รายงานวิจัยฉบับสมบูรณ์

โครงการ

เทคโนโลยีชีวภาพสำหรับการใช้เอนไซม์ในการสังเคราะห์สาร ดี-เฟนิลกลัยซีน อีแนนทิโอเมอร์บริสุทธิ์

Biotechnology for Enzymatic Synthesis of Enantiomerically Pure D-Phenylglycine

โดย

นายสุเทพ ไวยครุฑธา

มิถุนายน 2554

สัญญาเลขที่ RSA4780014

รายงานวิจัยฉบับสมบูรณ์

โครงการ

เทคโนโลยีชีวภาพสำหรับการใช้เอนไซม์ในการสังเคราะห์สาร ดี-เฟนิลกลัยซีน อีแนนทิโอเมอร์บริสุทธิ์

Biotechnology for Enzymatic Synthesis of Enantiomerically Pure D-Phenylglycine

นายสุเทพ ไวยครุฑธา

ภาควิชาจุลชีววิทยา
คณะวิทยาศาสตร์
มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

กิตติกรรมประกาศ

ผู้วิจัยขอขอบคุณ สำนักงานกองทุนสนับสนุนการวิจัย (สกว) ที่ให้ทุนสนับสนุนการวิจัย ขอบคุณนักศึกษาที่เข้าร่วมศึกษาวิจัยในโครงการ และขอขอบคุณ ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล ที่อำนวยความสะดวกในด้านต่างๆ ช่วยให้การวิจัยในครั้งนี้สามารถดำเนินการสำเร็จได้ด้วยดี

บทคัดย่อ

รหัสโครงการ : RSA4780014

ชื่อโครงการ : เทคโนโลยีชีวภาพสำหรับการใช้เอนไซม์ในการสังเคราะห์สาร ดี-เฟนิลกลัยซีน อีแนนทิโอเมอร์บริสุทธิ์

ชื่อนักวิจัย : นายสุเทพ ไวยครุฑธา

ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล

E-mail Address : scsvy@mahidol.ac.th

ระยะเวลาโครงการ : กันยายน 2547 - มิถุนายน 2554

D-phenylglycine aminotransferase (D-PhgAT) จากแบคทีเรีย *Pseudomonas stutzeri* ST-201 เร่งปฏิกิริยา ''stereo-inverting'' transamination ที่สามารถใช้สำหรับสังเคราะห์ enantiopure D-phenylglycine หรือ D-4-hydroxyphenylglycine (ซึ่งเป็น side-chains สำคัญที่มีความต้องการสูงในอุตสาหกรรมการผลิตยาปฏิชีวนะกลุ่ม β-lactam) โดยใช้ปฏิกิริยาของเอนไซม์ขั้นตอนเดียว และใช้ L-glutamate ซึ่งมีราคาถูก เป็นสารให้หมู่ amino โครงการวิจัยนี้ได้ใช้การพัฒนาทางเทคโนโลยีชีวภาพหลายด้าน เพื่อให้กระบวนการสังเคราะห์สารด้วยเอนไซม์ D-PhgAT มีประสิทธิภาพใช้งานได้ดี โดยได้ทำ directed mutagenesis ต่อยีน *dpgA* เพื่อเปลี่ยน serine ที่ปลายสายด้าน C ของ D-PhgAT ทำให้สามารถตรึงเอนไซม์กับ thiol-containing matrix ได้อย่างรวดเร็ว โดยที่ enzyme ยังมี activity ดีและคงตัวมากขึ้น ได้ใช้เอนไซม์ที่ตรึงนี้ในปฏิกิริยาที่มี benzoylformate ดูดซับอยู่บน Amberlite (IRA400) และปลดปล่อยออกมาอย่างช้าๆ พบว่าได้สาร D-phenylglycine 20.25 กรัมต่อลิตร ได้พัฒนาระบบการแสดงออกของยีนเพื่อการผลิต D-PhgAT ในเซลล์เจ้าบ้าน 2 ชนิด ระบบแรกใช้ *Escherichia coli* โดยมี *E. coli* chaperones แสดงออกร่วมด้วย สามารถผลิต D-PhgAT ได้เพิ่มขึ้นจาก 0.44 เป็น 1,768 unit.L^{-1}.OD^{-1} ระบบที่สองใช้ยีสต์ *Pichia pastoris* โดยมี *E. coli* chaperones แสดงออกร่วมด้วย สามารถผลิต D-PhgAT ได้ 14,717 unit.L^{-1}.OD^{-1} ได้แก้ปัญหาของ D-PhgAT ที่มีค่าการละลายต่ำ ด้วยการทำ structure-guided mutagenesis ซึ่งทำให้เพิ่มการละลายของ D-PhgAT จาก 11.5 เป็น 51 มก/มล ในโครงการยังได้มีการพัฒนาวิธีการใหม่สำหรับการพิสูจน์ stereospecificity ของการถ่ายทอดโปรตอนบน C-4' ของ coenzyme โดย aminotransferase รวมทั้งการประยุกต์ใช้เอนไซม์ D-PhgAT ในการตรวจวัดปริมาณ L-glutamate ด้วยวิธีหมุนเวียนสารตั้งต้น ซึ่งเป็นวิธีการวัดที่รวดเร็ว วัดได้ละเอียด และมีการรบกวนน้อย การวิจัยต่อเนื่องที่ควรทำในอนาคต ได้แก่ การดัดแปลงโครงสร้างโมเลกุลของ D-PhgAT เพื่อลดการยับยั้งจาก substrate การพัฒนากระบวนการตรวจวัดและเติม substrate ที่เหมาะสม และวิธีการดึง product ออกจากระบบ เพื่อให้ปฏิกิริยาการสังเคราะห์ด้วยเอนไซม์ D-PhgAT มีอัตราเร็วเพิ่มขึ้น และทำได้ยาวนานมากขึ้นไปอีก

คำหลัก : อะมิโนทรานส์เฟอเรส, ดี-ฟีนิลกลัยซีน, การสังเคราะห์สารด้วยเอนไซม์

Abstract

Project code: RSA4780014

Project Title : Biotechnology for Enzymatic Synthesis of Enantiomerically Pure D-Phenylglycine

Investigator : Dr Suthep Wiyakrutta

Department of Microbiology, Faculty of Science, Mahidol University

E-mail Address : scsvy@mahidol.ac.th

Project Period : September 2004 - June 2011

D-phenylglycine aminotransferase (D-PhgAT) from *Pseudomonas stutzeri* ST-201 catalyzes the ‘‘stereo-inverting’’ transamination that can be used to synthesize enantiopure D-phenylglycine or D-4-hydroxyphenylglycine, the two important side-chains in high demand for the β-lactam antibiotics industry, in a single enzymatic step using L-glutamate as a low-cost amino donor. Biotechnological development in various aspects were attempted to make this enzymatic synthesis process effective and viable. Directed mutagenesis of *dpgA* gene to replace the C-terminus serine with cysteine could facilitate rapid and site specific immobilization of the D-PhgAT(S453C) on thiol-containing matrix while maintaining enzyme activity and enhancing its stability. Biocatalysis using the immobilized D-PhgAT(S453C) in a controlled-release system of Amberlite (IRA400)-adsorbed benzoylformate yielded a final D-phenylglycine concentration of 10.25 $g.L^{-1}$. Two systems for D-PhgAT high-expression were developed. The first system was based on *Escherichia coli* host with chaperones co-expressions which improved the D-PhgAT yield from 0.44 to 1,768 $unit.L^{-1}.OD^{-1}$. In the second system, codon-optimized synthetic *dpgA* gene was expressed in *Pichia pastoris* with bacterial chaperones co-expressions which yielded D-PhgAT at 14,717 $unit.L^{-1}.OD^{-1}$. Problem of the inherently low solubility of the wild-type D-PhgAT was alleviated by structure-guided mutagenesis which could increase the enzyme solubility from 11.5 to 51 mg/mL. During investigation of molecular and catalytic property of the D-PhgAT, a new method for determination of aminotransferase stereospecificity for C-4' hydrogen transfer on the coenzyme was developed. A spectrophotometric enzymatic cycling method using L-glutamate dehydrogenase and D-PhgAT for determination of L-glutamate in foods is another aspect of D-PhgAT application developed during this project. Genetically modification of the D-PhgAT to relief substrate inhibition, developing effective and logical systems for substrate addition and product removal are important research topics that should be done in the future.

Keywords : aminotransferase, D-phenylglycine, enzymatic synthesis

สารบัญ

Introduction

ในปัจจุบันอุตสาหกรรมการสังเคราะห์ตัวยาและสารเคมี ได้เริ่มเข้าสู่ยุคที่เรียกว่า Green Chemistry คือมีการพัฒนาให้ได้กระบวนการผลิตที่ สิ้นเปลืองพลังงานน้อย (energy efficiency) ใช้สารเคมีอย่างมีประสิทธิภาพ (atom economy) ใช้กระบวนการผลิตที่ไม่ก่ออันตราย ใช้สารตั้งต้นที่เป็น renewable feedstocks และที่สำคัญคือกระบวนการผลิตจะต้องไม่ก่อผลเสียต่อสิ่งแวดล้อม และผลิตภัณฑ์ที่ได้ควรจะย่อยสลายได้หลังการใช้ โดยไม่ก่อมลพิษ ซึ่งแนวโน้มในอนาคตจะมีการกำหนดให้ใช้หลักการเหล่านี้ในการผลิตสินค้าเคมีภัณฑ์มากขึ้น และอาจถูกนำไปใช้เป็นเหตุผลในการกีดกันทางการค้าได้ ในอนาคตเคมีภัณฑ์ใดที่ผลิตโดยไม่ใช้กระบวนการที่เป็น green chemistry อาจไม่สามารถนำไปจำหน่ายในบางประเทศได้ ดังเช่นที่ได้เกิดขึ้นกับสินค้าทั้งด้านเกษตรและอุตสาหกรรมหลายๆกรณี อย่างที่ทราบกันดีโดยทั่วไป

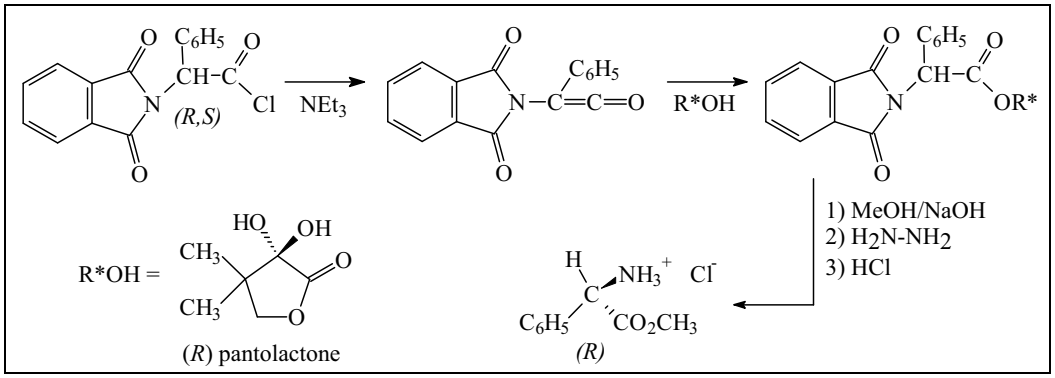
การใช้เอนไซม์เป็น biocatalyst เพื่อให้กระบวนการสังเคราะห์ทางเคมี มีความเป็น green chemistry มากขึ้น เป็นเรื่องที่ประเทศที่เจริญทางอุตสาหกรรม และบริษัทผลิตเคมีภัณฑ์ใหญ่ๆ ให้ความสนใจ ศึกษาวิจัยกันอย่างมาก ในปัจจุบัน (โปรดดู Green Chemistry Network website ที่ http://www.greenchemistrynetwork.org/) เนื่องจากเอนไซม์มีข้อดีหลายประการ ดังเป็นที่ทราบกันดีในวงวิชาการ แต่สำหรับในประเทศไทย การศึกษาวิจัย การพัฒนาองค์ความรู้ รวมทั้งการประยุกต์ใช้เอนไซม์ ในงาน chemical synthesis มีน้อยมาก ในขณะเดียวกันผู้เสนอโครงการมีความเห็นว่า ประเทศไทยน่าจะมีศักยภาพสูงทางด้านนี้ ในแง่ที่ว่าเรามีทรัพยากรชีวภาพ โดยเฉพาะจุลินทรีย์ ที่มีความหลากหลายทางชีวภาพสูงมาก จึงน่าจะเป็นแหล่งที่มีเอนไซม์ที่หลากหลาย ที่สามารถพัฒนานำมาใช้ประโยชน์ได้ หากว่าเราได้มีการพัฒนาองค์ความรู้และขีดความสามารถ และการร่วมมือกันของนักวิชาการในด้านนี้มากเพียงพอ

ผู้เสนอโครงการได้ศึกษาเอนไซม์ชื่อ D-phenylglycine aminotransferase มาระยะหนึ่ง โดยค้นพบเอนไซม์ชนิดนี้ จากแบคทีเรียที่แยกได้จากดินในประเทศไทย และสามารถ clone gene และผลิตเอนไซม์ปริมาณมากโดยใช้ recombinant host ได้แล้ว จากการศึกษาพบว่าเอนไซม์นี้มีคุณสมบัติเป็น stereo-inverting aminotransferase ที่มีความจำเพาะสูง และสามารถนำมาประยุกต์ใช้ในการสังเคราะห์สาร D-phenylglycine และ D-4-hydroxyphenylglycine ซึ่งเป็น industrial chemicals ที่มีความสำคัญ เช่น ใช้เป็น side-chain ในโมเลกุลของยาปฏิชีวนะในกลุ่ม semi-synthetic penicillins และ cephalosporins หลายชนิดที่มีความสำคัญในการรักษาโรคติดเชื้อแบคทีเรีย ได้แก่ยา Ampicillin, Bacampicillin, Cephalexin, Cefaclor, Cephaloglycin, Amoxicillin, Cefadroxyl, และ Cefatrizine เป็นต้น นอกจากนี้ D-phenylglycine และ D-4-hydroxyphenylglycine ยังใช้เป็นสาร chiral feedstock ในอุตสาหกรรมเคมีสังเคราะห์อื่นๆด้วย ซึ่งมีความต้องการใช้รวมกันเป็นปริมาณหลายพันเมตริกตันในแต่ละปี

D-phenylglycine และ D-4-hydroxyphenylglycine เป็นสาร unnatural amino acid ซึ่งไม่พบในสิ่งมีชีวิตและจุลชีพโดยทั่วๆไป ดังนั้นจึงไม่สามารถผลิตได้ด้วยกระบวนการ de novo fermentation โดยใช้ common industrial microorganisms เหมือนดังการผลิต L-amino acid ชนิดอื่นๆ เช่น L-glutamic acid, L-lysine, L-proline, L-aspartic acid etc. แต่เนื่องจากการที่ D-phenylglycine เป็นสารที่มีความสำคัญในเชิงอุตสาหกรรม จึงมีการพัฒนาวิธีการผลิตสารดังกล่าวนี้ขึ้นมาหลายวิธี ได้แก่

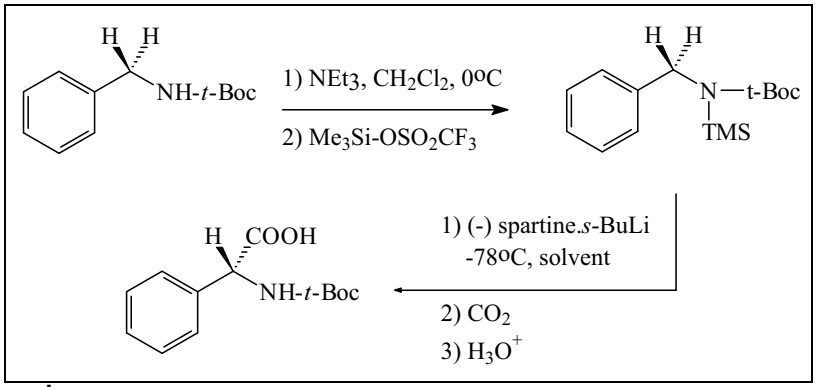
1. วิธีสังเคราะห์ทางเคมี เช่น

1.1 สังเคราะห์ R-phenylglycine methyl ester hydrochloride จาก racemic phenylglycine โดยผ่านขั้นตอน base catalysed diastereoselective addition ของ R pantolactone ไปเป็น N-phthalylprotected phenylglycine ketene (1) ดังแสดงใน รูปที่ 1



รูปที่ 1

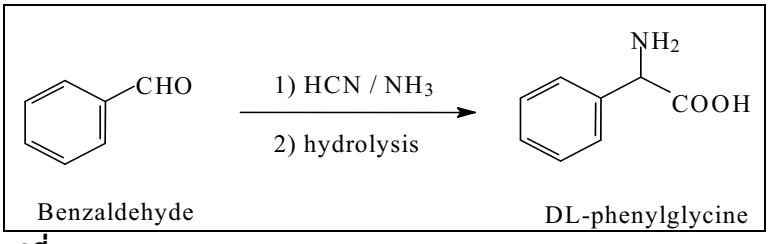
1.2 Enantioselective synthesis of phenylglycines using (-) sparteines-BuLi complex (2) มีขั้นตอนดังแสดงใน รูปที่ 2



รูปที่ 2

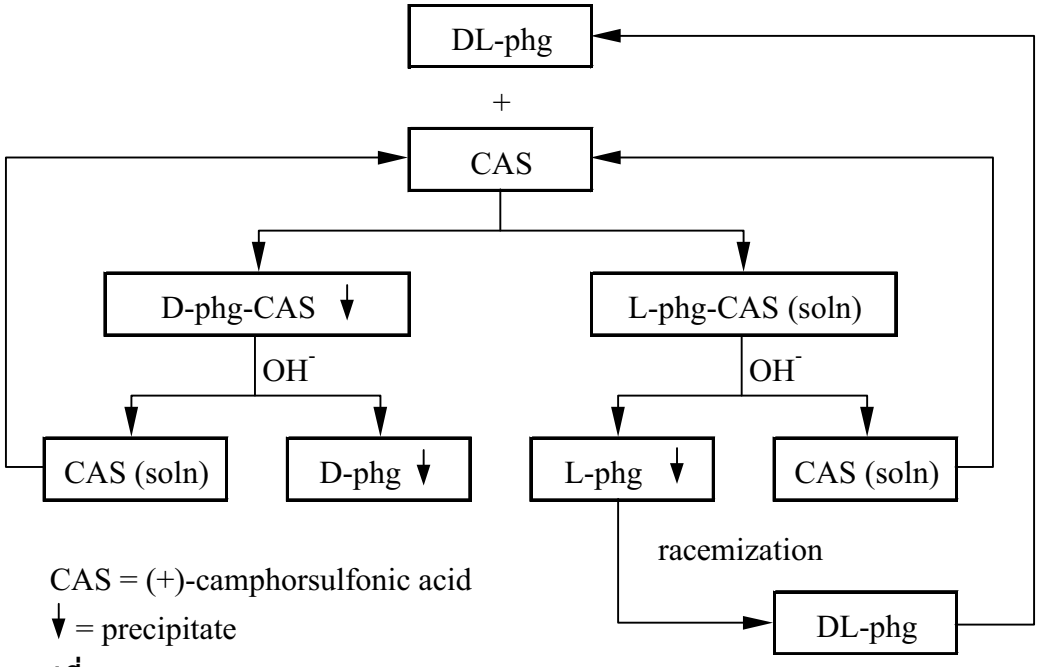
2. วิธี Chemical Resolution

2.1 Classical resolution of the racemate via diastereomeric salt formation with camphorsulphonic acid (3-4) เป็นวิธีที่ใช้ผลิตระดับอุตสาหกรรม เช่นบริษัท DSM-Andeno กระบวนการเริ่มการสังเคราะห์ racemic phenylglycine จาก benzaldehyde ด้วยปฏิกิริยา Strecker ดังรูปที่ 3



รูปที่ 3

จากนั้นทำ diastereomeric salt formation ด้วย (+) camphorsulphonic acid เพื่อแยกให้ได้ D-phenylglycine ดังแสดงใน รูปที่ 4



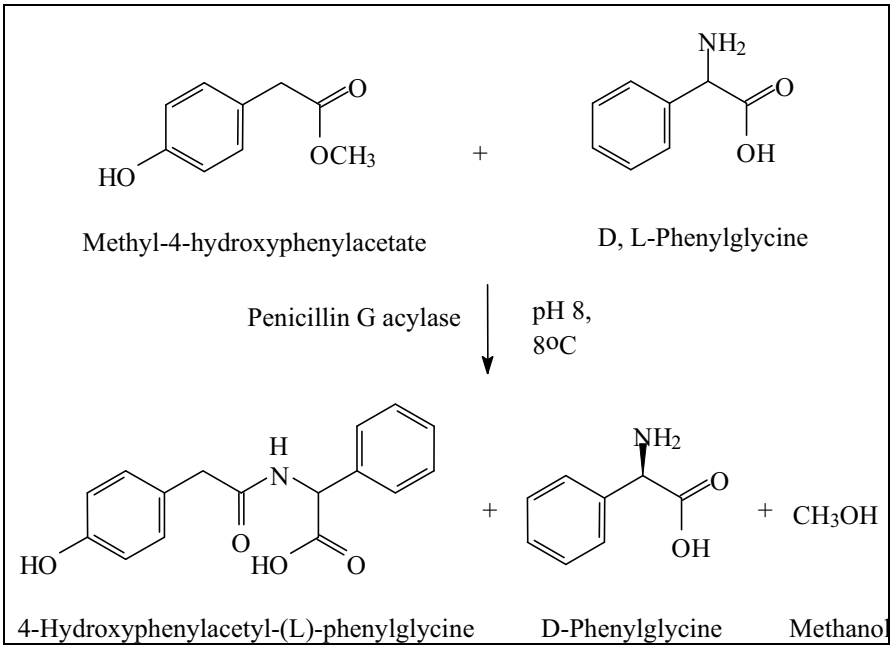
รูปที่ 4

3. วิธี Enzymatic Resolution

3.1 Penicillin G acylase Method (5)

เริ่มจาก racemic phenylglycine ใช้เอนไซม์ Penicillin G acylase (E.C.3.5.1.11) ทำให้เกิดปฏิกิริยา selective acylation reaction ต่อ L-phenylglycine โดยใช้ methyl-4-hydroxyphenylacetate ester เป็น acceptor ซึ่งผลของปฏิกิริยาจะได้สาร

4- hydroxyphenylacetyl-L-phenylglycine, D-phenylglycine และ methanol ดังแสดงใน รูปที่ 5



รูปที่ 5

วิธีที่ใช้หลักการ enzymatic resolution ด้วยเอนไซม์ Penicillin G acylase นี้ได้รับการพัฒนาต่อมาในหลายลักษณะ ได้แก่ การนำ multicompartment electrolyzer with isoelectric membrane (MIER) technology เข้ามาใช้ (6) และ การทำปฏิกิริยาใน organic solvent system (7)

