C2 (130 ppm, $^{3}J_{C\text{–}F} = 9$ Hz (C–F *meta*-coupling)) and C3 (116 ppm, $^{2}J_{C\text{–}F} = 22$ Hz (C–F *ortho*-coupling)) aromatic carbons were seen. The mass spectrum exhibited M + H, at $m/z = 170$. The stereochemistry of the amino acid was determined by chiral HPLC and in every case production of the desired (S) enantiomer was observed.

After considering both the yield and optical purity (Table 1) of the amino acid soil isolate 35, with glutamate as the amine donor, was selected for further development. Soil isolate 35 was cultivated at 30°C for 24 h in TSB supplemented with β-alanine. The cells were harvested by centrifugation. The supernate was then decanted off and the cell pellet suspended in 100 mM KH_2PO_4. After a second centrifugation, performed under the same conditions as the first one, the cell pellet was re-suspended in 100 mM KH_2PO_4, and burst under mechanical pressure. The lysate was separated from cell debris by centrifugation, assayed for protein content (7 mg ml^{-1}) and used without further purification for transaminase reaction. A mixture of 4-fluorophenylglyoxylic acid, sodium glutamate, pyridoxal 5-phosphate, lysate (7 mg ml^{-1} protein content) in 100 mM Tris buffer was incubated at 25°C with shaking. Samples were taken periodically and assayed for 4-fluorophenylglycine (Table 2). The best result afforded by the enzyme preparation for the conversion of the keto-acid to (S)-4-fluorophenylglycine was a 41% yield, 98% ee, over 71 h (Table 2). After 71 h the product concentration decreased, presumably due to other enzymes present in the cell free extract capable of utilising the amino acid. In order to screen for substrate specificity, several other substituted phenylglyoxylic acids were tested as substrates for the enzyme, however, the enzyme proved to be highly specific for 4-fluorophenyl glyoxylic acid (Table 3).

Table 2
Yield of 4-(S) fluorophenylglycine from enzyme preparation obtained from soil isolate 35

Time (h)	Product (mg l^{-1})	Yield (%)
23	252	12.5
47	552	27.6
71	820	41.0
94	605	30.3

Table 3
Activity of enzyme preparation for various keto-acids

Substrate	Product (yield)
4-Fluorophenylglyoxylic acid	4-(S)-fluorophenylglycine (41%)
4-Methoxyphenylglyoxylic acid[a]	–
3-Methylphenylglyoxylic acid[a]	–
2-Methylphenylglyoxylic acid[a]	–
4-Methoxyphenylglyoxylic acid[a]	–
4-Ethoxyphenylglyoxylic acid[a]	–
2-Thienylglyoxylic acid[b]	–
2-Furylglyoxylic acid[a]	–
3-Indoylglyoxylic acid[b]	–
3-Indoylmethaneglyoxylic acid[a]	–

[a] Keto-acid recovered.
[b] Numerous compounds observed by HPLC.

4. Conclusion

The highly stereospecfic conversion of 4-fluorophenylglyoxylic acid to (S)-4-fluorophenylglycine has been observed from eleven strains of the *Pseudomonas* and *Bacillus* genera. Soil isolate 35 was favoured for further development because of its high stereospecificity and high activity for the conversion of 4-fluorophenylglyoxylic acid to (S)-4-fluorophenylglycine.

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