รายงานวิจัยฉบับสมบูรณ์

โครงการวิจัยเรื่อง

การศึกษาเอนไซม์ที่พบใหม่ซึ่งแสดงคุณสมบัติ
D-Phenylglycine – L-Glutamate Aminotransferase: การแยกและเตรียมเอนไซม์บริสุทธิ์,
การศึกษาคุณลักษณะของเอนไซม์, และการทำ Gene Cloning

Study of a Potentially New and Useful Bacterial Enzyme,
D-Phenylglycine -L-Glutamate Amiotransferase : Enzyme isolation, Purification,
Physical-Enzymological Characterization, and Gene Cloning

รหัสโครงการ: BRG3980018

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กรกฎาคม 2552

สัญญาเลขที่ BR / 18 / 2539

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

คณะผู้วิจัยขอขอบคุณ สำนักงานกองทุนสนับสนุนการวิจัย (สกว) ที่ให้ทุนสนับสนุนการวิจัย และขอขอบคุณคณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล ที่อำนวยความสะดวกในด้านต่างๆ ช่วยให้การวิจัยในครั้งนี้สามารถดำเนินการสำเร็จได้ด้วยดี

บทคัดย่อ

แบคทีเรีย *Pseudomonas stutzeri* ST-201 แยกได้จากดินในประเทศไทยในการตรวจหาจุลินทรีย์ซึ่งมีเอนไซม์ที่สามารถนำมาใช้สังเคราะห์ D-phenylglycine เมื่อเพาะเลี้ยงบนอาหารที่มี D-phenylglycine เท่านั้นเป็นแหล่งคาร์บอนและไนโตรเจน แบคทีเรียนี้สร้างเอนไซม์ D-phenylglycine aminotransferase (D-PhgAT) ที่สามารถเปลี่ยน D-phenylglycine เป็น benzoylformate ซึ่งถูกสลายต่อไปโดยเอนไซม์อื่นๆ ให้เป็น common metabolites เพื่อการเจริญของแบคทีเรีย เมื่อทำให้เซลล์แตก เอนไซม์ D-PhgAT ได้ถูกแยกให้บริสุทธิ์โดยการตกตะกอนด้วยแอมโมเนียมซัลเฟท, isocratic phenyl agarose chromatography, LiChrospher TMAE 1000 anion-exchange chromatography และ SigmaChrom HIC-Phenyl hydrophobic interaction chromatography ตามลำดับ D-PhgAT ที่แยกได้มีความบริสุทธิ์สูงเมื่อวิเคราะห์ด้วย SDS-PAGE เอนไซม์ D-PhgAT ในสภาพธรรมชาติ มีน้ำหนักโมเลกุลปรากฏ (M_r) ประมาณ 92,000 ซึ่งประกอบด้วย 2 หน่วยย่อยที่มีน้ำหนักโมเลกุลเท่ากันคือ 47,500 และมีค่า isoelectric point (p*I*) เท่ากับ 5.0 เอนไซม์ชนิดนี้เร่งปฏิกริยา transamination ที่ผันกลับได้ ซึ่งจำเพาะสูงเฉพาะกับ D-phenylglycine หรือ D-4-hydroxyphenylglycine เท่านั้น โดยใช้ตัวรับหมู่อะมิโนที่จำเพาะคือ 2-oxoglutarate ซึ่งถูกเปลี่ยนเป็น L-glutamic acid โดยที่ D- และ L-isomers ของ phenylalanine, tyrosine, alanine, valine, leucine, isoleucine และ serine ไม่สามารถเป็นซับสเตรทสำหรับเอนไซม์นี้ได้ D-PhgAT มีอัตราการทำงานสูงสุดที่ pH 9-10 และที่อุณหภูมิ 35-45°ซ ค่า K_M ของเอนไซม์สำหรับ D-phenylglycine และ 2-oxoglutarate ที่อุณหภูมิ 35°ซ, pH 9.5 คือ 1.1 และ 2.4 มิลลิโมลาร์ ตามลำดับ กลไกของปฏิกิริยาเป็นแบบ Ping Pong Bi Bi เอนไซม์ D-PhgAT ถูกยับยั้งได้ดีด้วยตัวยับยั้งของเอนไซม์ที่มี pyridoxal phosphate เป็น co-enzyme โดยทั่วไป

การวิเคราะห์ลำดับกรดอะมิโนส่วนปลายสายด้าน N และภายในสาย ของโมเลกุล D-PhgAT บริสุทธิ์นำไปสู่การโคลน *dpgA* gene จาก genomic DNA ของ *P. stutzeri* ST-201 พบ promoter sequence และ ribosome binding site ที่ควบคุม *dpgA* gene ซึ่งมี 1362 นิวคลีโอไทด์ code สำหรับเอนไซม์ D-PhgAT ซึ่งประกอบขึ้นด้วยกรดอะมิโน 453 ตัว เมื่อนำ *dpgA* gene ไปแสดงออกใน *Escherichia coli* ภายใต้การควบคุมของ T7 promoter พบว่ามีการสร้าง PhgAT ที่ทำงานได้ และสมบัติเหมือน PhgAT ที่เตรียมจาก *P. stutzeri* ST-201 ทุกประการ

D-PhgAT ที่พบในการศึกษานี้เป็นเอนไซม์ใหม่ ที่ยังไม่เคยมีรายงานว่ามีการทำให้บริสุทธิ์และ มีการศึกษาคุณสมบัติต่างๆมาก่อน เอนไซม์นี้น่าสนใจทั้งด้าน วิทยาศาสตร์และ ด้านอุตสาหกรรม เนื่องจากเหตุผล 2 ประการ คือ ประการแรก เอนไซม์นี้มีคุณสมบัติพิเศษคือ “sterio-inverting” transamination activity ซึ่งไม่มีในกลุ่ม aminotransferases เท่าที่ทราบในปัจจุบัน ประการที่สอง D-PhgAT เป็นเอนไซม์ตัวใหม่ที่สามารถนำไปใช้ในการสังเคราะห์ enantiomerically pure D-phenylglycine หรือ D-4-hydroxyphenylglycine โดยใช้สารให้หมู่อะมิโนราคาถูก คือ L-glutamate และ สามารถกระทำได้โดยใช้ปฏิกริยาเอนไซม์ในขั้นตอนเดียว ซึ่งสาร D-phenylglycine และ D-4-hydroxyphenylglycine เป็น side-chains ที่มีความต้องการสูงในอุตสาหกรรมผลิตยาปฏิชีวนะกลุ่ม β-lactam

Keywords: อะมิโนทรานส์เฟอเรส, ดี-ฟีนิลกลัยซีน, ซูโดโมแนส

Abstract

A bacterium, identified as *Pseudomonas stutzeri* ST-201, was newly isolated from Thai soil under a screening program designed to search for microorganisms possessing of enzyme(s) applicable for D-phenylglycine synthesis. When grown on a minimal medium containing D-phenylglycine as the sole carbon and nitrogen source, the bacterium produced D-phenylglycine aminotransferase (D-PhgAT) that converted D-phenylglycine to benzoylformate which was further degraded to other common metabolites. Cell homogenate was prepared and the enzyme was purified by ammonium sulfate precipitation, isocratic phenyl agarose chromatography, LiChrospher TMAE 1000 anion-exchange chromatography and SigmaChrom HIC-Phenyl hydrophobic interaction chromatography. The purified D-PhgAT obtained was apparently homogeneous as analyzed by SDS-PAGE. The molecular weight (M_r) of the native enzyme was estimated to be 92,000. It was composed of two identical subunits, each with a molecular weight (M_r) of 47,500. The isoelectric point (p*I*) of the native enzyme was 5.0. The enzyme catalyzed reversible transamination reactions specific for D-phenylglycine or D-4-hydroxyphenylglycine in which 2-oxoglutarate was an exclusive amino group acceptor and was converted into L-glutamic acid. Neither the D- nor L-isomers of phenylalanine, tyrosine, alanine, valine, leucine, isoleucine or serine could serve as substrates. The enzyme was most active at alkaline pH with maximum activity at pH 9-10. The temperature for maximum activity was 35-45°C. The apparent K_M values for D-phenylglycine and for 2-oxoglutarate at 35°C, pH 9.5 were 1.1 mM and 2.4 mM, respectively. The transamination was found to proceed via a Ping Pong Bi Bi mechanism. The enzyme activity was strongly inhibited by typical inhibitors of pyridoxal phosphate-dependent enzymes.

N-terminal and internal amino acid sequences of purified D-PhgAT were determined leading to cloning of *dpgA* gene from genomic DNA of *P. stutzeri* ST-201. The *dpgA* gene was identified within an ORF of 1362 nucleotides downstream from a potential promoter sequence and a ribosome-binding site, and encodes the 453 amino acids-long D-PhgAT. Expression of the *dpgA* gene under a strong inducible T7 promoter in *E. coli* yielded active D-PhgAT having physical and catalytic properties identical to those of the enzyme produced by *P. stutzeri* ST-201.

The D-PhgAT found in this study is a new enzyme that has not been purified and characterized before. This enzyme is of both academic and industrial interest because it possesses a characteristic “stereo-inverting” transamination activity which is unusual among aminotransferases known to date. It allows utilization of L-glutamate, a cheap amino-group donor, for enzymatic synthesis of enantiomerically pure D-phenylglycine or D-4-hydroxyphenylglycine in a single enzymatic transamination reaction step. Both D-phenylglycine and D-4-hydroxyphenylglycine are important side-chains that are in high demand for the β-lactam antibiotics industry.

Keywords: aminotransferase, D-phenylglycine, *Pseudomonas*

สารบัญ

INTRODUCTION

Research and development of enzymatic process for production of the penicillin nucleus, 6-aminopenicillanic acid (6-APA), has been carried out in the Department of Microbiology and the Department of Biotechnology since early 1980s with considerable success and achievement (Meevootisom et al., 1983; Mevootisom and Saunders, 1987; Panbangred et al., 1990). 6-APA is commercially important in the pharmaceutical industry as a key intermediate from which almost all semisynthetic penicillins are derived. With the advances in enzyme technology, production of 6-APA by the enzymatic process is preferred over the chemical route in terms of economic, environmental and operational advantages. In the main bioconversion process, penicillin acylase (penicillin amidohydrolase, EC 3.5.1.11) is employed to catalyze hydrolysis of the amide bond of penicillin G to produce the β-lactam nucleus, 6-APA, and the corresponding side-chain, phenylacetic acid, is released as a by-product (Figure 1).

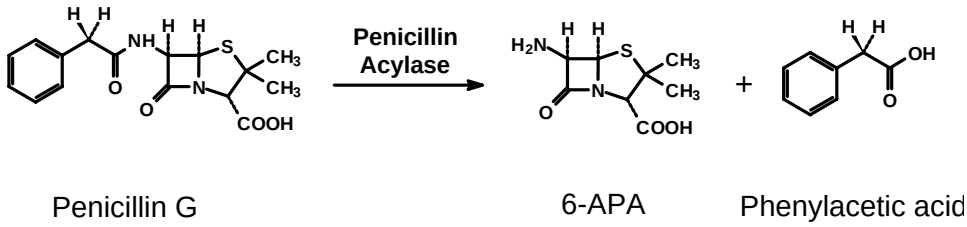

Figure 1. Production of 6-aminopenicillanic acid (6-APA) by penicillin acylase-catalyzed hydrolysis of penicillin G. Phenylacetic acid is released as a by-product.

Industrial scale production of 6-APA by enzyme hydrolysis of penicillin G leads to generation of a large amount of phenylacetic acid. In order to add more value to the 6-APA production process, it is of interest to convert the by-product phenylacetic acid into more useful compound(s). Target compounds which are structurally related to phenylacetic acid are D-(-)-α-phenylglycine and D-(-)-*p*-hydroxy-α-phenylglycine. These two compounds are useful as precursors for production of many semisyntheic penicillins and cephalosporins. D-(-)-α-phenylglycine is the side-chain of ampicillin, cephalexin, cephaloglycin, and cefaclor. D-(-)-*p*-hydroxy-α-phenylglycine is the side-chain of amoxycillin, cephadroxil, and cefatrizine (Figure 2).

To synthesize D-(-)-α-phenylglycine from phenylacetic acid, an amino group cannot be added directly to the α-carbon of phenylacetic acid but three successive reaction steps are required (Figure 3). Chemical or enzymatic modifications should be employed to create an oxo- functional group at the α-carbon of phenylacetic acid so that it can serve as an amino-group acceptor of a transamination reaction.

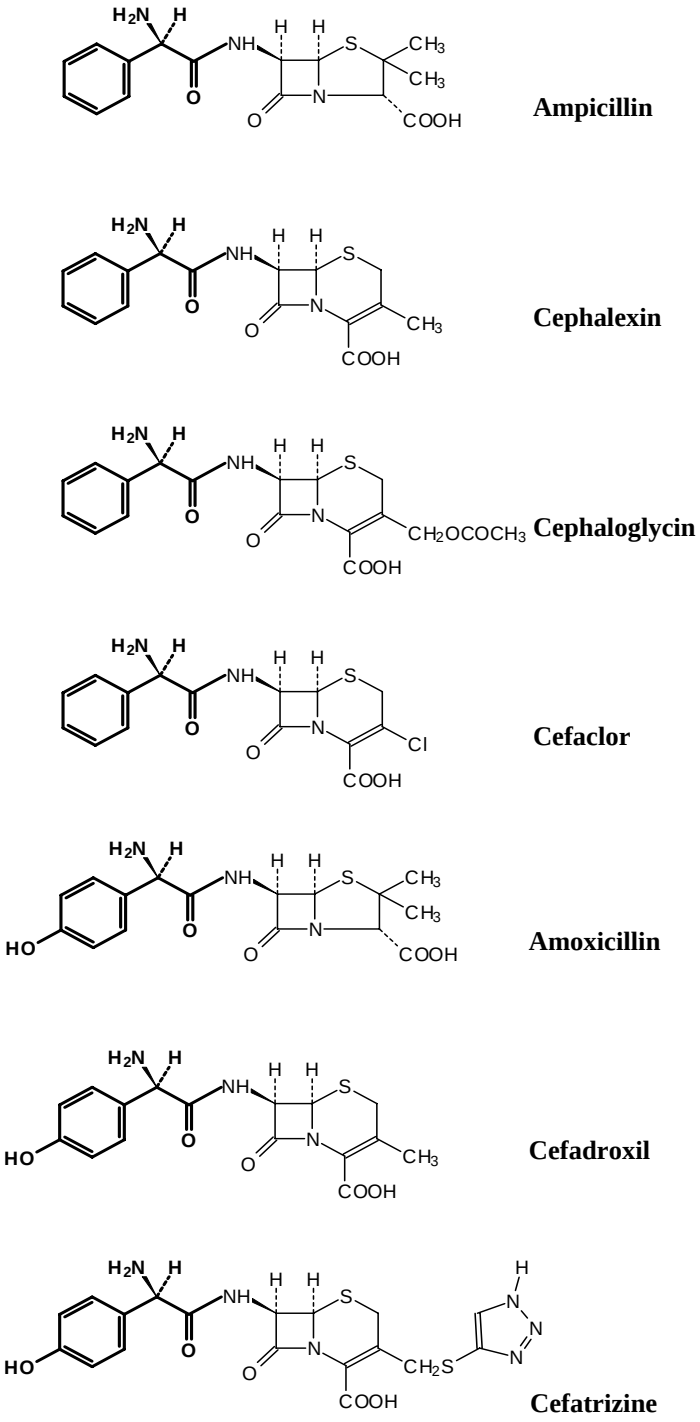

Figure 2. β-Lactam antibiotics having D-phenylglycine and D-*p*-hydroxyphenylglycine side-chains.

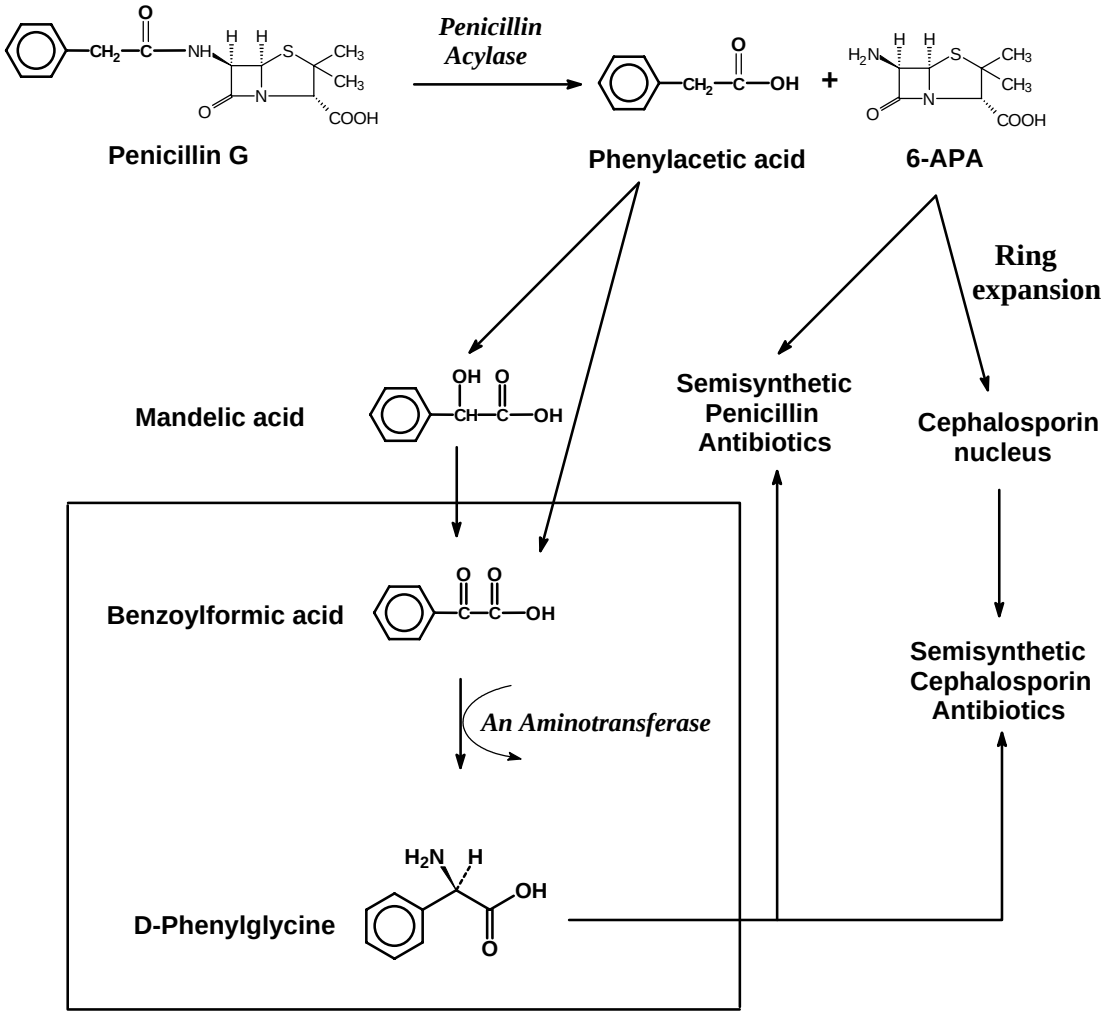

Figure 3. A proposed pathway for synthesis of D-phenylglycine from phenylacetic acid as an extension of the 6-APA production process. D-phenylglycine can be used as a side-chain for semi-synthetic penicillins and cephalosporins.

Thus, phenylacetic acid is first oxidized at the α-carbon to form an α-hydroxy acid (mandelic acid) and then a second oxidation at the α-carbon is required to convert mandelic acid to a 2-oxo acid (benzoylformic acid). In the third step, the benzoylformic acid produced is then converted to D-(-)-α-phenylglycine by a stereospecific transamination. To develop a complete process for production of D-(-)-α-phenylglycine from phenylacetic acid using the three reaction steps, namely formation of mandelic acid from phenylacetic acid, formation of benzoylformic acid from mandelic acid and formation of D-(-)-α-phenylglycine from benzoylformic acid, chemical and enzymatic methods are considered as follows.

For reaction step 1, the existence of enzymes required to convert phenylacetic acid to mandelic acid was evident from early studies on microbial metabolism of aromatic compounds. A basidiomycete fungus *Polyporus tumulosus* Cooke was found to convert tyrosine to *p*-hydroxyphenylpyruvic acid and further degradation to *p*-hydroxymandelic acid via *p*-hydroxyphenylacetic acid was suggested by Crowden (1967). More recently, there was a report of anaerobic degradation of phenylacetic acid by a mixed culture of various bacteria and a fungus showing that there was an accumulation of 10 to 15 mM of mandelic acid after 110-135 days of cultivation (Sembiring and Winter, 1989). This was a clear demonstration that microbial oxidation of phenylacetic acid to mandelic acid did occur. However, organism(s) or enzyme(s) responsible for this bioconversion have not been identified. Also, the need for anaerobic conditions and the long cultivation time required may limit its usefulness for development towards large scale operations. An ectomycorrhizal fungus *Pisolithus arrhizus* was reported to produce two antifungal compounds identified as (R)-(-)-*p*-hydroxymandelic acid and *p*-hydroxybenzoylformic acid which could inhibit phytopathogenic fungi (Kope et al., 1990). The fungus grew slowly and produced only small amounts of the compounds. The enzymes responsible for synthesis of these two compounds as well as their precursor molecule(s) have not been identified. Whether these two compounds are derived from phenylacetic acid or not, is not known.

There are reports that mammals such as humans, dogs, cats, rabbits and rats excrete mandelic acid as a major urinary metabolite after being dosed with ethylbenzene or styrene (Drummond et al., 1989 and 1990). However, the proposed routes for metabolism of ethylbenzene and styrene to mandelic acid by humans and these animals did not proceed via phenylacetic acid, as shown in Figure 4.

Mandelic acid and *p*-hydroxymandelic acid are naturally occurring compounds. The presence of a hydroxyl group at the benzylic carbon is also found in other natural bio-molecules such as octopamine and norepinephrine, which are products from enzymatic hydroxylation (benzylic-C oxidation) of *p*-tryptamine and dopamine, respectively (Figure 5). Bacterial oxidation of phenylacetic acid usually proceeds via ring oxidation and cleavage. The pathway for phenylacetic degradation through the formation of mandelic acid or *p*-hydroxymandelic acid seems to be a minor route. The existence of such a degradation pathway in bacteria has been speculated or suggested, but so far, the corresponding enzyme(s) that functions in oxidizing the phenylacetic acid to mandelic acid have not been identified or characterized. The name of such an enzyme does not appear in the current Enzyme List of the International Union of Biochemistry and Molecular Biology (IUBMB).

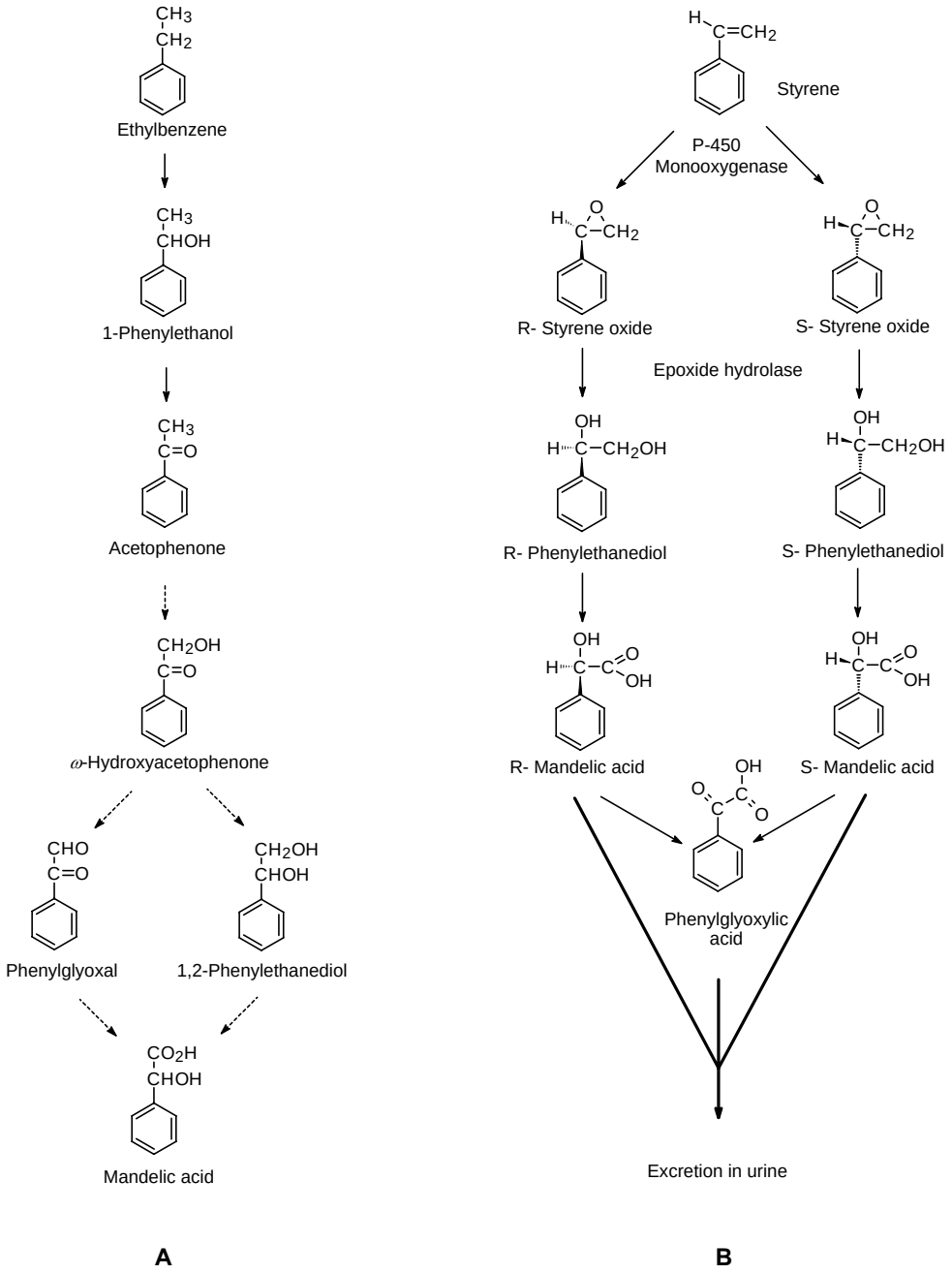

Figure 4. Proposed routes for metabolism of ethylbenzene to mandelic acid (A) (Drummond et al., 1990); and mammalian styrene metabolism (B) (Sumner and Fennell, 1994).

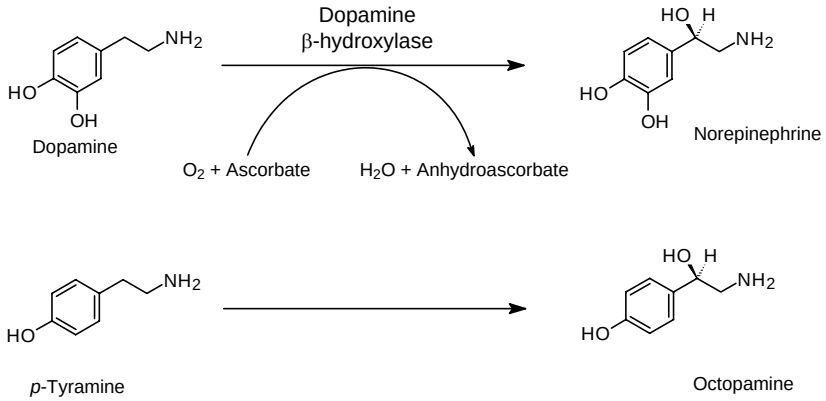

Figure 5. Enzymatic benzylic hydroxylation in biosynthesis of catecholamines.