4. วิธี Enzymatic Synthesis

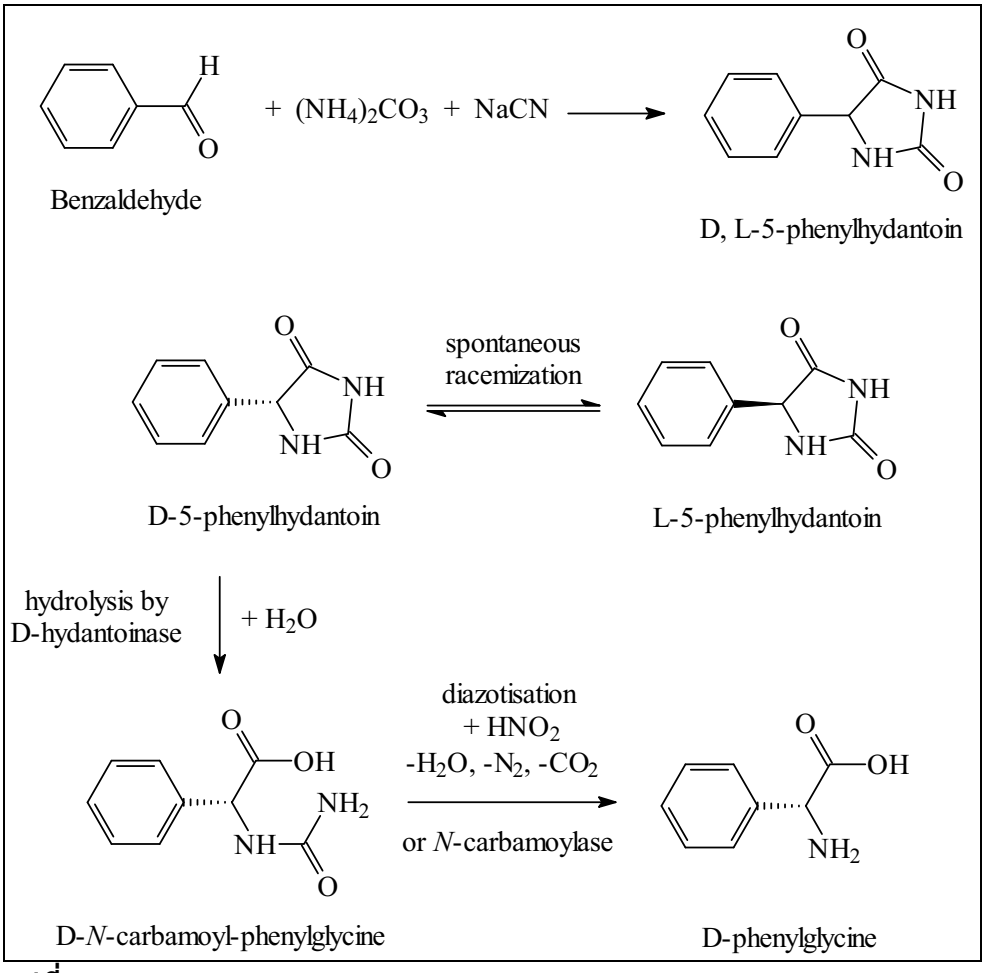
ปัจจุบันมีอยู่ 2 วิธีการหลักที่ใช้ในระดับอุตสาหกรรม ได้แก่

4.1 Hydantoinase process (8)

วิธีนี้ใช้ DL--5phenylhydantoin เป็นสารตั้งต้น ซึ่งเตรียมได้จาก benzaldehyde, ammonium bicarbonate และ sodium cyanide โดยใช้ปฏิกิริยา Bucherer-Berg reaction

เอนไซม์ D-hydantoinase (dihydropyrimidinase, E.C.3.5.2.2) มีความจำเพาะทำให้เกิดปฏิกิริยา hydrolytic ring cleavage เฉพาะกับ D- isomer ของ-5 substituted hydantoins เท่านั้น ในกรณีนี้คือ D--5phenylhydantoin ซึ่งจะถูกเปลี่ยนไปเป็น corresponding *N*-carbamoyl-D-amino acid คือ D-*N*-carbamoyl phenylglycine ซึ่งจะถูกเปลี่ยนต่อไปเป็น D-phenylglycine โดยทำปฏิกิริยา diazotisation โดยใช้ nitrous acid หรือใช้ เอนไซม์ตัวที่สองคือ N-carbamoylase enzyme ส่วน L--5phenylhydantoin ที่เหลืออยู่จะถูกเปลี่ยนมาเป็น D--5phenylhydantoin ได้

เรื่อยๆ โดยการเกิด spontaneous racemization จนในที่สุด DL--5phenylhydantoin จะถูกเปลี่ยนไปเป็น D-phenylglycine ได้จนหมด ขั้นตอนทั้งหมดของกระบวนการนี้ ดังแสดงใน รูปที่ 6

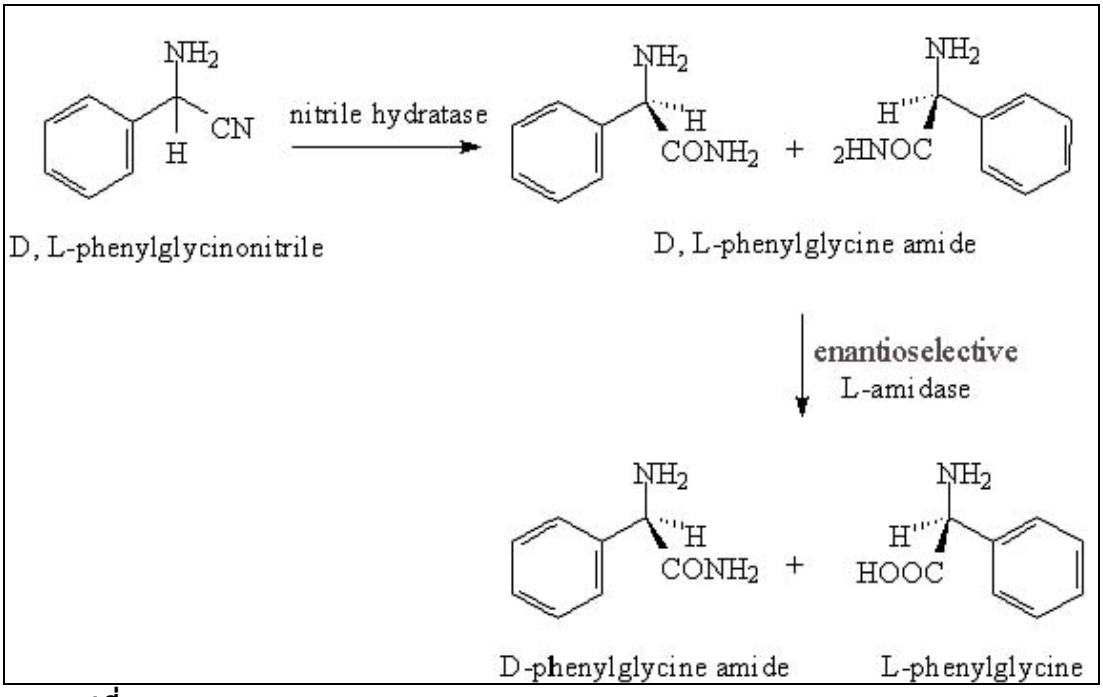


รูปที่ 6

4.2 D-phenylglycine amide production using nitrile hydratase and amidase (9)

วิธีนี้ใช้ DL-phenylglycinonitrile เป็นสารตั้งต้น ซึ่งเตรียมได้จากสาร aldehyde โดยใช้ปฏิกิริยา Strecker synthesis

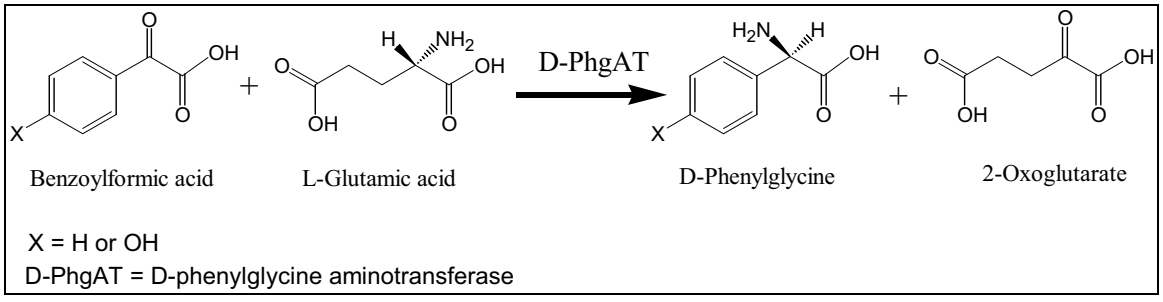
ใช้จุลินทรีย์ *Rhodococcus* ที่มีเอนไซม์ nitrile hydratase ซึ่งจะเปลี่ยน DL-phenylglycinonitrile ไปเป็น DL-phenylglycine amide ใน *Rhodococcus* ยังมีเอนไซม์ amidase ที่มีความจำเพาะต่อ L-enantiomer ของ phenylglycine amide ซึ่งจะเปลี่ยน L-phenylglycine amide ไปเป็น L-phenylglycine และได้ D-phenylglycine amide เป็นผลิตภัณฑ์สุดท้ายในปฏิกิริยา biotransformation นี้ ดังแสดงใน รูปที่ 7



รูปที่ 7

วิธีที่นำเสนอในโครงการวิจัยครั้งนี้

สำหรับกระบวนการผลิต enantiomerically pure D-phenylglycine ที่ผู้วิจัยได้ทำการศึกษาในโครงการววิจัยครั้งนี้ ใช้ปฏิกิริยาของเอนไซม์ D-phenylglycine aminotransferase โดยมีสารตั้งต้นคือ benzoylformic acid และ L-glutamic acid ดังปฏิกิริยาในรูปต่อไปนี้



วิธีนี้มีข้อดีคือ ใช้ปฏิกิริยาของเอนไซม์ ขั้นตอนเดียว สารตั้งต้น ทั้ง benzoylformic acid และ L-glutamic acid ละลายน้ำได้ดี ส่วน product คือ D-phenylglycine มีค่าการละลายต่ำจึงจะตกผลึกก่อน ทำให้แยก product ออกมาได้ง่าย และลดการเกิดปฏิกิริยาย้อนกลับ ส่วน co-product คือ 2-oxoglutarate ละลายน้ำได้ดี จึงต้องใช้ ion-exchange เป็นตัวดูดซับแยกออกมาจากปฏิกิริยา วิธีนี้

สารตั้งต้นที่ใช้เป็น amino donor คือ L-glutamic acid มีราคาถูก หาซื้อได้ง่ายในปริมาณมาก ส่วนสารตั้งต้นที่ใช้เป็น amino acceptor คือ benzoylformate นั้นสามารถสังเคราะห์ทางเคมีได้ง่ายโดยใช้สารตั้งต้นราคาถูก เช่น phenylacetic acid เป็นต้น

การสังเคราะห์สาร enantiopure D-phenylglycine โดยใช้เอนไซม์ D-phenylglycine aminotransferase นี้เป็นกระบวนการใหม่ที่ยังไม่มีผู้ใดใช้มาก่อน และผู้วิจัยพบว่ากระบวนการนี้มีข้อดีบางประการที่ดีกว่า กระบวนการสังเคราะห์แบบอื่นๆ ที่ใช้กันอยู่ในปัจจุบัน จึงได้เสนอโครงการวิจัยนี้เพื่อเป็นหัวข้อให้เกิดการสร้างองค์ความรู้ด้าน chemo-enzymatic synthesis ของประเทศไทยให้มากขึ้น และอาจพัฒนาไปสู่กระบวนการผลิตในเชิงอุตสาหกรรมได้ต่อไป

วัตถุประสงค์ของโครงการ

1) เพื่อดัดแปลงโมเลกุลของเอนไซม์ D-phenylglycine aminotransferase ด้วยวิธีการทางอณูพันธุวิศวกรรม ให้เหมาะสำหรับการการตรึงเอนไซม์ (enzyme immobilization)
2) เพื่อพัฒนาระบบในการทำ gene expression สำหรับการผลิต recombinant modified D-phenylglycine aminotransferase ที่สร้างขึ้นในข้อ 1
3) เพื่อพัฒนาวิธีแยก recombinant modified D-phenylglycine aminotransferase ออกจาก expression host และวิธีทำให้เอนไซม์บริสุทธิ์
4) เพื่อศึกษาเปรียบเทียบการตรึงเอนไซม์ ด้วยวิธีต่างๆ ให้ได้วิธีที่เหมาะสม
5) ศึกษาสมบัติของเอนไซม์ที่ตรึงแล้ว ในด้านต่างๆ เพื่อหาสภาวะที่เอนไซม์ทำงานได้ดีที่สุด
6) เพื่อสร้าง D-phenylglycine aminotransferase enzyme reactor ที่เหมาะสม
7) เพื่อพัฒนาวิธีการแยกสารผลิตภัณฑ์คือ D-phenylglycine ออกจาก reaction mixture
8) เพื่อพัฒนาวิธีการแยก co-product คือ 2-oxoglutarate ออกจาก reaction mixture
9) เพื่อพัฒนาและทดสอบระบบการผลิตที่ใช้ D-phenylglycine aminotransferase enzyme reactor ร่วมกับวิธีการที่ใช้แยกสารผลิตภัณฑ์ และ co-product สำหรับการสังเคราะห์สาร enantiopure D-phenylglycine

---

เอกสารอ้างอิง

1. Calmes, M., J. Daunis, N. Mai, and F. Natt. 1996. A convenient synthesis of optically active phenylglycine. Tetrahedron Lett. 37: 379-380.

2. Voyer, N., Roby, J., Chénard, S., and C. Barberis. 1997. Enantioselective synthesis of phenylglycines using (-) sparteines-BuLi complex. Tetrahedron Lett. 38: 6505-6508.

3. Sheldon, R. A. 1990. Industrial synthesis of optically active compounds. Chem. Ind. (London). 2 April, 212-219.

4. Sheldon, R. A. 1990. The industrial synthesis of pure enantiomers. Drug Inform. J., 24:129-139.

5. Rosell, C. M., R. Fernandez-Lafuente, and J. M. Guisan. 1993. Resolution of racemic mixtures by synthesis reaction catalysed by immobilized derivative of the enzyme penicillin G Aacylase. J. Mol. Catal. 84:365-371.

6. Bossi, A., M. Chiari, and P. G. Righetti. 1998. Production of D-phenylglycine from racemic (D,L)phenylglycine via isoelectrically-trap penicillin G acylase. Biotechnol Bioeng. 60:454-461.

7. Basso, A., P. Braiuca, L. De Martin, C. Ebert, L. Gardossi, and P. Linda. 2000. D-Phenylglycine and -4-hydroxyphenylglycine methyl esters via penicillin G acylase catalysed resolution in organic solvents. Tetrahedron-Asymmetr. 11:1789-1796.

8. Keil, O., M. P. Schneider, and J. P. Rasor. 1995. New hydantoinases from thermophilic microorganisms — Synthesis of enantiomerically pure D-amino acids. Tetrahedron-Asymmetr. 6: 1257-1260.

9. Timothy, M. B., and M. I. Page. 1998. Enantioselective biotransformations using *Rhodococci*. Antonie van Leeuwenhoek. 74:99-106.

10. Wiyakrutta, S. and V. Meevootisom. 1997. A stereo-inverting D-phenylglycine aminotransferase from *Pseudomonas stutzeri* ST-201: purification, characterization and application for D-phenylglycine synthesis. J. Biotechnol. 55:193–203.

EXPERIMENTS

Part I

Genetic Engineering of D-Phenylglycine Aminotransferase to Facilitate Immobilization and Application of the Immobilized Enzyme for D-Phenylglycine Synthesis

Materials and Methods

1. Plasmids, oligonucleotides and bacterial strains

Plasmid pEPL carrying the gene D-PhgAT encoding D-PhgAT from *Pseudomonas stutzeri* ST-201 was constructed and provided by P. Laowanapiban. The plasmid pET-17b (Figure 1) was used as both cloning and expression vector of the modified enzyme genes. For plasmid amplification, E. coli DH5α was used as the host; whereas for gene expression, *E. coli* BL21 (DE3) (Novagen) was employed. Bacterial cells harboring the plasmids were grown at 37°C in Luria-Bertani (LB) medium, containing 100 μg/ml ampicillin. The oligonucleotides used for mutagenesis PCR were from MWG Biotech (Germany).

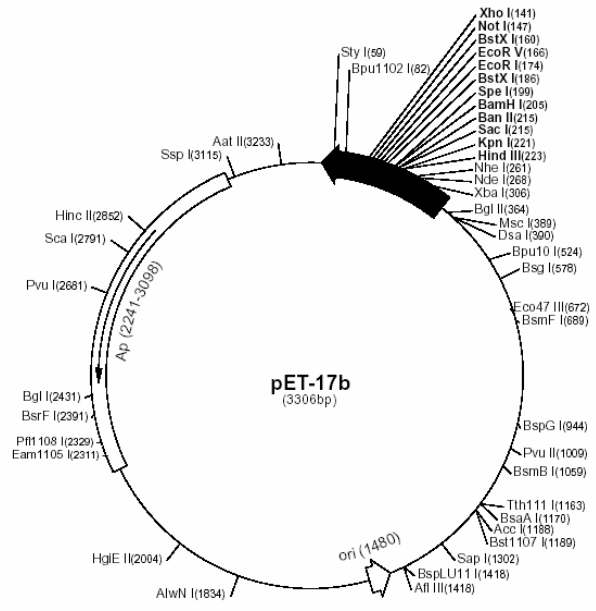


Figure 1. Map of pET-17b vector (Novagen). The 1362 bp D-PhgAT gene from *Pseudomonas stutzeri* ST-201 was inserted between NdeI-BamHI restriction sites.

2. Reagents

Restriction endonuclease enzymes were from New England Biolabs. Pfu DNA polymerase and T4 DNA ligase were from Promega. Isopropyl β-D-thiogalactopyranoside (IPTG), pyridoxal-5′-phosphate, Amberlite IRA-400(Cl) (trialkylbenzyl ammonium), a strong anion exchange resin, and D-phenylglycine were purchased from Sigma. Thiopropyl Sepharose 6B, EAH Sepharose 4B and ECH Sepharose 4B used for enzyme immobilization were obtained from Amersham Biosciences. All other chemicals were obtained from Fluka.

3. Preparation of D-PhgAT variants

3.1 *PCR-based mutagenesis*

The deduced amino acid sequence of D-PhgAT shows that it contains two cysteine residues (positions 266 and 327). Determination for the amount free sulfhydryl group indicated that they do not form a disulfide bond with each other (data not shown). At the position 453 of the C-terminus, one serine is located. In order to prepare D-PhgAT with increased reactivity towards thiol-containing matrix while preserving the catalytic activity, four D-PhgAT variants (CCC, CSS, SCS and SSC) consisting of cysteine and serine residues (positions 266, 327 and 453) at various combinations were prepared (Table 1).

Table 1. D-Phenylglycine aminotransferase and its variants prepared in this study.

D-phgAT wild type/variants	Position of amino acid		
	266	327	453
Wild type (CCS)	Cys	Cys	Ser
CCC	Cys	Cys	Cys
SCS	Ser	Cys	Ser
CSS	Cys	Ser	Ser
SSC	Ser	Ser	Cys

Cysteine/serine mutation was chosen because it is considered to be a conservative mutation since the two amino acids have very similar R-groups (–SH versus –OH). Also, the codons of the two amino acids have only one base different, that is, AGC (serine) and TGC

(cysteine). Thus, for the construction of CCC mutant where only the C-terminal serine 453 of the wild type was mutated to cysteine, simple mismatch PCR for a terminal mutation was used. To construct other D-PhgAT variants i.e. CSS, SCS and SSC whose internal mutations were introduced into the gene, at a distance from the protein chain terminus, the PCR-mediated recombination technique called “overlap extension PCR” was used (Figure 2). In this technique, two separate PCRs were run, using primers A and B and primers C and D to amplify overlapping sequences of the template DNA. Primers B and C have overlapping homologous regions containing the mutation of interest.

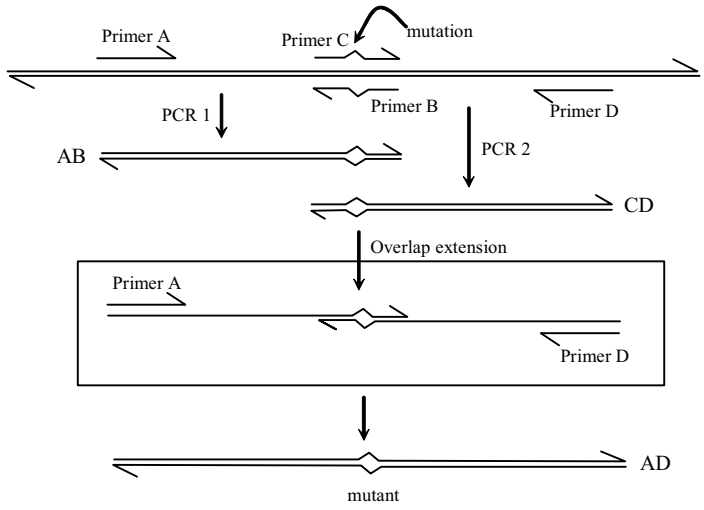


Figure 2. Site-directed mutagenesis by overlap extension PCR.

Following amplification, both products (AB and CD) were purified and fused together in a fusion PCR using the outermost primer A and D. PCR primers used in this study are shown in Tables 2 and 3.

Table 2. PCR primers used in the experiments.

Primer	Length (nt)	Sequence (5′→ 3′)	Mutagenic site / overlapping region / restriction site
Nde-N	21	GGA GCT CAT CAT ATG TCG ATC	*NdeI* site 5′-GGA GCT CAT CAT ATG TCG ATC-3′ Met Ser Ile->
WT-3′end	20	CTC GGA TCC TCA TGA TTG GT	*BamHI* site 3′-TG GTT AGT ACT CCT AGG CTC-5′ <- Ser stop
S453C	24	CTC GGA TCC TCA GCA TTG GTT TCC	*BamHI* 3′-CCT TTG GTT ACG ACT CCT AGG CTC-5′ <- Cys stop
C327S-L	18	CTT CGC GCT AAC ATC GTC	Ser -> C327S-R 5′-GAC GAT GTT AGC GCG AAG-3′ C327S-L 3′-CTG CTA CAA TCG CGC TTC-5′ <- Ser
C327S-R	18	GAC GAT GTT AGC GCG AAG	
C266S-L	19	TTA GCC AGG CTG GTG AGA T	Ser -> C266S-R 5′-CAG CCG GAT CTC ACC AGC CT -3′ C266S-L 3′-TA GAG TGG TCG GAC CGA TT-5′ <- Ser
C266S-R	20	CAG CCG GAT CTC ACC AGC CT	

Table 3. Templates and PCR primers used for the construction of mutant genes of the modified D-phenylglycine aminotransferases.