For conversion of mandelic acid to benzoylformic acid, the enzyme mandelate dehydrogenase was found in *Acinetobacter calcoaceticus* and *Pseudomonas putida* (Hoey and Fewson, 1990). Mandelic acid is a chiral compound since its α-hydroxy group can be in either the D- or L- configuration, so it can exist as D- or L-mandelic acid or as a racemate. [Note: D-mandelate = (R)-mandelate and L-mandelate = (S)-mandelate]. Consequently, there are two dehydrogenase enzymes which act on the α-hydroxy group of mandelic acid. These are D-mandelate dehydrogenase and the L-mandelate dehydrogenase which accept specifically only their respective stereo-isomeric substrates. Beside these two enzymes, there exists mandelate racemase (EC 5.1.2.2) which catalyzes the inter-conversion of D- and L- mandelic acid. Mandelate dehydrogenases are components of the mandelate (degradation) pathway which is involved in catabolism of aromatic compounds and is found among several strains of *pseudomonads*. D-mandelate dehydrogenase is an integral component of the cytoplasmic membrane and contains FAD as a prosthetic group. This enzyme is related to D-lactate dehydrogenases from *Escherichia coli* and *Acinetobacter calcoaceticus*, and the soluble D-lactate dehydrogenase from *Megasphaera elsdenii* (Hoey et al., 1987). On the other hand, L-mandelate dehydrogenase is a member of the FMN-dependent alpha-hydroxy acid dehydrogenase/oxidase family. FMN-containing oxidoreductases that act on alpha-hydroxy acids have been shown to be structurally related (Diep Le and Lederer, 1991). Enzymes in this group are such as: L-lactate dehydrogenase (EC 1.1.2.3) which catalyzes the conversion of L-lactate to pyruvate; L-lactate 2-monooxygenase (EC 1.13.12.4) (or lactate oxidase) found in *Mycobacterium smegmatis* which catalyzes the conversion of lactate and oxygen to acetate, carbon dioxide and water; glycolate oxidase (EC 1.1.3.15) [or (S)-2-hydroxy-acid oxidase], a peroxisomal enzyme that catalyzes the conversion of glycolate and oxygen to glyoxylate and hydrogen peroxide; long chain alpha-hydroxy acid oxidase (EC 1.1.3.-) a peroxisomal enzyme from rat kidney; and (S)-mandelate dehydrogenase from *Pseudomonas putida* ATCC 12633 (encoded by *mdlB* gene) which catalyzes the oxidation of (S)-mandelate to benzoylformate. Among the enzymes of this family, (S)-mandelate dehydrogenase is the only member that is the membrane-associated enzyme.

The gene for (S)-mandelate dehydrogenase from *Pseudomonas putida* ATCC 12633 was cloned and its DNA sequence was determined (Tsou et al., 1990). Mandelate dehydrogenases were also found in yeasts such as *Rhodotorula graminis* and *R. glutinis*. In these organisms, D-(-)-mandelate dehydrogenase was found to be an NAD(+)-dependent enzyme. L-(+)-mandelate dehydrogenase was found to contain heme and FMN, and was classified as a flavochrome b homologous with yeast L-(+)-lactate dehydrogenases.

To produce D-phenylglycine, a D- specific aminotransferase is required to catalyze amino-group transfer from an amino-group donor to benzoylformate in a stereospecific manner. It is generally known that D-amino-acid aminotransferase (EC 2.6.1.21) catalyzes the transamination of a wide spectrum of α-amino acids and 2-oxo acids. It is absolutely specific for D-isomer amino acids (Jones et al., 1985). Transaminations were found to occur between D-glutamate/2-oxoglutarate as a general substrate/product pair and many other D-amino acid/2-oxo acid pairs (Figure 6).

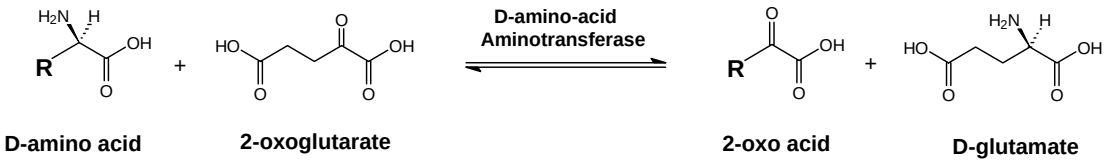

Figure 6. The overall transamination reaction catalyzed by D-amino-acid aminotransferase (Jones et al., 1985).

To apply D-amino-acid aminotransferase for D-phenylglycine production, D-glutamate would have to be used as the amino-group donor. The process would probably be impractical due to the high cost of D-glutamate. To overcome this problem, the cheap amino-group donor, L-glutamate, could be used with an additional enzyme, glutamate racemase. The racemase would convert L-glutamate to D-glutamate until the two enantiomers are at equal concentration. If D-amino-acid aminotransferase and the amino-group acceptor benzoylformate are present, amino-group transfer would occur from D-glutamate to benzoylformate and the desired product D-phenylglycine would be formed together with 2-oxoglutarate. The decrease in D-glutamate concentration caused by its utilization in the transamination reaction would disturb the racemase reaction equilibrium leading to conversion of more L-glutamate to D-glutamate. This, in turn, would shift the transmination equilibrium towards formation of more D-phenylglycine. While this process seems to work well, there is an inherent disadvantage to the use of both racemization and transamination reactions. The disadvantage lies in the fact that the equilibrium constant is equal to 1 for the racemization and is usually near unity for the transamination (Figure 7). This leads to incomplete conversion of the oxo acid, benzoylformate, to the desired product, D-phenylglycine. For example, by starting with equimolar amounts of L-glutamate and benzoylformate, the theoretical yield of D-phenylglycine would be approximately 41% at completion of the reaction. Increasing the initial concentration of L-glutamate by 50% would yield 9% more D-phenylglycine, but would still leave 50% of the bezoylformate unutilized in the reaction mixture.

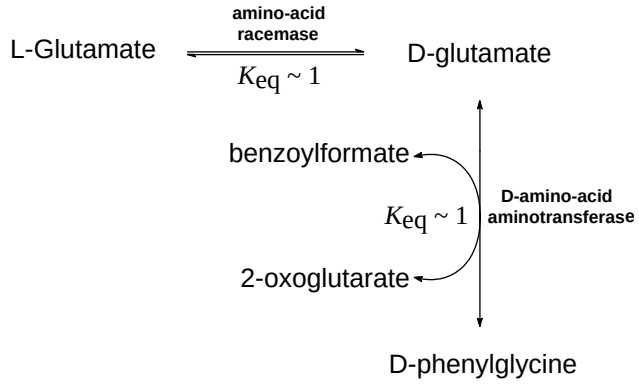

Figure 7. An inherent disadvantage of the use of racemization and transamination reactions for the production of amino acid is that their equilibrium constants are usually near unity. This leads to a low percentage of substrate transformation.

This inherently low conversion ratio is undesirable, not only because the yield is low, but also because the cost of the substrate, benzoylformate, is high. To force the reaction towards more complete conversion of benzoylformate, a larger amount of L-glutamate could be added to the reaction mixture. However, this can be done only up to a certain limit due to the nature of the equilibrium in reactions of this type. The increase in product yield is much less than the proportional increase in concentration of L-glutamate added, especially at high concentrations.

D-Amino-acid aminotransferase was first described in *Bacillus subtilis* by Thorne et al. (1954). It was subsequently found in many gram-positive bacteria. The probable physiological role of this enzyme in bacteria is to produce D-amino acids, and especially D-glutamic acid, which is required for synthesis of peptidoglycan of the bacterial cell wall. A thermophilic *Bacillus* sp. YM-1, was isolated and found to abundantly produce a thermostable D-amino-acid aminotransferase (Tanizawa et al., 1989). The corresponding gene for the D-amino acid aminotransferase was cloned and expressed in *E. coli*. This aminotransferase was applied for synthesis of various D-amino acids (Soda and Esaki, 1994). The synthetic process was rather complicated since it required up to four enzymes altogether as follows. A D-amino acid could be produced by D-amino-acid aminotransferase from D-alanine and the keto analogue of the desired D-amino acid. The pyruvate formed after transmination of D-alanine was aminated to form L-alanine by alanine dehydrogenase (EC 1.4.1.1). L-alanine was racemized by alanine racemase (EC 5.1.1.1) to re-form D-alanine. NADH required for the alanine dehydrogenase reaction was regenerated with formate by the catalytic action of formate dehydrogenase (EC 1.2.1.2) from *Candida* sp. This synthetic process could produce D-isomers of many proteinogenic amino acids, depending on the nature of the keto analogue used. Aromatic amino acids such as D-phenylalanine and D-tyrosine could be produced in high yields. By contrast, enzyme activity for production of D-phenylglycine was relatively very low. D-phenylglycine yield was reported to be much lower than those of the other amino acids despite very large amounts of D-amino-acid aminotransferase were employed. Since D-phenylglycine is a non-proteinogenic

amino acid, its metabolism may require a separate aminotransferase(s) which may differ from the general physiological D-amino-acid aminotransferase.

It would be preferable if the whole process for synthesis of D-phenylglycine from phenylacetic acid could be accomplished economically using enzymatic reactions. The enzymes required for conversion of phenylacetic acid to mandelic acid, and mandelic acid to benzoylformic acid are oxidative enzymes. Up to the present, there are some technical difficulties associated with the use of oxido-reductive enzymes making it impractical for use on a production scale. These enzymes require redox cofactors such as FAD, FMN, NAD(H), NADP(H), etc. which are consumed in the reactions. When the enzymes are isolated from their host organisms and used as free enzymes or immobilized enzymes, the cofactors must be regenerated. Cofactor regeneration can be accomplished if it is coupled with another oxido-reductive enzyme system requiring the same type of cofactor but operates in the opposite direction to the main reaction and utilizes a cheap substrate. However, cofactors for oxido-reductive enzymes are small organic molecules that function as “co-substrates” because they are not tightly bound but rather transiently associated with the enzyme molecules. This leads to a problem of cofactor loss during operation which would increase the total operational cost.

The use of microorganisms as a whole-cell biocatalyst may avoid the need to regenerate cofactors since it is expected that live microorganisms could, to a certain extent, maintain the balance between the oxidized and reduced forms of the cofactors. Such a biocatalyst may be constructed from microorganism possessing the two oxidative enzymes that can convert phenylacetic acid to benzoylformate or by cloning and expressing the corresponding genes (may be from different organisms) in the same host microorganism. The first oxido-reductive enzyme needed, the phenylacetic acid α-hydroxylase, has not yet been reported, but would be expected to be an intracellular (either cytosolic or membrane-bound) enzyme. The second oxido-reductive enzyme reqiured, the mandelate dehydrogenase, has already been reported to be associated with cytoplasmic membranes. The intracellular nature of these two enzymes might create difficulties for the use of whole-cells as a biocatalyst. It would require effective means to transport the phenylacetate substrate across the plasma membrane to meet the enzymes in the cytoplasm, and to transport the benzoylformate out of the cell. Alternatively, effective means to increase permeability of cell membranes for the two compounds with little or no adverse effects upon the cells would have to be devised since metabolically active cells would be essential for the two oxidative enzymes to function properly, and also for the regeneration of their coenzymes within the cells. In addition, accumulation of or exposure to high concentrations of phenylacetic acid, mandelic acid or benzoylformic acid might cause deleterious effects on living cells and thus, any organisms used should be able to tolerate the high concentrations of these compounds.

Synthesis of benzoylformic acid from phenylacetic acid by the enzymatic oxidations will not be economically feasible until a new appropriate technology can be developed to overcome the difficulties and problems mentioned above. At present, what seems to be the more practical methods for large scale production of benzoylformic acid is by using chemical oxidation. The heterogeneous permanganate oxidation method recently described by Zhao and Lee (1994) can be used to selectively oxidize arene compounds at the benzylic position yielding ketones if the α-carbon is secondary, and yielding alcohols if

the α-carbon is tertiary. This is one promising method that could be developed to produce benzoylformic acid from phenylacetic acid at a relatively low cost. Another interesting chemical alternative is the carboxylation of alkylhalides to form α-keto acids (Fell et al., 1985). However, the substrate for synthesis of benzoylformic acid by this method is not the halide-derivative of phenylacetic acid but rather of the related compound, benzoic acid.

All the evidence mentioned so far suggests that enzymatic synthesis of benzoylformic acid from phenylacetic acid may not be practical at present but it could be complemented by chemical methods. The remaining step towards production of D-phenylglycine, the amination of benzoylformic acid, may be carried out more competitively by using a stereospecific D-amino-acid aminotransferase than by the chemical methods. The chemical amination of oxo acids usually yields an amino acid racemate, necessitating an additional chiral selective separation process to recover the required D- enantiomer. By contrast, the stereospecific transamination catalyzed by a D-amino-acid aminotransferase yields an enantiomerically pure D-amino acid and the down-stream enantioselective separation is not required. However, there are some drawbacks associated with the use of the presently known D-amino-acid aminotransferases. Firstly, the D-amino-acid aminotransferases known to date exhibit very low activity towards formation of D-phenylglycine. Secondly, the amino-group donor required by these enzymes is D-glutamic acid which is either expensive or must be prepared by isomerization of its L- antipode using an additional enzyme, glutamate racemase.

The present study initially searched for D-amino-acid aminotransferases which were specific and effective in synthesizing D-phenylglycine. During the screening process, a characteristically distinct aminotransferase was found. It was named D-phenylglycine aminotransferase. The enzyme was purified and found to use L-glutamate as an amino-group donor. Upon transfer of the amino group to benzoylformate, D-(-)-α-phenylglycine was produced. Under optimized conditions, this aminotransferase was found to be active in catalyzing D-phenylglycine formation. This is the first report of a purified aminotransferase that can catalyze a stereo-inverting amino-group transfer between two different amino acids. Previously claims of similar conclusions were made with whole cells or crude extract which might have contained other enzymes such as racemases. The finding of this enzyme will have a positive impact on the enzymatic process for D-phenylglycine production using a transamination approach. L-glutamate is cheap and readily available in large quantity. Its use as an amino-group donor replacing the more expensive D-glutamate with this enzyme system is advantageous over the previously known D-amino-acid aminotransferase and would make the enzymatic process for production of D-(-)-α-phenylglycine competitive.

MATERIALS AND METHODS

1. Isolation of D-phenylglycine aminotransferase producing bacteria

Soil bacteria were isolated from growth on minimal D-phenylglycine (MDP) medium containing in 1 l of distilled water: 15 g agar, 3.40 g KH_2PO_4, 3.55 g Na_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 8 mg $CaCl_2.2H_2O$, 10 mg yeast extract, 1 ml trace metals solution (Beggs and Fewson, 1977) and 1.0 g of D-phenylglycine. Each isolate was cultured in liquid MDP medium at 30°C with shaking until the culture reached an $OD_{600\ nm}$, of approximately 1.0 after which the cells were collected and washed twice. A 10-mg wet weight portion was resuspended in 1 ml of 50 mM potassium phosphate buffer pH 7.0 containing 5 mM D-phenylglycine in the absence or presence of 15 mM neutralized (aminooxy)acetic acid. D-phenylglycine consumption at 30°C was monitored by HPLC. Bacterial isolates which degraded D-phenylglycine rapidly in the absence of, but more slowly in the presence of (aminooxy)acetic acid were selected. Clarified cell homogenates prepared from selected bacteria grown on MDP medium were tested for the presence of an enzyme that could produce D-phenylglycine from benzoylformate and L-glutamate. Taxonomic identification of the bacteria was carried out by investigating characteristic phenotypic properties according to Krieg and Holt (1984), and Smibert and Krieg (1994).

2. D-phenylglycine aminotransferase activity assays

2.1. Method 1

D-phenylglycine aminotransferase activity was determined by measuring D-phenylglycine formation from benzoylformate and L-glutamate. The reaction mixture was composed of 0.1 M CAPS buffer (pH 9.5), 60 mM L-glutamic acid (monosodium), 5 mM benzoylformate sodium, and 50 mM PLP. The mixture was pre-warmed at 35°C and the enzyme solution was added to initiate the reaction. After incubation at 35°C, aliquots of the reaction mixture were taken at intervals, heated in boiling water for 2 min, and kept on ice. D-phenylglycine produced in the enzymatic reaction was quantified by the stereoselective HPLC method of Gil-Av et al. (1980) with modifications. A reversed-phase analytical HPLC column (Spherisorb S5 ODS-2, 25 0.46 cm) was used with a pellicular C-18 guard column. The chiral mobile phase was composed of 2.4 mM copper(II) acetate, 5 mM L-proline and 3% methanol. The chromatograph (Bio-Rad 5000T HPLC system) was operated at room temperature (˜ 27°C) with a flow rate of 1.2 ml min^{-1}, and detection was with UV absorption at 210 nm. Under these conditions, the retention times of D- and L-phenylglycine were 7.8 and 13.2 min, respectively.

2.2. Method 2

Substrate specificity of the enzyme was studied by incubating the enzyme with various D- or L-amino acids as amino donors and 2-oxo acids as amino acceptors in question. Optical isomers of the amino acids consumed or produced in each reaction were analyzed by the HPLC method of Jegorov et al. (1988) with modifications. The analytical column was Spherisorb C-8, 5 mm (250 x 46 mm) with a C-8 guard column. The pre-column derivatization procedure and mobile phase composition were as described in the original method. Detection was with UV absorption at 360 nm. The mobile phase gradient profiles

were re-programmed to optimize for particular amino acids of interest. A constant flow of 0.9 ml min^{-1} was maintained during the analysis.

3. Purification of D-phenylglycine aminotransferase from native host

3.1. Enzyme induction, extraction and ammonium sulfate precipitation

The bacterium was cultured in LB medium (contained per l: 10 g tryptone, 5 g yeast extract and 10 g NaCl) at 30°C with shaking at 200 rpm until the culture reached an $OD_{600\ nm}$ of 2.0. The medium was then changed completely to the same volume of MDP medium containing 10 mM Dphenylglycine. Incubation was continued under the same conditions for a further 4 h and the cells were harvested by centrifugation. Cell pellet was resuspended in 9 volumes of ice-cold 50 mM potassium phosphate buffer pH 7.0 containing 1 mM EDTA, 0.01% mercaptoethanol, and 0.1 mM PLP. The cells were disrupted by sonication (Soniprep-150, MSE Gallenkamp) at 0–4°C. The resultant crude cell-extract was clarified by centrifugation and then subjected to ammonium sulfate fractional precipitation. The enzyme was purified by the following column chromatographic steps operated at room temperature. Protein elusion profiles were monitored by an in-line UV-absorption detector at 280 nm. Protein fractions collected were stored on ice immediately after being eluted. Protein content was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

3.2. Isocratic hydrophobic interaction chromatography

An aliquot of the active ammonuim sulfate precipitation pellet corresponding to a 250-ml cell culture was dissolved in 1 ml of PEMP buffer (50 mM potassium phosphate buffer pH 7.0, 50 mM EDTA, 0.005% mercaptoethanol, 50 mM PLP). The protein solution was applied onto a phenyl agarose column (glass, 1.5 50 cm, 80 ml bed volume) equilibrated with PEMP buffer, and eluted with the same buffer at 0.1 ml min 1. The active fractions were pooled and concentrated using a Centriprep-10 ultrafiltration cell (Amicon).

3.3. High-performance anion-exchange chromatography

The buffer of the concentrated pooled active fractions was replaced by TEMP buffer (20 mM Tris–HCl pH 7.5, 50 mM EDTA, 0.005% mercaptoethanol, 20 mMPLP) by means of ultrafiltration. The protein solution was applied onto a high-performance anion-exchange column (LiChrospher 1000 TMAE, 10 50) equilibrated with TEMP buffer. The elusion was performed at a flow rate of 0.5 ml min 1, starting with TEMP buffer for the first 10 min, and then with a linear salt gradient from 0 to 200 mM NaCl in the same buffer for the following 110 min. Active fractions were pooled and concentrated by ultrafiltration.

3.4. High-performance hydrophobic interaction chromatography

Ammonium sulfate was added to the concentrated pooled active fractions from the anion-exchange step to obtain a final concentration of 1.0 M. The protein solution was loaded onto a highperformance phenyl-agarose column (Sigma Chrom HIC-phenyl 7.5 75 mm) equilibrated with TEMP buffer containing 1.0 M ammonium sulfate. Elution was carried out at a flow rate of 0.5 ml min 1, starting with TEMP buffer containing 1.0 M ammonium sulfate for 10 min, followed by a steep descending gradient of ammonium sulfate from 1.0 to 0.5 M in 10 min, and then a linear gradient from 0.5 to 0 M in the following 60 min.

4. Molecular weight determinations

4.1. Native enzyme

The molecular weight of the native enzyme was determined by the method of Hedrick and Smith (1968) with modifications. Discontinuous non-denaturing polyacrylamide gel electrophoresis (native-PAGE) was carried out using a vertical slab gel apparatus with 3% stacking gel and 8, 10, 12, 14% separating gel concentrations. Protein molecular weight markers were: carbonic anhydrase (Mr-29 000); ovalbumin (Mr-45 000); bovine serum albumin monomer (Mr-66 000); and bovine serum albumin dimer (Mr-132 000). Protein bands were located by silver staining (Pharmacia Biotech).

4.2. Enzyme subunits

The molecular weight of the enzyme subunits was determined by discontinuous SDS-PAGE (Laemmli, 1970). Protein molecular weight markers were: a-lactalbumin (Mr-14 200); trypsin inhibitor (Mr-20 100); trypsinogen Mr-24 000); carbonic anhydrase (Mr-29 000); glyceraldehyde- 3-phosphate dehydrogenase (Mr-36 000); ovalbumin (Mr-45 000); bovine serum albumin (Mr-66 000); phosphorylase b subunit (Mr-97 400); b-galactosidase (Mr-116 000); and myosin (Mr-205 000). The purified enzyme was electrophoresed along with the standard markers in 3 different polyacrylamide gel concentrations (8% T, 3% C; 10% T, 3% C; and 12% T, 3% C). Protein bands were located by silver staining (Pharmacia Biotech).

5. Isoelectric point estimation

The isoelectric point of the purifiedenzyme was determined by a native isoelectric focusing (EF) method (Bollag and Edelstein, 1991) using carrier ampholytes (Sigma) at a pH range 3.5–10.0 on a polyacrylamide slab gel (5%T, 3%C). Standard proteins used were EF markers (range 3.6–9.3) in a kit obtained from Sigma. After focusing, the protein bands were located by silver staining (Pharmacia Biotech).

6. General DNA manipulation techniques

Isolation and purification of bacterial chromosomal and plasmid DNA, elution of DNA from agarose, and purification of PCR products were done by using the suitable Qiagen kits. Restriction endonuclease digestions and ligation were performed according to the instructions of the suppliers. General genetic and recombinant DNA techniques were as described by Sambrook et al. (1989). Oligonucleotide primers for PCR and DNA sequencing were synthesized by the BioService Unit, National Science and Technology Development Agency, Thailand using an Applied Biosystems 392 DNA/RNA Synthesizer.

7. Amino acid sequence determination

Protein fractions from the final chromatographic purification step were separated by SDS-PAGE. The protein in the gels were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P^{sq}, Millipore) using an electroblotter (Trans-blot SD, Bio-Rad) and stained with 0.1% Coomassie Brilliant Blue

R250. The single protein bands were excised from the membrane and the N-terminal amino acid sequence was determined using an Applied Biosystems Procise 492 Protein Sequencer at the Protein Sequencing Facility, Medical University of South Carolina, USA. The internal amino acid sequences were obtained by digesting the purified D-PhgAT with lysyl endopeptidase Lys-C at 37°C for 20 h. Peptides in the digest were separated and blotted onto a PVDF membrane strip using an Applied Biosystems Capillary HPLC (cLC) MicroBlotter 173A, and prominent fragments were selected for sequencing.

8. Purification of recombinant D-PhgAT from *E. coli*

Six grams of cell pellet were resuspended in 50 ml of 50 mM phosphate buffer pH 7.0 containing 1 mM PLP and 1 mM phenylmethylsulfonyl fluoride. The cells were disrupted by ultrasonication at 0 $^{\circ}$C and the crude homogenate was centrifuged at 11,000 ***g*** for 30 min at 4 $^{\circ}$C. The clear cell-free extract was subjected to ammonium sulphate precipitation where the D-PhgAT precipitated in the 25 - 45 % saturation fraction. After centrifugation at 11,000 ***g*** for 30 min at 4 $^{\circ}$C, the protein pellet containing D-PhgAT was collected, dissolved in 4 ml of TEMP buffer (20 mM Tris-HCl pH 7.5, 50 μM EDTA, 0.005 % mercaptoethanol, 20 μM PLP) plus 0.6 M ammonium sulphate and loaded onto a phenyl agarose CL-4B column (60-ml bed volume, 26-mm internal diameter) equilibrated in the same buffer. The column was eluted isocratically with the equilibration buffer at a flow rate of 1 ml min^{-1}. Fractions possessing D-PhgAT activity were pooled, concentrated and desalted by ultrafiltration (Centricon Plus-20, Millipore) and loaded onto a DEAE Sepharose Fast Flow column (60-ml bed volume, 26-mm internal diameter) equilibrated with TEMP buffer. The column was washed with a 1-column volume of TEMP buffer and eluted with a 6-column volume ascending linear gradient of NaCl in TEMP buffer, at a flow rate of 2 ml min^{-1}. Fractions containing the enzyme were pooled, concentrated and desalted by ultrafiltration as above. Purity of the enzyme preparation was determined by SDS-PAGE.

RESULTS

1. Screening for D-phenylglycine aminotransferase producers

Bacterial strains capable of utilizing D-phenylglycine as the sole source of carbon and nitrogen were isolated by enrichment culture on MDP agar medium. The possible presence of D-phenylglycine aminotransferase in these strains was tested by comparing rates of D-phenylglycine degradation in the presence and absence of (aminooxy)acetic acid (an inhibitor of pyridoxal phosphate dependent enzymes). Different inhibitory profiles were observed amongst the strains tested. Strain ST-201 was selected on the basis of possessing high D-phenylglycine degrading activity which was strongly inhibited by (aminooxy)acetic acid. Benzoylformate and benzoic acid were released into the culture medium at the early phase of Dphenylglycine degradation by the strain ST-201. Clarified homogenate prepared from D-phenylglycine induced cells of this bacterium could produce D-phenylglycine from benzoylformate and L-glutamate. However, L-phenylglycine was also formed at a higher rate, possibly due to normal cellular L-amino acid aminotransferases. The strain ST-20 1 was a gram-negative, motile, polar monotrichous flagellated rod, strictly aerobic, non-fermentative, non-spore forming, nonfluorescent, non-pigment producing, denitrifying bacterium identified as *Pseudomonas stutzeri*. The strain grew well at 30–35°C, but not above 42°C or below 8°C. D- and L-phenylglycine, benzoic acid, and 4-hydroxybenzoic acid could be used as the sole carbon and energy sources. Under the same conditions, no growth was observed with phenylacetic acid, D- or L- mandelic acid, and 2- or 3- hydroxybenzoic acid.

2. Purification of D-phenylglycine aminotransferase

Results from the purlfication procedure are summarized in Table 1 and Fig. 1. Both D-phenylglycine- and L-phenylglycine aminotransferase activities were found in clarified cell-extracts of D-phenylglycine induced *Pseudomonas stutzeri* ST-201. By ammonium sulfate fractional precipitation, most of the D-phenylglycine aminotransferase activity precipitated at 25–45% ammonium sulfate saturation while L-phenylglycine aminotransferase activity precipitation occurred from 20 to 70% with two maxima, one at 20–40% and a larger one at 50–70% ammonium sulfate saturation. Subsequent isocratic phenyl agarose chromatography removed more than 90% of unwanted proteins while the D-phenylglycine aminotransferase activity peak was eluted at 1.03 column volume with 80% recovery. L-phenylglycine aminotransferase activity was co-eluted in a smaller amount but could be completely removed by the following anion-exchange chromatography on a LiChrospher 1000 TMAE column. After the subsequent hydrophobic interaction chromatographic step using a SigmaChrom HIC-Phenyl column, the active fraction gave a single protein band on SDS-PAGE.

3. Molecular properties of D-phenylglycine aminotransferase

The molecular weight of the native D-phenylglycine aminotransferase enzyme was estimated to be 92 000 (Fig. 2) by the method modified from Hedrick and Smith (1968). By SDS-PAGE, the purified enzyme gave a single protein band with a molecular weight of 47 500 (Fig. 1). The isoelectric point (pI) of the enzyme deduced from an ampholyte polyacrylamide native-EF gel was 5.0 (picture not shown).

Table 1

Summary of the purification of D-phenylglycine aminotransferase from Pseudomonas stutzeri ST-201

Purification step	Total protein (mg)	Total activity (nkat)	Specific activity (nkat mg^{-1})	Yield (%)
(1) Clarified cell homogenate	1960	1008	0.51	100
(2) $(NH_4)_2SO_4$ precipitation	920	856	0.93	85
(3) Phenyl agarose	52.2	700	13.4	69.4
(4) LiChrospher 1000 TMAE	0.776	192	247.4	19.1
(5) Sigma ChromHIC-Phenyl	0.068	41.6	612	4.1

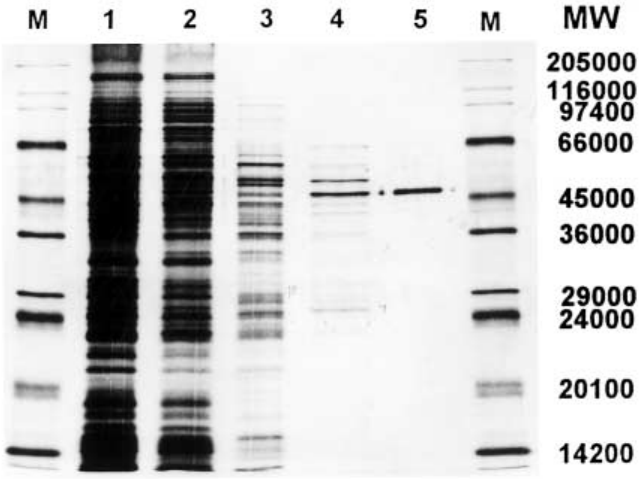

Fig. 1. SDS-PAGE analysis of enzyme purification. Lanes: M, molecular weight marker proteins; 1–5, protein samples from the purification steps in Table 1. MW, molecular weight.

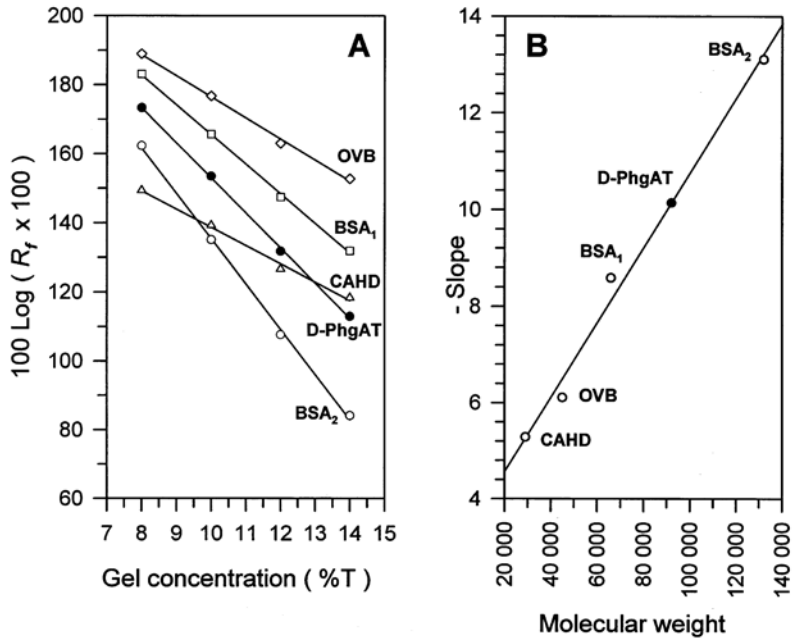

Fig. 2. Ferguson plots (A) and estimation of molecular weight of the native D-phenylglycine aminotransferase (D-phgAT) from slope vs. molecular weight plot (B). Molecular weight marker proteins: CAHD, carbonic anhydrase; OVB, ovalbumin; BSA1, bovine serum albumin monomer; BSA2, bovine serum albumin dimer.

4. Substrate specificity

The substrate specificity of the purified enzyme was studied on both sides of the transamination reaction. When benzoylformate was used as the amino-group acceptor, transamination occurred only when the amino-group donor was L-glutamate and the product was not L-phenylglycine but D-phenylglycine (Fig. 3). Neither D- nor L-phenylglycine formation from benzoylformate was detectable when D-glutamate, D-aspartate, L-aspartate, D-alanine or L-alanine were used as the amino-group donors. Similar results were obtained when 4-hydroxybenzoylformate was used as the amino group acceptor. That is, transamination was observed only with L-glutamate, and D-4-hydroxyphenylglycine was exclusively produced. Formation of D-phenylalanine or D-tyrosine from p-phenylpyruvate or p-hydroxyphenylpyruvate were not detectable with L-glutamate as the amino-group donor. In the opposite direction, where 2-oxoglutarate served as the amino-group acceptor, transamination occurred with D-phenylglycine and D-4-hydroxyphenylglycine. The amino acid product was L-glutamate, and not D-glutamate, in both cases. The enzyme activity towards D-4-hydroxyphenylglycine was found to be slightly higher than that for D-phenylglycine under our assay conditions. No L-glutamate or D-glutamate formation from 2-oxoglutarate could be detected when D-phenylalanine, D-tyrosine, D-alanine, D-aspartate, D-valine, Dleucine, DL-isoleucine, or DL-serine were used as amino-group donors.

The apparent K_m values determined according to Velick and Vavra (1962) for D-phenylglycine and 2-oxoglutarate at 35°C, pH 9.5 were 1.1 and 2.4 mM, respectively.

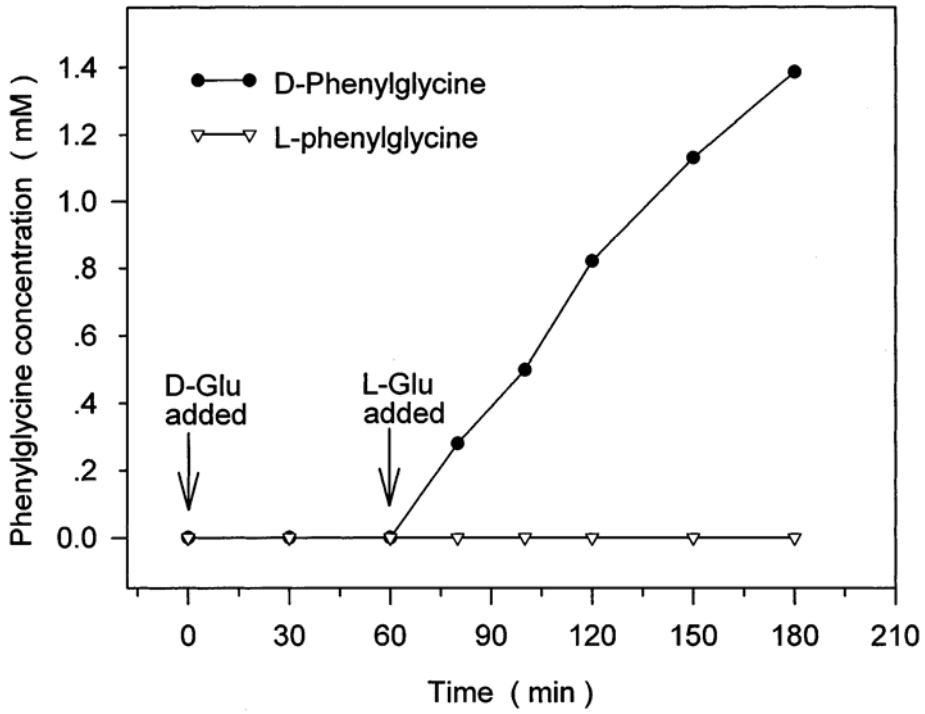

Fig. 3. Amino-group transfer to benzoylformate catalyzed by D-phenylglycine aminotransferase. Transamination occurred specifically from the L-isomer of glutamate and produced exclusively the D-isomer of phenylglycine.

5. Effect of pH and temperature on transamination activity

The effect of pH on transamination activity was examined in the direction of D-phenylglycine formation from L-glutamate and benzoylformate using the assay method 1. Enzyme activity was determined at 35°C in the presence of 100 mM of the following buffers: citrate-NaOH buffer (pH 5.0–6.0), piperazine-N,N%-bis[2-ethanesulfonic acid] (PIPES) (pH 6.5–7.0), tris[hydroxymethyl] aminomethane (Tris) (pH 7.5–9.0), 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) (pH 9.5–11.5). The pH-activity profile is shown in Fig. 4. The enzyme activity was low at neutral pH and almost no transamination activity was detectable below pH 6. The enzyme was highly active in the alkaline pH region with peak activity at pH 9–10. Activity dropped sharply when the pH was higher than 10.5 and there was no activity above pH 12.

The temperature effect on enzyme activity (Fig. 5) was determined at pH 9.5 where the enzyme was most active. The rate of D-phenylglycine formation was measured over a 30 min course of reaction at each designated temperature. Starting from 25°C, reaction velocity increased with increasing temperature and it reached a maximum at 35–45°C. Then it sharply declined as the temperature was raised higher than 50°C. No activity was measurable at 70°C where the enzyme was irreversibly inactivated.

Effect of temperature on enzyme stability (Fig. 6) was determined by incubating the enzyme in a neutral pH solution at each tested temperature for 10 min. Remaining activity was then assayed by the standard method at 35°C. The enzyme was found to be stable up to 50°C. Higher temperatures led to enzyme inactivation. There was no significant loss of activity of the purified enzyme preparation after storage unfrozen at 0°C for 2 months.

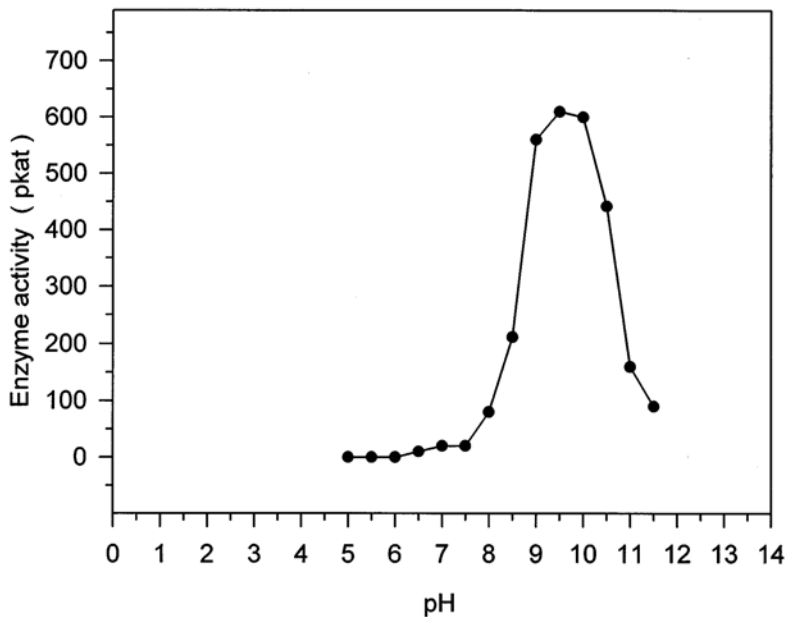

Fig. 4. pH-activity profile of D-phenylglycine aminotransferase. The enzyme activity was determined at 35°C in the direction of D-phenylglycine formation.

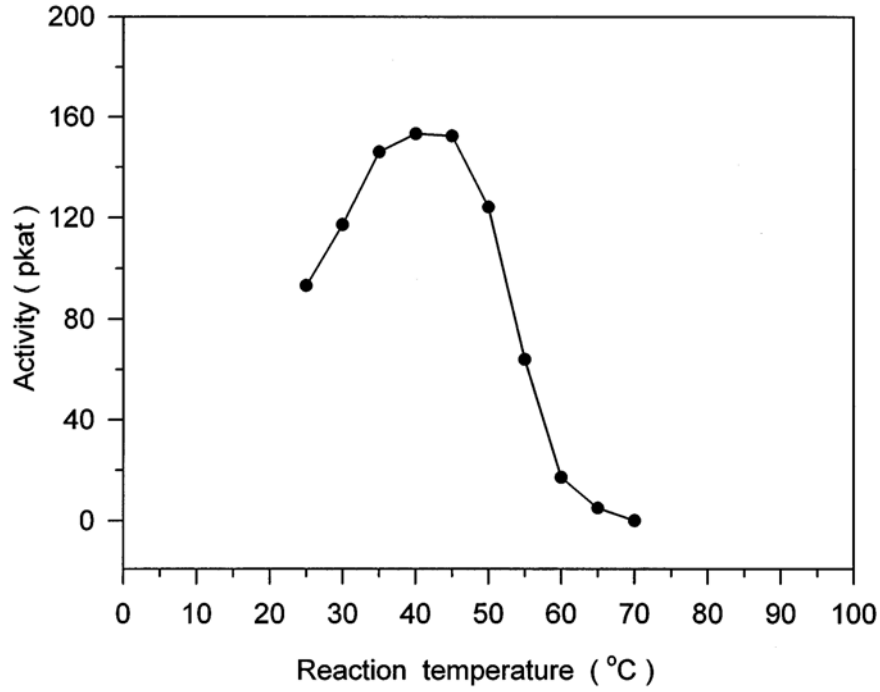

Fig. 5. Temperature-activity profile of D-phenylglycine formation catalyzed by D-phenylglycine aminotransferase.

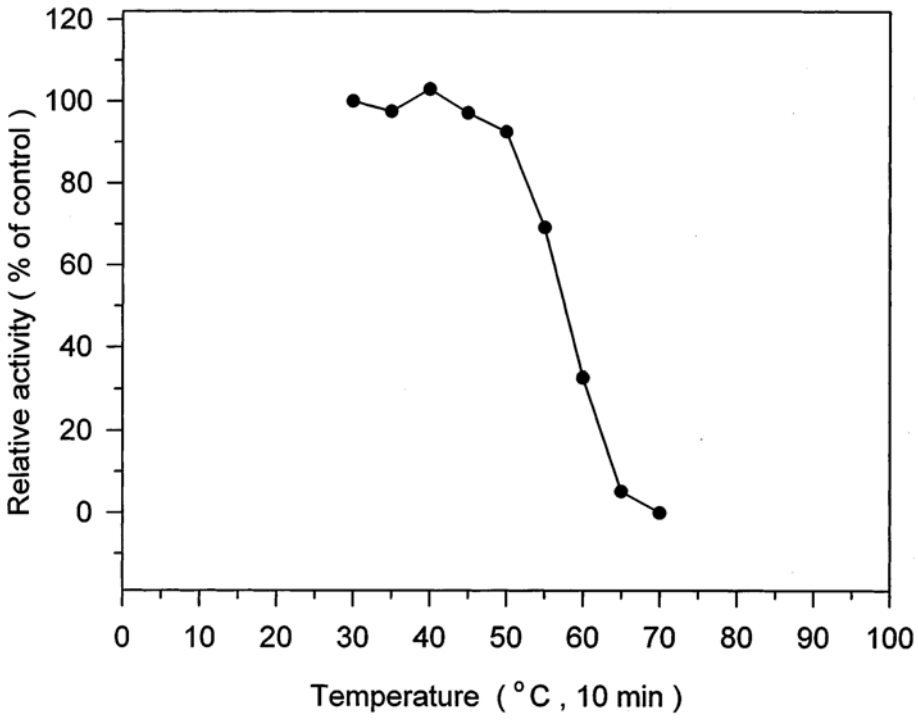

Fig. 6. Thermal stability of the D-phenylglycine aminotransferase. The enzyme was incubated at indicated temperatures for 10 min at pH 7.0. The remaining activity was determined by the standard assay at 35°C.

6. Inhibitors

Effects of possible inhibitors on the activity of the purified D-phenylglycine aminotransferase were tested by addition of a neutral solution of the substance to the reaction mixture at a final concentration of 1 mM. Compounds that showed little inhibitory effect at 1 mM were re-tested at 5 mM. Enzyme activity was determined by assay method 1 as described above, and the results are shown in Table 2. The enzyme was strongly inhibited by typical inhibitors of pyridoxal phosphate-dependent enzymes. These included (aminooxy)acetic acid, hydroxylamine, phenylhydrazine, and 3-methyl-2-benzothiazolinone hydrazone. Strong inhibition was also obtained with gabaculine, an irreversible GABA-transaminase inhibitor and D- and L-cysteine, which are known to form thiazolidine compounds with the PLP coenzyme. The thiolspecific reagents 5,5%-dithiobis(2-nitrobenzoic acid) and N-ethylmaleimide; the suicidal substrate of D-amino acid aminotransferase β-chloro-D-alanine and the compounds known to inhibit aminotransferases D-cycloserine, D- and L-penicillamine; moderately inactivated the enzyme. Among the heavy metal ions tested, mercuric ion was the most potent inhibitor. It completely inactivated the enzyme. Group I and group II metal ions and the chelating agent EDTA had no affect on transaminase activity.

Table 2

Effects of inhibitors on D-phenylglycine aminotransferase

Compounds	Concentration (mM)	Relative enzyme activity[a]
None		100
(Aminooxy)acetic acid	1	0
D-cycloserine	5	25
D-cysteine	1	0
L-cysteine	1	0
Gabaculine	1	0
Hydroxylamine	1	0
MBTH[b]	1	0
Phenylhydrazine	1	0
D-penicillamine	5	23
L-penicillamine	5	32
β-chloro-D-alanine	1	43
DTNB[b]	1	32
N-ethylmaleimide	1	66
$HgCl_2$	1	0
$ZnCl_2$	1	17
EDTA	1	100

[a] Remaining transaminase activity in the presence of compound in question in the reaction mixture.

[b] MBTH, 3-methyl-2-benzothiazolinone hydrazone; DTNB, 5,5%-dithio-bis(2-nitrobenzoic acid).

7. Enzymatic synthesis of enantiopure D-phenylglycine

The enzyme fraction obtained after the LiChrospher 1000 TMAE anion-exchange step was practically free from other unwanted enzyme activities or interfering substances. Thus, it could be used for synthesis of enantiopure D-phenylglycine. The reaction was carried out in a 5-ml mixture containing a 50 μg protein aliquot of the enzyme preparation, 10 mM PLP, 10 mM benzoylformate and 60 mM L-glutamate. The reaction mixture was adjusted to pH 9.0 with KOH and was incubated at 35°C for 3 h. The product was isolated by ultrafiltration (Centriprep 10, Amicon) and the enzyme was kept in an ice bath for re-use. D-phenylglycine was purified by semi-preparative reversed-phase HPLC (Spherisorb, S5 ODS2 25 2 cm) using pure water as the mobile phase. The phenylglycine peak was collected and evaporated to dryness. Infrared spectrum of the white crystalline product obtained was identical to that of the authentic D-phenylglycine standard. The phenylglycine produced was shown to be in the pure D- form by enantioselective HPLC. The same enzyme aliquot was used repeatedly to synthesize D-phenylglycine, one batch per day for 8 consecutive days. The aliquot was stored unfrozen at 0°C when not in use. The low cost substrate L-glutamate was used in excess to shift the reaction equilibrium so that most of the benzoylformate was converted into D-phenylglycine. With 10 mM benzoylformate present in the reaction, the maximum possible yield of D-phenylglycine (Mr-151.2) was 1.5 mg ml^{-1}. The use of six folds molar excess of L-glutamate over benzoylformate resulted in yields of 80% or more D-phenylglycine from the test batches (Fig. 7).

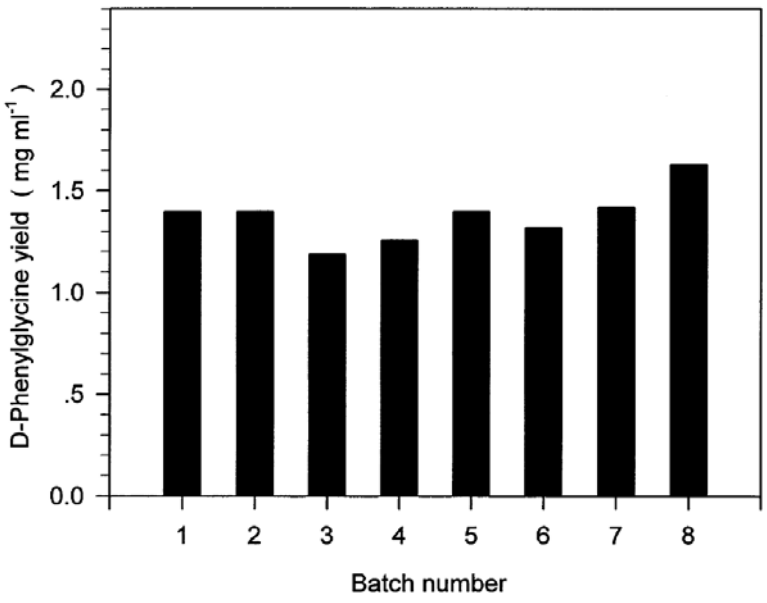

Fig. 7. Synthesis of enantiomerically pure D-phenylglycine by D-phenylglycine aminotransferase in an ultrafiltration cell operated as a 3-h batch once a day. The enzyme was used repetitively for 8 consecutive days, and was stored unfrozen at 0°C when not in use.

8. Cloning of gene encoding the D-phenylglycine aminotransferase

The amino acid sequences of the N-terminus and an internal peptide of purified D-phenylglycine aminotransferase were found to be SILNDYKRKTEGSVFWAQRARSVMPDGVTA and ASAGGLPGGILGGREDVMGVLSR, respectively. The degenerate PCR primers 5′ TACAAGCGIAAGACIGARGG 3′ (sense) and 5′ CAGCACACCCATTACATCYTC 3′ (antisense), designed from the least degenerate regions of the N-terminal peptide (YKRKTEG) and the internal peptide (EDVMGVL), respectively, amplified a 852 bp fragment from genomic DNA of *P. stutzeri* ST-201. Nucleotide sequence at the 5′ and 3′ ends of the 852 bp PCR product were in perfect agreement with the determined amino acid sequence of the N-terminal and the internal peptide of D-PhgAT, indicating that a fragment of the dpgA gene had been amplified. Screening the *P. stutzeri* ST-201 partial genomic library in *E. coli* with the 852 bp DIG-labelled probe led to extraction of plasmid pBPL-E6 from a positive clone containing a 6.5 kb EcoRI genomic DNA fragment. Restriction analysis of pBPL-E6 revealed the presence of the gene encoding the D-phenylglycine aminotransferase within a 1.7-kb PstI-HindIII fragment that was then subcloned into pBluescript II SK, resulting in pBPL-ph.

Upon sequencing on both strands, the DNA insert of pBPL-ph was found to be 1756-bp long (Fig. 8) containing an ORF of 1362 bp starting with an ATG (nt 301-303) start codon 300 bp downstream of the PstI site and ending with a TGA (nt 1660-1662) stop codon 94 bp upstream of the HindIII site. The deduced amino acid residues 2–31 and 270-292 were, respectively, identical to those of the N-terminal and the internal peptides determined from purified D-phenylglycine aminotransferase, confirming that the gene isolated corresponded with the purified enzyme. The ORF predicted a mature protein of 452 amino acids (excluding the initial Met) with a calculated molecular mass of 48.9 kDa. This was consistent with the 47.5 kDa subunit molecular mass of the purified D-phenylglycine aminotransferase as previously determined by SDS-PAGE. The gene encoding the D-phenylglycine aminotransferase was name the "*dpgA*" gene, and the DNA sequence was deposited in the GenBank (accession AY319935).

9. Expression of recombinant D-phenylglycine aminotransferase in *E. coli*

To obtain a good yield of the enzyme, the *dpgA* gene was cloned into the pET-17b expression vector and transformed into *E. coli* BL21(DE3) expression host. The recombinant *E. coli* host was first grown at 30^{O}C with 200 rpm shaking until the culture reached an OD_{600nm} of 2.0. The culture temperature was then shifted down to 25^{O}C, the shaking rate reduced to 100 rpm and IPTG added to a final concentration of 0.4 mM to induce gene expression during the following 3 h. Under these conditions, the D-phenylglycine aminotransferase specific activity in the cell extract was 30.62 nkat.mg^{-1} (Table 3), which was approximately 60 times greater than the endogenous D-phenylglycine aminotransferase activity produced by the *P. stutzeri* ST-201 native host. The recombinant D-phenylglycine aminotransferase was purified to a specific activity of 357 nkat mg^{-1} and the final yield of 46 % (Table 3).