D-phgAT variants	Template	Primers	
		AB fragment	CD fragment
CCC	WT	Nde-N, S453C (simple mismatch PCR)	
CSS	WT	Nde-N, C327S-L	C327S-R, WT-3′end
SCS	WT	Nde-N, C266S-L	C266S-R, WT-3′end
SSC	CSC	Nde-N, C266S-L	C266S-R, S453C
CSC	CCC	Nde-N, C327S-L	C327S-R, S453C
SCC	CCC	Nde-N, C266S-L	C266S-R, S453C

Reaction for a simple mismatch PCR or 1o overlap extension PCR (generating AB or CD fragment)

	Volume (μl) for 50-μl reaction
Distilled water	37.6
Pfu DNA polymerase 10X buffer with 20 mM $MgSO_4$	5.0
10 mM upstream primer	2.5
10 mM downstream primer	2.5
10 mM dNTP mix	1.0
20 ng/ml DNA template	1.0
3 U/ml *Pfu* DNA polymerase	0.4

Thermal cycling condition:

Step	Temperature (^{o}C)	Time (min)	Number of cycles
Initial denaturation	95	1:00	1
Denaturation	95	0:30	30
Annealing	50	0:30	
Extension	72	2:15	
Final extension	72	5:00	1
Soak	4	Indefinite	1

Reaction for 2° overlap extension PCR (fusion of AB and CD fragments)

	Volume (µl) for 50-µl reaction
Distilled water	36.6
Pfu DNA polymerase 10X buffer with 20 mM $MgSO_4$	5.0
10 mM upstream primer	2.5
10 mM downstream primer	2.5
10 mM dNTP mix	1.0
AB fragment	1.0
CD fragment	1.0
3 U/ml *Pfu* DNA polymerase	0.4

Thermal cycling condition:

Step	Temperature (°C)	Time (min)	Number of cycles
Initial denaturation	95	1:00	1
Denaturation	95	0:30	
Annealing	50	0:30	20
Extension	72	3:15	
Final extension	72	5:00	1
Soak	4	Indefinite	1

After each step of amplification, the PCR product was size-verified by agarose gel electrophoresis. The corresponding band of full-length PCR product was cut and then eluted from the gel. DNA fragment was end-cut with the restriction enzymes Nde I and BamH I and subsequently ligated to the vector pET-17b, which was previously cut similarly with the same enzymes.

3.2 DNA transformation

Competent *E. coli* was prepared according to Hanahan's method (1). DNA transformation was carried out following the manufacturer's instruction. Clones containing the plasmid were selected on LB agar plates containing 100 μg ml-1 ampicillin. Plasmid from selected clone was then extracted, re-cut with Nde I and BamH I and then size-verified by agarose gel electrophoresis to check whether it contained plasmid carrying the desired insert.

3.3 Protein synthesis

The transformed bacteria were cultured to synthesize the protein according to Novagen's instruction. Specifically, 200 ml of LB broth medium containing 100 $\mu g\ mL^{-1}$ ampicillin was inoculated with 0.2 ml of an overnight culture of the transformed *E. coli*, and incubated at 37°C with 200 rpm-shaking until $OD_{600\ nm}$ reached 0.8. Then, isopropylthio-β-D-galactoside (IPTG) was added to a final concentration of 0.4 mM to induce gene expression. After additional 3 h of incubation with low speed shaking (100 rpm) at 25°C, the cells were harvested by centrifugation at 8000 rpm (Sorvall RC 5C) for 5 min at 4°C. The harvested cells were washed twice with 0.85% NaCl solution. Cell aliquots were tested for enzyme activity by resuspending in 9X volume of Lysis buffer (0.1 M sodium phosphate buffer pH 7.6, 1 mM EDTA and 2.5 μM PLP) and incubated with 0.5 mg /ml lysozyme for 20 min. Cell disruption was done by sonication (Vibra cell, Sonics & Materials Inc. U.S.A.) on ice in cycle of 20-s burst with 20-s intermittent cooling until a translucent mixture was obtained. The clarified cell lysate after centrifugation was subjected to activity assay. The residual pellet was stored at –20°C for subsequent purification steps.

3.4 Enzyme purification

Clarified cell lysate was prepared according to the same method described in 3.3. It was then subjected to two chromatographic purification steps; anion exchange chromatography followed by hydrophobic interaction chromatography. For anion exchange chromatography, the column was packed with DEAE-Sepharose Fast Flow (Sigma) with a bed volume of 50 ml, pre-equilibrated with TEMP buffer pH 7.6 (20 mM Tris HCl, 1 mM EDTA, 0.01% β-mercaptoethanol and 2.5 μM PLP). The clarified cell lysate was directly passed through the column and the elution was performed at a flow rate of 2 ml min^{-1}. Initially, the unbound proteins were washed off with 50 ml (one column volume), then a

linear gradient of TEMP buffer containing 0.6 M sodium chloride was applied for 150 ml. D-PhgAT activity in eluted fractions was determined by spectrophotometric assay. The active fractions were pooled, concentrated using a centrifugal filter device (Centricon Plus-20 cut off at 10,000 Da, Amicon).

For hydrophobic interaction chromatography, phenylagarose CL-4B (Sigma) chromatographic medium was packed in a column XK16/40 (Pharmacia) with a bed volume of 60 ml. The column was pre-equilibrated with TEMP buffer pH 7.6 containing 1 M ammonium sulfate. The concentrated fraction from anion exchange chromatography was added with 4 M ammonium sulfate to a final of concentration of 1 M and then loaded into the column. Firstly, one column volume of the same buffer was used to elute the unbound proteins at a flow rate of 1 ml min^{-1}. Then, the enzyme was eluted with a linear gradient from 1 M to 0 M ammonium sulfate. The active fractions were pooled and concentrated using a centrifugal filter device (Centricon Plus-20 cut off at 10,000 D, Amicon). Upon centrifugation, the sample volume was reduced and the salt was eliminated at the same time. The desalting step was repeated once to ensure that the enzyme was free from salt. Purified enzyme was kept in TEMP buffer with 25% glycerol at $4^{\circ}C$ for further use.

Purity of the enzyme along the purification process was monitored by SDS-PAGE. Protein bands were visualized by Coomassie Brilliant Blue staining.

4. Enzyme immobilization

4.1 Enzyme immobilization on Thiopropyl Sepharose 6B

Enzyme immobilization via disulfide linkage on a commercially available Thiopropyl Sepharose 6B was carried out following the manufacturer's instruction. The characteristics for the gel were summarized in Table 4. Initially, dried bead was re-swollen and washed three times with Binding buffer (100 mM Tris buffer pH 7.5, 100 mM NaCl and 1 mM EDTA) that was saturated with nitrogen gas. The enzyme was reduced with Tris[2-carboxylethyl] phosphine hydrochloride (TCEP) to generate free sulfhydryl group by mixing the enzyme with an equal volume of immobilized TCEP disulfide reducing gel (Pierce, Rockford, IL, USA) in a microcentrifuge tube at room temperature. After 30 min, the slurry was centrifuged at 1000 rpm for 1 min to recover the supernatant containing the reduced enzyme. Subsequently, 3 ml of Binding buffer containing 3 mg of freshly reduced enzyme was incubated with 3 ml of gel. The reaction was carried out at $4^{\circ}C$ for 3 h with gentle shaking on a rotary shaker. The suspension was then centrifuged at 1,000 rpm and the beads were collected and rinsed with Binding buffer until no enzyme activity was detectable

in the rinsing fluid. Finally, the beads were resuspended in Binding buffer with 25% glycerol and kept at 4oC.

4.2 Enzyme immobilization on EAH Sepharose 4B and ECH Sepharose 4B

Enzyme immobilization on EAH Sepharose 4B or ECH Sepharose 4B was achieved via the coupling between the amino or carboxyl group on the enzyme to the carboxyl group (ECH Sepharose 4B) or amino group (EAH Sepharose 4B) on the support accordingly. The characteristics for both gels are shown in Table 6. The reaction was setup in a 1.6 ml total volume composed of 500 μl gel, 1 mg enzyme, 0.1 M N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and distilled water adjusted to pH 6. The mixture was mixed gently on a rotary wheel for 6 h at 4°C. Then the product was washed thoroughly with distilled water until no enzyme activity was found in the washing liquid. The beads were re-suspended in the 25% glycerol and stored at 4°C.

Table 4. Summary of characteristics of gels used for enzyme immobilization

Gel	Matrix	M_r	Size range (μm)	Spacer	Active group
Thiopropyl sepharose 6B	Agarose 6%	6×10^6	45-165	2-hydroxypropyl, 4 atoms	-SH
EAH Sepharose 4B	Agarose 4%	2×10^7	45-165	1,6-diaminohexane, 10 atoms	$-NH_2$
ECH Sepharose 4B	Agarose 4%	2×10^7	45-165	6-aminohexanoic, 9 atoms	-COOH

5. Characterization of soluble and immobilized enzyme

5.1 Determination of sulfhydryl group

Since D-PhgAT contained two cysteine residues on each molecule in which they tended to form an intermolecular disulfide linkage and did not react with 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB or Ellman's reagent), to determine its sulfhydryl content, the enzyme was initially reduced to generate free sulfhydryl by incubating with Immobilized TCEP Disulfide Reducing Gel (Pierce, Rockford, IL, USA) at room temperature. After shaking

the mixture on a rotary wheel for 30 min, the supernate containing the reduced enzyme was collected by centrifugation at 1,000 rpm for 1 min.

The thiol content of the enzyme was quantified by spectrophotometric determination of the released 2-nitro-5-thiobenzoic acid (TNB) when the free sulfhydryl group of the enzyme reacted with Ellman's reagent (2). In the assay, 250 μl of the sample (previously diluted so that the 250 μl sample had a sulfhydryl concentration less than 1.0 mM) was mixed with 2.5 ml of reaction buffer (0.1 M sodium phosphate buffer, pH 7.3, containing 1 mM EDTA and 6 M guanidinium chloride) and 50 μl Ellman's reagent. For a blank, 250 μl of reaction buffer was added instead of the sample. The reaction was mixed and incubated at room temperature for 15 min before subjected to spectrophotometric measurement at 412 nm. The net absorbance after subtracting the blank was used to calculate the concentration in molar amount of sulfhydryl group in the sample from the molar extinction coefficient of TNB (13,700 $M^{-1}.cm^{-1}$).

5.2 Determination of protein

Protein concentrations were determined with the Bradford assay (3) using bovine serum albumin as standard. Determination of protein amount bound to the matrix was done by calculating the difference between the amount initially added and that left in the supernate and in the washing liquids.

5.3 Determination of enzyme activity

Enzyme activity was determined with two methods. In the first method, a spectrophotometric assay was used to determine D-PhgAT activity in the direction of benzoylformate and L-glutamate synthesis. The assay was carried out in a 1 ml reaction mixture containing 5 μl soluble enzyme or 10 μl immobilized enzyme, 100 mM Tris buffer (pH 9.0), 1 mM D-phenylglycine, 1 mM 2-oxoglutarate, 25 μM PLP and 25 mM EDTA. When assayed with immobilized enzyme, the reaction mixture was regularly mixed by pipetting up and down throughout the assay to prevent the bead sedimentation. The rate of benzoylformate formation was measured as a function of time by monitoring the increase in absorbance at 254 nm using a Kontron Uvikon 922 spectrophotometer (Kontron Instruments, Watford, Bucks, UK). The second method was used for determining D-PhgAT activity in the direction of D-phenylglycine synthesis. 100 mM Tris buffer (pH 9.0), benzoylformate, L-glutamate, 25 μM PLP and 25 mM EDTA were incubated with 100 μl of immobilized

enzyme in a 1 ml reaction mixture at 37°C. Aliquots were taken at time intervals, heated in boiling water for 2 min and filtered through a membrane (0.45 μm). The clear filtrate was subsequently assayed for D-phenylglycine by HPLC.

5.4 Enzyme kinetic study

The Michaelis constants (KM) of D-PhgAT wild type and variants for D-phenylglycine and 2-oxoglutarate were determined by assaying the enzyme activity using standard spectrophotometric method (D-phenylglycine degradation) except that the reaction mixtures contained various concentrations of D-phenylglycine and 2-oxoglutarate in a range of 0.8 - 1.25 mM in 100 mM Tris buffer. The substrate mixture in a cuvette was incubated for 5 min at 35°C before being added with the enzyme and the mixture was mixed well by pipetting. Initial enzyme velocities were calculated from the slope of the absorbance-time plot at $OD_{254\ nm}$ over 1.5 min- period with the kinetic mode measurement. Double reciprocal plots of the initial velocities (Vi) against substrate concentrations were constructed when the concentration of one substrate was fixed and the other was varied and K_M was determined from the second Lineweaver-Burk plots according to the method of Velick and Varva (4).

5.5 High performance liquid chromatography analysis

Concentrations of D-phenylglycine were analyzed by HPLC using System GoldTM The PersonalTM Chromatograph (Beckman Instrument, Inc., Fullerton, CA, USA) or HP 1100 series (Hewlett Packard, USA). 20 μl of sample were injected onto a Spherisorb ODS2 column (250 x 46 mm) (Waters, Milford, MA, USA) operating at 25°C. Assay conditions were; mobile phase: isocratic 50 mM potassium phosphate buffer (pH 7.0) at a flow rate of 1.0 ml min-1; UV Abs detection at 254 nm.

5.6 Determination of pH effects on enzyme activity and stability

Effects of pH on the enzyme activity were examined using the spectrophotometric method for activity determination. Initial enzyme activity was measured at 37°C at a range of pH values between 5 and 12 using 100 mM of the following buffers: citrate buffer (pH 5, 6), PIPES buffer (pH 6-8), Tris buffer (pH 7-9) and CAPS buffer (pH 9-12). Activity measurements were made over a 1.5-min time period to ensure a linear product-time profile.

The effect of pH on the stability of the immobilized enzyme was measured by incubating the enzyme at a range of pH values between 7.5 and 9.5, 37oC for 6 h. At time

intervals, aliquots of enzyme were assayed for residual activity using the standard spectrophotometric method.

5.7 Determination of temperature effects on enzyme activity and stability

Effects of temperature on enzyme activity were examined by the standard spectrophotometric assay in the range of 20-80^{O}C at pH 8.0. Activity measurements were made over a 1.5-min time period to ensure a linear product-time profile. Thermal stability was studied by incubating the enzyme in 100 mM PIPES buffer (pH 7.5) at 37, 40, 50 and 60^{O}C, for 6 h. Then, the heated samples were cooled down to 30^{O}C for 5 min in a 30^{O}C water bath before being used as the enzyme sources in the assay for their residual activities using the standard spectrophotometric method.

5.8 Determination of the effects of substrate concentration on enzyme activity

Effects of substrate concentration on the reaction rate were determined by incubating 100 μl immobilized enzyme in 100 mM PIPES buffer (pH 7.5) containing benzoylformate and L-glutamate at a range of initial concentrations between 50 and 1500 mM at 37^{O}C. The rate of D-phenylglycine formation was measured over a 30 min reaction time.

5.9 Study on storage stability

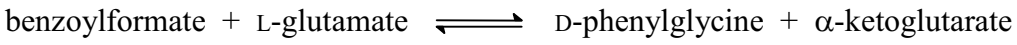
To compare long term storage stability, the initial rates of reaction by soluble and immobilized enzyme were determined over a period of 28 days. For these studies, the enzymes were stored at room temperature or 4^{O}C in the presence or absence of 25% glycerol. The residual activity computed by dividing the initial rate of reaction at any day by the rate at day 1 was plotted against the number of days and used as the indicator of the storage stability of the enzyme.

6. Development of a process for D-phenylglycine synthesis using immobilized enzyme

6.1 Determination of equilibrium constant of D-phenylglycine synthesis reaction

The reactions composed of substrates and/or products of various known concentrations ranging from 3.5 to 10 mM were setup in 1-mL tube using soluble enzyme as catalyst. After shaking the reaction at 37^{O}C for 18 h to reach the equilibrium, the

concentrations of substrates and products were re-analyzed using HPLC. The equilibrium constant of the overall D-phenylglycine synthesis reaction was calculated using the equation;



$$K_{eq} = \frac{[\text{D-phenylglycine}][\alpha\text{-ketoglutarate}]}{[\text{benzoylformate}][\text{L-glutamate}]}$$

6.2 Investigation of the solubility of D-phenylglycine

The saturated solutions of D-phenylglycine were prepared at pH 5-9 and kept at 4, 25, 37°C for 24 h. The clear supernate was carefully taken using 0.45 μm-membrane syringe to prevent solid D-phenylglycine contamination and then diluted. The diluted samples were then subjected to concentration analysis using the spectrophotometric method.

6.3 Study of adsorption of the substrates and products onto the resin

Prior to use, 50 g anion exchange resin Amberlite IRA-400 was washed sequentially with 100 ml of 1 M HCl solution, deionized water, 1 M NaOH solution, deionized water, 1 M HCl solution, deionized water (until the pH equaled 7) and dried at 60°C in a controlled temperature oven for 24 h. One gram of the dried resin was added to 10 ml of 30 mM D-phenylglycine, benzoylformate, L-glutamate or 2-oxoglutarate at pH 5, 7, 7.5, 8 and 9. Subsequently, all the samples were incubated in a shaking incubator at 200 rpm, 37°C for 12 h. The residual (unadsorbed) concentration was measured using HPLC, from which the amount of adsorbed compounds (per gram dried resin) was calculated.

6.4 Use of the Amberlite IRA-400 resin-adsorbed benzoylformate in D-phenylglycine synthesis

In order to prepare resin adsorbed-benzoylformate, 20 g of the previously washed and dried Amberlite IRA-400 resin was added to 40 ml 2 M benzoylformate (pH 7.5) at 37°C while agitating at 200 rpm. After 6 h, the adsorbed resin was filtered and dried at 60°C for 24 h. The amount of benzoylformate released from the resin was investigated by shaking 0.5 g benzoylformate-adsorbed resin in 25 ml 100 mM PIPES buffer (pH 7.5) at 200

rpm. Periodically, the supernatant was withdrawn and subjected to spectrophotometric measurement of benzoylformate. The desorption was repeated using fresh buffer until no further release was observed and the results were used to calculate the cumulative amount of benzoylformate released.

Determination on appropriate amount of benzoylformate adsorbed resin required in D-phenylglycine synthesis was studied by adding varying amounts of the resin to a 1 ml reaction containing 100 mM PIPES buffer (pH 7.5), 1 M L-glutamate, 25 μM PLP and 100 μl immobilized enzyme. The reaction proceeded at 37°C under agitation at 200 rpm for 12 h. A control reaction with no benzoylformate adsorbed resin was conducted using benzoylformate solution instead at a final concentration of 100 mM. Upon sampling, sufficient distilled water was added to the reaction to dissolve the product. Immobilized enzyme and Amberlite IRA-400 resin were excluded by using a 0.45 μm-syringe filter and the filtrate was diluted with distilled water prior to analysis of D-phenylglycine and benzoylformate concentrations by HPLC.

Results

1. Random immobilization of wild-type D-PhgAT on agarose matrixes

The wild-type D-PhgAT was immobilized on three different commercially available agarose-based matrixes through –NH2, –COOH or –SH. Because there are a number of these functional groups scattered on the enzyme molecule, thus, the attachment points between the enzyme molecule and the matrixes are in random fashion. It was found that after the enzyme was immobilized on EAH Sepharose 4B and Thiopropyl Sepharose 6B, the activity of the immobilized enzyme dropped to 15.58 and 19.92 % of that of the free enzyme, respectively (Table 5). The wild type enzyme could not be successfully immobilized on ECH Sepharose 4B since it was apparently precipitated once EDC was added to the coupling reaction. As a result, the enzyme activity was undetectable in the supernate as well as on the bead. The drastic decrease in catalytic activity led us to investigate whether or not site-specific immobilization could yield higher specific activity.

Table 5. Immobilization of the wild-type D-PhgAT on agarose-based matrixes.

Matrix	**No. of potentially active residues per one enzyme subunit**	**Amount of bound protein (mg/g dry matrix)**	*Specific activity* **(units/mg protein)**	**Relative activity* (%)**
No matrix (soluble enzyme)	-	-	20.73	100
EAH Sepharose 4B	29 Asp & 21 Glu	30.01	3.23	15.58
ECH Sepharose 4B	10 Lys	Immobilization failed, protein precipitated		
Thiopropyl Sepharose 6B	2 Cys	19.60	4.13	19.92

* Relative activity is defined as the specific activity of D-PhgAT variants related to the specific activity of the wild type.

2. Preparation of modified D-PhgAT

2.1 Site-directed mutagenesis

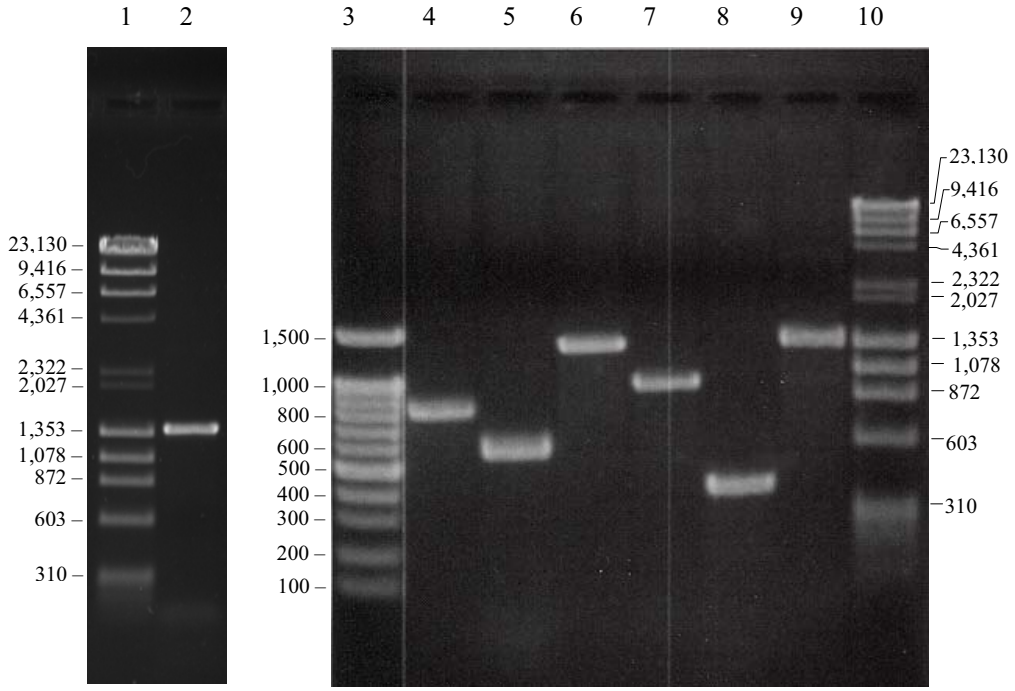
D-PhgAT variants possessing cysteines at various positions were constructed using PCR-based site-directed mutagenesis. The amplified D-PhgAT gene with S453C mutation constructed by simple mismatch PCR and electrophoretically separated on 0.75% agarose gel is shown in Figure 3a. By using the overlap extension PCR, mutations at specific points were introduced in order to construct other desired variants. Figure 3b shows the amplified primary DNA fragments (AB and CD) and full-length fusion products (AD) on 1% agarose gel. Sizes of all products observed on the agarose gel were in good agreement with that expected. The mutated D-PhgAT was, then, inserted into the expression vector, pET-17b, which was then transformed into *E. coli* BL21(DE3) to express the modified enzymes.

2.2 Purification of D-PhgAT variants

After disruption of the induced cells, the clarified lysate was used as a source for enzyme purification using anion exchange chromatography, followed by hydrophobic interaction chromatography. The examples of chromatograms representing protein elution pattern from each chromatographic step are illustrated in Figures 4 and 5. Along the purification process, the purity of the enzyme was monitored using SDS-PAGE. The results from SDS-PAGE after Coomassie Blue staining are shown in Figure 6. The purified enzyme gave a single band of a protein, satisfactorily for further characterization and immobilization purposes.

2.3 Determination of sulfhydryl groups

Quantity of sulfhydryl groups of cysteine on each enzyme molecule was determined by reducing the purified enzymes with TCEP, then, total sulfhydryl content was determined using Ellman's reagent under the denaturing condition (in the presence of guanidinium chloride). From the change in the absorbance at 412 nm, the average number of –SH group on each enzyme molecule was calculated (Table 6). Results of the studies with the wild-type and mutated D-PhgAT demonstrated that numbers of cysteine residues were correctly introduced each D-PhgAT variant.