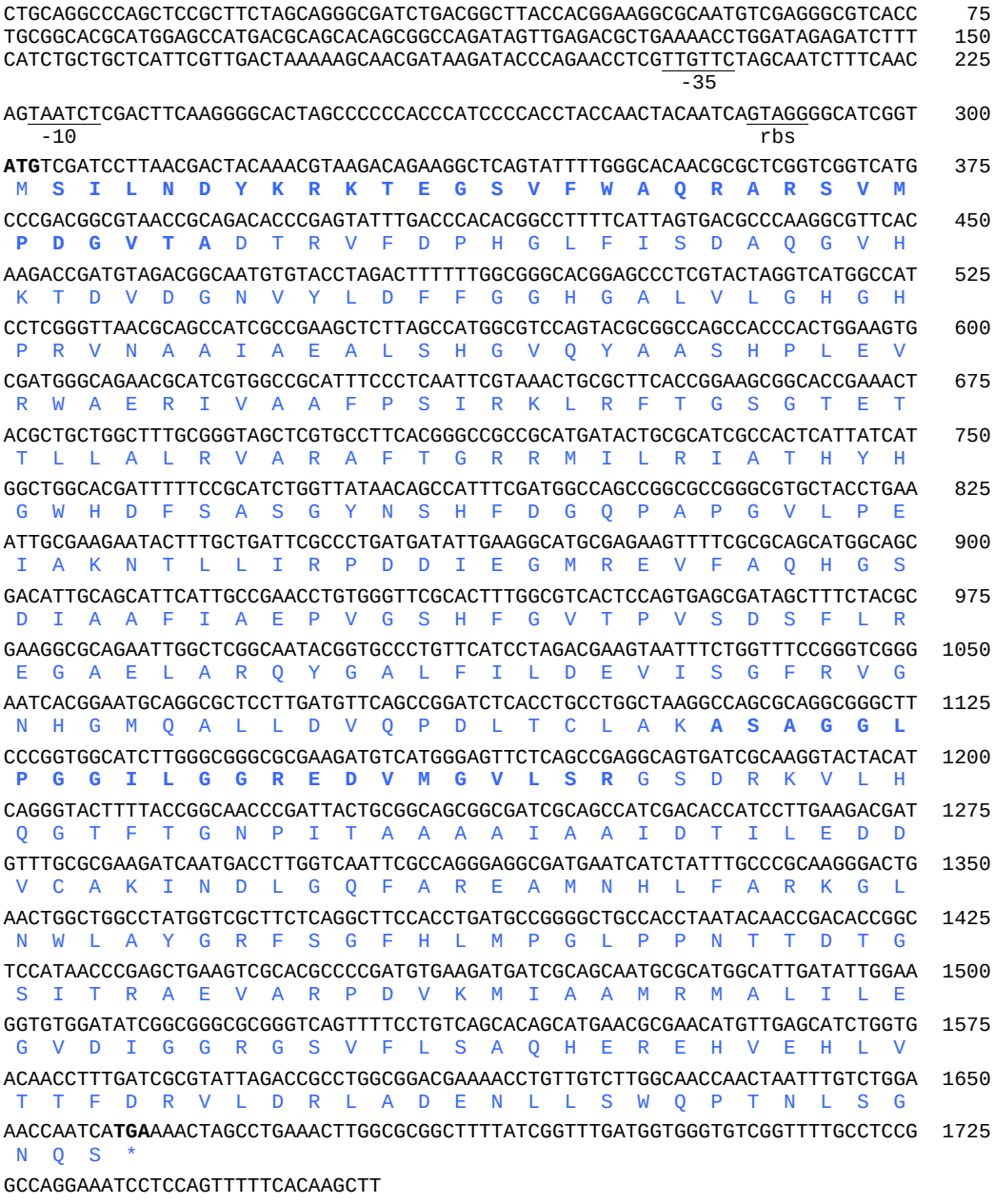

Fig. 8. Nucleotide sequence of *dpgA* gene. The putative promoter (-35 and -10) and the ribosome binding site (rbs) are shown. The deduced amino acid sequence of D-phenylglycine aminotransferase is shown below the nucleotide sequence. The stop codon is indicated by an asterisk. The N-terminal and internal amino acid sequences identical to those obtained from sequencing of the purified D-PhgAT are shown in bold letters.

SDS-PAGE (12 % gel) analysis (Fig. 9) of protein from cell extracts revealed that *E. coli* BL21(DE3) carrying *dpgA* gene under the control of T7 RNA polymerase promoter system of pET-17b expression vector produced an observable amount of D-PhgAT under a non-induced condition (Fig. 9, lane 3) when compared with the background proteins (Fig. 9, lane 2) of the host carrying the pET-17b plasmid without the *dpgA* gene. After induction for 3 h, D-PhgAT protein expression increased to approximately 14 % of total cellular proteins (Fig. 9, lane 4) as determined by gel scanning and calculation of the band intensity. The purified recombinant D-phenylglycine aminotransferase was found to have an apparent molecular mass of 48 kDa (Fig. 9,lane 7) which was identical to that of endogenous D-phenylglycine aminotransferase purified from *P. stutzeri* ST-201. It was also in good agreement with the calculated molecular mass of 48.9 kDa inferred from the amino acid sequence. The K_m values for D-phenylglycine and 2-oxoglutarate determined at 30^{o}C, were essentially the same for both the recombinant and the endogenous D-phenylglycine aminotransferase.

Table 3.

Purification of D-PhgAT from *E. coli* BL21(DE3) cells expressing *dpgA* gene in pET-17b plasmid

Step	**Total protein (mg)**	**Total activity (nkat)**	**Specific activity (nkat mg^{-1})**	**Purification (fold)**	**Yield (%)**
Clarified cell extract	578	17701	30.62	1	100
Ammonium sulfate	269	15046	55.93	1.83	85
Phenyl agarose	31.6	10092	319.4	10.4	57
DEAE Sepharose	23.0	8212	357.0	11.7	46

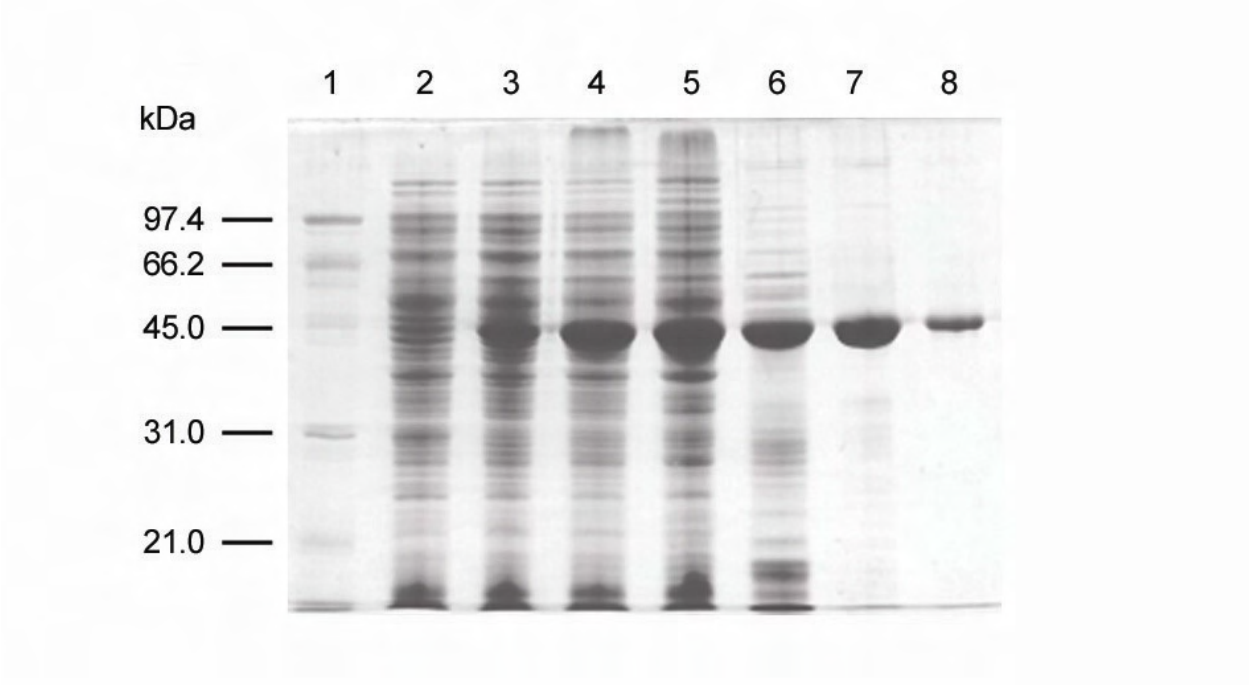

Fig. 8. Analysis of expression and purification of recombinant D-PhgAT

The proteins from cell extracts and purification steps were separated by SDS-PAGE (12 %, w/v) and Coomassie-blue-stained. Molecular masses of standard proteins are given in kDa on the left side. Lanes: 1, standard proteins; 2, crude extract of E. coli BL21(DE3)/pET17-b (without the insert); 3-7, proteins from *E. coli* BL21(DE3)/ pEPL (dpgA gene under the T7 RNA polymerase promoter system); 3, crude extract of cells without induction; 4, extract of cells after 3-h induction; 5, ammonium sulfate precipitate (25–45%); 6, phenyl agarose pool; 7, DEAE Sepharose FF pool; 8, endogenous D-PhgAT purified from *P. stuzeri* ST-201.

DISCUSSION

The initial step of D-phenylglycine degradation by microorganisms isolated using D-phenylglycine as the sole source of carbon and nitrogen can occur in several ways. They may attack D-phenylglycine by the action of D-amino-acid dehydrogenase, D-amino-acid oxidase, D-amino-acid aminotransferase, amino-acid racemase, or hydroxylases acting on the phenyl side-group. The strong inhibitory effect of (aminooxy)acetic acid upon D-phenylglycine degradation by strain ST-201 was evidence for involvement of aminotransferase. Additionally, the presence of benzoylformate (an immediate product) and benzoic acid in the culture medium strongly suggested that transamination occurred at the first step. Benzoylformate could then be converted to benzaldehyde and to benzoic acid by the successive action of benzoylformate decarboxylase and benzaldehyde dehydrogenase, which are components of the mandelate degradation pathway (Hoey and Fewson, 1990). The bacterium may dissimilate benzoate via cathecol and the β-ketoadipate pathway (Neidle et al., 1987) which is commonly found in pseudomonads. However, *Pseudomonas stutzeri* ST-201 could not grow with D- or L-mandelate as the sole carbon source, possibly because it lacked mandelate dehydrogenase.

The D-phenylglycine aminotransferase purified from *Pseudomonas stutzeri* ST-201 has characteristic properties common among aminokansferases; i.e. it is a homodimeric enzyme, strongly inhibited by typical pyridoxal-dependent enzyme inhibitors, and more active in alkaline pH. It differs, however, from other aminotransferases previously described in the following aspects. Firstly, previous reports (Walter et al., 1975; Soper and Manning, 1978, 1981, 1982) indicated that during transamination, L-amino acid aminotransferases and D-amino acid aminotransferases catalyzed hydrogen exchange at the β-carbon (β-elimination) of their amino acid substrates. There is a requirement for a D-amino acid to be a substrate of a D-amino-acid aminotransferase that it should possess at least two protons on the β-carbon of the molecule (Jones et al., 1985). By contrast, the necessity for the presence of at least two b-protons on the D-amino acid substrates was, apparently, not required by D-phenylglycine aminotransferase. Its substrates, D-phenylglycine and 4-hydroxyphenylglycine, have no hydrogen atom attached to the β-carbon. However, the presence of two β-protons in L-glutamate does not exclude it from being a substrate of D-phenylglycine aminotransferase. Secondly, commonly known L-amino-acid aminotransferases accept L-amino acids as substrates and the products are L-amino acids while D-amino acid aminotransferases accept D-amino acids as substrates and produce alternate D-amino acids (Jones et al., 1985). By contrast, D-phenylglycine aminotransferase from the strain ST-201 accepted specifically only D-phenylglycine or D-4-hydroxyphenylglycine as amino-group donors and the amino-group acceptor was 2-oxoglutarate which was converted into L-glutamate upon accepting the amino group. In the reverse direction, L-glutamate was the specific amino-group donor. The aminated enzyme then accepted benzoylformate or 4-hydroxybenzoylformate as oxo acid substrates and converted them into D-phenylglycine or D-4-hydroxyphenylglycine, respectively. No other amino acids apart from the three mentioned (D-phenylglycine, D-4-hydroxyphenylglycine and L-glutamate) were found to be substrates for this enzyme. In this respect, D-phenylglycine aminotransferase is different from typical L-amino acid aminotransferases and D-amino acid aminotransferases. Nor is D-phenylglycine aminotransferase an

amino acid racemase, because the D- and L-amino acids on the two sides of the transamination reaction have structurally different side-groups.

The narrow substrate range of D-phenylglycine aminotransferase is advantageous for its host bacterium growing on D-phenylglycine as the sole source of carbon and nitrogen. While actively taking part in D-phenylglycine dissimilation, the aminotransferase may not interfere with normal metabolism of other amino acids which are essential for cell growth. Additionally, by the 'stereo-inverting' transamination property of this aminotransferase, the amino nitrogen can be channeled directly from the D-phenylglycine substrate into L-glutamate which is a central molecule in cellular nitrogen metabolism. This is accomplished in one step without the need for additional enzymes to racemize the phenylglycine or glutamate. How such stereo-inverting transamination proceeds is not known. Domain motions and the existence of open and closed conformations are evident in aspartate aminotransferase (McPhalen et al., 1992), whose substrate-product pairs are very similar in structure and size. Larger conformational changes would be expected with D-phenylglycine aminotransferase to accommodate its two substrate-product pairs which are much different in size, shape, polarity of the side-chains and configuration of amino group at the α-carbon. While it seems that D-phenylglycine aminotransferase may have evolved to specialize in D-phenylglycine utilization, data on the natural abundance of D-phenylglycine or its derivatives is lacking. Two organisms are known to produce D-4-hydroxyphenylglycine, yet in small amounts. A strain of *Streptomyces fungicidicus* (Hatano et al., 1984) produces enduracidins which are cyclic peptide antibiotics containing D-4-hydroxyphenylglycine, L-4-hydroxyphenylglycine and L-3,5-dichloro-4-hydroxyphenylglycine. *Nocardia uniformis* subsp. *tsuamanensis* (ATCC 21 806) produces a series of monocyclic D-lactam antibiotics called nocardicins (Hosoda et al., 1977) which contain D-4-hydroxyphenylglycine moieties. L-4-hydroxyphenylglycine was found to be the true immediate precursor and the inversion into D- configuration took place at the time the precursor was used to assemble the nocardicin molecules (Townsend and Brown, 1981).

Enzymatic transamination has not been applied for production of D-phenylglycine and D-4-hydroxyphenylglycine, probably due to the following reasons. Firstly, because of the high cost of the 2-oxo acid substrates, benzoylformate and 4-hydroxybenzoylformate. Secondly, commonly known D-amino acid aminotransferases have very low transamination activity towards D-phenylglycine (Soda and Esaki, 1994), and the amino-group donors accepted are strictly only D-amino acids. Direct application of D-amino acids as amino-group donors is not practical because of their high cost. An alternative way is to generate D-amino acids in situ from their L-isomers using a corresponding amino-acid racemase as an extra enzyme added into the transamination system.

With the recent advance in chemical synthetic methods (Fell et al., 1985; Zhao and Lee, 1994), many 2-oxo acid precursors, including benzoylformate and 4-hydroxybenzoylformate, may now be prepared at a relatively low cost. This, together with the unique substrate specificity of D-phenylglycine aminotransferase from ST-201 have made the aminotransferase approach become a more attractive alternative process for Dphenylglycine synthesis. With this D-phenylglycine aminotransferase, L-glutamate can be used directly as an amino-group donor without the need for an amino-acid racemase to isomerize the L-amino acid amino-group donor, as required with typical D-amino-acid aminotransferases which

accept only D-amino acid substrates. Our preliminary data demonstrated that D-PhgAT can be used, with reasonable stability, to synthesize enantiopure D-phenylglycine from benzoylformate and L-glutamate. By a similar process, D-4-hydroxyphenylglycine can also be produced.

The *dpgA* gene encoding the D-phenylglycine aminotransferase has successfully been cloned, its DNA sequence was determined and analyzed. Expression of the *dpgA* gene in recombinant *E coli* yielded high amount of functional D-phenylglycine aminotransferase.

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Journal of Biotechnology 55 (1997) 193–203

JOURNAL OF Biotechnology

A stereo-inverting D-phenylglycine aminotransferase from *Pseudomonas stutzeri* ST-201: purification, characterization and application for D-phenylglycine synthesis

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Received 8 October 1996; accepted 7 May 1997

Abstract

D-phenylglycine aminotransferase (D-PhgAT) from a newly isolated soil bacterium, *Pseudomonas stutzeri* ST-201, was purified to electrophoretic homogeneity and characterized. The molecular weight (M_r) of the native enzyme was estimated to be 92 000. It is composed of two subunits identical in molecular weight ($M_r = 47\,500$). The isoelectric point (p*I*) of the native enzyme was 5.0. The enzyme catalyzed reversible transamination specific for D-phenylglycine or D-4-hydroxyphenylglycine in which 2-oxoglutarate was an exclusive amino group acceptor and was converted into L-glutamic acid. Neither the D- nor L-isomer of phenylalanine, tyrosine, alanine, valine, leucine, isoleucine or serine could serve as a substrate. The enzyme was most active at alkaline pH with maximum activity at pH 9–10. The temperature for maximum activity was 35–45°C. The apparent K_m values for D-phenylglycine and for 2-oxoglutarate at 35°C, pH 9.5 were 1.1 and 2.4 mM, respectively. The enzyme activity was strongly inhibited by typical inhibitors of pyridoxal phosphate-dependent enzymes. Possible application of this enzyme for synthesis of enantiomerically pure D-phenylglycine was demonstrated. © 1997 Elsevier Science B.V.

Keywords: Pseudomonas stutzeri; D-phenylglycine; D-4-hydroxyphenylglycine; Aminotransferase; Purification; Characterization

1. Introduction

Aminotransferases are pyridoxal-dependent enzymes catalyzing high fidelity stereospecific amino group transfers by a reversible Ping Pong Bi Bi kinetic mechanism (Kirsch et al., 1984). L-amino-acid aminotransferase abstracts an amino group from a specific L-amino acid substrate via the pyridoxal-5′-phosphate (PLP) coenzyme to generate the pyridoxamine-5′-phosphate (PMP) form, and the corresponding oxo acid is released as a

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product of the first half-reaction. In the second half-reaction, the amino group from PMP coenzyme is transferred to a different oxo acid specific for the enzyme, to produce the corresponding L-amino acid and to regenerate the PLP form of the holoenzyme ready for catalyzing the next round of transamination. D-amino-acid aminotransferases act by an analogous mechanism, but are absolutely specific for D-isomers of amino acids (Jones et al., 1985).

It is generally known that amino group transfer catalyzed by aminotransferase is strictly stereoconserved (del Pozo et al., 1989; Sugio et al., 1995). Little work has been done on enzymes capable of transferring amino groups of opposite configuration between two different amino acids. There are reports of such transaminase activity in plants (Mapson et al., 1969) and bacteria (van den Tweel et al., 1986, 1988). However, the enzyme preparations employed in those studies were rather heterogeneous and the transaminases in question have not been purified and characterized.

We have isolated a strain of *Pseudomonas stutzeri* from Thai soil and found an aminotransferase specific for D-phenylglycine or D-4-hydroxyphenylglycine on one side and specific for L-glutamate on the other side of the transamination reaction. In this communication, we describe the purlfication and characterization of this unique enzyme. Potential application of the enzyme in D-phenylglycine synthesis using L-glutamate as an amino group donor was demonstrated.

2. Materials and methods

2.1. Materials

Chemicals and solvents were purchased from Sigma (St. Louis, USA) and E. Merck (Darmstadt, Germany). Phenyl agarose (P3693) and a SigmaChrom HICPhenyl HPLC column were obtained from Sigma. A LiChrospher 1000 TMAE Superformance (10 × 50) column was supplied by E. Merck. Spherisorb S5 ODS2 and S5 C8 (46 × 250) HPLC columns were from Phase Sep (Clwyd, UK). Chromatographic separations were carried out using a Bio-Rad 5000T biocompatible HPLC system (Bio-Rad, USA). A Mini Protean II vertical slab gel (Bio-Rad) was used for performing electrophoresis.

2.2. Isolation of D-phenylglycine aminotransferase producing bacteria

Soil bacteria were isolated from growth on minimal D-phenylglycine (MDP) medium containing in 1 l of distilled water: 15 g agar, 3.40 g KH_2PO_4, 3.55 g Na_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 8 mg $CaCl_2.2H_2O$, 10 mg yeast extract, 1 ml trace metals solution (Beggs and Fewson, 1977) and 1.0 g of D-phenylglycine. Each isolate was cultured in liquid MDP medium at 30°C with shaking until the culture reached an $OD_{600\ nm}$, of approximately 1.0 after which the cells were collected and washed twice. A 10-mg wet weight portion was resuspended in 1 ml of 50 mM potassium phosphate buffer pH 7.0 containing 5 mM D-phenylglycine in the absence or presence of 15 mM neutralized (aminooxy)acetic acid. D-phenylglycine consumption at 30°C was monitored by HPLC. Bacterial isolates which degraded D-phenylglycine rapidly in the absence of, but more slowly in the presence of (aminooxy)acetic acid were selected. Clarified cell homogenates prepared from selected bacteria grown on MDP medium were tested for the presence of an enzyme that could produce D-phenylglycine from benzoylformate and L-glutamate.

Taxonomic identification of the bacteria was carried out by investigating characteristic phenotypic properties according to Krieg and Holt (1984), and Smibert and Krieg (1994).

2.3. Enzyme activity assays

2.3.1. Method 1

D-phenylglycine aminotransferase activity was determined by measuring D-phenylglycine formation from benzoylformate and L-glutamate. The reaction mixture was composed of 0.1 M CAPS buffer (pH 9.5), 60 mM L-glutamic acid (monosodium), 5 mM benzoylformate sodium, and 50 μM PLP. The mixture was pre-warmed at 35°C and the enzyme solution was added to initiate the reaction. After incubation at 35°C,

aliquots of the reaction mixture were taken at intervals, heated in boiling water for 2 min, and kept on ice. D-phenylglycine produced in the enzymatic reaction was quantified by the stereoselective HPLC method of Gil-Av et al. (1980) with modifications. A reversed-phase analytical HPLC column (Spherisorb S5 ODS-2, 25 × 0.46 cm) was used with a pellicular C-18 guard column. The chiral mobile phase was composed of 2.4 mM copper(II) acetate, 5 mM L-proline and 3% methanol. The chromatograph (Bio-Rad 5000T HPLC system) was operated at room temperature (~27°C) with a flow rate of 1.2 ml min^{-1}, and detection was with UV absorption at 210 nm. Under these conditions, the retention times of D- and L-phenylglycine were 7.8 and 13.2 min, respectively.

2.3.2. Method 2

Substrate specificity of the enzyme was studied by incubating the enzyme with various D- or L-amino acids as amino donors and 2-oxo acids as amino acceptors in question. Optical isomers of the amino acids consumed or produced in each reaction were analyzed by the HPLC method of Jegorov et al. (1988) with modifications. The analytical column was Spherisorb C-8, 5 μm (250 × 46 mm) with a C-8 guard column. The pre-column derivatization procedure and mobile phase composition were as described in the original method. Detection was with UV absorption at 360 nm. The mobile phase gradient profiles were re-programmed to optimize for particular amino acids of interest. A constant flow of 0.9 ml min^{-1} was maintained during the analysis.

2.4. Enzyme purification

2.4.1. Enzyme induction, extraction and ammonium sulfate precipitation

The bacterium was cultured in LB medium (contained per l: 10 g tryptone, 5 g yeast extract and 10 g NaCl) at 30°C with shaking at 200 rpm until the culture reached an $OD_{600\ nm}$ of 2.0. The medium was then changed completely to the same volume of MDP medium containing 10 mM D-phenylglycine. Incubation was continued under the same conditions for a further 4 h and the cells were harvested by centrifugation. The cell pellet was resuspended in 9 volumes of ice-cold 50 mM potassium phosphate buffer pH 7.0 containing 1 mM EDTA, 0.01% mercaptoethanol, and 0.1 mM PLP.

The cells were disrupted by sonication (Soniprep-150, MSE Gallenkamp) at 0–4°C. The resultant crude cell-extract was clarified by centrifugation and then subjected to ammonium sulfate fractional precipitation.

The enzyme was purified by the following column chromatographic steps operated at room temperature. Protein elusion profiles were monitored by an in-line UV-absorption detector at 280 nm. Protein fractions collected were stored on ice immediately after being eluted. Protein content was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

2.4.2. Isocratic hydrophobic interaction chromatography

An aliquot of the active ammonuim sulfate precipitation pellet corresponding to a 250-ml cell culture was dissolved in 1 ml of PEMP buffer (50 mM potassium phosphate buffer pH 7.0, 50 μM EDTA, 0.005% mercaptoethanol, 50 μM PLP). The protein solution was applied onto a phenyl agarose column (glass, 1.5 × 50 cm, 80 ml bed volume) equilibrated with PEMP buffer, and eluted with the same buffer at 0.1 ml min^{-1}. The active fractions were pooled and concentrated using a Centriprep-10 ultrafiltration cell (Amicon).

2.4.3. High-performance anion-exchange chromatography

The buffer of the concentrated pooled active fractions was replaced by TEMP buffer (20 mM Tris–HCl pH 7.5, 50 μM EDTA, 0.005% mercaptoethanol, 20 μM PLP) by means of ultrafiltration. The protein solution was applied onto a high-performance anionexchange column (LiChrospher 1000 TMAE, 10 × 50) equilibrated with TEMP buffer. The elusion was performed at a flow rate of 0.5 ml min^{-1}, starting with TEMP buffer for the first 10 min, and then with a linear salt gradient from 0 to 200 mM NaCl in the same buffer for the following 110 min. Active fractions were pooled and concentrated by ultrafiltration.

2.4.4. High-performance hydrophobic interaction chromatography

Ammonium sulfate was added to the concentrated pooled active fractions from the anion-exchange step to obtain a final concentration of 1.0 M. The protein solution was loaded onto a high-performance phenyl-agarose column (Sigma Chrom HIC-phenyl 7.5×75 mm) equilibrated with TEMP buffer containing 1.0 M ammonium sulfate. Elution was carried out at a flow rate of 0.5 ml min^{-1}, starting with TEMP buffer containing 1.0 M ammonium sulfate for 10 min, followed by a steep descending gradient of ammonium sulfate from 1.0 to 0.5 M in 10 min, and then a linear gradient from 0.5 to 0 M in the following 60 min.

2.5. Molecular weight determinations

2.5.1. Native enzyme

The molecular weight of the native enzyme was determined by the method of Hedrick and Smith (1968) with modifications. Discontinuous non-denaturing polyacrylamide gel electrophoresis (native-PAGE) was carried out using a vertical slab gel apparatus with 3% stacking gel and 8, 10, 12, 14% separating gel concentrations. Protein molecular weight markers were: carbonic anhydrase ($M_r = 29\,000$); ovalbumin ($M_r = 45\,000$); bovine serum albumin monomer ($M_r = 66\,000$); and bovine serum albumin dimer ($M_r = 132\,000$). Protein bands were located by silver staining (Pharmacia Biotech).

2.5.2. Enzyme subunits

The molecular weight of the enzyme subunits was determined by discontinuous SDS-PAGE (Laemmli, 1970). Protein molecular weight markers were: α-lactalbumin ($M_r = 14\,200$); trypsin inhibitor ($M_r = 20\,100$); trypsinogen $M_r = 24\,000$); carbonic anhydrase ($M_r = 29\,000$); glyceraldehyde-3-phosphate dehydrogenase ($M_r = 36\,000$); ovalbumin ($M_r = 45\,000$); bovine serum albumin ($M_r = 66\,000$); phosphorylase b subunit ($M_r = 97\,400$); β-galactosidase ($M_r = 116\,000$); and myosin ($M_r = 205\,000$). The purified enzyme was electrophoresed along with the standard markers in 3 different polyacrylamide gel concentrations (8% T, 3% C; 10% T, 3% C; and 12% T, 3% C). Protein bands were located by silver staining (Pharmacia Biotech).

2.6. Isoelectric point estimation

The isoelectric point of the purifiedenzyme was determined by a native isoelectric focusing (EF) method (Bollag and Edelstein, 1991) using carrier ampholytes (Sigma) at a pH range 3.5–10.0 on a polyacrylamide slab gel (5%T, 3%C). Standard proteins used were EF markers (range 3.6–9.3) in a kit obtained from Sigma. After focusing, the protein bands were located by silver staining (Pharmacia Biotech).

3. Results

Screening for D-phenylglycine aminotransferase producers.

Bacterial strains capable of utilizing D-phenylglycine as the sole source of carbon and nitrogen were isolated by enrichment culture on MDP agar medium. The possible presence of D-phenylglycine aminotransferase in these strains was tested by comparing rates of D-phenylglycine degradation in the presence and absence of (aminooxy)acetic acid (an inhibitor of pyridoxal phosphate dependent enzymes). Different inhibitory profiles were observed amongst the strains tested. Strain ST-201 was selected on the basis of possessing high D-phenylglycine degrading activity which was strongly inhibited by (aminooxy)acetic acid. Benzoylformate and benzoic acid were released into the culture medium at the early phase of D-phenylglycine degradation by strain ST-201. Clarified homogenate prepared from D-phenylglycine induced cells of this bacterium could produce D-phenylglycine from benzoylformate and L-glutamate. However, L-phenylglycine was also formed at a higher rate, possibly due to normal cellular L-amino acid aminotransferases.

The strain ST-20 1 was a gram-negative, motile, polar monotrichous flagellated rod, strictly aerobic, non-fermentative, non-spore forming, non-fluorescent, non-pigment producing, denitrifying bacterium identified as *Pseudomonas stutzeri*. The

Table 1
Summary of the purification of D-phenylglycine aminotransferase from *Pseudomonas stutzeri* ST-201

Purification step	Total protein (mg)	Total activity (nkat)	Specific activity (nkat mg^{-1})	Yield (%)
(1) Clarified cell homogenate	1960	1008	0.51	100
(2) $(NH_4)_2SO_4$ precipitation	920	856	0.93	85
(3) Phenyl agarose	52.2	700	13.4	69.4
(4) LiChrospher 1000 TMAE	0.776	192	247.4	19.1
(5) Sigma ChromHIC-Phenyl	0.068	41.6	612	4.1

strain grew well at 30–35°C, but not above 42°C or below 8°C. D- and L-phenylglycine, benzoic acid, and 4-hydroxybenzoic acid could be used as the sole carbon and energy sources. Under the same conditions, no growth was observed with phenylacetic acid, D- or L- mandelic acid, and 2- or 3- hydroxybenzoic acid.

3.1. Purification of D-phenylglycine aminotransferase

Results from the purlfication procedure are summarized in Table 1 and Fig. 1. Both D-phenylglycine- and L-phenylglycine aminotransferase activities were found in clarified cell-extracts of D-phenylglycine induced *Pseudomonas stutzeri* ST-201. By ammonium sulfate fractional precipitation, most of the D-phenylglycine aminotransferase activity precipitated at 25–45% ammonium sulfate saturation while L-phenylglycine aminotransferase activity precipitation occurred from 20 to 70% with two maxima, one at 20–40% and a larger one at 50–70% ammonium sulfate saturation. Subsequent isocratic phenyl agarose chromatography removed more than 90% of unwanted proteins while the D-phenylglycine aminotransferase activity peak was eluted at 1.03 column volume with 80% recovery. L-phenylglycine aminotransferase activity was co-eluted in a smaller amount but could be completely removed by the following anion-exchange chromatography on a LiChrospher 1000 TMAE column. After the subsequent hydrophobic interaction chromatographic step using a SigmaChrom HIC-Phenyl column, the active fraction gave a single protein band on SDS-PAGE.

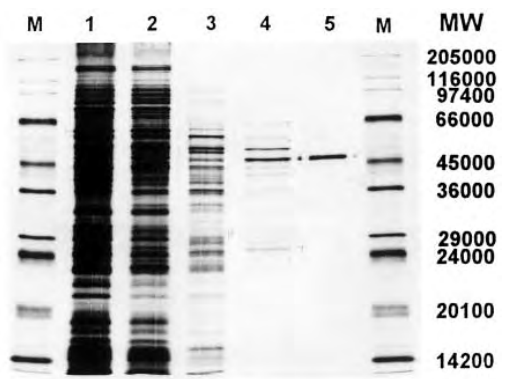

Fig. 1. SDS-PAGE analysis of enzyme purification. Lanes: M, molecular weight marker proteins; 1–5, protein samples from the purification steps in. MW, molecular weight.

3.2. Molecular properties

The molecular weight of the native D-phenylglycine aminotransferase enzyme was estimated to be 92 000 (Fig. 2) by the method modified from Hedrick and Smith (1968). By SDS-PAGE, the purifiedenzyme gave a single protein band with a molecular weight of 47 500 (Fig. 1). The isoelectric point (p*I*) of the enzyme deduced from an ampholyte polyacrylamide native-EF gel was 5.0 (data not shown).

3.3. Substrate specificity

The substrate specificity of the purifiedenzyme was studied on both sides of the transamination reaction. When benzoylformate was used as the amino-group acceptor, transamination occurred only when the amino-group donor was L-glutamate and the product was not L-phenylglycine but D-phenylglycine (Fig. 3). Neither D- nor L-phenyl-

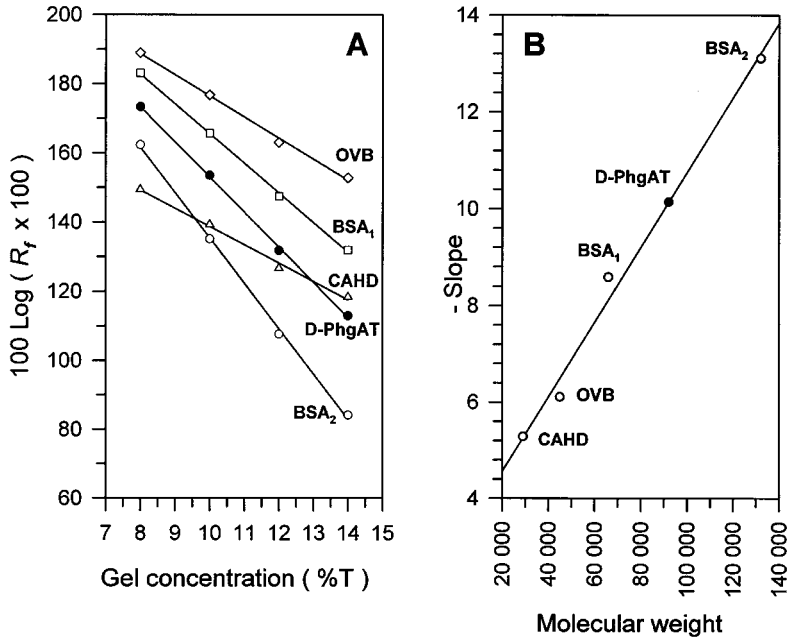

Fig. 2. Ferguson plots (A) and estimation of molecular weight of the native D-phenylglycine aminotransferase (D-phgAT) from -slope vs. molecular weight plot (B). Molecular weight marker proteins: CAHD, carbonic anhydrase; OVB, ovalbumin; BSA_1, bovine serum albumin monomer; BSA_2, bovine serum albumin dimer.

glycine formation from benzoylformate was detectable when D-glutamate, D-aspartate, L-aspartate, D-alanine or L-alanine were used as the amino-group donors. Similar results were obtained when 4-hydroxybenzoylformate was used as the aminogroup acceptor. That is, transamination was observed only with L-glutamate, and D-4-hydroxyphenylglycine was exclusively produced. Formation of D-phenylalanine or D-tyrosine from *p*-phenylpyruvate or *p*-hydroxyphenylpyruvate were not detectable with L-glutamate as the amino-group donor. In the opposite direction, where 2-oxoglutarate served as the amino-group acceptor, transamination occurred with D-phenylglycine and D-4-hydroxyphenylglycine. The amino acid product was L-glutamate, and not D-glutamate, in both cases. The enzyme activity towards D-4-hydroxyphenylglycine was found to be slightly higher than that for D-phenylglycine under our assay conditions. No L-glutamate or D-glutamate formation from 2-oxoglutarate could be detected when D-phenylalanine, D-tyrosine, D-alanine, D-aspartate, D-valine, D-leucine, DL-isoleucine, or DL-serine were used as amino-group donors.

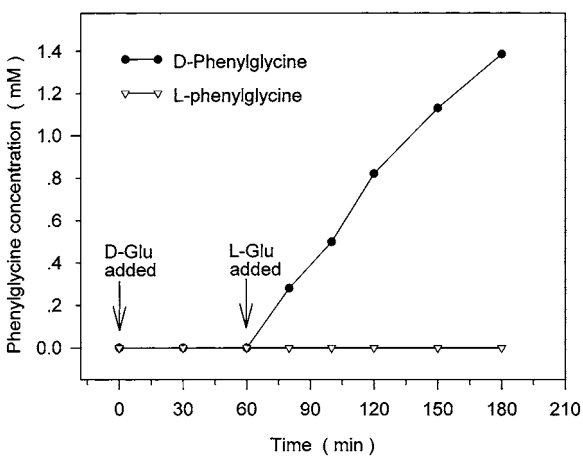

Fig. 3. Amino-group transfer to benzoylformate catalyzed by D-phenylglycine aminotransferase. Transamination occurred specifically from the L-isomer of glutamate and produced exclusively the D-isomer of phenylglycine.

The apparent K_m values determined according to Velick and Vavra (1962) for D-phenylglycine

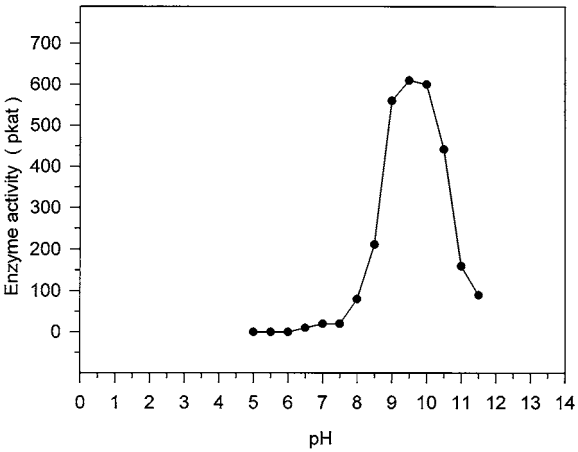

Fig. 4. pH-activity profile of D-phenylglycine aminotransferase. The enzyme activity was determined at 35°C in the direction of D-phenylglycine formation.

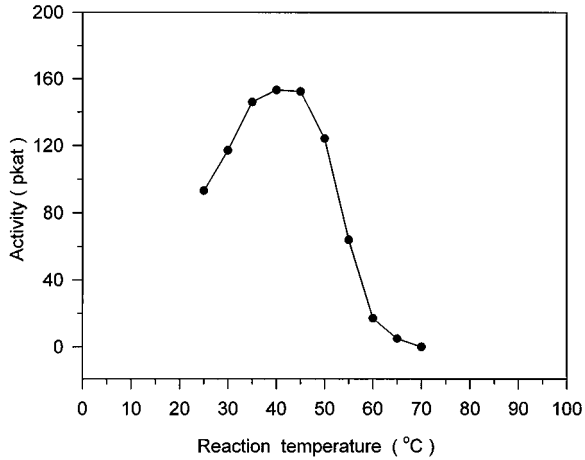

Fig. 5. Temperature-activity profile of D-phenylglycine formation catalyzed by D-phenylglycine aminotransferase.

and 2-oxoglutarate at 35°C, pH 9.5 were 1.1 and 2.4 mM, respectively.

3.4. Effect of pH and temperature

The effect of pH on transamination activity was examined in the direction of D-phenylglycine formation from L-glutamate and benzoylformate using assay method 1. Enzyme activity was determined at 35°C in the presence of 100 mM of the following buffers: citrate-NaOH buffer (pH 5.0–6.0), piperazine-*N*,*N*′-bis[2-ethanesulfonic acid] (PIPES) (pH 6.5–7.0), tris[hydroxymethyl]aminomethane (Tris) (pH 7.5–9.0), 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) (pH 9.5–11.5). The pH-activity profile is shown in Fig. 4. The enzyme activity was low at neutral pH and almost no transamination activity was detectable below pH 6. The enzyme was highly active in the alkaline pH region with peak activity at pH 9–10. Activity dropped sharply when the pH was higher than 10.5 and there was no activity above pH 12.

The temperature effect on enzyme activity (Fig. 5) was determined at pH 9.5 where the enzyme was most active. The rate of D-phenylglycine formation was measured over a 30 min course of reaction at each designated temperature. Starting from 25°C, reaction velocity increased with increasing temperature and it reached a maximum at 35–45°C. Then it sharply declined as the temperature was raised higher than 50°C. No activity was measurable at 70°C where the enzyme was irreversibly inactivated.

Effect of temperature on enzyme stability (Fig. 6) was determined by incubating the enzyme in a neutral pH solution at each tested temperature for 10 min. Remaining activity was then assayed by the standard method at 35°C. The enzyme was

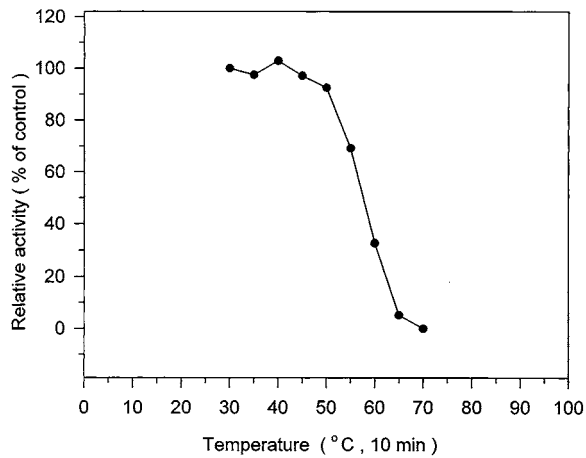

Fig. 6. Thermal stability of the D-phenylglycine aminotransferase. The enzyme was incubated at indicated temperatures for 10 min at pH 7.0. The remaining activity was determined by the standard assay at 35°C.

Table 2
Effects of inhibitors on D-phenylglycine aminotransferase

Compounds	Concentration (mM)	Relative enzyme activity[a]
None		100
(Aminooxy)acetic acid	1	0
D-cycloserine	5	25
D-cysteine	1	0
L-cysteine	1	0
Gabaculine	1	0
Hydroxylamine	1	0
MBTH[b]	1	0
Phenylhydrazine	1	0
D-penicillamine	5	23
L-penicillamine	5	32
β-chloro-D-alanine	1	43
DTNB[b]	1	32
N-ethylmaleimide	1	66
$HgCl_2$	1	0
$ZnCl_2$	1	17
EDTA	1	100

[a] Remaining transaminase activity in the presence of compound in question in the reaction mixture.
[b] MBTH, 3-methyl-2-benzothiazolinone hydrazone; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid).

found to be stable up to 50°C. Higher temperatures led to enzyme inactivation. There was no significant loss of activity of the purified enzyme preparation after storage unfrozen at 0°C for 2 months.

3.5. Inhibitors

Effects of possible inhibitors on the activity of the purified D-phenylglycine aminotransferase were tested by addition of a neutral solution of the substance to the reaction mixture at a final concentration of 1 mM. Compounds that showed little inhibitory effect at 1 mM were re-tested at 5 mM. Enzyme activity was determined by assay method 1 as described above, and the results are shown in Table 2. The enzyme was strongly inhibited by typical inhibitors of pyridoxal phosphate-dependent enzymes. These included (aminooxy)acetic acid, hydroxylamine, phenylhydrazine, and 3-methyl-2-benzothiazolinone hydrazone. Strong inhibition was also obtained with gabaculine, an irreversible GABA-transaminase inhibitor and D- and L-cysteine, which are known to form thiazolidine compounds with the PLP coenzyme. The thiolspecific reagents 5,5′-dithio-bis(2-nitrobenzoic acid) and *N*-ethylmaleimide; the suicidal substrate of D-amino acid aminotransferase β-chloro-D-alanine and the compounds known to inhibit aminotransferases D-cycloserine, D- and L-penicillamine; moderately inactivated the enzyme. Among the heavy metal ions tested, mercuric ion was the most potent inhibitor. It completely inactivated the enzyme. Group I and group II metal ions and the chelating agent EDTA had no affect on transaminase activity.

3.6. Enzymatic synthesis of enantiopure D-phenylglycine

The enzyme fraction obtained after the LiChrospher 1000 TMAE anion-exchange step was practically free from other unwanted enzyme activities or interfering substances. Thus, it could be used for synthesis of enantiopure D-phenylglycine. The reaction was carried out in a 5-ml mixture containing a 50 μg protein aliquot of the enzyme preparation, 10 μM PLP, 10 mM benzoylformate and 60 mM L-glutamate. The reaction mixture was adjusted to pH 9.0 with KOH and was incubated at 35°C for 3 h. The product was isolated by ultrafiltration (Centriprep 10, Amicon) and the enzyme was kept in an ice bath for re-use. D-phenylglycine was purified by semi-preparative reversed-phase HPLC (Spherisorb, S5 ODS2 25 × 2 cm) using pure water as the mobile phase. The phenylglycine peak was collected and evaporated to dryness. Infrared spectrum of the white crystalline product obtained was identical to that of the authentic D-phenylglycine standard. The phenylglycine produced was shown to be in the pure D- form by enantioselective HPLC. The same enzyme aliquot was used repeatedly to synthesize D-phenylglycine, one batch per day for 8 consecutive days. The aliquot was stored unfrozen at 0°C when not in use. The low cost substrate L-glutamate was used in excess to shift the reaction equilibrium so that most of the benzoylformate was converted into D-phenylglycine. With 10

mM benzoylformate present in the reaction, the maximum possible yield of D-phenylglycine ($M_r = 151.2$) was 1.5 mg ml^{-1}. The use of sixfold molar excess of L-glutamate over benzoylformate resulted in yields of 80% or more D-phenylglycine from the test batches (Fig. 7).

4. Discussion

The initial step of D-phenylglycine degradation by microorganisms isolated using D-phenylglycine as the sole source of carbon and nitrogen can occur in several ways. They may attack D-phenylglycine by the action of D-amino-acid dehydrogenase, D-amino-acid oxidase, D-amino-acid aminotransferase, amino-acid racemase, or hydroxylases acting on the phenyl side-group. The strong inhibitory effect of (aminooxy)acetic acid upon D-phenylglycine degradation by strain ST-201 was evidence for involvement of aminotransferase. Additionally, the presence of benzoylformate (an immediate product) and benzoic acid in the culture medium strongly suggested that transamination occurred at the first step. Benzoylformate could then be converted to benzaldehyde and to benzoic acid by the successive action of

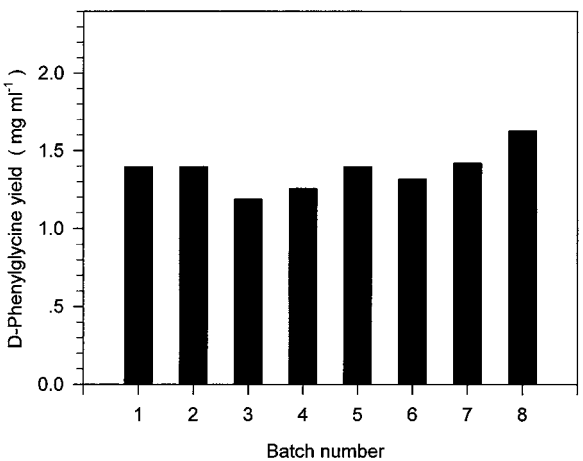

Fig. 7. Synthesis of enantiomerically pure D-phenylglycine by D-phenylglycine aminotransferase in an ultrafiltration cell operated as a 3-h batch once a day. The enzyme was used repetitively for 8 consecutive days, and was stored unfrozen at 0°C when not in use.

benzoylformate decarboxylase and benzaldehyde dehydrogenase, which are components of the mandelate degradation pathway (Hoey and Fewson, 1990). The bacterium may dissimilate benzoate via cathecol and the β-ketoadipate pathway (Neidle et al., 1987) which is commonly found in pseudomonads. However, *Pseudomonas stutzeri* ST-201 could not grow with D- or L-mandelate as the sole carbon source, possibly because it lacked mandelate dehydrogenase.

The D-phenylglycine aminotransferase (D-PhgAT) purified from *Pseudomonas stutzeri* ST-201 has characteristic properties common among aminokansferases; i.e. it is a homodimeric enzyme, strongly inhibited by typical pyridoxal-dependent enzyme inhibitors, and more active in alkaline pH. It differs, however, from other aminotransferases previously described in the following aspects. Firstly, previous reports (Walter et al., 1975; Soper and Manning, 1978, 1981, 1982) indicated that during transamination, L-amino-acid aminotransferases and D-amino acid aminotransferases catalyzed hydrogen exchange at the β-carbon (β-elimination) of their amino acid substrates. There is a requirement for a D-amino acid to be a substrate of a D-amino-acid aminotransferase that it should possess at least two protons on the β-carbon of the molecule (Jones et al., 1985). By contrast, the necessity for the presence of at least two β-protons on the D-amino acid substrates was, apparently, not required by D-PhgAT. Its substrates, D-phenylglycine and 4-hydroxyphenylglycine, have no hydrogen atom attached to the β-carbon. However, the presence of two β-protons in L-glutamate does not exclude it from being a substrate of D-PhgAT. Secondly, commonly known L-amino-acid aminotransferases accept L-amino acids as substrates and the products are L-amino acids while Damino-acid aminotransferases accept D-amino acids as substrates and produce alternate D-amino acids (Jones et al., 1985). By contrast, D-PhgAT from the strain ST-201 accepted specifically only D-phenylglycine or D-4-hydroxyphenylglycine as amino-group donors and the amino-group acceptor was 2-oxoglutarate which was converted into L-glutamate upon accepting the amino group. In the reverse direction, L-glutamate was the specific

amino-group donor. The aminated enzyme then accepted benzoylformate or 4-hydroxybenzoylformate as oxo acid substrates and converted them into D-phenylglycine or D-4-hydroxyphenylglycine, respectively. No other amino acids apart from the three mentioned (D-phenylglycine, D-4-hydroxyphenylglycine and L-glutamate) were found to be substrates for this enzyme. In this respect, D-PhgAT is different from typical L-amino-acid aminotransferases and D-amino-acid aminotransferases. Nor is D-PhgAT an amino acid racemase, because the D- and L-amino acids on the two sides of the transamination reaction have structurally different side-groups.

The narrow substrate range of D-PhgAT is advantageous for its host bacterium growing on D-phenylglycine as the sole source of carbon and nitrogen. While actively taking part in D-phenylglycine dissimilation, the aminotransferase may not interfere with normal metabolism of other amino acids which are essential for cell growth. Additionally, by the 'stereo-inverting' transamination property of this aminotransferase, the amino nitrogen can be channeled directly from the D-phenylglycine substrate into L-glutamate which is a central molecule in cellular nitrogen metabolism. This is accomplished in one step without the need for additional enzymes to racemize the phenylglycine or glutamate. How such stereo-inverting transamination proceeds is not known. Domain motions and the existence of open and closed conformations are evident in aspartate aminotransferase (McPhalen et al., 1992), whose substrate-product pairs are very similar in structure and size. Larger conformational changes would be expected with D-PhgAT to accommodate its two substrate-product pairs which are much different in size, shape, polarity of the sidechains and configuration of amino group at the α-carbon.

While it seems that D-PhgAT may have evolved to specialize in D-phenylglycine utilization, data on the natural abundance of D-phenylglycine or its derivatives is lacking. Two organisms are known to produce D-4-hydroxyphenylglycine, yet in small amounts. A strain of *Streptomyces fungicidicus* (Hatano et al., 1984) produces enduracidins which are cyclic peptide antibiotics containing D-4-hydroxyphenylglycine, L-4-hydroxyphenylglycine and L-3,5-dichloro-4-hydroxyphenylglycine. *Nocardia uniformis* subsp. *tsuamanensis* (ATCC 21 806) produces a series of monocyclic D-lactam antibiotics called nocardicins (Hosoda et al., 1977) which contain D-4-hydroxyphenylglycine moieties. L-4-hydroxyphenylglycine was found to be the true immediate precursor and the inversion into Dconfiguration took place at the time the precursor was used to assemble the nocardicin molecules (Townsend and Brown, 1981).

Enzymatic transamination has not been applied for production of D-phenylglycine and D-4-hydroxyphenylglycine, probably due to the following reasons. Firstly, because of the high cost of the 2-oxo acid substrates, benzoylformate and 4-hydroxybenzoylformate. Secondly, commonly known D-amino-acid aminotransferases have very low transamination activity towards D-phenylglycine (Soda and Esaki, 1994), and the amino-group donors accepted are strictly only D-amino acids. Direct application of D-amino acids as amino-group donors is not practical because of their high cost. An alternative way is to generate D-amino acids in situ from their L-isomers using a corresponding amino-acid racemase as an extra enzyme added into the transamination system.

With the recent advance in chemical synthetic methods (Fell et al., 1985; Zhao and Lee, 1994), many 2-oxo acid precursors, including benzoylformate and 4-hydroxybenzoylformate, may now be prepared at a relatively low cost. This, together with the unique substrate specificity of D-PhgAT from ST-201 have made the aminotransferase approach become a more attractive alternative process for Dphenylglycine synthesis. With this D-PhgAT, L-glutamate can be used directly as an amino-group donor without the need for an amino-acid racemase to isomerize the L-amino acid amino-group donor, as required with typical D-amino-acid aminotransferases which accept only D-amino acid substrates. Our preliminary data demonstrated that D-PhgAT can be used, with reasonable stability, to synthesize enantiopure D-phenylglycine from benzoylformate and L-glutamate. By a similar process, D-4-hydroxyphenylglycine can also be produced.

Acknowledgements

We wish to thank Prof. T.W. Flegel for his critical reading of the manuscript. Financial support from The Thailand Research Fund which covered part of this work is gratefully acknowledged.

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electronic reprint

Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray crystallographic analysis of D-phenylglycine aminotransferase from *Pseudomonas stutzeri* ST201

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Crystallization and preliminary X-ray crystallographic analysis of D-phenylglycine aminotransferase from *Pseudomonas stutzeri* ST201

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Received 19 September 2002
Accepted 20 March 2003

D-Phenylglycine aminotransferase (D-PhgAT) catalyzes the reversible transamination of D-phenylglycine to L-glutamate with 2-oxoglutarate as the amino-group acceptor. Crystals of substrate-free *Pseudomonas stutzeri* D-PhgAT bound to the cofactor pyridoxal-5′-phosphate (PLP) were obtained by the hanging-drop vapour-diffusion method using ammonium sulfate as a precipitant. The crystals belong to space group $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 75.155$, $c = 147.554$ Å. The asymmetric unit contains one molecule of D-PhgAT and has a solvent content of 50.0%. A complete native X-ray diffraction data set was collected from a single crystal at 100 K to a resolution of 2.3 Å.

1. Introduction

D-Phenylglycine aminotransferase (D-PhgAT), a vitamin B_6-dependent enzyme, catalyzes the stereospecific amino-group transfer of D-phenylglycine to 2-oxoglutarate to yield L-glutamate by a reversible ping-pong kinetic mechanism (Wiyakrutta & Meevootisom, 1997; Kirsch *et al.*, 1984). The enzyme requires pyridoxal phosphate (PLP) as a coenzyme and has substrate specificity for D-phenylglycine and D-4-hydroxyphenylglycine. However, neither D- nor L-aromatic nor branch-chained amino acids can be utilized as substrates. Using the 'stereo-inverting' transamination property of D-PhgAT, the amino nitrogen can be channelled directly between D-phenylglycine and L-glutamate, a central molecule in cellular nitrogen metabolism, without the need for additional amino-acid racemases for phenylglycine or glutamate. D-PhgAT, found as a homodimer of molecular mass 92 kDa, has been purified from *Pseudomonas stutzeri* ST201 (Wiyakrutta & Meevootisom, 1997). The enzyme is most active at alkaline pH, with maximum activity at pH 9–10, and can be inhibited by typical inhibitors of pyridoxal phosphate-dependent enzymes. The D-PhgAT encoding gene, isolated from the plasmid pBPL-ph, was cloned into the expression vector pET-17b and the expression host BL21(DE3) (Laowanapiban, 2001). From a BLASTp search (Altschul *et al.*, 1997) using the deduced amino-acid sequence, the 453-residue D-PhgAT was found to be homologous to the aminotransferase subgroup II (Mehta *et al.*, 1993) or aminotransferase subclass II (Schneider *et al.*, 2000), including glutamate-1-semialdehyde aminomutase (GSAAT) and ornithine aminotransferase (OAT) with sequence identity of 25–30 and 21%, respectively. Notably, D-PhgAT lacks sequence homology to D-amino-acid aminotransferase (DAAT), having a sequence identity of only 16%.