(a) (b)

Figure 3. Agarose gel electrophoresis of PCR products obtained from site-directed mutagenesis of D-PhgAT gene by (a) simple mismatch PCR and (b) overlap extension PCR.

Lane 1 and 10 : ϕX174 DNA/Hae III and λ DNA/Hind III markers

Lane 2 : D-PhgAT gene with S453C mutation

Lane 3 : SibEnzyme® DNA markers 100 bp + 1.5 Kbp

Lane 4 : AB fragment for construction of D-PhgAT (C266S)

Lane 5 : CD fragment for construction of D-PhgAT (C266S)

Lane 6 : Full-length D-PhgAT (C266S)

Lane 7 : AB fragment for construction of D-PhgAT (C327S)

Lane 8 : CD fragment for construction of D-PhgAT (C327S)

Lane 9 : Full-length D-PhgAT (C327S)

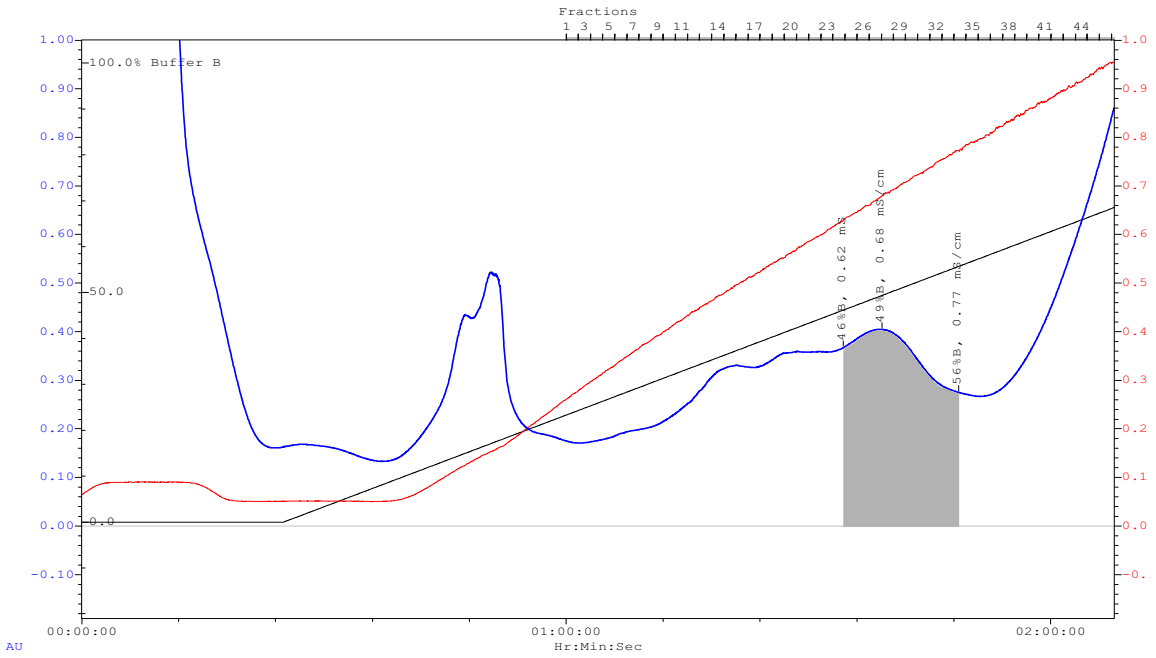


Figure 4. Protein elution pattern and sodium chloride gradient of DEAE anion exchange chromatography. Shading area represents the D-PhgAT fractions collected for the next HIC chromatographic step.

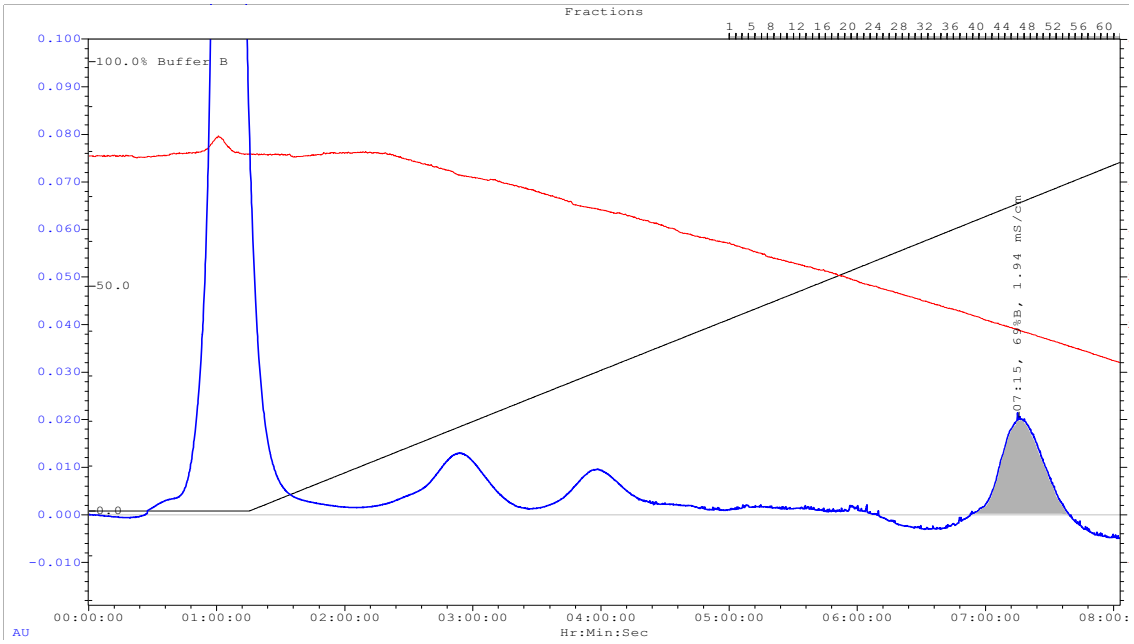


Figure 5. Protein elution pattern and ammonium sulfate gradient of phenyl agarose CL-4B hydrophobic interaction chromatography. Shading area represents the D-PhgAT fractions collected for desalting and concentration step.

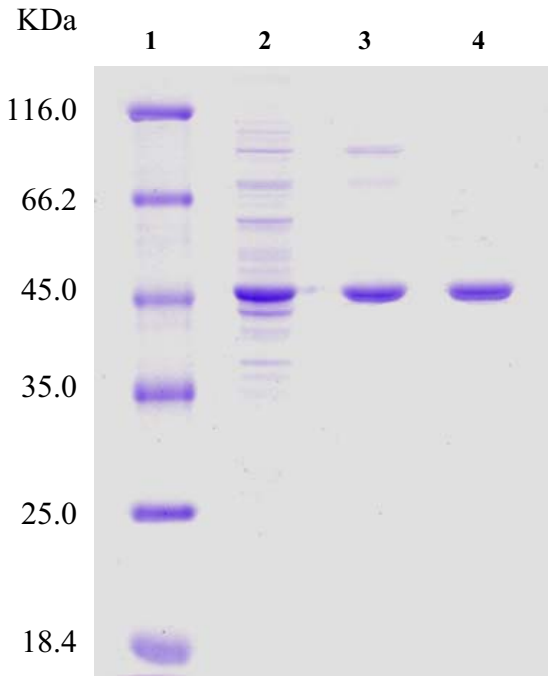


Figure 6. SDS-PAGE analysis of D-PhgAT(S453C) from the purification process.

Lane 1 : Molecular weight markers with their molecular weight indicated

Lane 2 : Crude lysate after cell disruption

Lane 3 : Pooled fractions after DEAE anion exchange chromatography

Lane 4 : Pooled fractions after phenyl agarose HIC chromatography

2.4 Specific activity and kinetic parameters of soluble D-PhgAT variants

Specific activity and kinetic parameters of D-PhgAT mutants were compared to that of the wild-type enzyme, which was expressed and purified using the same procedures. As shown in Table 6, all variants had different specific activities and kinetic parameters. Except for D-PhgAT (S453C), they all had lower specific activity and higher Michaelis constant (KM) than that of the wild type. The decrease in activity was in line with the number of native cysteine residues that was mutated to serine. D-PhgAT (S453C) whose only serine 453 was mutated to cysteine while the two native cysteine were still intact, had a comparable specific activity and KM to that of the wild type. Thus, it was chosen for further immobilization and subsequent studies.

Table 6. Number of cysteines present per enzyme subunit, activity and kinetic parameters of D-PhgAT wild type and variants.

Enzyme	No. of cysteines*	Specific activity (units/mg protein)	K_M (mM)	
			D-phg	α-KG
Wild type (CCS)	1.85	20.73	0.384	1.640
CCC	2.89	18.23	0.309	1.648
CSS	0.82	11.45	0.420	1.732
SCS	0.85	8.00	0.651	1.881
SSC	0.88	6.28	0.622	1.960

* Number of cysteine residues for each protein subunit determined by using the Ellman's reagent

3. Immobilization of D-phgAT (S453C) onto Thiopropyl Sepharose 6B

The reduced enzyme was mixed and incubated with Thiopropyl Sepharose 6B for various periods of time at 4oC. The immobilization was monitored as a function of activity of the unbound enzyme left in the supernate versus the incubation time (Figure 7). The reactions containing soluble enzyme in the absence of gel were set up under the same condition in order to demonstrate that the decrease in activity was not due to the enzyme instability. After approximately 1 h, complete immobilization of D-PhgAT (S453C) onto the bead was achieved as indicated by no further reduction of free enzyme in the supernatant. In contrast, the immobilization of wild-type enzyme was much slower and did not reach the saturation over a period of 12 h. This finding indicated that D-PhgAT (S453C) was much more reactive towards thiol groups than the wild type. Similarly, the amount of D-PhgAT (S453C) bound to the matrix was found to be higher than that of the wild type (Table 7).

The immobilized enzymes obtained from various time of incubation were assayed for the activity. The immobilized D-PhgAT (S453C) had approximately twice higher activity than that of immobilized wild type (Figure 8). The activity of immobilized D-phgAT (S453C) was highest after 1 h of incubation and then slightly decreased afterward. In contrast, the activity of bead bound with wild-type enzyme slowly increased with time after one hour of incubation.

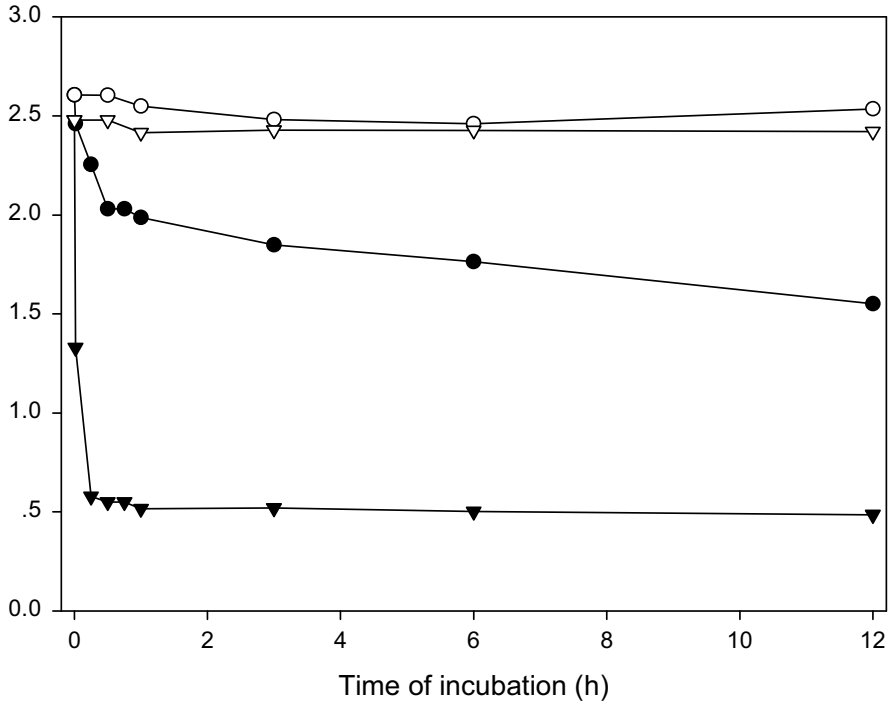


Figure 7. Time course of the immobilization of D-PhgAT wild type (●) and D-PhgAT(S453C) (▼), monitored by measuring the activity of the unbound enzyme left in the supernate. Open symbols represent the activity of soluble wild type (O) and D-phgAT(S453C) (∇) incubated in the absence of matrix under the same condition.

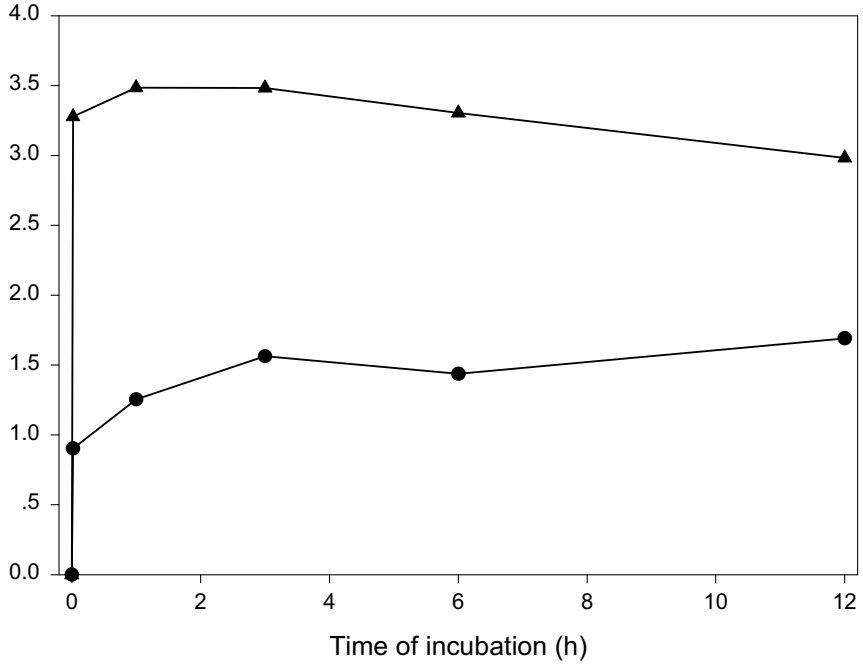


Figure 8. Activity of immobilized wild type (●) and D-phgAT(S453C) (▼) obtained from various time of incubation.

Table 7. Amount of protein immobilized on Thiopropyl Sepharose 6B and the specific activity of immobilized enzyme compared to that of soluble enzyme.

Enzyme	*Amount immobilized* (mg/g dry matrix)	Specific activity (units/mg protein)	Relative activity* (%)
D-PhgAT(S453C)			
Soluble	-	18.23	-
Immobilized	34.62	5.53	30.33
Wild-type D-phgAT			
Soluble	-	20.73	-
Immobilized	19.60	4.13	19.92

* Relative activity is defined as the specific activity of immobilized enzyme relative to the specific activity of the soluble enzyme.

4. Characterization of immobilized D-phgAT (S453C)

4.1 Effects of pH on enzyme activity and stability

To study pH effects on enzyme activity and stability, initial activity was measured over a brief period of 1.5 min to negate the effects of any instability, especially at extremes of pH. It was obviously found that both immobilized (Figure 9a) and soluble (Figure 9b) D-PhgAT (S453C) were highly active in alkali conditions with the highest activity at pH 9 in Tris or CAPS buffer. Differences in enzyme activity were observed when different types of buffer were used, in particular, higher enzyme activity was observed in PIPES buffer than that in Tris buffer at the same pH. These results were similar to the previously reported profile of soluble wild-type enzyme, which was most active at pH 9–10 (5).

Despite the increased activity when the pH was raised from 7 to 9, the stability of the enzyme was significantly decreased (Figure 10). Immobilized D-PhgAT (S453C) which had been incubated at pH above 9 lost all activity in 5-6 h. Interestingly, whereas incubation of the soluble D-PhgAT (S453C) enzyme at pH 7.5 over a period of 6 h led to nearly 30% loss of its initial activity, its immobilized form could maintain 90% of its initial activity under this condition. Consequently, PIPES buffer at pH 7.5 was chosen for further D-phenylglycine synthesis experiments.

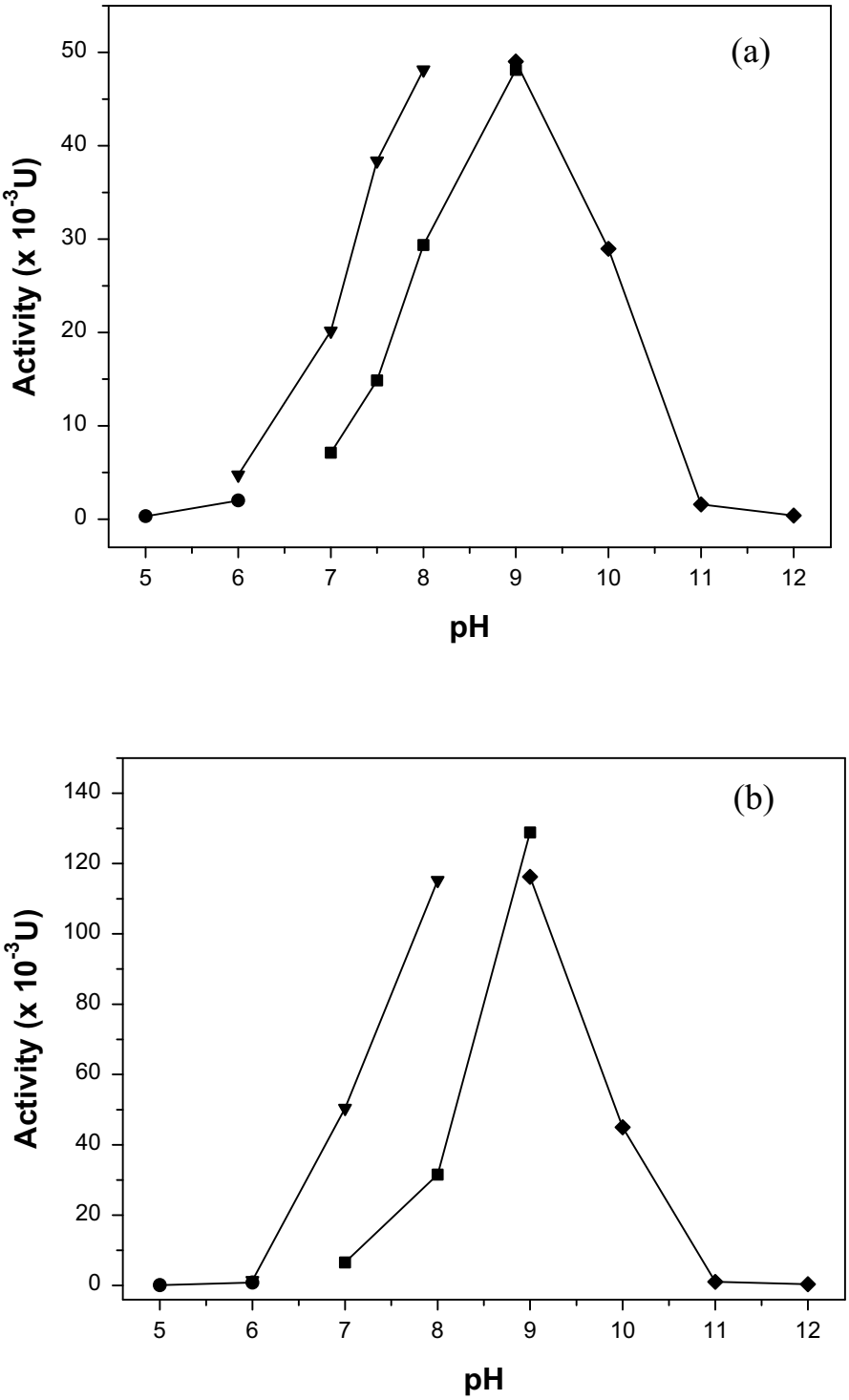
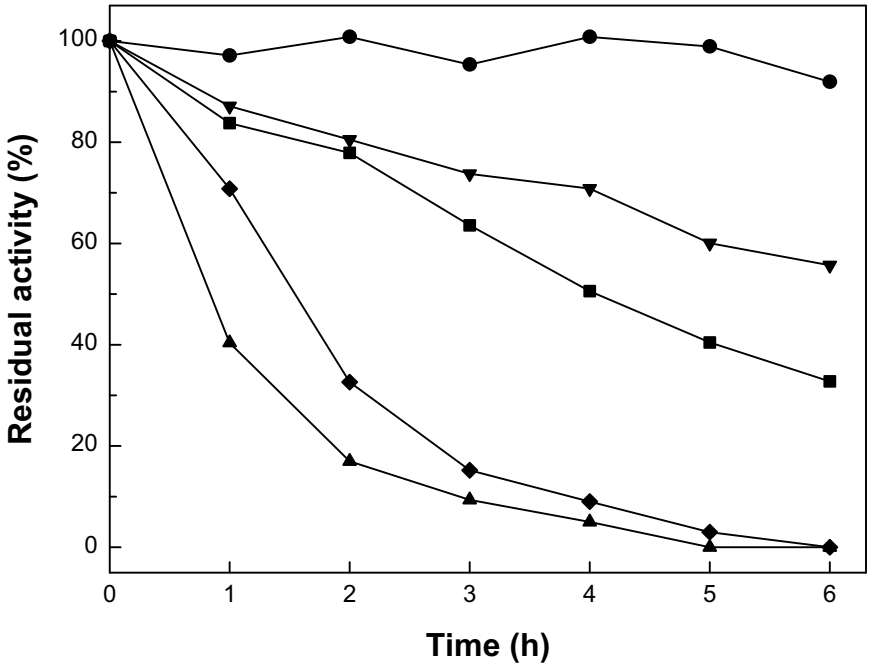


Figure 9. Effects of pH on the activity of D-PhgAT (S453C) (a) immobilized and (b) soluble enzyme determined at 37°C in 100 mM different buffers: (●) citrate buffer, (▼) PIPES buffer, (■) Tris buffer and (◆) CAPS buffer.



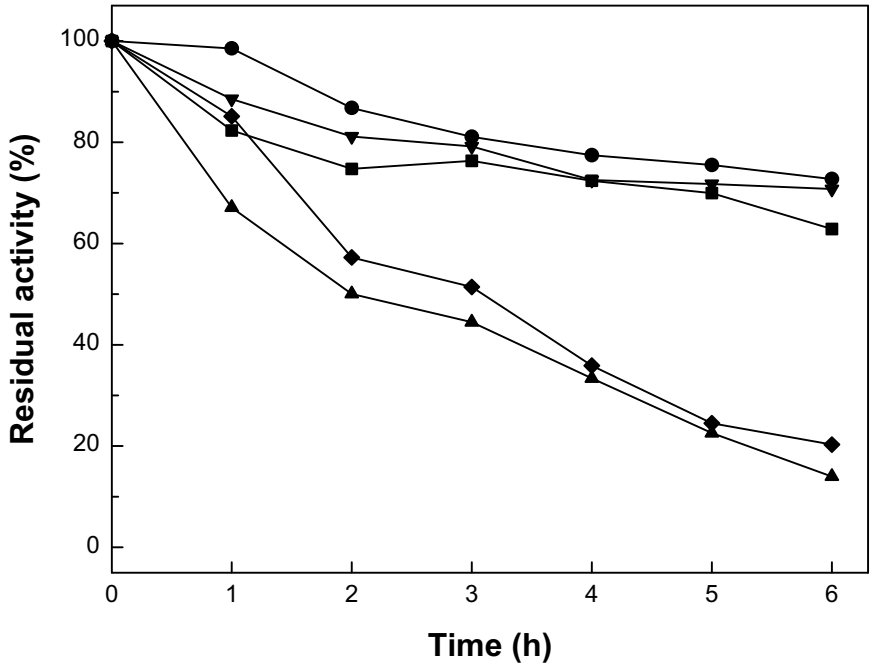


Figure 10. Effects of pH on stability of D-PhgAT (S453C) (a) immobilized and (b) soluble enzyme. Residual activities were determined periodically after incubation of the enzyme at 37°C at: (●) pH 7.5 and (▼) pH 8.0 in 100 mM PIPES buffer, (■) pH 8.5 and (◆) pH 9.0 in 100 mM Tris buffer and (▲) pH 9.5 in 100 mM CAPS buffer.