In general, the amino-group transfer reaction catalyzed by aminotransferases is highly stereo-conserved (Sugio *et al.*, 1995; Peisach *et al.*, 1998) and there is little information regarding transamination reactions between two amino acids which have opposite configuration (van den Tweel *et al.*, 1986, 1988). Therefore, we have initiated a crystallographic investigation of the stereospecific reaction mechanism, as well as of the substrate specificity of the D-PhgAT enzyme.

2. Experimental

2.1. Overexpression and purification

pEPL-transformed *E. coli* BL21(DE3) cells were grown in LB medium with 50 mg l^{-1} ampicillin at 303 K. When the culture reached an absorbance at 600 nm of 2.0, IPTG was added to 0.4 m*M* and the culture was further incubated for 3 h. The cells were harvested by centrifugation, resuspended in 50 m*M* phosphate buffer pH 7.0 with 1 m*M* PLP and 1 m*M* PMSF and subsequently disrupted by a sonicator. The protein was purified by ammonium sulfate precipitation at 25–45% saturation, followed by chromatography on a phenyl-agarose CL-4B column and a DEAE anion-exchange column. The combined active fractions were desalted and concentrated to about 10 mg ml^{-1} using a Centricon Plus-20 ultrafiltration membrane (Amicon, Beverly, MA, USA).

2.2. Crystallization and X-ray data collection

Initial crystallization conditions were obtained using the vapour-diffusion technique

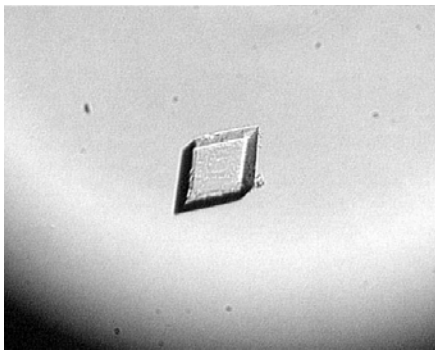

Figure 1
Photograph of a recombinant D-PhgAT trigonal crystal (approximate dimensions 0.2 × 0.2 × 0.2 mm).

in a 24-well tissue-culture plate at 277 and 298 K. In each drop, 2 µl of 10 mg ml^{-1} D-PhgAT solution in 10 m*M* Tris pH 7.8, 100 m*M* NaCl, 1 m*M* EDTA was mixed with an equal volume of reservoir solution. Promising conditions were discovered using $(NH_4)_2SO_4$ and pH-screening grids at 277 K and were further optimized with respect to pH and precipitant concentration. The optimal condition was found to consist of 200 m*M* phosphate buffer pH 6.2 and 28–30% saturated $(NH_4)_2SO_4$. These rhombohedral crystals typically grew to 0.2 × 0.2 × 0.2 mm within a few weeks (Fig. 1).

2.3. Data collection

A 2.3 Å resolution data set from D-PhgAT in complex with PLP was collected at the National Synchrotron Light Source (NSLS) at Brookhaven National Laboratory on beamline X8C. A single crystal (0.1 × 0.1 × 0.1 mm), cryoprotected by a 60 s soak in a reservoir solution containing 35% $(NH_4)_2SO_4$, 200 m*M* phosphate buffer pH 6.2 and 30% glycerol, was flash-frozen in the cryogenic nitrogen stream using a nylon loop. The crystals of D-PhgAT complexed with PLP possessed trigonal symmetry, with unit-cell parameters $a = b = 75.155$, $c = 149.554$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. The reflection data were collected on an ADSC Quantum-4 CCD detector using X-ray radiation ($\lambda = 0.9795$ Å) with a crystal-to-detector distance of 200 mm and a 0.5° oscillation angle per image, with an exposure time of 60 s for each image and a total of 360 frames (Fig. 2). The diffraction data were integrated, scaled and reduced with *MOSFLM* and *SCALA* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994) (Table 1). The Laue symmetry and systematic absences showed a clear threefold screw axis, identifying the space group as either $P3_121$ or $P3_221$. The data set was 99.8% complete, with a scaling R_{sym} of 0.065 for 22 083 unique reflections in the resolution range 20.0–2.3 Å (Table 2). Although D-PhgAT exists as a dimeric protein in solution, there is one molecule of D-PhgAT in the asymmetric unit. This revealed that in the crystalline state each monomer of the dimeric enzyme adopts a similar conformation, unlike the asymmetric dimeric crystal structures of glutamine-1-semialdehyde aminomutase (Hennig *et al.*, 1997) and ornithine aminotransferase (Shen *et al.*, 1998). Assuming one monomer of 47.5 kDa D-PhgAT in the asymmetric unit, the calculated volume per unit mass, V_M (Matthews, 1968), is 2.46 Å^3 Da^{-1}, corresponding to a solvent content of 50.0%.

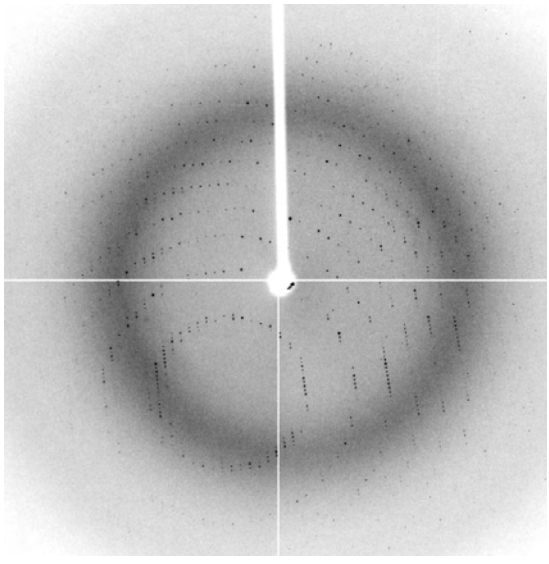

Figure 2
A typical 0.5° oscillation X-ray diffraction pattern of a D-PhgAT crystal.

Preliminary phases for D-PhgAT have been obtained by a molecular-replacement technique using a modified GSA-AT structure. A full description of the procedure for obtaining phase information will be published elsewhere.

The authors wish to thank the Thailand Research Fund for financial support (RSA15/2544). We acknowledge the use of the X8C beamline at the National Synchrotron Light Source (Brookhaven, USA), Dr Herbert E. Klei (Bristol-Myers Squibb) for his assistance during the data collection and Professor William N. Lipscomb for critical comments. CS is supported by a scholarship from the Postgraduate Education and Research in Chemistry.

Table 1
Unit-cell parameters and data-collection statistics.

Space group	$P3_121$ or $P3_221$
Unit-cell parameters (Å)	$a = b = 75.155$, $c = 147.554$
Resolution (Å)	20–2.3
Temperature	100
Wavelength (Å)	0.9795
Total No. of reflections	487524
No. of unique reflections	22083
Data completeness	99.8
R_{sym}	0.065
Average $I/\sigma(I)$	8.7
Multiplicity	10.7
No. of molecules in asymmetric unit	1
V_M (Å^3 Da^{-1})	2.46
Solvent content (%)	50.0

Table 2
Statistics of data collection from a D-PhgAT crystal by resolution shell.

Resolution (Å)	No. of unique reflections	Completeness (%)	R_{sym}
7.27	753	95.1	0.040
5.14	1346	100	0.041
4.20	1688	100	0.040
3.64	1985	100	0.045
3.25	2218	100	0.056
2.97	2444	100	0.082
2.75	2660	100	0.112
2.57	2826	99.9	0.152
2.42	2960	99.9	0.203
2.30	3203	100	0.270

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Specific detection of L-glutamate in food using flow-injection analysis and enzymatic recycling of substrate

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Received 7 April 2004; received in revised form 20 May 2004; accepted 20 May 2004

Abstract

A flow injection analysis (FIA) system for specific determination of L-glutamate in food samples based on a bi-enzymatic amplification system has been developed. The content of L-glutamate in the sample was amplified by cycling between L-glutamate dehydrogenase (GlDH) and a novel enzyme, D-phenylglycine aminotransferase (D-PhgAT). In this system, GlDH converts L-glutamate to 2-oxoglutarate with concomitant reduction of NAD^+ to NADH. D-PhgAT transfers an amino group from D-4-hydroxyphenylglycine to 2-oxoglutarate, thus recycling L-glutamate. Accumulation of NADH in the course of the enzymatic recycling was monitored both by fluorescence and UV absorbance and used for quantification of L-glutamate. The assay was characterized by high long-term stability (at least 70 days) and good reproducibility (within-day and between-day RSDs were 4.3–7.3% and 8.9%). The fluorimetric assay was slightly more sensitive with a L-glutamate detection limit of 0.4 μM and linear range of 2.5–50 μM. The assay was specific for L-glutamate, with recoveries between 95–103% in the presence of 17 different amino acids tested one by one. The method was applied to analysis of real food samples and results were correlated with a commercial Boehringer Mannheim assay kit.

Keywords: L-Glutamate; Flow injection analysis; D-Phenylglycine aminotransferase; Enzymatic cycling

1. Introduction

Monosodium L-glutamate (MSG) has been used for several decades to enhance the flavour and taste of food throughout the world. Nevertheless, questions have been raised since the early 1980s about the safety of MSG. The debate concerns whether MSG contributes to the adverse reactions described as Chinese Restaurant Syndrome [1–3] or more recently referred to as MSG symptom complex, which occurs in certain groups of people after ingestion of food containing high amount of MSG. L-Glutamate is thus considered an important analyte to determine in food. Besides the safety of food, L-glutamate quantification is also an essential part of quality control in such industries as fish sauces production [4], especially, in Thailand and other Southeast Asian countries, where the annual production of fish sauces for local consumption and worldwide export is large-scaled.

Different kinds of food, particularly, fish sauces and soy sauces contain various amino acids in relatively high concentration compared to L-glutamate [5–7], which results in impaired recovery values. Thus, the development of an assay with improved selectivity and sensitivity for measuring L-glutamate is important for the fish sauce industry as well as for other food industries where the content of free L-glutamate in the products has to be quantified.

Numerous analytical methods for determination of L-glutamate have been developed using different detection techniques and assay formats, e.g. spectrophotometry [8,9], fluorescence [10], chromatography [11], enzyme based electrode [12], and flow injection analysis (FIA) [13–23].

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doi:10.1016/j.aca.2004.05.048

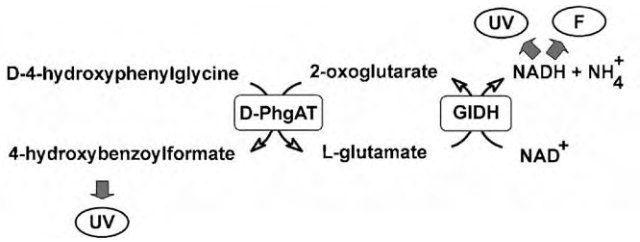

Fig. 1. Enzymatic cycling for the determination of low levels of L-glutamate using GlDH and D-PhgAT. Each turn of the cycle generates one molecule each of NADH and 4-hydroxybenzoylformate, which were detected by UV absorbance at 340 nm. NADH was also detected fluorometrically ($\lambda_{ex} = 340$ nm, $\lambda_{em} = 460$ nm).

Commercially available and the most common method for determination of L-glutamic acid is the end-point colorimetric method [8], which is considered to be time-consuming and laborious. An alternative to batch methods is flow injection analysis (FIA), which has become of widespread use, since the method can be completely automated and offers excellent reproducibility, minimized analysis time, reduced consumption of reagents, and in certain configurations, very high sensitivity. Among the FIA methods reported for determination of L-glutamate [13–23], those based on enzymatic recycling of the substrate (L-glutamate) have shown to be superior in terms of enhanced sensitivity [17,22,23]. We recently isolated and characterized a novel enzyme D-phenylglycine aminotransferase (D-PhgAT), which has unique substrate specificity towards D-phenylglycine as an amino group donor [24]. D-PhgAT coupled to L-glutamate dehydrogenase (GlDH) provides a recycling system, in which L-glutamate is amplified with high specificity (Fig. 1). Earlier we applied this approach to spectrophotometric determination of L-glutamate in batch with high sensitivity and high selectivity [25].

In this work we developed a FIA method for determination of L-glutamate in food samples based on the recycling of L-glutamate by a co-immobilized GlDH/D-PhgAT reactor. The NADH, amplified in the reactor according to the reaction cycle shown in Fig. 1, was monitored both by fluorescence and UV detection and correlated to the concentration of L-glutamate in the sample.

2. Experimental section

2.1. Chemicals and buffers

D-phenylglycine aminotransferase (D-PhgAT) was purified from a recombinant *E. coli* strain expressing the cloned gene encoding the D-PhgAT from *Pseudomonas stutzeri* ST201 as previously described [26].

The following reagents were purchased from Fluka Chemie AG (Buchs, Switzerland): β-NAD^+; pyridoxal-5′-phosphate (PLP); adenosine-5′-diphosphate disodium salt (ADP); D-phenylglycine; D-4-hydroxyphenylglycine; 2-oxoglutaric acid; benzoylformic acid; and L-glutamate dehydrogenase (GlDH, EC 1.4.1.3, from bovine liver containing in 50% glycerol). L-Glutamic acid, glycine, glycerol, EDTA, Trisma® base, glutaraldehyde, sodium cyanoborohydride ($NaBH_3CN$), Bradford reagent, bovine serum albumin (BSA), and L-amino acids used in the interference studies (asparagine, aspartate, alanine, cysteine, histidine, leucine, isoleucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, valine) were from Sigma (St. Louis, MO). Aminopropyl controlled-pore glass (AMP-CPG, 240 Å pore size, 80–120 mesh) was purchased from CPG Inc. (Fairfield, NJ) online. The L-glutamic acid assay kit was from Boehringer Mannheim (Mannheim, Germany). All other chemicals were of analytical grade, and HPLC grade water was prepared in a Milli-Q system (Millipore, Bedford, MA). Fish sauce (Tiparos), soy sauce (Healthy Boy) and oyster sauce (Maekrua) were products from Thailand, which were purchased from a local food stores in Sweden.

Phosphate buffer used for immobilization was 0.1 M potassium phosphate, pH 7.0. The buffer used as carrier in FIA and for enzyme activity measurements was 0.1 M Tris–HCl, pH 8.0.

2.2. Instrumentation

The automatic FIA system, shown in Fig. 2, consisted of a Gilson ASTED 233XL autosampler (Villiers-le-Bel, France) with a Rheodyne six-port injector valve (model 7010, Berkeley, CA) equipped with a 10 μl injection loop. The autosampler was controlled by the Gilson System Interface Module (model 506C), which enabled data acquisition using the UniPoint 3.0 software (Gilson). Carrier buffer, D-4-hydroxyphenylglycine and NAD^+ were supplied via Gilson Minipuls 3 peristaltic pump (Villiers-le-Bel, France). A 473 μl knitted coil was introduced for adequate mixing of the reagents prior to the injection. A 50 μl flow through column (0.5 cm long, 3.5 mm i.d.) filled with CPG with immobilized enzymes was connected to the injection valve. Analytical signals were simultaneously registered by a Spectra FL2000 fluorescence detector (Spectra-Physics, Milford, MA) and by a Gilson UV–vis detector (model 118). Excitation and emission wavelengths for the fluorescence detector were set at 340 and 460 nm, respectively. UV absorbance

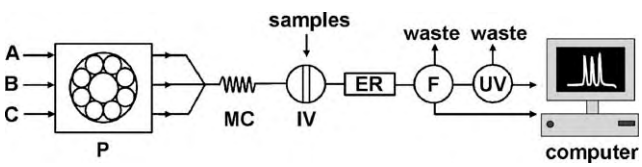

Fig. 2. Schematic diagram of the flow injection system. A: Tris–HCl buffer (0.1 M, pH 8.0); B: 10 mM D-4-hydroxyphenylglycine, containing 1 μM PLP; C: 2 mM NAD^+; P: peristaltic pump; MC: mixing coil; IV injection valve; ER: enzyme reactor; F: Fluorescence detector; UV: UV detector.

was registered at 340 nm. All connections were made using PEEK tubings (i.d. 0.25 mm, Alltech, Deerfield, IL).

Absorbance measurements for enzyme activity and Bradford assay were performed using a Hitachi U-1100 (Tokyo, Japan) spectrophotometer.

2.3. Preparation of immobilized enzyme reactor

Enzymes were covalently immobilized on AMP-CPG. 50 mg dry weight of CPG was activated by glutaraldehyde (GA) in two different ways: (1) "wet" activation, 2.5% (v/v) GA in 0.1 M phosphate buffer, pH 7.0, incubated for 1 h under reduced pressure at room temperature (RT), and (2) "dry" activation, exposed to the vapour of 1 ml of 25% (v/v) GA in a sealed Petri dish for 24 h. After activation with GA, the beads were washed extensively with Milli-Q water and then with 0.1 M phosphate buffer pH 7.0. Unless stated otherwise, 6 units of GlDH and 6 units of D-PhgAT (ratio 1:1) in 1 ml of phosphate buffer were mixed and used for immobilization on the GA activated CPG in the presence of 3 mM ADP and 0.5 mM PLP since there are evidence that ADP can enhance the activity of free [27] and immobilized [19] GlDH. Immobilization of enzymes on CPG was performed overnight under agitation at 4 °C. The enzyme activated CPG was collected and washed with phosphate buffer, and then residual aldehyde groups were blocked with 100 mM glycine solution at 4 °C for 1.5 h. The resulting Schiff bases were reduced by addition of $NaBH_3CN$ to the final concentration of 3.5 mg ml^{-1} and the reaction was let to proceed for 1.5 h. The enzyme-activated support was then thoroughly washed with phosphate buffer and packed into a 50 μl flow through reactor. When not in use, the enzyme reactor was stored at 4 °C in phosphate buffer pH 7.0 in the presence of 20% glycerol and 0.5 mM EDTA.

2.4. Enzyme activity assay

D-PhgAT activity was assayed as previously described [25]. Briefly, 20 μl of D-PhgAT was added to 980 ml of a mixture containing 0.1 M Tris–HCl pH 8.0, 10 mM D-phenylglycine, 25 mM 2-oxoglutarate, 5 mM PLP, and 5 mM EDTA. The rate of benzoylformate formation upon transamination of D-phenylglycine with 2-oxoglutarate as an amino group acceptor was monitored spectrophotometrically at 254 nm at 25 °C. One unit of the D-PhgAT activity was defined as the amount of enzyme capable of producing one micromole of benzoylformate per minute under these conditions.

The activity of GlDH was measured based on the degradation of ammonia, and concomitant oxidation of NADH. The depletion of NADH was monitored spectrophotometrically at 340 nm. One activity unit for GlDH corresponded to the amount of enzyme that catalyzed the oxidation of one micromole of NADH per minute under the defined conditions.

2.5. Protein assay

The protein concentration in the supernatant before and after immobilization was determined by the Bradford method [28] using BSA as a standard. The resulting enzyme concentrations were used to calculate the coupling yield as the ratio of total protein amount (D-PhgAT and GlDH) before and after immobilization.

2.6. Flow injection analysis procedure

The carrier buffer and the two substrate buffers 2 mM NAD^+ and 10 mM D-4-hydroxyphenylglycine containing 1 μM PLP were supplied by the peristaltic pump and mixed in a mixing coil at a total flow rate of 0.2 ml min^{-1}. L-Glutamate standard solutions at concentrations between 0 and 100 μM were introduced into the FIA system via the ASTED autosampler. When food samples (fish, oyster and soy sauces) were analyzed these were first diluted 1000, 4000 or 10,000 times with the carrier buffer. When the standard or the sample reached the enzyme reactor, the flow was stopped for 3 min, allowing the incubation of the analyte with enzymes. The time when the sample reached the reactor was determined by injecting bromocresol green and measuring the time between the injection and the color development in the column. Fluorescence signals for NADH and UV absorbance signals for NADH and 4-hydroxybenzoylformate were used to quantify the analyte concentration in the sample. To estimate the interference from other amino acids, a mixture containing 10 μM L-glutamate and 10 μM L-amino acid was injected and the peak height was compared with that from 10 μM L-glutamate alone.

2.7. L-glutamate analysis using the Boehringer Mannheim kit

The measurement of L-glutamate using the Boehringer Mannheim kit was performed according to the manufacturer's instructions with a minor modification, adjusting the volume to 1.0 ml. For detection of L-glutamate in fish, soy, and oyster sauces the samples were first diluted 100–500 times and then analyzed in the same way as L-glutamate standards.

3. Results and discussion

3.1. Enzymatic cycling of L-glutamate using GlDH and D-PhgAT

Cycling assays to measure L-glutamate in foodstuff and biological samples were previously reported [9,10,17,19,22,23,29]. The most common approaches are based on a combination of GlDH with amino acid aminotransferases [10,19,29] or L-glutamate oxidase [17,22,23]. Any cycling assay involves amplification of a reaction product signal,

thus allowing the detection of analytes with higher sensitivity. However due to the broad substrate specificity of the enzymes used and the presence of interfering compounds in samples, most glutamate assays suffer from insufficient selectivity. For example, L-glutamate oxidase uses both L-glutamate and L-aspartate as substrates [9], thus both amino acids will be amplified and detected when combined with GlDH. The same is true for L-alanine aminotransferase and L-aspartate aminotransferase used in L-glutamate recycling assays.

D-PhgAT from *Pseudomonas stutzeri* ST201 catalyzes the transamination reaction between D-phenylglycine or D-4-hydroxyphenylglycine and 2-oxoglutarate, resulting in the formation of L-glutamate and benzoylformate or 4-hydroxybenzoylformate [24]. The D-specific aminotransferase (D-PhgAT) was used in this work in order to improve the selectivity for L-glutamate detection combined with high sensitivity due to amplification through recycling. D-PhgAT and GlDH were co-immobilized on CPG, packed in a reactor and inserted into the FIA set up shown in Fig. 2. The L-glutamate recycling assay (Fig. 1) generates two accumulating products, NADH and 4-hydroxybenzoylformate. Both NADH and 4-hydroxybenzoylformate strongly absorb at 340 nm ($\varepsilon_{340\,\mathrm{nm}} = 6.22 \times 10^3$ and $8.90 \times 10^3\,\mathrm{l\,mol^{-1}\,cm^{-1}}$, respectively) [25], and thus the signal amplification effect of the cycling reactions is further enhanced. The GlDH/D-PhgAT enzyme reactor was connected to two detectors in series: fluorescence, registering accumulation of NADH, and absorbance, registering the accumulation of both NADH and 4-hydroxybenzoylformate. The detection of L-glutamate using both detection techniques was evaluated and compared.

3.2. Optimization of co-immobilized enzyme ratios

The sensitivity of the cycling assay is dependent on the activity of the two co-immobilized enzymes. To achieve the best performance, the ratio between co-immobilized GlDH and D-PhgAT was optimized. CPG with GlDH:D-PhgAT activity ratios of 1:5, 1:2.5, 1:1, 5:1, and 10:1 (unit $\mathrm{ml^{-1}}$) were prepared and the immobilization efficiencies calculated from the protein content before and after the immobilization step. Low coupling yield (36%) was obtained for the reactors with the GlDH:D-PhgAT ratios of 5:1 and 10:1, whereas high coupling yield (90%) was observed for the reactors with ratios of 1:2.5 and 1:5. The coupling yield for the reactor with 1:1 ratio was 70%. Since GlDH is much larger (hexamer, 336 kDa) [30] than D-PhgAT (dimer, 90 kD) [24,26] the low coupling yields at high GlDH content might be explained by steric hindrance that affects the penetration of enzymes into the pores and thus influences the immobilization efficiency, when the content of GlDH increases.

Further, enzyme reactors with the different activity ratios were tested in the FIA system where the fluorescence intensity of 15 μM L-glutamate was measured. As seen in Fig. 3, the signal increases with the amount of D-PhgAT (GlDH:D-PhgAT ratios between 1:2.5 and 10:1), which indicates that D-PhgAT provides an efficient contribution to the cycling of L-glutamate in the system. The highest amplification was observed for the GlDH:D-PhgAT ratio of 1:2.5, however, upon the subsequent stability study of each reactor, the 1:2.5 ratio showed a 30% decrease of activity between two days of measurement. This decrease was less dramatic for the 1:1 and 1:5 ratios (14 and 18% respectively), at the same conditions for the same period of time, hence the 1:1 activity ratio was chosen for all further experiments.

3.3. Stability of the enzyme reactor

The operational stability of the enzyme reactor with a GlDH:D-PhgAT ratio of 1:1 was evaluated by repetitive injections (100 times) of 10 μM L-glutamate during one day. The decrease of activity after the first 50 injections was 20%, and after another 50 injections the total decrease in activity was 25% (results not shown).

Long-term storage stability of the enzyme reactors was evaluated by intermittent injection of different concentrations of L-glutamate. We found that after 7–10 days the signal had decreased to about 50% of the initial value, but then it remained constant over at least 70 days for all L-glutamate concentrations tested (Fig. 4).

Excessive cross-linking with GA may lead to aggregation, precipitation, and distortion of the three dimensional enzyme structures and thus result in dramatic decrease of activity [31]. To potentially improve the stability of the immobilized enzymes, two types of CPG activation procedures were tested for the 1:1 activity ratio: (1) “wet” activation with 2.5% GA and (2) “dry” activation with vapour (25% GA). Vapour GA activation is a milder procedure, and has shown to be less harmful to immobilized proteins [32]. Comparison of the two procedures revealed that vapour GA treatment did yield higher initial enzyme activity (36%), which however decreased and reached the level of the activity of the “wet” reactor in a week (data not shown). In the longer perspective (70 days) we did not observe any difference in the performance of “wet” and “dry” reactors.

The stability of all reactors was followed until a constant signal was reached (50% of the initial values), which, as stated above, took place after 7–10 days. At this point the reactors were used for the further experiments described below.