4.2 Effects of temperature on enzyme activity and stability

Effects of temperature on enzyme activity were investigated by measuring an initial activity over a brief period of 1.5 min in order to negate the effects of any instability. Above 20^{O}C, enzyme activity of both soluble and immobilized D-PhgAT (S453C) increased with increasing temperature. The soluble enzyme showed an activity maximum at 45-55^{O}C and then declined severely as the temperature was raised above 65oC (Figure 11). However, when the enzyme was immobilized onto Thiopropyl Sepharose 6B, the temperature optimum was shift to 50-65^{O}C and a large drop in activity was found when the immobilized enzyme was assayed at the temperature higher than 70^{O}C. A range of 37-60^{O}C where the immobilized enzyme had good activity was chosen for the study of thermal stability over a period of 6 h and the result is illustrated in Figure 12. Results showed that high temperature were detrimental for the immobilized enzyme. At 60^{O}C where the immobilized enzyme was most active, no residual activity was found after 4-h incubation. Therefore, the chosen temperature for carrying out the reaction for all subsequent experiments was 37^{O}C.

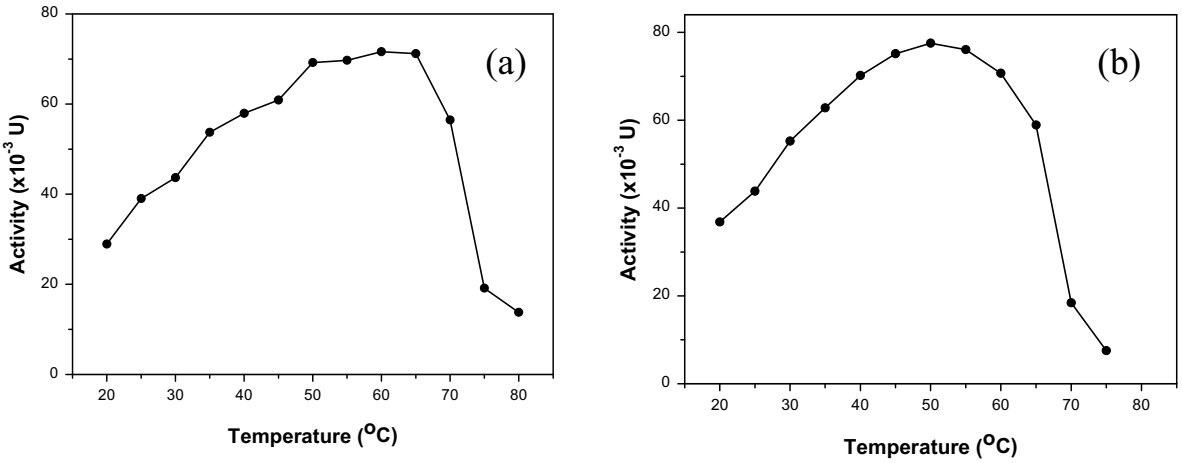


Figure 11. Effects of temperature on activity of D-PhgAT (S453C) (a) immobilized and (b) soluble enzyme determined in 100 mM PIPES buffer, pH 8.0.

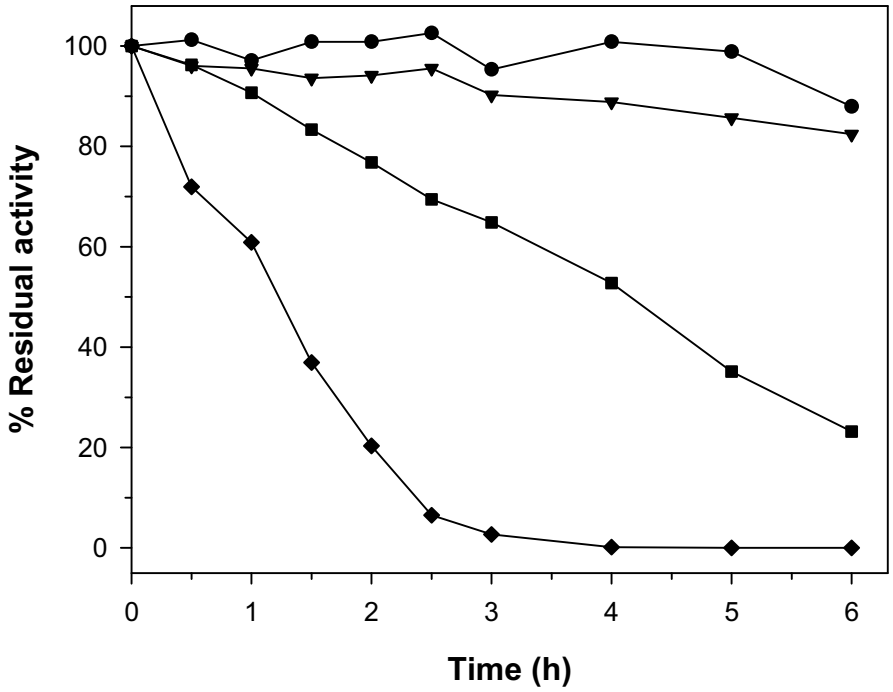


Figure 12. Effects of temperature on stability of immobilized D-PhgAT (S453C). Residual activities were determined periodically after incubating the enzyme in 100 mM PIPES buffer, pH 8.0 at: (●) 37°C, (▼) 40°C, (■) 50°C and (◆) 60°C.

4.3 Effects of substrate concentration on enzyme activity

Benzoylformate showed a notable inhibitory effect towards the enzyme activity (Figure 13a). By contrast, the reaction rate increased proportionally to L-glutamate concentration, up to 1.25 M (Figure 13b). However, at higher L-glutamate concentrations, the reaction rate fell sharply indicating that substrate inhibition did occur.

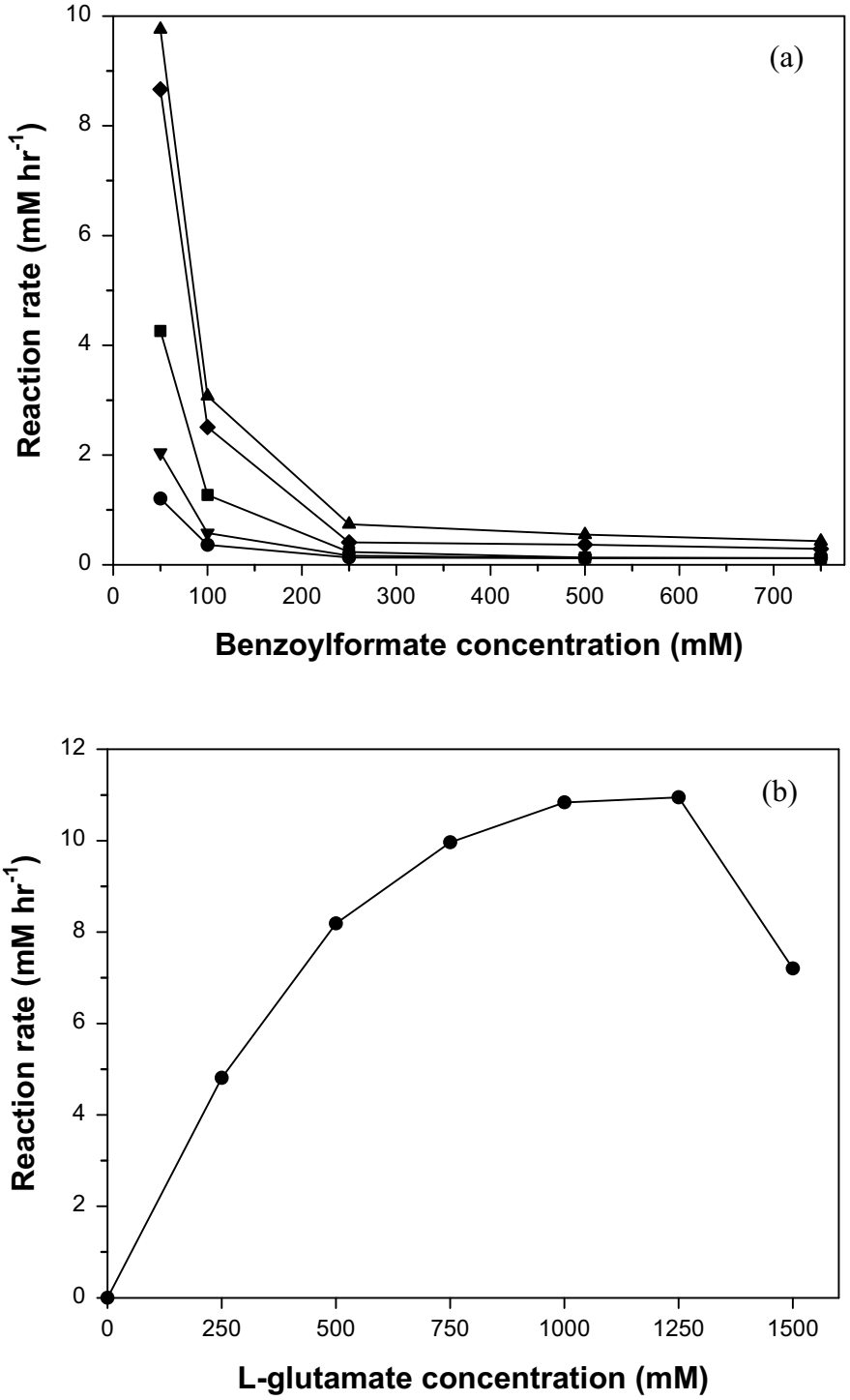


Figure 13. Effects of substrate concentration on the D-phenylglycine synthesis rate. (a) Effects of benzoylformate at different L-glutamate concentrations (●) 50 mM, (▼) 100 mM, (■) 250 mM, (◆) 500 mM and (▲) 750 mM, and (b) Effects of L-glutamate concentrations at 50 mM benzoylformate.

4.4 Storage stability of soluble and immobilized enzymes

Since long-term stability of immobilized enzymes is one of the key factors that determines their practical applications, it was evaluated over a 28-day period when the enzymes were kept under various conditions. Results showed that stability of both soluble and immobilized enzyme was superior when they were stored in 25% glycerol at 4°C, with about 80% remaining of their original activities after 28 days. Without 25% glycerol, the enzymes showed lower stability even when kept at 4°C. Effects of long-term storage and temperature were significant when the enzymes were kept at room temperature for 28 days. The immobilized and the soluble enzymes maintained their activities after 28 days at 18% and 5%, respectively.

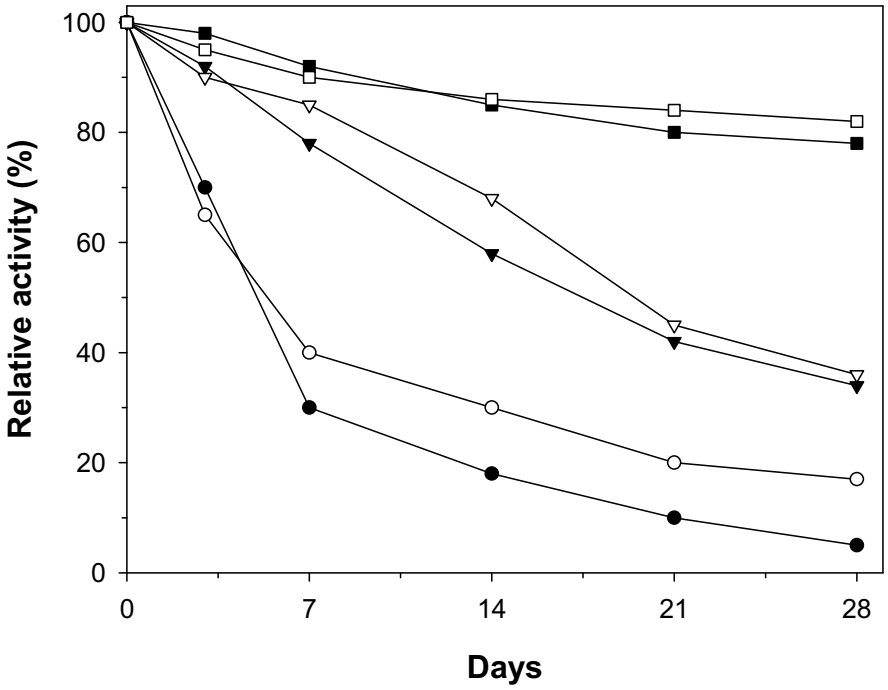


Figure 14. Storage stability of soluble and immobilized D-PhgAT(S453C). The soluble enzyme was stored at (●) room temperature, (▼) 4°C and (■) 4°C in the presence of 25 % glycerol and compared with immobilized enzyme stored at (O) room temperature,(∇) 4°C and (□) 4°C in the presence of 25 % glycerol.

5. D-Phenylglycine synthesis and productivity enhancement by resin approach

5.1 Equilibrium constant of D-phenylglycine synthesis reaction

The equilibrium constant (K_{eq}) of the overall D-phenylglycine synthesis reaction was determined by chemical analysis of all substrate and product concentrations at equilibrium as in the equation below. K_{eq} in this study was found to be 0.075 $\pm$ 0.004.

$$K_{eq} = \frac{[\text{D-phenylglycine}][\alpha\text{-ketoglutarate}]}{[\text{benzoylformate}][\text{L-glutamate}]}$$

5.2 Solubility of the product D-phenylglycine

Solubility of D-phenylglycine was investigated as a function of pH and temperature. As shown in Figure 15, D-phenylglycine has low water solubility at acidic to neutral pH and low temperature.

5.2 Solubility of the product D-phenylglycine

Solubility of D-phenylglycine was investigated as a function of pH and temperature. As shown in Figure 25, D-phenylglycine has low water solubility at acidic to neutral pH and low temperature.

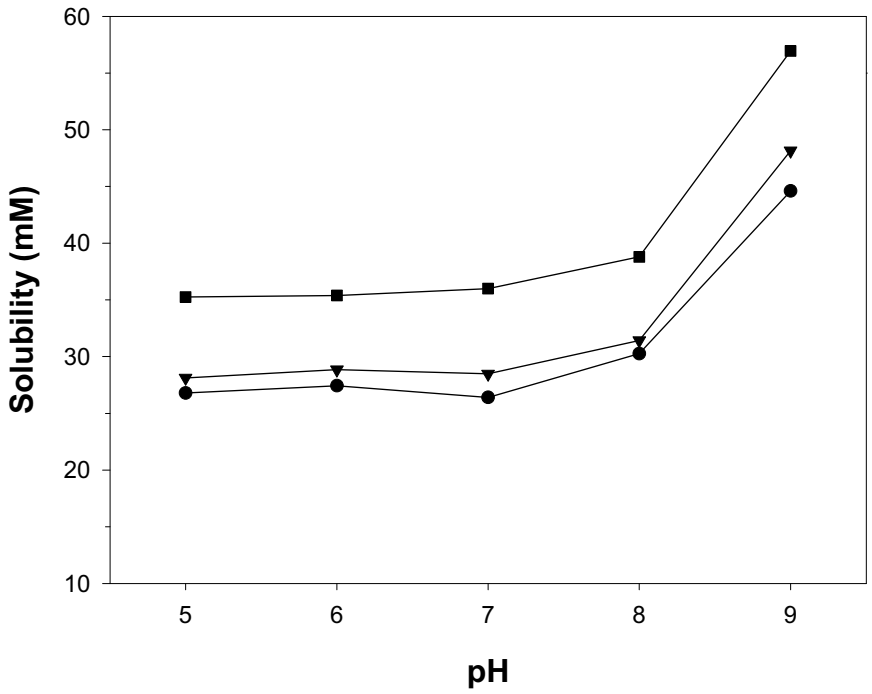


Figure 15. Solubility of D-phenylglycine. The experiment was done by analyzing the concentration of saturated D-phenylglycine solution prepared at various pH's and at (■) 37, (▼) 25 and (●) 4 $^{\circ}$C.

5.3 Adsorption of substrates and products onto resins

Amberlite IRA-400 was examined for its capabilities to adsorb the substrates and products. This was done in order to find a potential means of controlled-release substrates and/or potentially in situ product removal to minimize the substrate and product inhibitory effects. It was found that Amberlite IRA-400 showed promising characteristics and was most suitable for the system. At pH 7.5 at which the synthesis reaction was conducted, the adsorption of L-glutamate as well as the desired product D-phenylglycine onto Amberlite IRA-400 was very low even after 12 h of incubation (Figure 16). By contrast, the resin could efficiently adsorb benzoylformate, the substrate, whose concentration should be kept low in the reaction to minimize the substrate inhibitory effect.

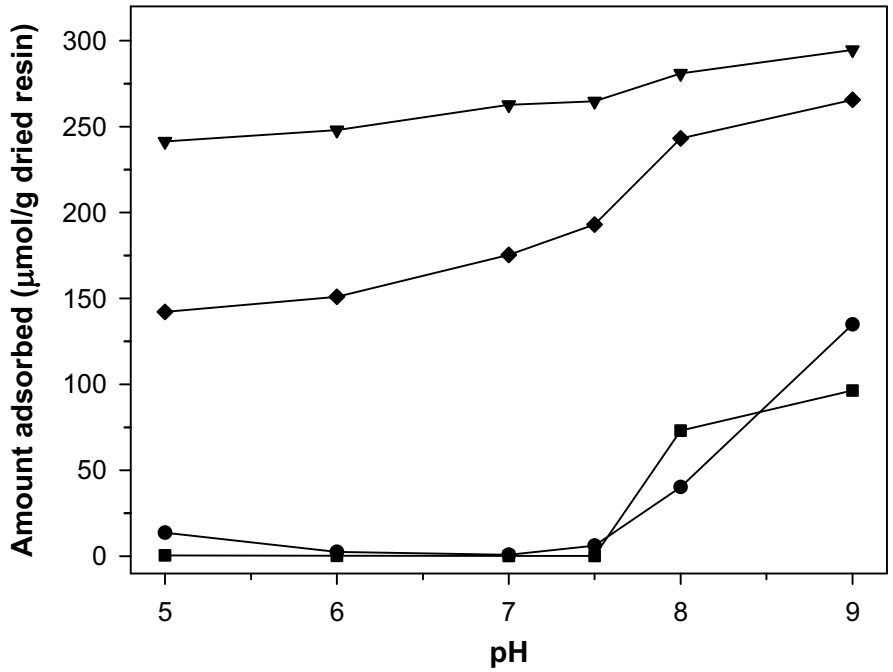


Figure 16. Adsorption to Amberlite IRA-400 under agitation at 200 rpm, 37^{O}C for 12 h with 30 mM solution of: (■) D-phenylglycine, (●) L-glutamate, (◆) 2-oxoglutarate and (▼) benzoylformate.

Additionally, Amberlite IRA-400 was found to adsorb 2-oxoglutarate relatively well, in which it might be employed for removal of this by-product while the reaction was in progress. However, as the resin was to be used for adsorbing benzoylformate for the substrate controlled-release purpose, if it is to be used for adsorbing 2-oxoglutarate in the same reaction a competitive binding should occur. Thus, a study to determine whether there was a competitive binding or not was done by adding 0.1 g of Amberlite IRA-400 into 1 ml solution containing both 15 mM benzoylformate and 15 mM 2-oxoglutarate. The mixture was subjected to continuous shaking at 200 rpm, 37°C for 18 h. Results showed that the resin could adsorb both compounds quite well whether or not they were present singly or in combination (Table 8). Thus, this showed that the benzoylformate-adsorbed resin, after releasing the substrate, could adsorb the by-product 2-oxoglutarate.

Table 8. Adsorption of 2-oxoglutarate and benzoylformate onto 0.1 g Amberlite IRA-400 when the compounds were present singly or in combination.

Compound and starting amount	Amount adsorbed (μmole)	adsorption %
2-oxoglutarate		
30 μmole alone	19.31	64.35
15 μ mole in combination with	9.37	62.46
15 μmole benzoylformate		
benzoylformate		
30 μmole alone	26.47	88.24
15 μ mole in combination with	12.22	88.12
15 μ mole2-oxoglutarate		

5.4 Enhancement of D-phenylglycine productivity by benzoylformate-adsorbed Amberlite IRA-400 resin

Amberlite IRA-400 resin-adsorbed benzoylformate was prepared in order to supply substrate via a controlled-release mechanism in the enzyme reaction. One gram of the adsorbed resin prepared as previously described released approximately one mmole of

benzoylformate at pH 7.5 (Figure 17). When different amounts of the adsorbed resin (0.05-0.4 g) were used in the 1-ml synthesis reaction, they significantly enhanced both the D-phenylglycine synthesis rate and yield as compared to that without using the resin (Figure 18a). When less amounts of the adsorbed resin were used, the initial rate of reaction was faster but D-phenylglycine synthesis ceased rapidly, possibly due to the low amount of benzoylformate left to drive the reaction. The addition of more benzoylformate-adsorbed resin into the reaction at this stage could regain the product formation (Figure 19). When D-phenylglycine was formed at a concentration greater than 5 mg/ml (approximate aqueous solubility of D-phenylglycine at pH 7.5, 37^{O}C), solid product was conspicuously precipitated from the reaction mixture (Figure 20). After 6 h, the reaction with 0.1 g benzoylformate-adsorbed resin yielded the highest D-phenylglycine amount of 10.25 mg/ml (68% yield) while the reaction in the absence of the resin (but 100 mM benzoylformate was fed instead) yielded only 2.73 mg/ml (16% yield). Similar reaction profiles were obtained from the reaction without and with 0.5 g resin, although the initial concentrations of benzoylformate in both reactions were 110 and 158 mM, respectively (Figure 18b).

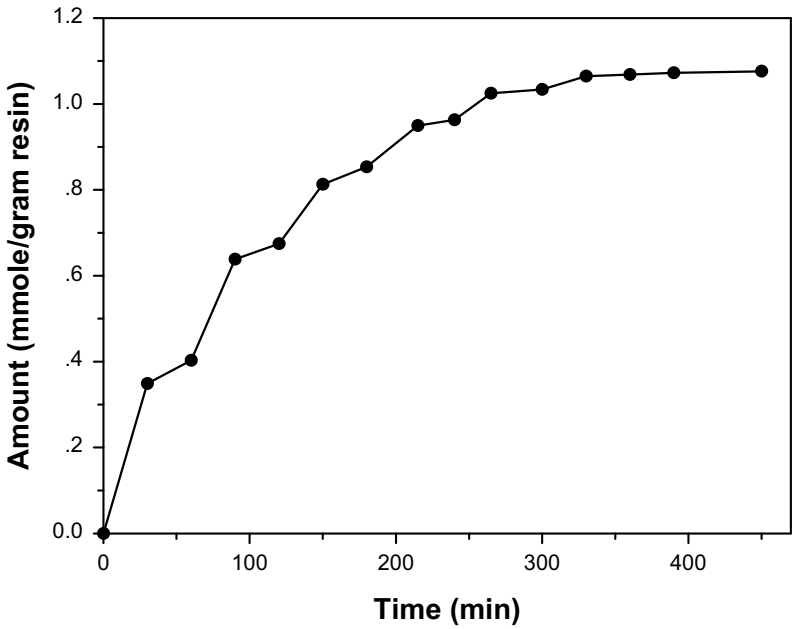


Figure 17. The release of benzoylformate from Amberlite IRA-400. The experiment was done with repeated change of fresh buffer until no further release was observed.

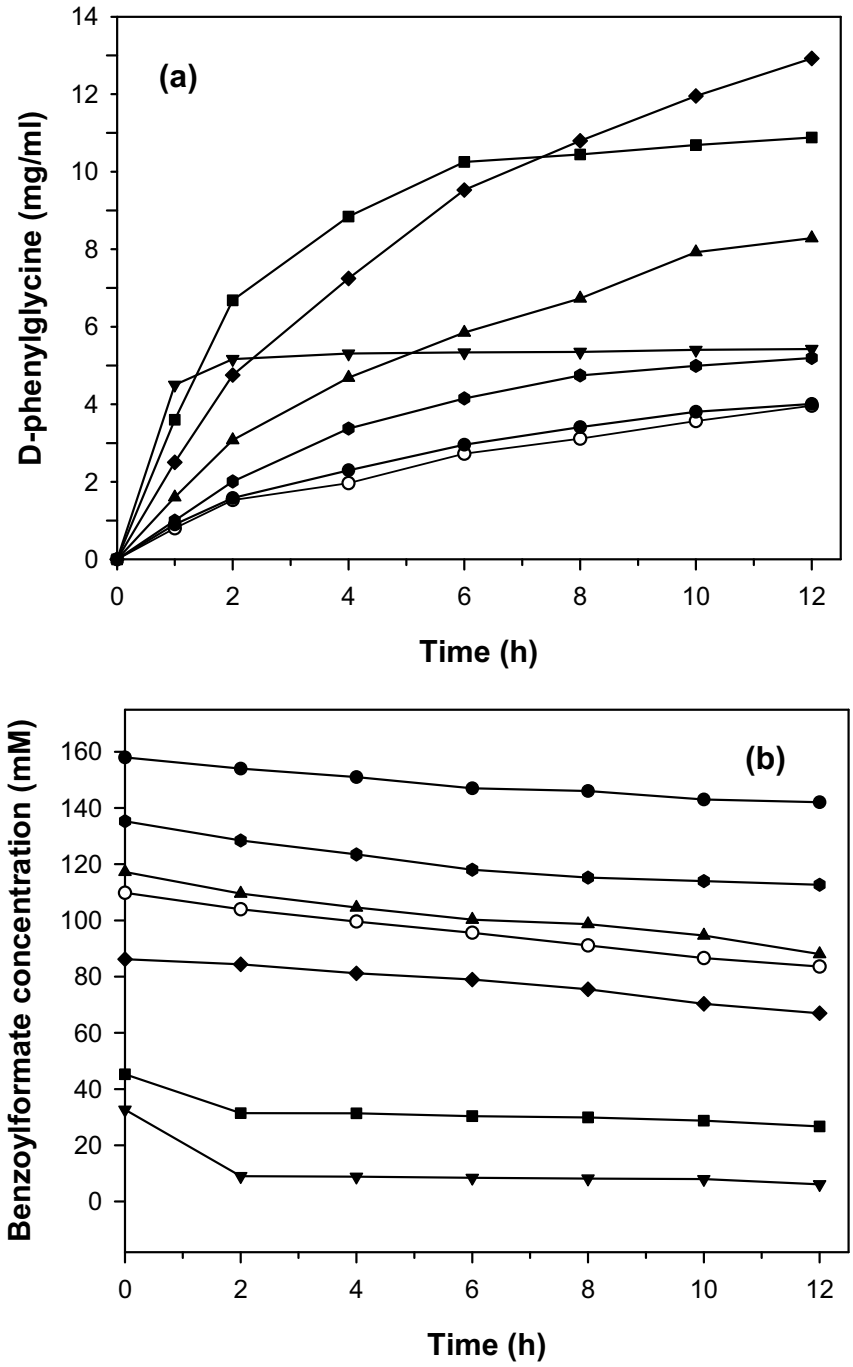


Figure 18. Relationship between the amounts of benzoylformate-adsorbed resin used in the enzymatic reaction and the D-phenylglycine produced. (a) D-phenylglycine synthesis using various amounts of benzoylformate-adsorbed resin and (b) Amount of benzoylformate detected in the supernate. The reaction in one ml contained in addition to varying amounts of benzoylformate-adsorbed resin: (O) 0 g, (▼) 0.05 g, (■) 0.1 g, (◆) 0.2 g, (▲) 0.3 g, (⬢) 0.4 g and (●) 0.5 g.

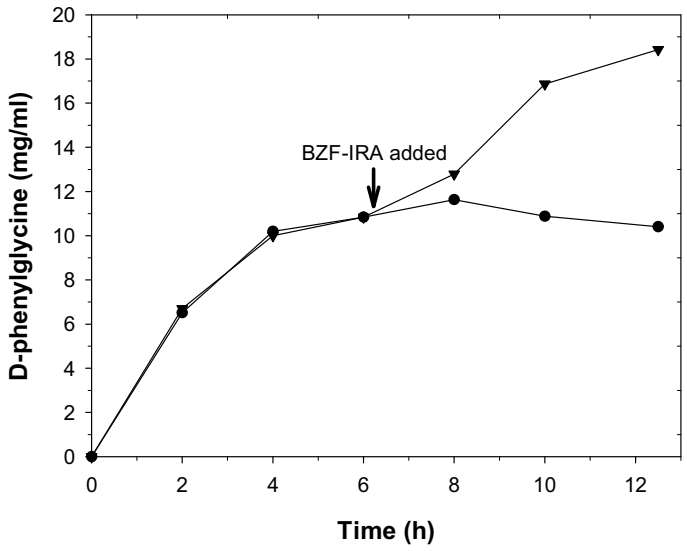


Figure 19. D-Phenylglycine synthesis with an additional supply of benzoylformate-adsorbed resin at 6 h. At 0 h, both 1-ml reactions were started with 100 mM Tris buffer (pH 7.5), 1 M L-glutamate, 0.1 g benzoylformate-adsorbed resin, 25 μM PLP and 100 μl immobilized enzyme. At 6 h, 0.1 g more benzoylformate-adsorbed resin was added to the reaction mixture (▼), and no benzoylformate-adsorbed resin was added to the reaction mixture (●).

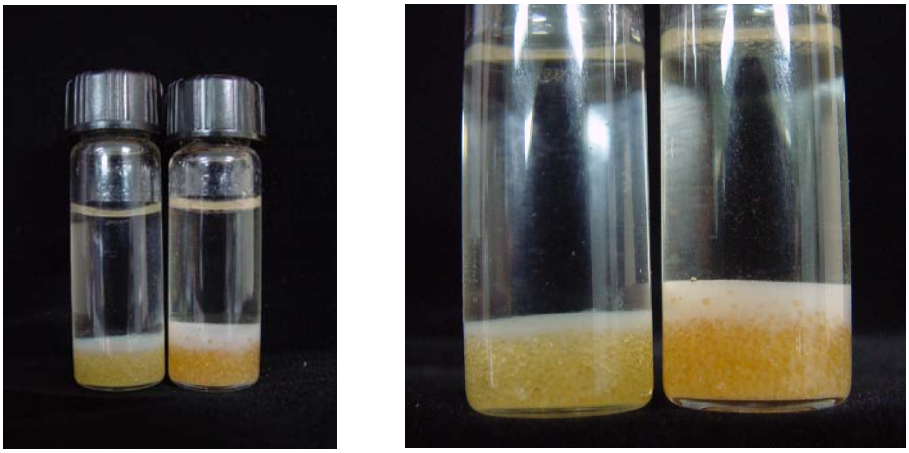


Figure 20. Batch reaction for the synthesis of D-phenylglycine. The left bottle showed the 3 ml mixture containing 100 mM Tris buffer (pH 7.5), 1 M L-glutamate, 0.1 g benzoylformate-adsorbed resin, 25 μM PLP and 300 μl immobilized enzyme at time zero (before the enzymatic reaction began) and the right bottle showed changes in characteristics of the mixture after the reaction was completed with the clearly seen white precipitate of D-phenylglycine and bead of immobilized enzyme on the top layer above the yellow layer of Amberlite IRA-400.

เอกสารอ้างอิง

1. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning, a laboratory manual, 2nd edition. New York: Cold Spring Harbor Laboratory Press;1989.
2. Riddles PW, Blakeley RL, Zerner B. Reassessment of Ellman's reagent. Method in Enzymol. 1983;91:49-60.
3. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248-54.
4. Velick SF, Varva J. A kinetic and equilibrium analysis of the glutamic oxaloacetate transaminase mechanism. J Biol Chem 1962;237:2109-22.
5. Wiyakrutta S, Meevootisom V. A stereo-inverting D-phenylglycine aminotransferase from *Pseudomonas stutzeri* ST-201: purification, characterization and application for D-phenylglycine synthesis. J Biotechnol 1997; 55(3): 193-203.

Part II

High-Expression of Functional Recombinant D-Phenylglycine Aminotransferase in *Escherichia coli*

Objective

To improve the yield of functional D-phenylglycine aminotransferase expressed in *E. coli*.

Materials and Methods

1. Bacterial strains and plasmids

E. coli BL21(DE3) harboring pEPL which carries the *dpgA* gene encoding D-PhgAT (1). Takara's chaperone plasmid set was purchased from Takara Bio Inc.

2 Materials

Restriction enzyme, *Bam*HI was obtained from New England Biolabs. Isopropyl β-D-thiogalactopyranoside (IPTG), the coenzyme, pyridoxal-5′-phosphate (PLP), D-phenylglycine (D-Phg), α-ketogrutarate, L-arabinose, tetracycline, ampicillin and chloramphinicol, were purchased from Sigma. Pyridoxine hydrochloride (vitamin B6) and benzyl alcohol were from Fluka, and Bradford reagent was from Bio-Rad.

3. Transformantion of chaperone plasmid into *E. coli* BL21 (DE3) harboring pEPL

Competent cells of *E. coli* BL21 (DE3) harboring pEPL was prepared by using the Inoue method (2). Transformation of the resulting competent cells with chaperone plasmid, pG-KJE8 was performed as described by Sambrook and Russell (2). The positive transformants were obtained by plating the transformants on selection plates containing 50 $\mu g.mL^{-1}$ ampicillin and 20 $\mu g.mL^{-1}$ chloramphinicol for pEPL and pGKJE8 selection, respectively. Confirmation of the positive clones were done by culturing the selected clones in liquid medium supplemented by the same antibiotics at the same concentrations and the plasmids of the selected clones were extracted and subjected to restriction enzyme analysis. Finally, the retransformed strain harboring both pEPL and pG-KJE8 was obtained and verified by agarose gel electrophoresis.

4. Culture conditions, expression, and co-expression

Single colonies of the retransformed strain was inoculated into 3 mL of Luria-Bertani medium containing the antibiotics for selection of pEPL and the chaperone plasmid, pG-KJE8 as described above. The culture was incubated at 37°C until 0.4 $OD._{600nm}$ was reached, then, the culture was added with L-arabinose and tetracycline to the final concentration of 0.5 $mg.mL^{-1}$ and 5 $ng.mL^{-1}$, respectively, to induce chaperone proteins expression. One h later, IPTG induction of D-PhgAT expression was carried out for 4 h. Cells were harvested by centrifugation and DPhgAT activity in the cellular protein extract was determined.

The study on chemical induction of chaperones by benzyl alcohol was done with *E. coli* BL21(DE3) harboring pEPL that was cultured at 37°C until the cell density of 0.4 $OD._{600nm}$ was reached. Then, benzyl alcohol was added to a final concentration of 10 mM and one h later IPTG at the final concentration of 0.4 mM was added to induce D-PhgAT production at 25°C. Similarly, the study on activity gaining by addition of pyridoxine hydrochloride to the final concentration of 5 mM, then, one hour later IPTG at the final concentration of 0.4 mM was added to induce D-PhgAT production at 25°C. The experiments were performed in triplicates.

5. Soluble proteins analysis

To lyse the cells, the induced cell pellets were re-suspended in a lysis buffer containing 100 mM potassium phosphate buffer pH 7.6, 1 mM EDTA, 0.01% β-mercaptoethanol, and 0.1 mM PLP to a cell density of 7.5 O.D.600nm. The cells were subjected to disruption by ultrasonication (Vibra cell, Sonics & Materials Inc. U.S.A.). Sample tubes were placed in an ice-bath throughout the process and the disruption was carried out in the cycles of 9.9 sec burst with 9.9 sec intermittent cooling. Cell debris was separated by centrifugation at 12,000 rpm (Eppendorf centrifuge 5417R) for 30 min at 4°C. Supernatant was transferred into new microcentrifuge tube and used as a soluble fraction. The soluble fraction was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

6. *D*-Phenylglycine aminotransferase (*D*-PhgAT) activity assay

D-PhgAT activity of the soluble fractions was determined according to the modified method as described by Khampha et al (3). Briefly, the enzyme activity was determined using a spectrophotometric enzyme kinetic assay by measuring the amount of 4-hydroxy

benzolyformic acid (4-OHBZF) formed upon transamination of D-4-OHPhg using 2-oxoglutarate as an amino acceptor. The reaction mixture (980 μl) contained 10 mM D-4-OHPhg, 10 mM α-ketoglutarate, 5 μM PLP, 5 μM EDTA and 50 mM 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid buffer, pH 9.5. A 20 μl volume of appropriately diluted D-PhgAT solution was added to the reaction mixture and quickly and thoroughly mixed to start the reaction. The rate of 4-OHBZF formation is monitored by recording the increase in UV absorption at 340 nm for 180 sec using a spectrophotometer (Unicam Heλios Alpha). One unit of D-PhgAT activity was defined as the amount of enzyme that catalyzes the formation of 1 μmole of 4-OHBZF min^{-1}. Molar absorption coefficient of 4-OHBZF at 340 nm (ε_{340nm}) is 24×10^{3} $M^{-1}cm^{-1}$ (4).

Results

1. Transformantion chaperone plasmid into E. coli BL21 (DE3) harboring pEPL

The transformants carrying both pEPL plasmid which contained dpgA gene encoding the D-PhgAT, and pG-KJE8 plasmid which contained molecular chaperone genes, *dnaK-dnaJ-grpE* and *groEL-groES* were selected on culture plates containing 50 $\mu g.mL^{-1}$ ampicillin and 20 $\mu g.mL^{-1}$ chloramphenicol. They were verified by restriction enzyme digestion with BamHI and the desired clones having both pEPL and pG-KJE8 corresponding bands were selected (data not shown) and used for production of D-PhgAT.

2. Effects of benzyl alcohol, pyridoxine, and DnaK-DnaJ-GrpE and GroEL-GroES co-expression on active D-PhgAT yield

After 4-h induction with IPTG, the D-PhgAT activity in cell free extracts from all the experiment were as shown in Fig. 1. Compared with *E. coli* BL21(DE3) harboring pEPL plasmid alone (experiment 2), benzyl alcohol addition resulted in only 1.3-fold increase of the D-PhgAT activity (experiment 3) while pyridoxine addition showed 2.2-fold increase (experiment 4). When both pyridoxine and benzyl alcohol were added, around 2.7-fold increase in the enzyme activity was observed (experiment 5). The yield of the enzyme obtained with co-expression of DnaK-DnaJGrpE and GroEL-GroES chaperones was dramatically increased to around 3,107-fold (experiment 6). When the chaperones co-expression was done in the presence of added pyridoxine, further improvement to 4,067-fold (experiment 7) increase in the D-PhgAT activity yield was obtained. SDS-PAGE profiles of

soluble cellular proteins of *E. coli* from all the experiments with the protein band corresponded to D-PhgAT are shown in Fig 2. D-PhgAT was found to constitute nearly 50% of total soluble cellular proteins in *E. coli* cells from experiment 7 (lane 7) which yielded the highest D-PhgAT activity.

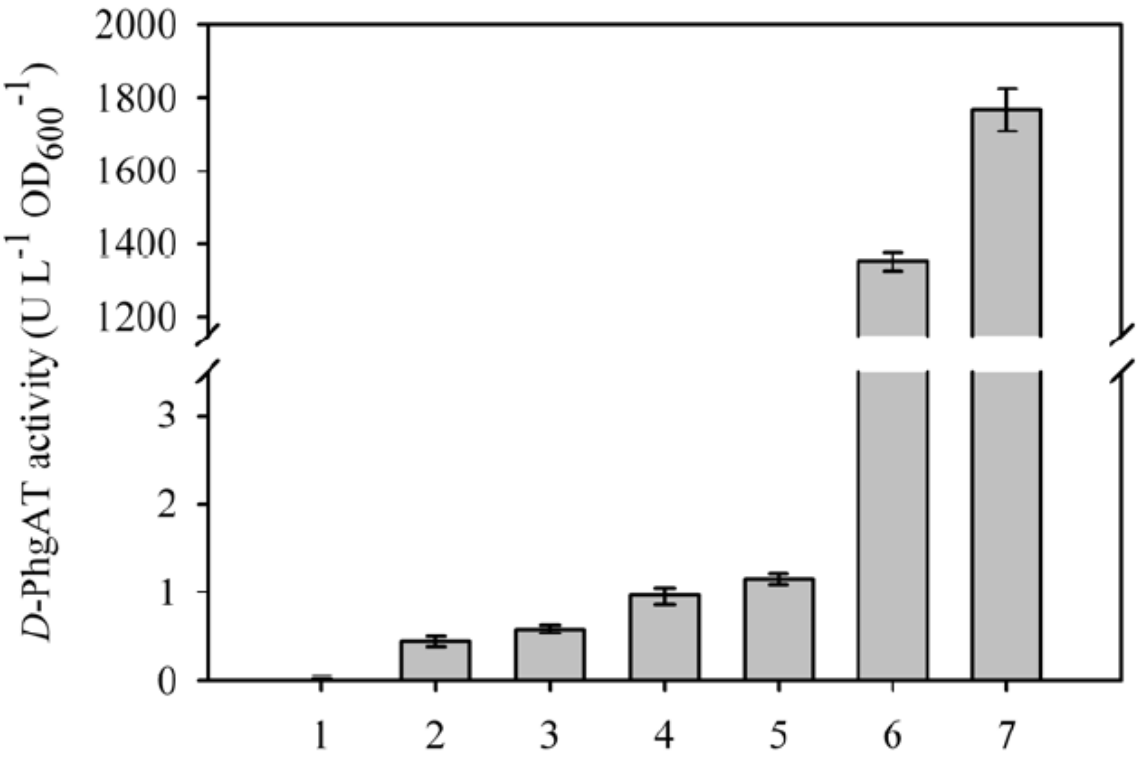


Fig. 1 D-PhgAT activity in cell free extract of *E. coli* BL21 (DE3) harboring pEPL (1-5) or harboring both pEPL and pG-KJE8 (6-7). (1) no induction, (2) induced by IPTG, (3) induced by IPTG in the presence of benzyl alcohol, (4) induced by IPTG in the presence of pyridoxine hydrochloride, (5) induced by IPTG in the presence of both pyridoxine hydrochloride and benzyl alcohol, (6) induced by IPTG, and Larabinose and tetracycline (for chaperones), and (7) induced by IPTG, and Larabinose and tetracycline (for chaperones) in the presence of pyridoxine hydrochloride.

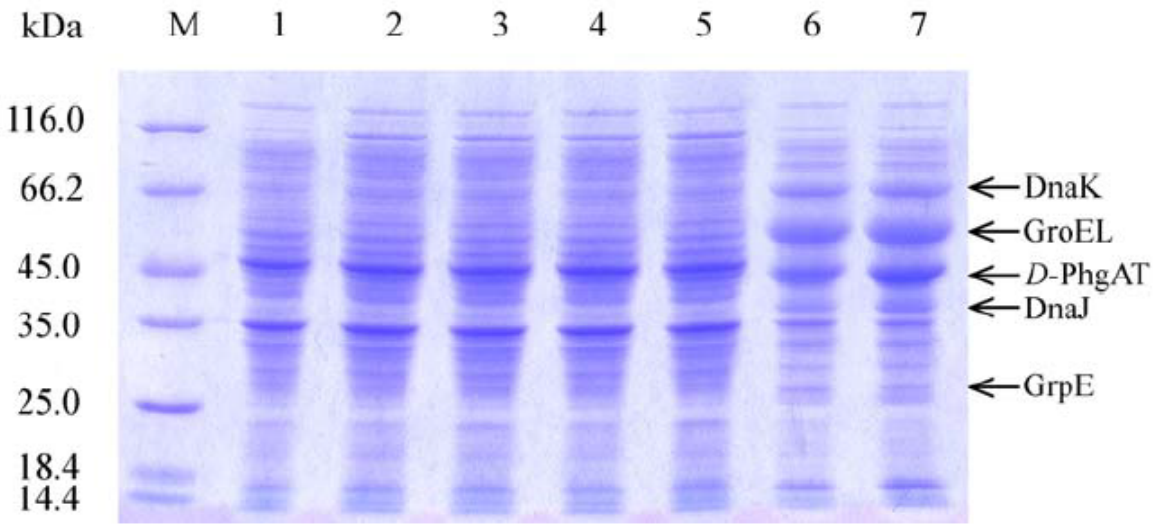


Fig. 2 SDS-PAGE analysis of cell free extract of *E. coli* BL21 (DE3) harboring pEPL (1-5) or harboring both pEPL and pG-KJE8 (6-7). Lane numbers correspond with the experiment number in Fig. 1. Proteins in lane M are standard molecular weight markers.

Discussion and Conclusion

D-PhgAT is known to form inclusion body in the over-expression host. This resulted in low yield of the soluble active enzyme despite the high amount of the protein was produced. Treating the cells with benzyl alcohol known to cause changes in fluidity of the cell membrane which in turn induced expression of endogenous chaperones showed no positive effect. This might be because the chaperones induced were not suitable or not sufficient for assisting D-PhgAT folding. The effect of benzyl alcohol was reported to be more pronounced when used at higher temperature (30 ºC) (5) so at the temperature of 25ºC employed in this study this compound may not be sufficiently effective. The high yield of active D-PhgAT was obtained with the co-expression of chaperones, DnaK-DnaJ-GrpE and GroES-GroEL, alone or in combination with the addition of pyridoxine hydrochloride indicating that the chaperones set was very helpful. The data might also indicate the importance of GroES-GroEL over the DnaK-DnaJ-GrpE in the folding of D-PhgAT enzyme since the latter set of chaperones did not help in the experiment with benzyl alcohol induction (5). It should be pointed out here that the amount of chaperone present intracellularly may be important while in this study no quantitation of these intracellular chaperones were determined. Optimization of the level of chaperones co-expression may

further increase the over-expressed D-PhgAT yield. The co-expression of molecular chaperones in the presence of pyridoxine at 5 mM concentration yielded higher amount of functionally active D-PhgAT in comparison with the co-expression alone indicating that the cofactor plays an important role in the folding. Since there are more chaperones of *E. coli* such as trigger factor (TF), ClpA/ClpB, and IbpA/IbpB (6) which may be helpful in the folding of D-PhgAT, therefore, they should be tested either individual or in combination. Other chaperones are interesting to be co-expressed with our target proteins because each chaperone assists protein folding in different stages (7). As such experiment, appropriate chaperones will be used to improve a target protein production with the highest yield. In addition, the GroESGroEL as suggested above should be investigated to see whether they are alone that give the benefit to D-PhgAT folding. Furthermore, the concentration of the coenzyme precursor, pyridoxine, may be optimized to give even a higher yield of the active DPhgAT.