3.4. Cycling efficiency of the enzyme reactor

A factor that enhances the assay sensitivity is the substrate recycling time, which is governed by the residence time in the enzyme reactor. With this principle, a small amount of L-glutamate can be recycled in the reactor, maintaining the analyte concentration nearly constant, while at the same time amplifying the concentration of detectable products (NADH

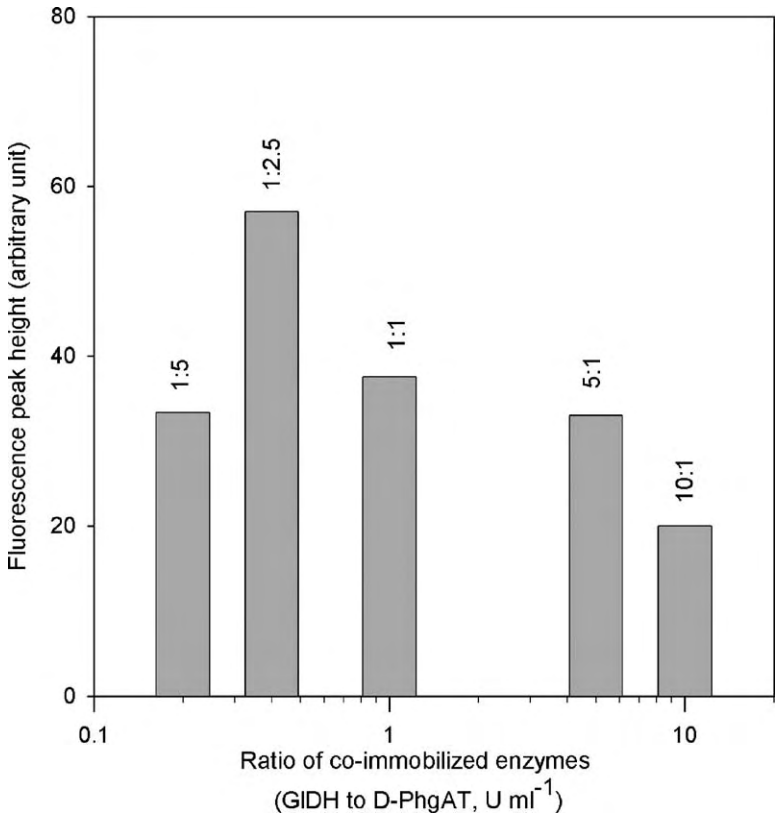

Fig. 3. Influence of activity ratio between co-immobilized GlDH and D-PhgAT on the intensity of fluorescence peak. L-Glutamate 15 μM, incubation time 3 min. Other conditions are as in Fig. 2.

and 4-hydroxybenzoylformate in our case). Thus, the longer the residence time, the more substrate cycles are performed, which leads to the accumulation of detectable product(s). In this report, a reactor with a total bed-volume of 50 μl was used. To achieve 1 min substrate recycling in this reactor, a flow rate as low as 0.05 ml min^{-1} must be used. However, under these conditions, undesired dispersion of the analyte with resulting huge peak broadening was observed (data not shown). To avoid this, the flow rate was increased to 0.2 ml min^{-1} and sufficient amplification of the product was

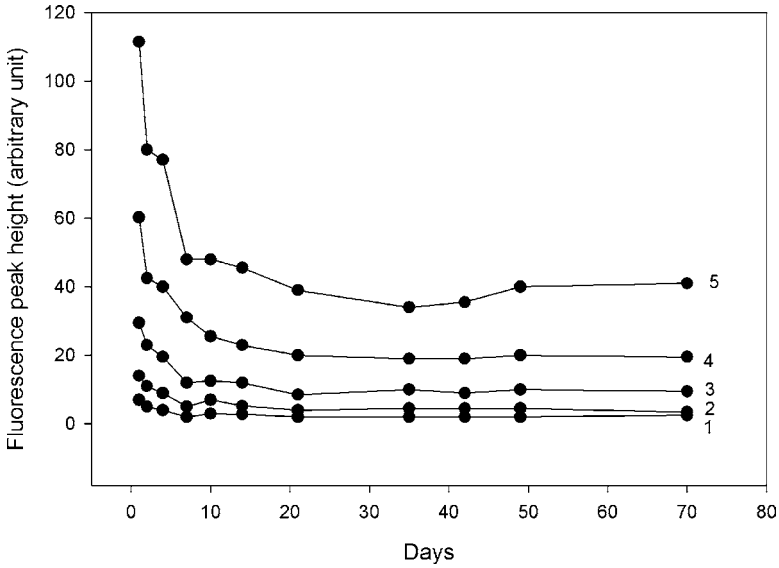

Fig. 4. Long-term stability of enzyme reactor. Fluorescence signals obtained from various concentrations of L-glutamate. (1) 6.25 μM, (2) 12.5 μM, (3) 25 μM, (4) 50 μM, and (5) 100 μM. Conditions: GlDH:D-PhgAT 1:1, incubation time 0 min. Other conditions are as in Fig. 2.

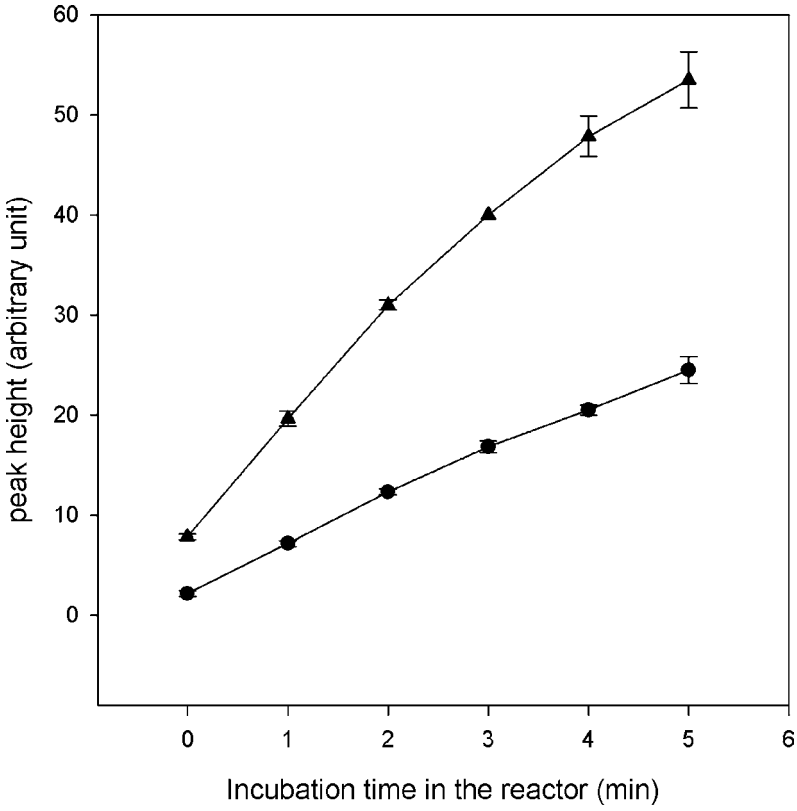

Fig. 5. Effect of the residence time of the L-glutamate standard in the enzyme reactor on the intensity of fluorescence and UV signals. 15 μM L-glutamate, GlDH:D-PhgAT ratio 1:1. (▲) fluorescence; (●) UV absorbance. Each point is the mean value from triplicate assays; the error bar represents the standard deviation. Other conditions are as in Fig. 2.

achieved by stopping the flow, incubating the analyte in the reactor for the desired time. Fig. 5 shows the effect of varying the residence time (between 0 and 5 min) on the intensity of the fluorescence and UV signal of the product. A residence time of 3 min was optimal in terms of intensity of the product signal and assay time for one sample, and was thus selected for further experiments.

3.5. Calibration graph characteristics

Calibration curves for L-glutamate using fluorescence and UV detection were recorded for analyte concentrations between 2.5 and 100 μM. Linearity of the calibration curve was observed up to 50 μM ($r = 0.998$), however for the convenience of analyzing food samples (see Section 3.7) the range of 2.5–20 μM ($r = 0.999$) was chosen for further measurement. Another reason for why injection of high L-glutamate concentration was avoided was because it exhausted the enzymes and resulted in a decrease of reactor activity.

Fig. 6 shows L-glutamate calibration curves (average of nine different calibrations during three days) using fluorescence and UV detection. In the absence of D-4-hydroxyphenylglycine, L-glutamate is not recycled and the signal is, as seen, not amplified. In this so-called non-cycling FIA system the sensitivity was significantly less compared to the recycling system. As seen, UV detection was also significantly less sensitive compared to fluorescence, despite the fact that two amplified products (NADH and 4-hydroxybenzoylformate) were monitored by UV, whereas only one (NADH) was fluorescent. The detection limits (signal-to-noise ratio of 3) for L-glutamate using fluorescence and UV detection were however rather similar, namely 0.4 and 0.7 μM, respectively.

The reproducibility of the assay was evaluated from nine different calibration curves performed three-times during a day over three days. Within-day and between-day relative standard deviation (RSD) values were calculated based on all data in the curves, which resulted in the RSD interval for the analyzed concentration range. The resulting RSD values for within-day and between-day were 4.3–7.3% and 8.9%, respectively, using fluorescence detection. For UV detection within-day and between-day RSDs were 8.4–10.9% and 13.0%, respectively.

3.6. Interference from other amino acids

The interference effect from various amino acids commonly used in the food industry on the determination of L-glutamate was investigated. With the aim to study assay specificity, L-glutamate recovery tests were carried out in the presence of other amino acids. Solutions containing 10 μM L-glutamate and 10 μM amino acid were injected in the

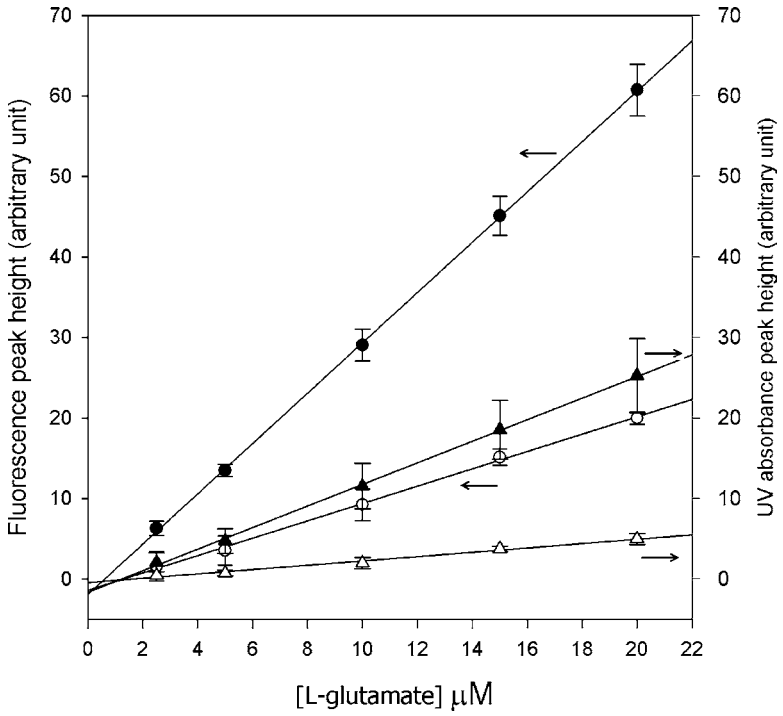

Fig. 6. Calibration curves for L-glutamate using cycling and non-cycling FIA system. Fluorescence detection (●), (○), UV absorbance detection (▲), (△). Solid symbols represent cycling assay, and open non-cycling assay. Data are averages between nine independent calibration experiments. The error bars show the standard deviation. Linear regression for cycling assay: $y = 1.3425x - 1.6440$ (UV), $y = 3.1265x - 1.8747$ (fluorescence detection). Conditions are as in Fig. 2.

enzyme reactor and the analytical signals were compared with those obtained in the presence of 10 μM L-glutamate alone. As shown in Table 1, the selectivity of this assay for L-glutamate in the presence of other L-amino acids and ascorbic acid is excellent (L-glutamate recovery was between 95 and 103%). Such high specificity is observed owing to the unique property of D-PhgAT, not catalyzing the conversion of other L-amino acids, except L-glutamate, and its high specificity towards D-phenylglycine or D-4-hydroxyphenylglycine as amino group donors [20], which are non-natural amino acids not found in food.

Table 1
The influence of various L-amino acids and ascorbic acid on the determination of L-glutamate

Tested interferences	Recovery (%)
None	100.0 ± 0.3
Ascorbic acid	98.0 ± 0.1
L-Asparagine	95.3 ± 0.7
L-Aspartate	102.1 ± 0.1
L-Alanine	100.0 ± 0.3
L-Cysteine	96.6 ± 0.9
L-Glutamine	102.8 ± 0.4
L-Histidine	100.7 ± 0.5
L-Leucine	94.7 ± 0.0
L-Isoleucine	100.7 ± 0.5
L-Lysine	98.9 ± 0.3
L-Methionine	103.2 ± 0.3
L-Phenylalanine	98.7 ± 0.4
L-Proline	97.3 ± 0.9
L-Serine	100.0 ± 0.1
L-Threonine	98.0 ± 0.6
L-Tryptophan	101.1 ± 0.0
L-Valine	99.3 ± 0.3

Conditions as in Fig. 2.

3.7. Application to the analysis of real food samples

Based on the need to determine L-glutamate in foodstuffs, three kinds of food samples were selected for this study: fish-, oyster- and soy sauces. The only pre-treatment was that the samples were diluted before injection into the FIA system. The samples were then analyzed by the proposed method, using L-glutamate standards to determine the actual content of the analyte in the sample. To optimize the sample dilution, sauces were first diluted until a colourless solution was obtained, after that the samples were further diluted so that the signal fell into the linear range of the L-glutamate assay. Optimal dilutions for oyster-, fish-, and soy sauces were 1000, 4000 and 10,000 times, respectively. The results obtained by the recycling FIA method were compared with those obtained by a commercial spectrophotometric method supplied by Boehringer Mannheim. As shown in Fig. 7, good correlation between Boehringer Mannheim and the proposed FIA methods was observed ($r^2 = 0.998$, the relative error less than 4%).

Having a linear range of 2.5–20 μM the proposed flow method is very practical for detection of L-glutamate in food

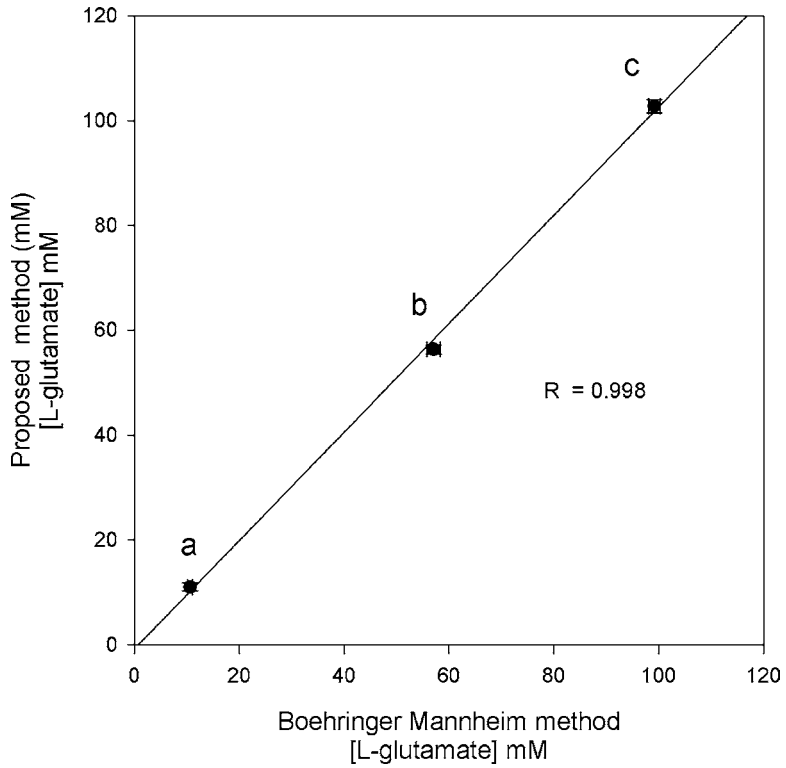

Fig. 7. Comparison of the results obtained from the analysis of real food samples, (a) oyster sauce; (b) fish sauce; (c) soy sauce, by the commercial Boehringer Mannheim and the proposed FIA method using fluorescence detection. Data in triplicates are shown. The error bars represent the standard deviation.

samples. The average content of free L-glutamate in Thai fish sauces is around 14.89 g l^{-1} [6] or 80 mM, which means that the sample must be diluted 8000 times to get L-glutamate concentration in the midpoint of the standard curve. Most of the matrix interferences from the original samples are thus diminished, and therefore the assay is highly specific.

4. Conclusions

A new FIA method for determination of L-glutamate in foodstuff, based on bi-enzymatic recycling, has been developed. L-glutamate was quantified at the sub micromolar level using a co-immobilized GlDH and D-PhgAT reactor combined with fluorescence and UV detection. Our work demonstrates that the unique substrate specificity of D-PhgAT enables very selective determination of L-glutamate in the presence of interfering compounds and in food containing various amino acids, e.g., fish sauces produced in Southeast and East Asian countries. The developed FIA method correlates well with the commercial Boehringer Mannheim L-glutamate assay kit ($r = 0.998$, bias <4%). Sensitive detection of L-glutamate is achieved using either fluorescence or UV detection. The latter is especially useful since common relative low cost instruments can be utilized for detection in the proposed FIA.

Acknowledgements

This work was supported by The Swedish Foundation for International Cooperation in Research and Higher Education (STINT), The Swedish Research Council (Vetenskapsrådet), and Thailand Research Fund (TRF) through the Royal Golden Jubilee Ph.D. program (grant no. PHD/0144/2542).

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Isolation and characterization of a benzoylformate decarboxylase and a NAD^+/$NADP^+$-dependent benzaldehyde dehydrogenase involved in D-phenylglycine metabolism in *Pseudomonas stutzeri* ST-201

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Received 6 July 2007; received in revised form 7 August 2007; accepted 8 August 2007
Available online 25 August 2007

Abstract

Following induction with D-phenylglycine both D-phenylglycine aminotransferase activity and benzoylformate decarboxylase activity were observed in cultures of *Pseudomonas stutzeri* ST-201. Induction with benzoylformate, on the other hand, induced only benzoylformate decarboxylase activity. Purification of the benzoylformate decarboxylase, followed by N-terminal sequencing, enabled the design of probes for hybridization with *P. stutzeri* ST-201 genomic DNA libraries. Sequencing of two overlapping genomic DNA restriction fragments revealed two open reading frames which were denoted *dpgB* and *dpgC*. Sequence alignments suggested that the genes encoded a thiamin-diphosphate-dependent decarboxylase and an aldehyde dehydrogenase, respectively. Both genes were isolated and expressed in *Escherichia coli*. The *dpgB* gene product was confirmed as a benzoylformate decarboxylase while the *dpgC* gene product was characterized as a NAD^+/$NADP^+$-dependent benzaldehyde dehydrogenase. In keeping with their high sequence identities (both greater than 85%) the kinetic properties of the two enzymes were similar to those of the homologous enzymes in the mandelate pathway of *Pseudomonas putida* ATCC 12633. However, *Pseudomonas stutzeri* ST-201 was unable to grow on either isomer of mandelate, and sequencing indicated that the *dpgB* gene did not form part of an operon. Thus it appears that the two enzymes form part of a D-phenylglycine, rather than mandelate, degrading pathway.

Keywords: Gene cloning; Southern hybridization; Histidine tag; Mandelate pathway; Enzyme; D-phenylglycine pathway

1. Introduction

Benzoylformate decarboxylase (BFDC; EC 4.1.1.7) is a thiamin diphosphate (ThDP)-dependent enzyme that catalyzes the decarboxylation of benzoylformate forming benzaldehyde and carbon dioxide [1]. BFDC was initially identified as a member of the mandelate pathway, which enables bacteria to utilize mandelate as a sole carbon source. In this pathway, benzoylformate is formed via the oxidation of *S*-mandelate (Fig. 1) [2–4]. To date, the enzyme has been found in *Pseudomonas* and *Acinetobacter* species [5,6], as well as *Neurospora crassa* [7], with the most detailed studies being carried out on the BFDC from *Pseudomonas putida* ATCC 12633 (*Pp*BFDC). These include enzyme purification and characterization [3,4], gene cloning [8], kinetic and mechanistic studies [9–11], and X-ray crystallography [12,13]. Over the past few years *Pp*BFDC has become increasingly useful for the synthesis of enantiomerically pure pharmaceutical and chemical compounds [14–16]. Consequently, there is commercial interest in identifying BFDCs with an expanded substrate range whether by protein engineering [17–19] or from alternative sources [20].

Recently, benzoylformate was reported as the product of the reaction catalyzed by a D-phenylglycine aminotransferase

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doi:10.1016/j.bbagen.2007.08.007

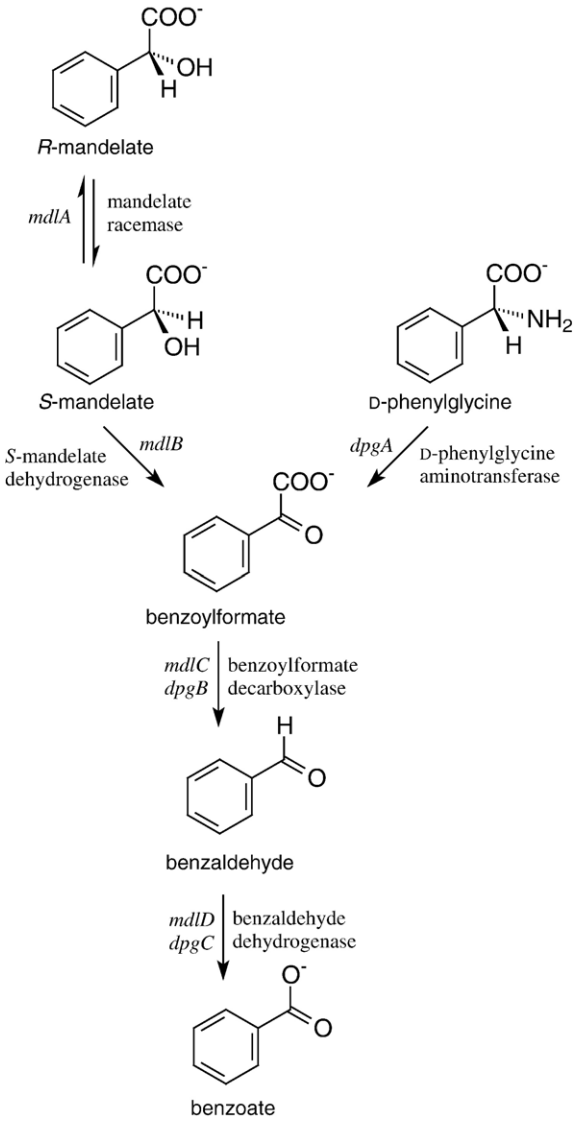

Fig. 1. The mandelate pathway in *P. putida* ATCC 12633 and the proposed pathway for D-phenylglycine degradation in *P. stutzeri* ST-201.

(D-PhgAT) in *Pseudomonas stutzeri* ST-201, a newly isolated soil bacterium found in Thailand [21]. Although *P. stutzeri* ST-201 had not been reported to possess BFDC activity, it had been shown to release both benzoylformate and benzoate into the culture medium during the early stages of D-phenylglycine degradation [21]. Therefore, it was reasonable to hypothesize that this strain could produce both BFDC and benzaldehyde dehydrogenase (BADH) for subsequent metabolism of benzoylformate and benzaldehyde, respectively. Here we confirm the presence of a BFDC in *P. stutzeri* strain ST-201 (*Ps*BFDC) and report the isolation of fragments of *P. stutzeri* ST-201 genomic DNA containing the genes encoding *Ps*BFDC (*dpgB)* and *Ps*BADH (*dpgC*). We describe the expression of *dpgB* and *dpgC* in *Escherichia coli* and the initial characterization of their gene products. Moreover, we suggest that these enzymes form part of a catabolic pathway, which we have termed the D-phenylglycine degradation pathway (Fig. 1).

2. Materials and methods

2.1. Materials

P. stutzeri ST-201 was available from a previous study [21]. *E. coli* strains, restriction enzymes, ligases, DNA polymerase and other molecular biology reagents were obtained from New England Biolabs, Promega, Stratagene or Novagen. All other chemicals were of the highest quality commercially available.

2.2. Screening for enzymes involved in mandelate and D-phenylglycine degradation

To verify the existence of enzymes catabolizing substrates of interest, *P. stutzeri* ST-201 was tested for growth on minimal medium (MM) agar containing (in 1 l): 15 g agar, 3.4 g KH_2PO_4, 3.6 g Na_2HPO_4, 0.2 g $MgSO_4$, 8 mg $CaCl_2$, 10 mg yeast extract and 1 ml trace metals solution [22]. The MM was supplemented, as appropriate, with the following carbon sources at a concentration of 10 mM: *R*-mandelate, *S*-mandelate, D-phenylglycine, benzoylformate or benzoate. For growth on benzaldehyde, a disk containing benzaldehyde (10 μl) was placed on the agar. With the exception of MM containing D-phenylglycine, 0.4 g NH_4NO_3 was included as the nitrogen source. The plates were examined for evidence of growth after incubation for 48 h at 30 °C.

To explore the induction of D-PhgAT and BFDC activity, *P. stutzeri* ST-201 was shaken in 200 ml Luria-Bertani (LB) medium at 30 °C until the culture reached an OD_{600} of 2.8. At that time the LB was exchanged for MM containing one of glucose, D-phenylglycine or benzoylformate (10 mM). Incubation was continued under the same conditions for a further 20–160 min with cell samples being harvested every 20 min. Enzyme activity in crude cell extracts was then determined using standard assays (below).

2.3. Purification of wt PsBFDC

P. stutzeri ST-201 was grown in LB (2 l) at 30 °C until the culture reached an OD_{600} ~2.8. At that time the LB was exchanged for MM containing 10 mM benzoylformate. Shaking continued at 30 °C for 9 h after which time the cells were harvested by centrifugation and the pellet resuspended in a buffer comprising 50 mM potassium phosphate, pH 6.0, 0.1 mM ThDP and 0.1 mM phenylmethylsulfonyl fluoride. Following sonication and centrifugation the cell-free extract (CFE) was subjected to stepwise ammonium sulfate fractionation. The ammonium sulfate fraction containing BFDC was dissolved in buffer A (25 mM potassium phosphate, pH 6.0, 0.1 mM ThDP, 0.1 mM $MgCl_2$, 10% glycerol) and applied to a Phenyl Sepharose FF column which had been equilibrated with buffer B (0.1 M sodium phosphate, pH 6.0 containing 0.1 M $(NH_4)_2SO_4$ and 0.1 mM ThDP). The BFDC was then eluted with buffer B at a flow rate of 2 ml min^{-1}. The active fractions were pooled, concentrated and exchanged into buffer C (25 mM Tris–HCl, pH 7.5, 0.1 mM ThDP) using a Centricon Plus-20 filtration unit (Amicon). The concentrate was loaded onto a Q Sepharose FF column equilibrated with buffer C, and the enzyme was eluted with buffer C containing NaCl (0.75 M). After the purity and molecular weight of the active fractions were checked by SDS-PAGE, a sample was blotted onto a polyvinylidine fluoride membrane and subjected to N-terminal sequencing (University of Newcastle, Australia).

2.4. Construction of subgenomic library and cloning of the dpgB gene

The degenerate forward primer, BFDC_F (Table 1), was designed based on the N-terminal amino acid sequence of *Ps*BFDC isolated in this work (above). The reverse primer, BFDC_R, was designed using the conserved amino acid sequences of BFDCs from *P. putida* (gi:3915757) and *P. aeruginosa* PAO1 (gi:15600094). With these primers a 1.28-kb DNA fragment containing part of the *dpgB* gene was amplified by PCR from the genomic DNA of *P. stutzeri* ST-201. The PCR products were cloned into pGEM-T Easy (Promega) and used to generate a digoxigenin-labeled *dpgB*-specific probe for Southern and colony hybridization.

Table 1
Primers used in this study

Primer	Sequence
BFDC_F[a]	5′-GGCATHGAYACCGTITTCGG-3′
BFDC_R	5′-TAGTTGGCCGAGCCGTCG-3′
BFDC_N[b]	5′-ATATCC**CATATG**GCATCGGTACACAGC-3′
BFDC_C[b]	5′-AAATC**AAGCTT**CACAGGCTGACCGTGCTGAC-3′
BFDC_X[c]	5′-GCACGGTCAGC**CTcgag**AGCTTGGTACCGAGCTCGG-3′
BADH_F[b]	5′-CCGAATAGAGGGATTG**CATATG**AATTATCTGTCCCC-3′
BADH_R[b]	5′-GCGCGCTCCGCGT**CTCGAG**TTACGGCACAATCC-3′

[a] Degenerate primer where H=A, C or T; Y=C or T; I=inosine.
[b] Introduced restriction sites are in bold.
[c] The new *Xho*I site is in bold and the lowercase letters indicate a base change from the wild-type sequence.

For subgenomic library construction, genomic DNA isolated from *P. stutzeri* ST-201 was subjected to digestion with various restriction enzymes, followed by agarose gel electrophoresis and Southern hybridization. Positive DNA fragments were eluted from the gel and ligated into pBluescript II SK which had been digested with the corresponding restriction enzyme(s). The ligation products were transformed into *E. coli* JM109 by electroporation and plated on LB agar containing 50 μg/ml ampicillin, 20 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 80 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). White colonies, containing a DNA insert, were screened by colony hybridization to identify individual colonies carrying a plasmid containing the *dpgB* gene. The two plasmids identified in this manner were designated pBCH1 and pBCH2.

2.5. DNA sequencing and gene analysis

The nucleotide sequences of both strands of the DNA fragment in pBCH1 and pBCH2 were determined at the National Science and Technology Development Agency, Thailand. The open reading frames (ORFs), DNA and protein alignment were analyzed using either VectorNTI (Invitrogen) or BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The nucleotide and deduced amino acid sequences were compared to the sequences in the GenBank and SwissProt databases at the National Center for Biotechnology Information (Bethesda, MD) using the BLAST network server.

The nucleotide sequences determined in this work have been deposited under GeneBank accession no EF419244.

2.6. Expression of P. stutzeri dpgB gene in E. coli and purification of recombinant PsBFDC

The *dpgB* gene was PCR amplified using pBCH1 as the template. The forward and reverse primers, BFDC_N and BFDC_C (Table 1) introduced *Nde*I and *Hin*dIII sites, respectively. The PCR product was digested with *Nde*I and *Hin*dIII and ligated into pET17b. The resulting plasmid, pET17b*Ps*BFDC, was transformed into *E. coli* strain BL21(DE3). For expression of the recombinant *Ps*BFDC, the transformants were grown at 37 °C in LB to an A_{600} of 0.8 and induced by the addition of 0.4 mM IPTG. After a 6-h incubation the cells were harvested by centrifugation. Purification of the recombinant *Ps*BFDC was accomplished using the protocol described above for the wt enzyme. The purified enzyme, which showed a single band on SDS-PAGE, was stored at 4 °C in a buffer containing 50 mM phosphate, pH 6.0, 0.2 mM ThDP and 0.1 mM $MgCl_2$. The protein concentration was determined by the Bradford method using bovine serum albumin as the standard [23].

2.7. Expression and purification of histidine-tagged PsBFDC

Using pET17b*Ps*BFDC as the template, the QuikChange (Stratagene) methodology was employed to introduce an *Xho*I restriction site at the C-terminus of the *dpgB* gene. The sequence of the forward primer, BFDC_X, is shown in Table 1. The resultant plasmid was digested with *Nde*I and *Xho*I and the 1.6-kb fragment ligated into *Nde*I- and *Xho*I-digested pET24b, to provide a plasmid, denoted pET24b*Ps*BFDHis. The fidelity of the mutagenesis was confirmed by sequencing at the University of Michigan core facility.

pET24b*Ps*BFDHis was transformed into *E. coli* strain BL21(DE3)pLysS (Promega) and a single colony was used to inoculate 50 ml of LB broth containing kanamycin (50 μg/ml) and chloramphenicol (25 μg/ml). This culture was grown overnight at 37 °C and used to inoculate 1 l of fresh medium. The fresh culture was grown at 37 °C until OD_{600} reached 0.8. The cells were cooled to 25 °C, and the protein expression was induced by the addition of 1 mM IPTG. The cells were grown for an additional 12 h at 25 °C prior to harvesting by centrifugation. The cell pellet was resuspended in buffer D (50 mM potassium phosphate pH 8.0, 500 mM NaCl, 0.1 mM ThDP) containing 0.1 mM phenylmethanesulfonyl fluoride, disrupted by sonication and the cell debris was removed by centrifugation. The CFE was applied to a HIS-select™ Nickel Affinity column (Sigma) previously equilibrated in buffer D. After the column was washed with buffer D containing 20 mM imidazole, the enzyme was eluted with buffer D containing 250 mM imidazole. The fractions of highest purity, as judged by SDS-PAGE, were pooled and the buffer was exchanged for storage buffer (100 mM potassium phosphate buffer, pH 6.0, 1 mM $MgSO_4$, 0.5 mM ThDP, 10% glycerol) using Econo-Pac 10 DG desalting columns (Bio-Rad). The protein was concentrated using Amicon Ultra centrifugal filters (Millipore) before being stored at −20 °C. Protein concentrations were determined by either the Bradford [23] or a UV method [24].

2.8. Preparation and purification of histidine-tagged PsBADH

Based on the sequence of the open reading frame in pBCH2, two primers were designed to amplify the *dpgC* gene. The forward (BADH_F) and reverse (BADH_R) primers (Table 1) added *Nde*I and *Xho*I sites, respectively. Amplification was achieved with *PfuUltra* DNA polymerase (Stratagene), using pBCH2 as the template. The PCR product was purified, digested and ligated into pET19b (Novagen) previously digested with *Nde*I and *Xho*I. This construction added a 10× histidine tag to the N-terminus of the enzyme and generated the expression vector, pET19b*Ps*BADH-His. Once the fidelity of the amplification was verified by sequencing, the plasmid was transformed into *E. coli* strain BL21(DE3)pLysS (Promega) for expression.

Expression and preparation of a CFE of his-tagged *Ps*BADH were carried out as described for *Ps*BFDC-his. The extract was applied on HIS-Select™ Nickel Affinity column previously equilibrated in buffer E (50 mM potassium phosphate buffer, pH 8.0, 300 mM NaCl, 2 mM DTT) containing 10 mM imidazole. After washing with buffer E containing 40 mM imidazole, the enzyme was eluted in buffer E containing 250 mM imidazole. The fractions of highest purity were pooled and the buffer was exchanged for storage buffer (100 mM HEPES pH 7.5, 100 mM KCl, and 2 mM DTT) using Econo-Pac 10 DG desalting columns (Bio-Rad). The *Ps*BADH-his was then concentrated using Amicon Ultra centrifugal filters (Millipore) before being stored at −20 °C.

2.9. Measurement of enzyme activity

In crude cell extracts, D-PhgAT activity was determined using a spectrophotometric assay in which the rate of benzoylformate formation was measured by monitoring the increase in absorbance at 254 nm [25]. The assay was carried out in a buffer (1 ml) containing Tris–HCl (100 mM, pH 9.0), D-phenylglycine (1 mM), α-ketoglutarate (1 mM), pyridoxal-5′-phosphate (25 μM) and EDTA (25 mM). BFDC activity in crude cell extracts was determined using a continuous assay based on the decrease in absorbance at 334 nm as benzoylformate was converted to benzaldehyde [4]. The reaction mixture (1 ml) contained benzoylformate (8.33 mM) and ThDP (40 μM) in sodium phosphate buffer (66 mM, pH 6.0). Both assays were performed at 25 °C.

In addition to the direct method described for the induction studies, the decarboxylation of benzoylformate was also followed using a more sensitive coupled assay [9]. Here the assay was performed at the appropriate temperature in 100 mM potassium phosphate buffer pH 6.0, containing 1.0 mM $MgCl_2$, 0.5 mM ThDP, 0.3 mM NADH and 0.25 U/ml of horse liver alcohol dehydrogenase. The benzoylformate concentration was varied from 0.3 to 3× K_m and the reaction was initiated by the addition of BFDC. The decrease in absorbance at 340 nm was measured on a Cary 50 spectrophotometer (Varian) equipped with a temperature-controlled cell holder. Initial velocity data were

fitted to the Michaelis–Menten equation using the Enzyme Kinetics module of SigmaPlot® (SPSS Inc.). Kinetic parameters were determined per monomer using molecular masses of 56,302 for *Ps*BFDC or 57,367 for *Ps*BFDC-his.

Benzaldehyde dehydrogenase activity was monitored spectrophotometrically by observing the increase in A_{340} due to the reduction of NAD^+ [26]. Routine activity assays were carried out at 25 °C in a 1 ml reaction mixture containing 100 mM TAPS buffer, pH 8.5, 100 mM KCl, 1 mM DTT, 1 mM NAD^+ and 1 mM benzaldehyde. K_m(app) values for benzaldehyde were determined by fixing NAD^+ or $NADP^+$ at a concentration of 1 mM and varying the benzaldehyde concentration as required. For determination of the apparent K_m values for NAD^+ and $NADP^+$, benzaldehyde was maintained at a saturating concentration (1 mM) and the NAD^+ or $NADP^+$ concentration varied appropriately. Kinetic data were fitted to the Michaelis–Menten equation using the Enzyme Kinetics module of SigmaPlot® (SPSS Inc.). Kinetic parameters were determined per monomer using a molecular mass of 50278 Da for *Ps*BADH-his.

3. Results and discussion

3.1. Implication of a benzoylformate decarboxylase in D-phenylglycine degradation

With the exception of *R*- and *S*-mandelate, *P. stutzeri* ST-201 was able to grow on MM agar containing each of the tested carbon sources. The results demonstrated that the bacteria could produce enzymes capable of catabolizing D-phenylglycine, benzoylformate, benzaldehyde and benzoate. Further, the results showed that *P. stutzeri* ST-201 lacked, at the least, a *S*-mandelate dehydrogenase such as that found in the mandelate pathway of *P. putida* ATCC 12633 (Fig. 1, [3]). Therefore it was reasonable to assume that the benzoylformate found in cultures of *P. stutzeri* ST-201 grown on D-phenylglycine [21] was the product of the transamination of D-phenylglycine (Fig. 1).

In *P. putida* ATCC 12633 some of the enzymes of the mandelate pathway are coordinately regulated. BFDC activity, for example, can be induced by the addition of either *R*- or *S*-mandelate or benzoylformate (but not benzoate) to the culture medium [3]. In *P. stutzeri* ST-201 neither D-PhgAT nor BFDC activity was observed in MM containing glucose. However, when the culture medium was changed from LB to MM containing D-phenylglycine, D-PhgAT activity was detected after 40 min and was followed by BFDC activity which appeared after 100 min (Fig. 2A). Conversely, in MM with benzoylformate as sole carbon source there was no evidence for D-PhgAT activity but BFDC activity was observed after 60 min (Fig. 2B). Clearly, benzoylformate can induce only the expression of the BFDC gene whereas D-phenylglycine brings about an increase in the levels of both enzymes. What is not clear is whether this is because D-phenylglycine actually induces the genes for both enzymes or whether the gene for D-PhgAT is induced and the benzoylformate subsequently produced induces the BFDC gene. Based on the times at which the two enzyme activities are observed the latter is the most likely explanation. Regardless, the sequential expression of the enzymes substantiates the involvement of BFDC in the pathway in a step following D-phenylglycine degradation.

The decrease in the absorbance due to benzoylformate, as well as the presence of the characteristic odor of benzaldehyde, in the BFDC assays both pointed to the *P. stutzeri* ST-201 extracts having BFDC activity. Nevertheless, the presence of a

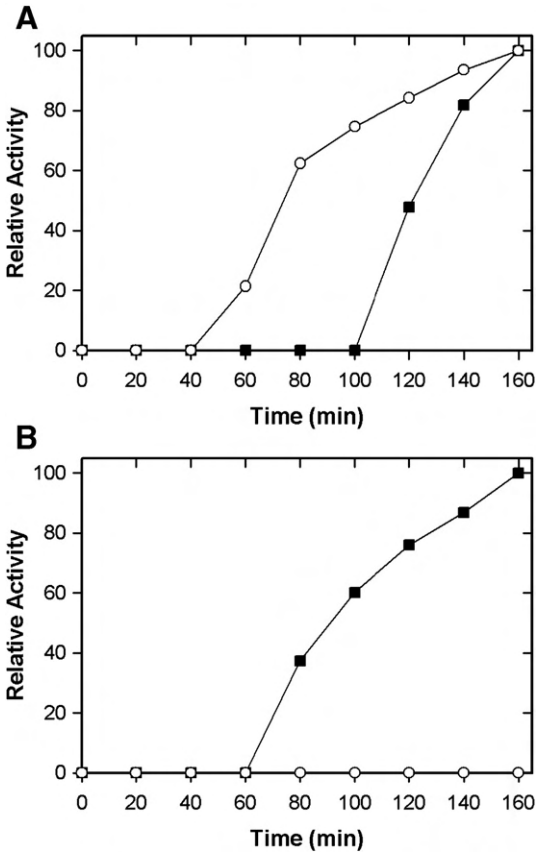

Fig. 2. Time course for the appearance of D-phenylglycine aminotransferase (○) and benzoylformate decarboxylase (■) activity. At $t=0$ the culture of *P. stutzeri* ST-201 was changed to a minimal medium containing either (A) D-phenylglycine or (B) benzoylformate at a concentration of 10 mM. Enzyme activity was measured as described in Materials and methods and normalized to activity at $t=160$ min.

*Ps*BFDC could only be confirmed by purification of the enzyme. This was achieved in a series of steps including ammonium sulfate fractionation, followed by hydrophobic and ion-exchange chromatography. SDS-PAGE analysis suggested a molecular weight of ~55 kDa. Sequencing of the purified enzyme provided an N-terminal sequence of ASVHSITYELLRRQGIDTVFGNP which was employed to generate primers (Table 1) for use in hybridization experiments.

3.2. Construction of subgenomic library and identification of the dpgB and dpgC genes

Southern hybridization of *P. stutzeri* ST-201 genomic DNA which had been digested with *Eco*RI and *Hin*dIII gave a positive band of ca. 5–7 kb. A subgenomic DNA library was constructed and, of 400 recombinant clones, one was positive to colony hybridization. That clone carried a plasmid with a 6-kb insert which was designated as pBCH1. Sequencing of pBCH1 revealed an open reading frame (ORF) of 1581 bp which was denoted *dpgB*, and which encoded a protein of 526 amino acids with a deduced molecular mass of 56 kDa. This was in good agreement with the 55 kDa observed on SDS-PAGE for the BFDC purified from induced *P. stutzeri* ST-201 cells. In addition, the deduced amino acid sequence of the N-terminal 23

amino acids of *dpgB* was identical to that of the native *Ps*BFDC lacking the N-terminal methionine.

BLASTp analysis of the deduced amino acid sequence of *dpgB* revealed significant homology to other bacterial benzoylformate decarboxylases, with the highest sequence identity being 91% to *Pp*BFDC (gi:3915757). Those active site residues implicated in the catalytic mechanism of *Pp*BFDC, including Ser26, Glu47, His70 and His281 [11], were all conserved. Analysis of the DNA sequence upstream of *dpgB* identified a putative ribosome binding site and −10 and −35 promoter regions. No ORFs were identified in the 300-bp sequence upstream from *dpgB* while downstream of the ORF a putative ρ-independent transcriptional terminator was also identified (data not shown). In addition a partial ORF was identified about 250 bp downstream of *dpgB* (Fig. 3A). Overall these results suggested that *dpgB* was transcribed monocistronically, unlike the gene encoding *Pp*BFDC which was arranged and transcribed in the *mdl*CBA operon (Fig. 3B) [8].

In addition to the *Eco*RI–*Hin*dIII digests described above, Southern hybridization of *P. stutzeri* ST-201 genomic DNA digested with *Pst*I also gave a positive band. Colony hybridization with a second subgenomic library identified a clone carrying a plasmid with a 4-kb insert. This plasmid was designated as pBCH2. Sequencing of pBCH2 identified a region which corresponded to part of the *dpgB* gene. However, this insert also carried the full-length ORF of 1311 bp downstream of *dpgB*. The ORF, denoted *dpgC*, was transcribed in the same direction as *dpg*B and encoded a protein of 436 amino acids with a deduced molecular mass of 47.7 kDa.

The evidence for BFDC activity coupled with the observations that *P. stutzeri* ST-201 was able to grow on benzaldehyde

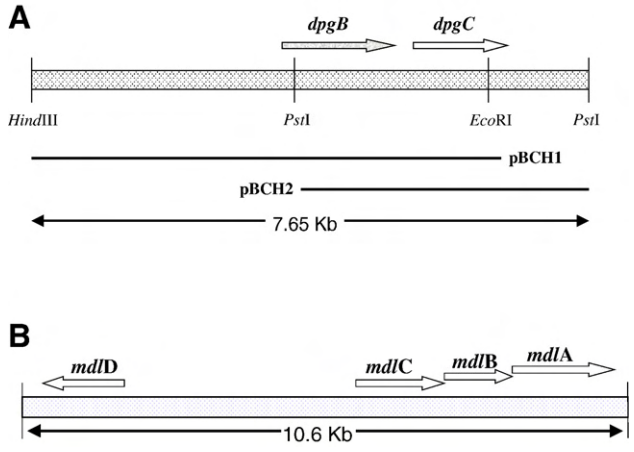

Fig. 3. (A) Restriction fragment of the genomic DNA from *P. stutzeri* ST-201 carrying the genes for benzoylformate decarboxylase (*dpgB*) and benzaldehyde dehydrogenase (*dpgC*). The fragment (7.65 kb) shown is the combination of two overlapping genomic DNA fragments contained in pBCH1 and pBCH2. The arrows show the position and orientation of the genes, and relevant restriction sites are indicated. The sequence of the combined fragment has been deposited under GeneBank accession no. EF419244. (B) Organization of the mandelate pathway genes based on GeneBank accession no. AY143338. Benzoylformate decarboxylase (*mdlC*), *S*-mandelate dehydrogenase (*mdlB*) and mandelate racemase (*mdlA*) make up the *mdlCBA* operon while benzaldehyde dehydrogenase (*mdlD*) is transcribed independently.

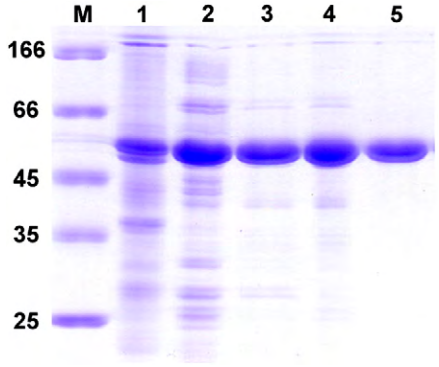

Fig. 4. SDS-PAGE analysis of the recombinant *Ps*BFDC during the purification process. Lanes: M, molecular mass standards (kDa); 1, crude cell lysate; 2, 45-65% ammonium sulfate fraction; 3, following phenyl sepharose chromatography; 4, following ultrafiltration; 5, following Q-Sepharose chromatography.

and that benzoate could be found in the culture medium suggested the likely presence of a BADH. Consequently, it was not surprising when a BLAST search of the full-length amino acid sequence indicated that the *dpgC* gene product was likely to be an aldehyde dehydrogenase, probably of the class 3 type. Dehydrogenases of this class are characterized by the ability to utilize both NAD^+ and $NADP^+$ [27]. The sequence was 86% identical to that of the BADH from *P. putida* ATCC 12633 [26]. The catalytic residues, including the catalytic thiol (Cys249) and the general base (Glu337), were conserved as was the aspartic acid residue (Asp253) that links conserved domains in class 3 aldehyde dehydrogenases [28]. *Ps*BADH also shared 30–40% sequence identity with other aldehyde dehydrogenases from pseudomonads, including the *p*-hydroxybenzaldehyde dehydrogenase from *P. putida* NCIMB 9866 [29]. However, although they are closely related phylogenetically, the other pseudomonad aldehyde dehydrogenases are not class 3 enzymes [27].

3.3. Expression and purification of recombinant PsBFDC

BFDC activity was found in the CFE from IPTG-induced *E. coli* BL21 (DE3) harboring the plasmid pET17b*Ps*BFDC. The majority of the BFDC activity precipitated at 45–65% ammonium sulfate saturation. As shown in Fig. 4, following hydrophobic interaction chromatography, ultrafiltration and ion-exchange chromatography, the enzyme ran as a single band by SDS-PAGE analysis.

The molecular mass of the recombinant enzyme, ~56 kDa, was in good agreement with the molecular mass calculated from the *dpgB* gene sequence. Gel-filtration experiments provided a native molecular mass of ~220 kDa (data not shown). This indicated that BFDC from *P. stutzeri* ST-201 associates as a homotetramer, a characteristic common in bacterial BFDCs [5,13]. The enzyme showed maximum activity at 35–50 °C and at pH 6.0 (data not shown) which was similar to results for the BFDCs from *N. crassa* [7] and *P. putida* [4], respectively.

Michaelis–Menten parameters were determined for *Ps*BFDC under optimal conditions (35 °C and pH 6.0, Table 2). The K_m value for benzoylformate of 0.69 mM was similar to

Table 2
Kinetic parameters for *Ps*BFDC [a]

Enzyme	Temperature (°C)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
wt *Ps*BFDC	35	0.69±0.10	222±3	3.2×10^5
*Ps*BFDC-his	35	0.44±0.07	284±12	6.4×10^5
*Ps*BFDC-his	30	0.43±0.05	213±7	5.0×10^5
*Pp*BFDC-his [b]	30	0.54±0.05	342±13	6.3×10^5

[a] Experiments were carried out at pH 6.0 as described in Materials and methods.
[b] Data from Lingen et al. [17].

Table 3
Kinetic parameters for *Ps*BADH [a]

Variable substrate	Fixed substrate [b]	K_m (app) (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
Benzaldehyde	NAD^+	7.1±0.6	57±8	8.0×10^6
Benzaldehyde	$NADP^+$	6.9±0.4	6.4±0.4	9.3×10^5
NAD^+	Benzaldehyde	997±111	176±9	1.8×10^5
$NADP^+$	Benzaldehyde	6140±375	40±3	6.5×10^3

[a] Experiments were carried out at 25 °C and pH 8.5 as described in Materials and methods. The data are the mean of at least 3 individual experiments and are reported as ±S.D.
[b] Concentration of the fixed substrate was maintained at 1 mM.

that for *Pp*BFDC which was not surprising given that there is 91% sequence identity between the two enzymes, and those changes in sequence are located at some distance from the active site. However, at 222 s^{-1} the k_{cat} value for *Ps*BFDC was 35% lower than that of *Pp*BFDC even though the latter was determined at a lower temperature (30 °C). ThDP-dependent enzymes are often purified as his_6-tagged variants to avoid loss of activity during multi-step purifications [14,30]. So, to facilitate a more rapid purification, *Ps*BFDC was also prepared with a C-terminal histidine tag, and a homogenous preparation was obtained in a single step (Fig. 5A). This preparation showed an increase in k_{cat} to 284 s^{-1} and a decrease in K_m value to 0.43 mM. At 30 °C *Ps*BFDC had both K_m and k_{cat} values lower than those of its *P. putida* counterpart although the k_{cat}/K_m values were virtually identical (Table 2). Predictably, as suggested by the high sequence identity, preliminary experiments indicate that the substrate range for *Ps*BFDC is unlikely to be different from that of *Pp*BFDC (data not shown).

3.4. Cloning, expression, purification and characterization of benzaldehyde dehydrogenase

The X-ray structure of the class 3 aldehyde dehydrogenase from rat liver [31] shows that the C-terminal residues interact with other regions of the protein whereas the N-terminal residues are fully accessible. Based on these data a N-terminal histidine tag has been successfully employed in this laboratory to purify *Pp*BADH-his. The kinetic constants for this variant were essentially identical to those of the wt enzyme (C. Yeung and M. McLeish, unpublished results). Given the 86% sequence identity between *Pp*BADH and the putative *Ps*BADH it seemed reasonable to use a N-terminal histidine tag to facilitate the purification of the *dpgC* gene product. Accordingly the *dpgC* gene was amplified from pBCH2 and placed in the expression vector pET19b which adds an N-terminal His_{10}-tag. The enzyme was purified in a single step using a nickel affinity column. The purified protein showed a single band on SDS-PAGE with an estimated molecular mass consistent with that calculated from the gene sequence (Fig. 5B).

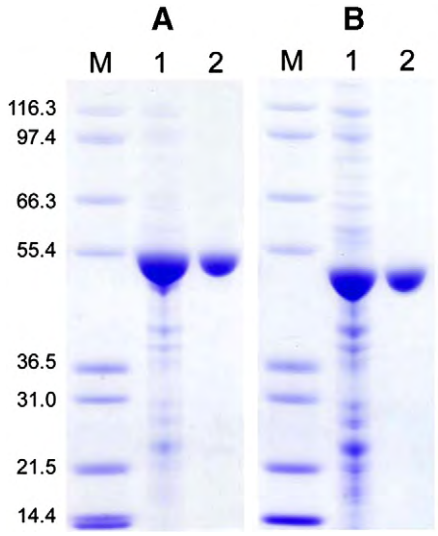

Fig. 5. Single step purification of (A) *Ps*BFDC-his and (B) *Ps*BADH-his. In each case the lanes are: M, molecular mass standards (kDa); 1, crude cell lysate; 2, after His-Select nickel affinity chromatography.

As predicted, the enzyme was able to oxidize benzaldehyde using either NAD^+ or $NADP^+$ as the cofactor. *Ps*BADH prefers NAD^+ over $NADP^+$ which is similar to both the rat aldehyde dehydrogenase [32] and *Pp*BADH [26]. The K_m(app) values for NAD^+ and $NADP^+$ were obtained at saturating benzaldehyde concentration and, therefore, are indicative of the true K_m values. However, for comparison with data for *Pp*BADH [26], K_m(app) values for benzaldehyde were obtained at 1 mM NAD(P)$^+$ which is less than saturating. As with *Pp*BADH, it was likely that benzaldehyde was the natural substrate for *Ps*BADH as its apparent K_m values were in the low micromolar range (Table 3). Overall, in keeping with their relative sequence identities, the kinetic properties of *Ps*BADH and *Pp*BADH are very similar.

4. Conclusions

We have shown that growth of *P. stutzeri* ST-201 on D-phenylglycine results initially in the induction of D-PhgAT and subsequently in the induction of BFDC. Conversely, benzoylformate is only able to induce BFDC. We have cloned and sequenced the *dpgB* and *dpgC* genes and characterized the gene products as a BFDC and a BADH, respectively. The two enzymes share a high degree of sequence identity with the homologous enzymes in the mandelate pathway of *P. putida*. Moreover, their kinetic parameters are similar, suggestive of comparable metabolic roles. It is possible that *Ps*BFDC and *Ps*BADH are components of the mandelate pathway yet *P. stutzeri* ST-201 is unable to utilize mandelate as a carbon

source. Further the genes are organized quite differently. In *P. putida* ATCC 12633, the gene encoding BFDC (*mdlC*) forms part of an operon with those encoding mandelate racemase (*mdlA*) and *S*-mandelate dehydrogenase (*mdlB*), while the BADH gene (*mdlD*) is located upstream of the *mdlCBA* operon and is transcribed in the opposite direction. In *P. stutzeri* ST-201, the BFDC gene (*dpgB*) is independently transcribed, and the BADH gene (*dpgC*) is located downstream of, and transcribed in the same direction as, *dpgB*. Taken together the data suggest that, in combination with D-phenylglycine aminotransferase, the two enzymes form a pathway for the degradation of D-phenylglycine with the product, benzoate, presumably entering the β-ketoadipate pathway [6]. Whether benzoate is initially converted to 4-hydroxybenzoate or to catechol, and whether the subsequent cleavage occurs by the ortho or meta pathway [6,33], is the subject of ongoing experiments.

Acknowledgements

This work is supported in part by the Commission on Higher Education and Faculty of Graduate Studies, Mahidol University, and by the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (Grant No.PHD/0023/2548). The work was also supported by the National Science Foundation (EF 0425719).

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