References

1. Laowanapiban, P. (2001). D-Phenylglycine aminotransferase from *Pseudomonas stutzeri* ST-201: gene cloning, sequence analysis, comparative structural modeling and over-expression in Escherichia coli. Ph.D. Thesis, Faculty of Science, Department of Microbiology, Mahidol University.
2. Sambrook, J., & Russell, D. W. (2001). Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
3. Khampha, W., Wiyakrutta, S., & Meevootisom, V. (2004). Spectrophotometric enzymatic cycling method using glutamate dehydrogenase and D-phenylglycine aminotransferase for determination of L-glutamate in foods. Anal Chim Acta, 520(1-2), 133-139.
4. Jomrit, J. (2010). Stereo-inverting property of D-Phenylglycine aminotransferase. M.Sc. Thesis, Faculty of Science, Department of Biotechnology, Mahidol University.
5. Shigapova, N., Torok, Z., Balogh, G., Goloubinoff, P., Vigh, L., & Horvath, I. (2005). Membrane fluidization triggers membrane remodeling which affects the thermotolerance in *Escherichia coli*. Biochem Biophys Res Commun, 328(4), 1216-1223.
6. Agashe, V. R., & Hartl, F. U. (2000). Roles of molecular chaperones in cytoplasmic protein folding. Semin Cell Dev Biol, 11(1), 15-25.
7. Hartl, F. U., & Hayer-Hartl, M. (2002). Molecular chaperones in the cytosol: from nascent chain to folded protein. Science, 295(5561), 1852-1858.

Part III

High-Expression of Functional Recombinant D-Phenylglycine Aminotransferase in *Pichia pastoris*

Objective

To construct *Pichia pastoris* expression system that produce functional D-phenylglycine aminotransferase in higher yield than the *E. coli* system

Materials and Methods

1. Strains, plasmids, oligonucleotides, media, chemicals

The *E. coli* DH5α and *P. pastoris* KM71 were obtained from Novagen (Madison, WI, USA) and Invitrogen (San Diego, CA, USA), respectively. The *Pichia* expression plasmids pPIC3.5K, pAO815, pGAPZ B and pPICZ B were purchased from Invitrogen. A wild-type *dpgA* gene was codon-optimized for matching to that of the *Pichia* expression system, and then synthesized by GenScript (Piscataway, NJ, USA). Primers for gene amplifying and real-time PCR were shown in Table 2. Restriction endonucleases, DNA polymerase and T4 DNA ligase were supplied from New England Biolabs (Ipswich, MA, USA). Culture media for bacteria and yeast cells (e.g. LB, YPD, BMGY, BMMY and MD) were from BD (Franklin Lakes, NJ, USA). The antibiotic Ampicillin and Zeocin were from Bio Basic (Ontario, Canada) and Invitrogen, respectively. Methanol and all other chemicals were obtained from Sigma (St. Louis, MO, USA).

2. Generation of *Pichia pastoris* strain expressing intracellularly recombinant D-PhgAT

The synthetic gene encoded for D-PhgAT was amplified by PCR using *Pfu* DNA polymerase and pUC57_D-PhgAT served as a DNA template. Forward and reverse primers contained *Bam*HI and *Eco*RI sites, respectively for cloning into pPIC3.5K and subsequently transformed into *E. coli* DH5α. The resulting plasmid pPIC3.5K_D-PhgAT was linearized with *Sal*I and integrated into *P. pastoris* KM71 by electroporation at 1.5 kV, 25 μF and 200 Ω. The transformants were selected on Minimal Dextrose (MD) without histidine plates (1.34 % YNB with ammonium sulphate without amino acids, 4 × 10-5 % biotin, and 2 % dextrose). Then, the pooled transformants was spread on YPD containing 0.25 mg mL^{-1} Geneticin to

screen for the strain which contained the multiple copies of D-PhgAT gene. The verification of D-PhgAT gene insertion in *Pichia* chromosome was done by PCR using gene specific primer. Eight verified recombinant clones were tested for expression level of D-phgAT and the highest expressing clone was applied in this study. The DNA sequencing of expression cassette was done to confirm the correctness.

3. *In vitro* mutimerization of D-PhgAT, GroEL and GroES genes in single plasmid and generation the *Pichia pastoris* strains co-producing D-PhgAT, GroEL and GroES

The D-PhgAT gene was amplified and cloned into pAO815 at *Eco*RI site to construct pAO_D-PhgAT. The two genes encoded for GroEL and GroES were individually amplified using pGro7 as a DNA template and the forward and reverse primers for both genes contained *Eco*RI and *Xba*I sites, respectively for cloning into pGAPZ B. The plasmid pGAPZ B_GroES was cut with *Bgl*II and *Bam*HI and the resulted 1.2 kb of GroES expression cassette was ligated into the *Bam*HI-digested pGAPZ B_GroEL to generate pGAPZ B_GroEL_GroES. Then, the combined expression cassette of GroEL and GroES was cloned into the *Bam*HI-digested pAO815_D-PhgAT by the same way as previous to create the pAO_D-PhgAT_GAP_ELS. The construction of pAO_D-PhgAT_AOX_ELS was additionally done by the similar procedure except that the GroEL and GroES genes were cloned into pAO815 instead of pGAPZ B to form the expression cassettes under the control of AOX1 promoter. Two recombinant plasmids pAO_D-PhgAT_GAP_ELS and pAO_D-PhgAT_AOX_ELS were linearized with *Stu*I before transformation into *P. pastoris* KM71. The transformants were selected on Minimal Dextrose (MD) without histidine plates. PCR analysis using gene-specific primers to detect the presence of s*dgpA*, *groEL* and *groES* genes in *P. pastoris* genome was made.

4. *In vivo* generation of *P. pastoris* KM71 containing multiple copies of *dpgA*, *groEL* and *groES* genes

The GroEL and GroES expression cassettes were amplified by PCR method using pGAPZ B_GroEL_GroES as a DNA template and forward and reverse primers contained *Xho*I and *Not*I, respectively for cloning into pPICZ_26s rDNA. The resulting pPICZ_26s rDNA_GAP_GroELS was linearized with either *Sac*I or *Sfo*I to integrate consecutively at 5'AOX1 or 26s rDNA locus in the chromosome of recombinant *Pichia* KM71 contained pPIC3.5K_D-PhgAT. The transformants were primarily selected on YPD plates containing 25 µg mL-1 of Zeocin. Subsequently, the resistant clones were re-streaked on YPD plates with

higher concentration of Zeocin (100, 500, 1000, 2000 and 5000 μg mL^{-1}) to select for clones having that multiple copies of *groEL* and *groES* genes. PCR analysis using gene-specific primers was made to confirm the gene integration.

5. Real-time PCR

Quantitative real time-PCR was performed in duplicates using SYBR Green in 20 μL reactions. The reaction mixture was composed of 2 ng of genomic DNA and 0.5 μM of each primer. The thermal cycling conditions were 15 min at 95°C followed by 40 cycles of 30 s at 95°C, 30 s at 60°C and 45 s at 72°C. After the amplification, a melting curve analysis with a temperature gradient of 0.1°C s^{-1} from 65°C to 95°C was examined to exclude the amplification of unspecific products. For correct determination of the starting copy quantity, the reference gene actin was also quantified in parallel. The normalized copy number was calculated by relative quantification as described by Livak and Schmittgen (1) with following formula; $n = 2^{-\Delta\Delta Ct}$ where $\Delta\Delta$Ct = (Ct target sample – Ct reference sample) – (Ct target calibrator – Ct reference calibrator). Ct is defined as the point at which the fluorescence level rises above the baseline. The *P. pastoris* KM71 containing pAO_D-PhgAT_GAP_ELS which contained single copy of each gene was used as the calibrator.

6. Expression of D-PhgAT and GroEL-ES in *P. pastoris* KM71

The single colony of each strain was grown in 100 mL of BMGY (1 % yeast extract, 2 % peptone, 100 mM potassium phosphate pH 6.0, 1.34 % YNB, 4 × 10-5 % biotin and 1 % glycerol) medium at 30°C 200 rpm until culture reached an OD_{600nm} of 6. Cells were harvested by centrifugation at 2000 × g for 5 min at room temperature and re-suspended with 20 mL of BMMY (0.5 % methanol) medium. The incubation was continued to induce the expression of the recombinant D-PhgAT and GroEL-ES. At every 24 h, the expression culture was harvested and methanol was added to final concentration of 0.5 % in the remaining culture.

7. Preparation of cell-free extracts

Protein extraction was accomplished by using a French Press. Cell pellets were washed once and re-suspended with chilled lysis buffer (50 mM sodium phosphate pH 7.4, 1 mM PMSF, 1 mM EDTA and 5 % glycerol) with ratio of cell wet weight to buffer volume of 1:4. The samples were applied at 16,000 psi cell pressure for 4 passes. The cell lysates

were centrifuged at 12,000 × g for 10 min at 4°C to collect the supernatants for determining the D-PhgAT activity.

8. Determination of D-Phenylglycine aminotransferase activity

The assay was performed in a 1 mL reaction by mixing 20 μL of soluble enzyme fraction to a 980 μL of reaction mixture containing 100 mM CAPSO pH 9.0, 10 mM D-4-hydroxy phenylglycine, 10 mM α-ketoglutaric acid, 25 μM EDTA and 25 μM PLP. The rate of benzoylformate formation was measured as a function of time by monitoring the increase in absorbance (340 nm) using a spectrophotometer (Biospec-1601, Shimadzu Corp., Japan).

9. SDS-Polyacrylamide Gel Electrophoresis

The sample was quantified the protein concentration by NanoDrop and, then mixed with the sample buffer before boiling for 10 min. The 30 μg protein was loaded into gel which was made of 12 % separation gel with a 4 % stacking gel using Mini-Protein II Dual Slab Cell (BIO-RAD). Besides, total cell protein was analyzed by preparing the sample as follows (2). A 3 mg wet weight of cell pellet was washed once and re-suspended in 0.3 mL of distilled water, and then an equal volume of 0.2 M of NaOH is added. Next, cells were collected after 5 min of incubation at room temperature, and the supernatant is carefully removed. A 70 μL of SDS-sample buffer was added to the pellet, and the cells are re-suspended by repeated pipetting. The samples are boiled for 3 min and centrifuged briefly. A 15 μL of the extract was loaded. The gel electrophoresis was run at 150 V for 1 h and the gel was stained with Coomassie Brilliant Blue.

Results

1. Intracellular expression of recombinant D-PhgAT in *P. pastoris* KM71

The inducing condition with 0.5 % methanol was carried out at 30°C for 144 h to examine the yield and optimal time for harvesting the D-PhgAT. As we estimated the intensity of protein bands, the recombinant D-PhgAT was consistently expressed up to 30 % of total cellular proteins at all induction time (Figure 1). However, majority of the expressed protein aggregated as inclusion bodies (Figure 2). Soluble protein fraction was found to contain D-PhgAT enzymatic activity at 0.00152 U mg^{-1}. Several strategies lead to the native folding of over-expressed including lower temperature, increase the cellular concentrations of

osmolytes and molecular chaperones (3) were attempted in this study. By using 0.5 M sorbitol, 0.4 to 4 mM benzyl alcohol and even though 20°C, the solubility of D-PhgAT was not significantly enhanced and the inclusion bodies did not obviously alleviated as judged from the intensity of protein bands (Figure 3).

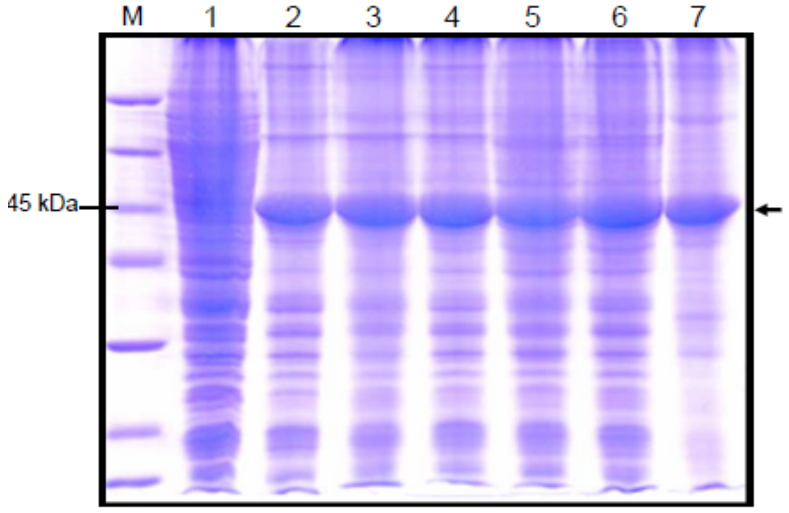


Figure 1. Expression of D-PhgAT in *P. pastoris* KM71 at 30°C. SDS-PAGE analysis of total cellular proteins of recombinant *P. pastoris*. Lane M, molecular mass marker; Lane 1, 24 h of induction of *P. pastoris* KM71 harboring the parent vector pPIC3.5K; Lane 2-7, Induction of *P. pastoris* KM71 harboring pPIC3.5K_D-PhgAT for 24, 48, 72, 96, 120 and 144 h, respectively. The molecular mass of D-PhgAT (45 kDa) is indicated.

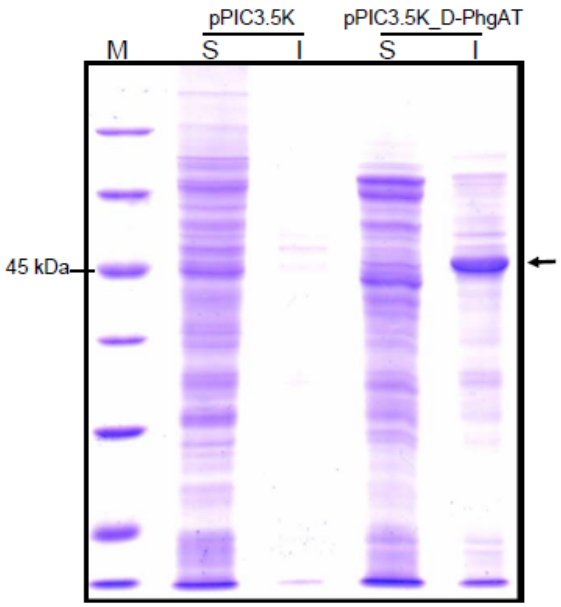


Figure 2. Expression of recombinant *P. pastoris* KM71 at 30°C for 24 h. SDS-PAGE analysis of soluble (S) and insoluble (I) fractions of cells containing the pPIC3.5K and pPIC3.5K_D-phgAT. Lane M, molecular mass marker. The recombinant D-PhgAT is indicated with arrow.

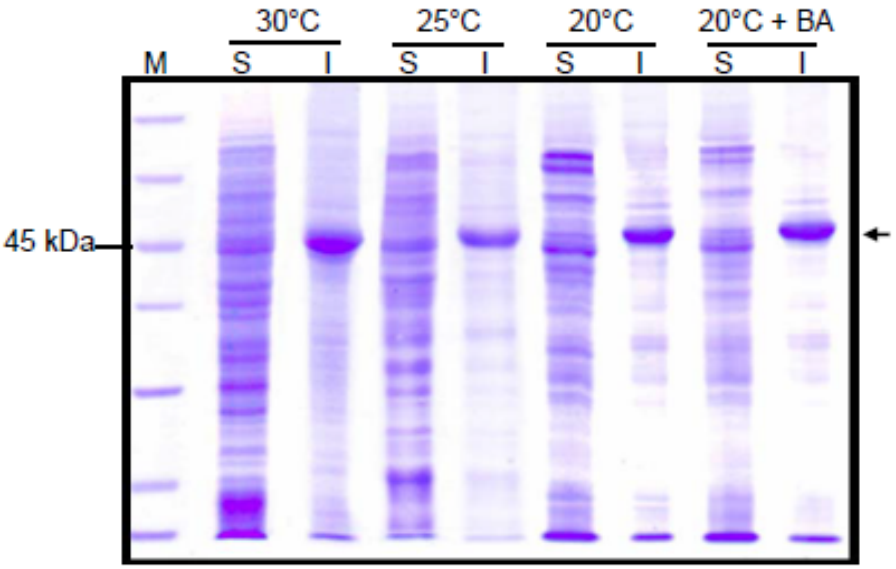


Figure 3. Effect of temperature and benzyl alcohol (BA) on soluble D-PhgAT production. SDS-PAGE analysis of soluble (S) and insoluble (I) fractions from *P. pastoris* KM71 containing pPIC3.5K_D-PhgAT were induced at 30°C, 25°C, 20°C and the combination of 20°C with 0.4 mM benzyl alcohol. Lane M, molecular mass marker. The arrow indicates the band of D-PhgAT.

2. Co-expression of GroEL-GroES chaperones with D-PhgAT in *P. pastoris* KM71

As inducing the molecular chaperones in *P. pastoris* KM71 by addition of benzyl alcohol did not assist the native folding of D-PhgAT, the co-expression of bacterial chaperonin GroEL-GroES was investigated. Since we previously achieved the noticeably high yield of soluble D-PhgAT by co-producing with GroEL-GroES in *E. coli* system, therefore, the in vitro multimerization of single copy gene encoded for D-PhgAT, GroEL and GroES was constructed in pAO815 (Figure 4). The D-PhgAT was produced under the control of AOX1 promoter whereas GroEL-GroES was that of both GAP and AOX1 promoter to examine the effect of former or co- producing of recombinant chaperonin on the quantity of soluble D-PhgAT. The expression of recombinant proteins was carried out at 30ºC. Strikingly, the functionally active enzyme was found which the highest specific activity was reached at 24 h of induction (Figure 5). Moreover, the result was correlated with the absence of D-PhgAT inclusion body when compared with the strain without chaperonin co-expression (Figure 6). However, there was no significantly increase in the amount of soluble active D-

PhgAT even though the GroEL-GroES level was elevated and no detectable inclusion body in the strain harboured pAO_D-PhgAT_AOX_ELS as determined by SDS-PAGE.

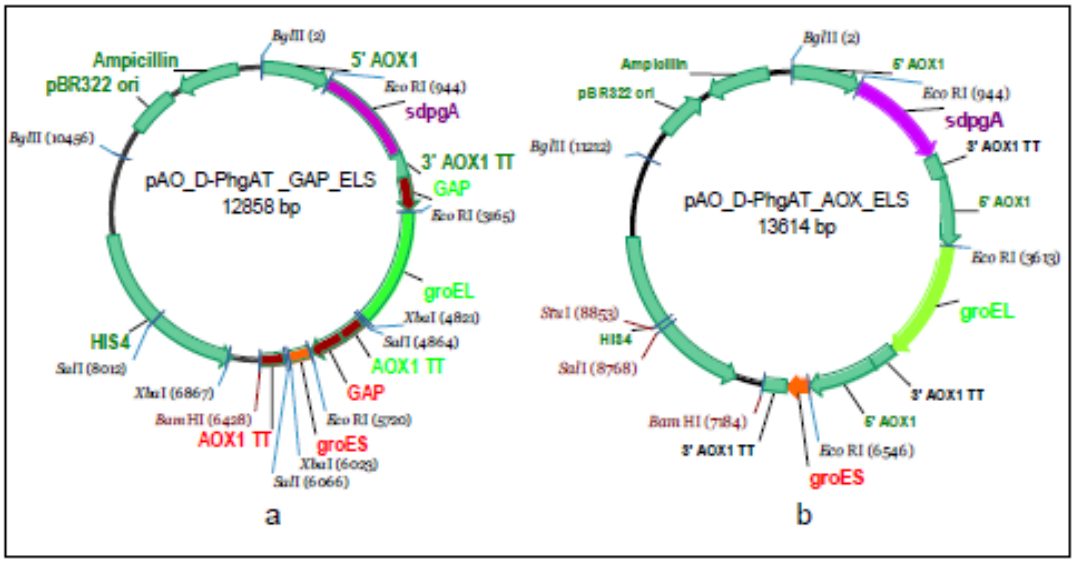


Figure 4. The maps of pAO_D-PhgAT_GAP_ELS and pAO_D-PhgAT_AOX_ELS. The D-PhgAT was under the control of AOX1 promoter. Both GroEL and GroES were under the control of the same promoter which is either GAP (a) or AOX1 (b).

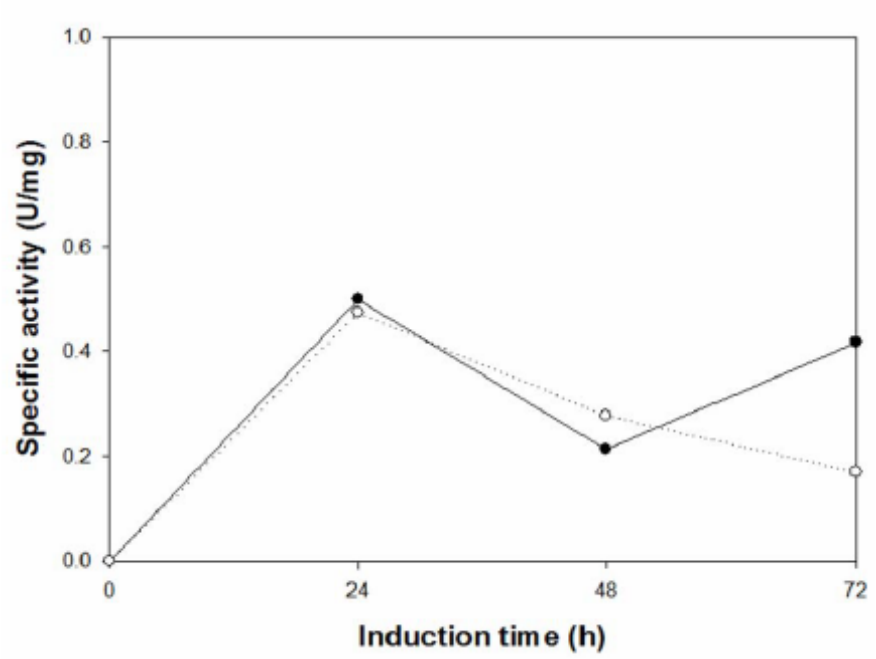


Figure 5. Specific activity of D-PhgAT from recombinant *P. pastoris* KM71 co-expressing the GroEL-GroES at 30°C. The recombinant strains containing pAO_D-PhgAT_AOX_ELS (white circle) and pAO_D-PhgAT_GAP_ELS (black circle) were induced for 3 days with 0.5% methanol.

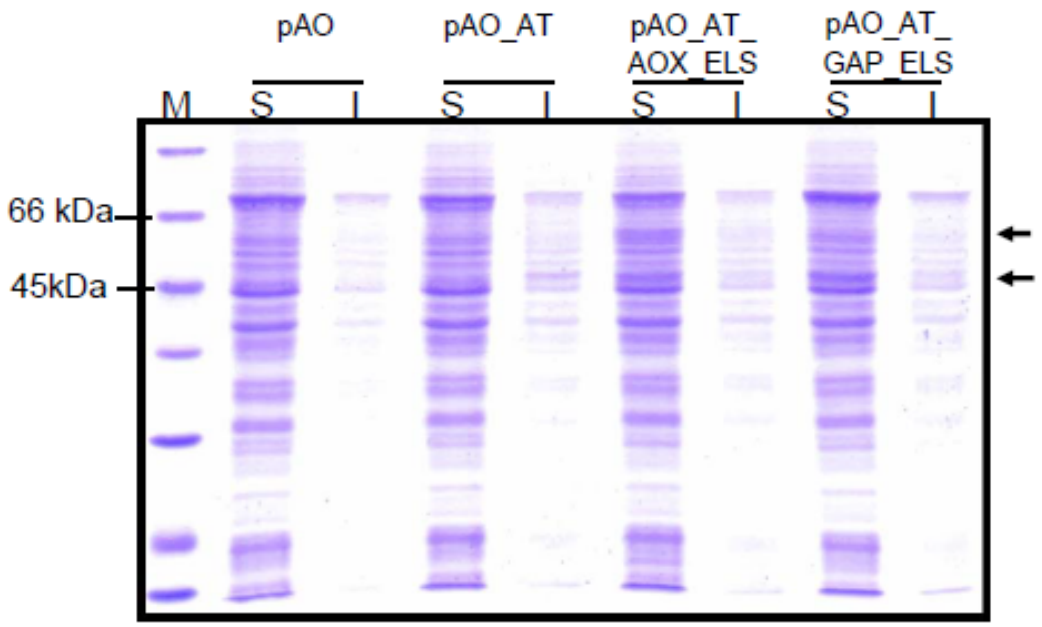


Figure 6. Co-expression of GroEL-GroES with D-PhgAT in *P. pastoris* KM71 at 30°C for 24 h. SDS-PAGE analysis of soluble (S) and insoluble (I) fractions from 4 recombinant *Pichia* strains harbored parent vector pAO815, pAO_D-PhgAT, pAO_D-PhgAT_AOX_ELS and pAO_D-PhgAT_GAP_ELS, consecutively. Lane M, molecular mass marker. The protein bands corresponding to D-PhgAT (45 kDa) and GroEL (60 kDa) are arrow indicated.

3. Expression of multiple copies of *dpgA*, *groEL* and *groES* genes in *P. pastoris* KM71

Although we obtained the functionally active D-PhgAT from the in vitro multimerization of single copy gene *dpgA*, *groEL* and *groES*, the high amount of enzyme was not achieved even if the GroEL-GroES level was increased under the control of AOX1 promoter. Therefore, the expression of multiple copy genes was investigated. For the multi-copy of *dpgA* gene, the recombinant strain which chromosomally contained pPIC3.5K_D-PhgAT was used to co-express with the GroEL-GroES. The insertion of pPICZ_26s rDNA_GAP_GroELS at 5'AOX1 was made to generate the multiple copies of groEL and groES genes in this strain. Furthermore, the integration at 26s rDNA site was also done since it is the repetitive and ubiquitous region which possibly provide the numerous copies of insert. After screening the transformants on YPD plates containing Zeocin, there were four clones showed the resistance up to 5000 μg mL^{-1} of Zeocin and subsequently selected for the expression of soluble D-PhgAT. The induction was performed at 30ºC in methanol medium for 24 h. Remarkably, four multi-copy strains showed the specific activity of D-PhgAT ranging from 2 to 12 times higher than that of the former strain which carrying the

single copy gene (Table 1). Moreover, an elevated amount of soluble active D-PhgAT was obtained whereas no inclusion body appeared in the insoluble fractions from any multi-copy strains as judged by the intensity of protein bands (Figure 7). The copy number of *dpgA* and *groEL* genes was quantified by real time PCR method. We assumed that the copy number of groES was equal to that of groEL gene because both genes were constructed in the same plasmid pPICZ_26s rDNA_GAP_GroELS (Figure 8). The maximum copy of groEL and groES genes was generated when the integration occurred at 26s rDNA locus and resulted to the highest yield of active D-PhgAT (Table 1).

Table 1. Specific activity of D-PhgAT in recombinant *P. pastoris* clones with different copy number of genes

Host strain	**Integrated plasmid**	**Integration site**	**Specific activity of D-PhgAT (U/mg)**	**Copy number**	
				dphgAT	***groELS***
KM71	pPIC3.5K_ D-phgAT	*HIS4*	0.0004	3	0
KM71	pAO815_ D-phgAT_ GAP _ELS	*HIS4*	0.779	1	1
KM71_D-PhgAT	pPICZ_26s_ GAP_ELS	5′*AOX1*	7.697	4	2
		5′*AOX1*	6.130	3	2
		26s rDNA	1.596	1	3
		26s rDNA	9.288	2	10

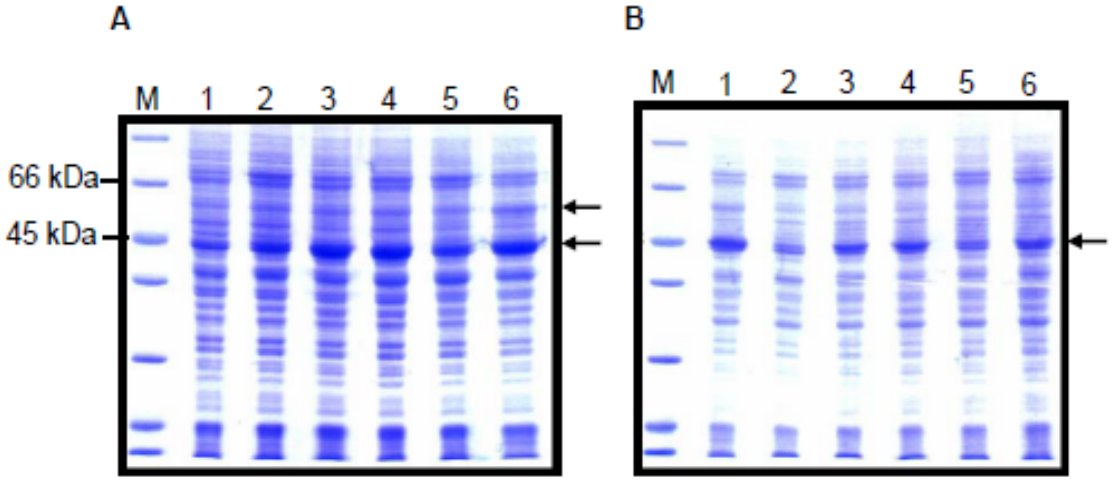


Figure 7. Expression of recombinant *P. pastoris* KM71 harbored multi-copy genes at 30°C for 24 h. SDS-PAGE analysis of soluble (A) and insoluble (B) fractions from 6 recombinant strains. Lane M, molecular mass marker; Lane 1, strain harbored pPIC3.5K_D-PhgAT; Lane 2; strain harbored pAO_D-PhgAT_GAP_ELS; Lane 3-6; 4 recombinant strains harbored pPIC3.5K_D-PhgAT which re-transformed with pPICZ_26s rDNA_GAP_GroELS. The protein bands corresponding to D-PhgAT (45 kDa) and GroEL (60 kDa) are arrow indicated.

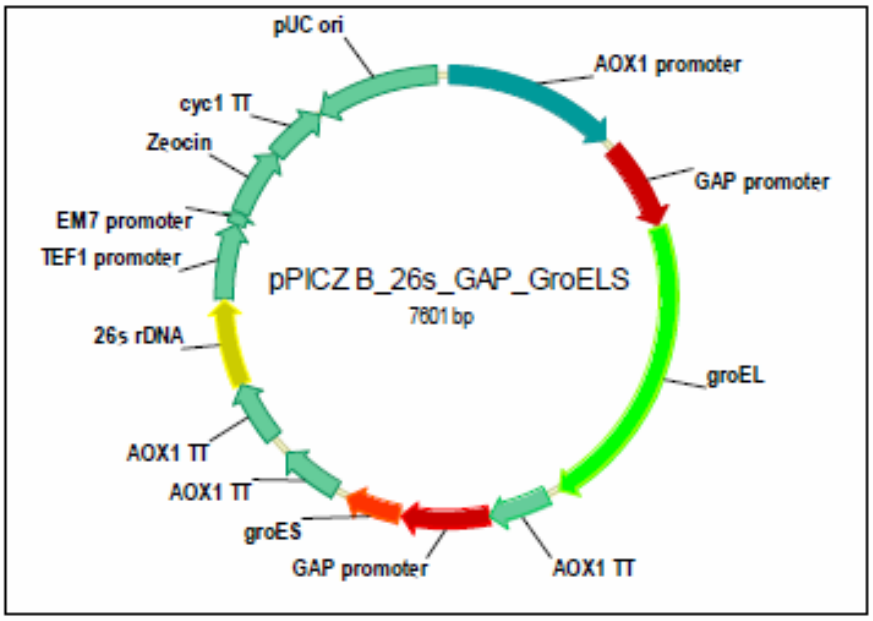


Figure 9. The map of pPICZ_26s rDNA_GAP_GroELS. Two expression cassettes of groEL and groES were inserted behind 5' AOX1 promoter whereas the 26s rDNA fragment was that of AOX1 terminator.

References

1. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta_{CT}}$ method. Methods 2001, 25:402-408.
2. Matsuo Y, Asakawa K, Toda T, Katayama S: A rapid method for protein extraction from fission yeast. Biosci Biotechnol Biochem 2006, 70:1992-1994.
3. de Marco A, Vigh L, Diamant S, Goloubinoff P: Native folding of aggregation-prone recombinant proteins in Escherichia coli by osmolytes, plasmid- or benzyl alcohol-overexpressed molecular chaperones. Cell Stress Chaperones 2005, 10:329-339.

Part IV

Improving *in vitro* solubility of D-phenylglycine aminotransferase by structure-guided mutagenesis

Objective

To genetically engineer the molecular structure of D-phenylglycine aminotransferase to increase its *in vitro* solubility

Materials and Methods

1. Bioinformatics analyses

The target residues for molecular modification by site-directed mutagenesis were chosen by analysis of the crystal structure for the D-PhgAT (PDB 2CY8) from RCSB Protein Data Bank. BetaTPred2 was applied to predict β-turn in the protein structure. GETAREA and ArealMol were applied to predict solvent accessible surface area or percentage of solvent expose. Analysis of crystal contacts was performed by CryCo server. The residues that act as the cofactor-binding, the inter-domain or the inter-subunit residues were excluded from the candidates for prevent the damage to catalytic performance and conformational structure of the enzyme.

2. Preparation of mutant plasmids

The wild-type D-PhgAT has been previously genetically modified by inserting hexa-histidine (H6) and a cysteine (C) residue at the C-terminal using PCR-based mutagenesis. Primers for site-directed mutagenesis were ordered from 1st BASE, Singapore. Site-directed mutagenesis was performed using QuikChange® Lightning Site-Directed Mutagenesis Kit (Stratagene, California, USA). Mini-preps of the mutant plasmids were performed using the QIAprep® Spin Miniprep Kit (Qiagen, Hilden, Germany). Sequencing of the mutant plasmids was done at BioDesign, Thailand. The genes encoding D-PhgAT mutants were placed under a tightly controlled expression system of the pET-11a vector (Novagen®, Darmstadt, Germany) and a high stringency expression host, *E. coli* Tuner (DE3) pLysS (Novagen®, Darmstadt, Germany).

3. Overexpression and purification of D-PhgAT

The wild-type D-PhgAT was over-expressed as previously described and purified by ammonium sulfate precipitation at 25-45% saturation, followed by chromatography on a Phenyl Sepharose 6 Fast Flow (high sub) column (Amersham Pharmacia Biotech, Uppsala, Sweden) and a DEAE SepharoseTM column (GE Healthcare, Uppsala, Sweden). For the expression of the mutant enzymes, *E. coli* Tuner (DE3) pLysS cells harboring the mutant plasmids were grown with shaking in LB broth supplemented with 50 μg/mL of ampicillin and 34 μg/mL of chloramphenicol at 37 °C. A final concentration of 0.4 mM IPTG was added to the cultures at an A_{600nm} of 0.6 to induce the expression of proteins. The cultures were further incubated with shaking at 20 °C for 16 h. The mutant enzymes with hexa-histidine and a cysteine tag (H_6C) at C-terminal were purified from the cell extracts by a Talon® metal affinity column charged with Co^{2+} (Clontech, California, USA). The mutant enzymes without the H_6C tag at C-terminal were purified by the same method of the wild-type enzyme as described above. The combined active fractions were desalted and concentrated using Amicon® Ultra-15 centrifugal filter devices (Millipore, County Cork, Ireland). Enzyme purity was determined by SDS-PAGE. Purified enzymes were kept in 20 mM Tris-HCl buffer (pH 7.6) containing 2.5 μM PLP, 1 mM EDTA, and 0.01% β-mercaptoethanol (TEMP buffer) at 4°C in Protein LoBind tube (Eppendorf, Hamburg, Germany) until use.

4. In vitro protein solubility measurement

All measurements were performed at room temperature (25°C). The in vitro protein solubility was determined by concentration method. The in vitro solubility assay was measured at the neutral pH for enzyme storage (20 mM TEMP buffer, pH 7.6) and the optimal pH for enzyme activity application (50 mM CAPSO buffer pH 9.5). The enzyme solution was concentration in Microcon® YM-30 centrifugal filter devices (Millipore, Massachusetts, USA) at 4,000 g until the retained volume remained constant or a phase change was observed (precipitations or other solid forms). The enzyme solution was agitated regularly during concentration to prevent membrane blockage. The soluble phase was collected and centrifuged to remove any solid forms at 16,000 ***g*** for 15 min. Enzyme quantitation in the soluble phase was determined by NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Delaware, USA) with the absorption at 280 nm and the molar absorption coefficient (ε) of 38,055 $M^{-1}cm^{-1}$. The measurements were done in triplicates.

5. Enzyme activity assay

A spectrophotometric assay was used to determined enzyme activity in the direction of p-OHBZF synthesis. The assay was carried out in 1 ml reaction mixture containing 20 µl of soluble enzyme, 10 mM D-4-hydroxyphenylglycine, 10 mM α-ketoglutarate, 50 mM CAPSO buffer (pH 9.5), 5 µM PLP and 5 µM EDTA. The rate of p-OHBZF formation was measured as a function of time by monitoring the increase in absorbance (340 nm) using Heλios α spectrophotometer (Spectronic Unicam, Cambridge, UK). Activity measurements were made over a 1.5 min time period to ensure a linear product-time profile. The measurements were done in triplicates.

6. Determination of pH effect on enzyme activity

The effect of pH on the activity of enzyme was examined using the same method of enzyme activity determination. Enzyme activity was measured at a range of pH values between 5.0 and 11.0 using 50 mM of the following buffers: Citrate buffer (pH 5.0-6.0), PIPES buffer (pH 6.0-7.5), Tris buffer (pH 7.5-8.5), CAPSO buffer (pH 8.5-10.0) and CAPS buffer (pH 10.0-11.0). Activity measurements were made over a 1.5 min time period to ensure a linear product-time profile. The measurements were done in triplicates.

7. Circular dichroism spectroscopy

Far-ultraviolet circular dichroism spectra were obtained by a Jasco J-715 spectropolarimeter purged with oxygen-free nitrogen (Jasco, Maryland, USA). The instrument was calibrated with 1.0 mg/mL (+)-10-camphorsulphonic acid, yielding an intensity ratio between 192 and 290 nm more than 2.0. Protein samples in 20 mM TEMP buffer (pH 7.6) were measured at protein concentration of 1.5 mg/mL over wavelength range from 190 nm to 300 nm, at 25ºC, using 0.02 cm path length cylindrical quartz cuvette (Hellma, Müllheim, Germany). Scanning rate was set at a rate of 20 nm/min, 1.0 s response time, and 50 millidegree sensitivity. All spectra measurement were done in triplicates and subtracted by baseline spectra of 20 mM TEMP buffer (pH 7.6).

8. Determination of thermal stability

Thermal stability values were considered as the melting temperature (Tm) of the protein samples which is determined from thermal denaturation curves. The thermal denaturation curves were performed using Jasco J-715 spectropolarimeter purged with

oxygen-free nitrogen which the protocols utilized for instrument, the cuvette and sample concentration were the same as described above. Thermal denaturation curves were monitored from 25 to 85 ºC by the ellipticity at 222 nm and the Tm values are determined as the temperature at which 50% of protein molecules are unfolded, as measured by that ellipticity. All spectra measurement were done in triplicates and subtracted by baseline spectra of 20 mM TEMP buffer (pH 7.6).

9. Determination of isoelectric point value (pI)

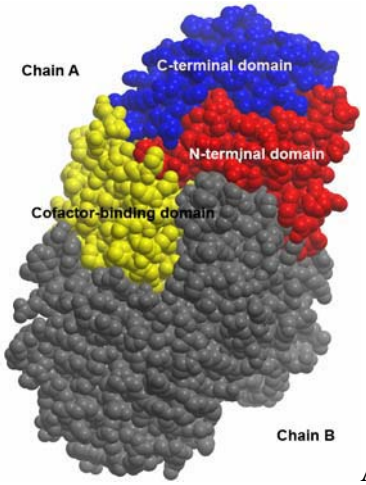
The isoelectric point values of the enzymes were determined by application of 2-D electrophoresis method. First-dimension isoelectric focusing was performed using 7 cm ImmobilineTM DryStrips pH 3-10 NL (GE Healthcare Bio-Sciences, Uppsala, Sweden) and running on EttanTM IPGphorTM (Amersham Pharmacia Biotech, California, USA). The second-dimension SDS-PAGE was performed using a Mini-Protein II Dual Slab Cell (BIO-RAD, California, USA) and visualized by silver staining.

Results

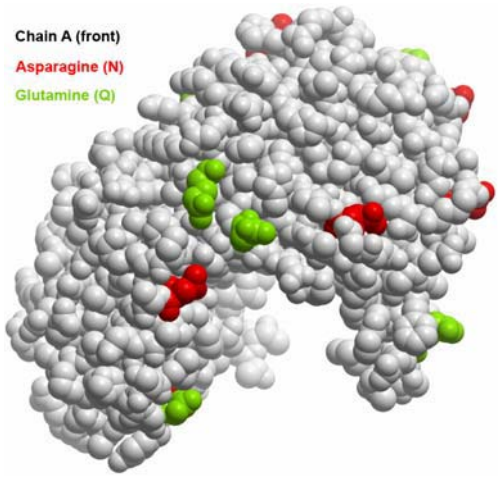
1. Construction of plasmids pEPL-N439D-Hc, pEPL-Q444E-Hc, and pEPL-N439D Q444E-Hc containing genes encoding the engineered D-PhgAT

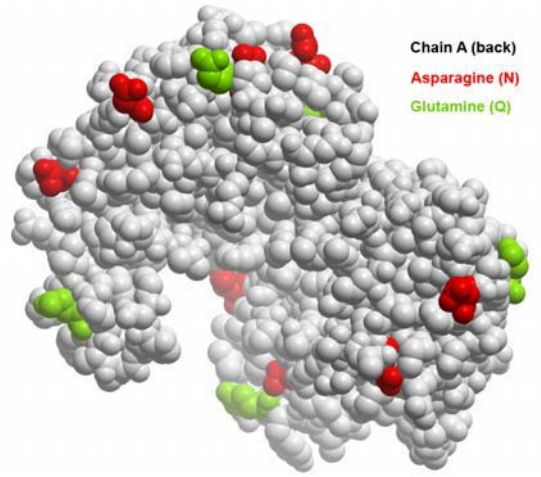
1.1 Bioinformatics analysis

Asparagine (N) and glutamine (Q) at β-turn were chosen as the target for mutation. Crystal structure of D-PhgAT (PDB: 2CY8) is showed in Figure 1A. The D-PhgAT consists of 2 identical subunits. Each subunit can be divided into 3 domains which are N-terminal domain (residue 2 - 75), cofactor-binding domain (residue 76 - 325), and C-terminal domain (residue 325 - 453). The N and Q residues in D-PhgAT are shown in Figure 1B, 1C and the positions were in Table 1.



A





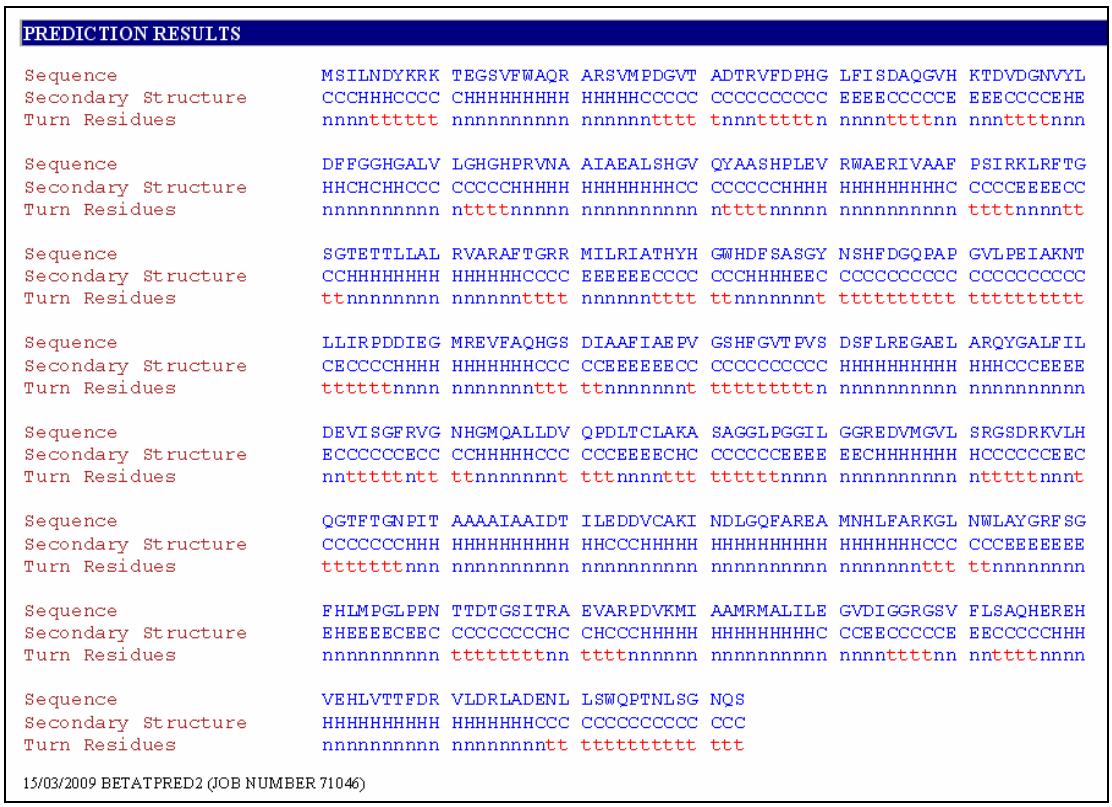
B C

Figure 1. (A) Crystal structure D-PhgAT (PDB: 2CY8). The enzyme consists of 2 identical monomers. Each monomer can be divided into 3 domains which are N-terminal domain, cofactor-binding domain, and C-terminal domain (B, C) The asparagines (N) colored in red and glutamines (Q) colored in green were identified in each subunit. The figure was generated using the ICM-Browser 3.6 program.

Table 1. Identification of asparagine (N) and glutamine (Q) residues in D-PhgAT

Amino acid	Position in each monomer	Total of residues in each monomer
N	5, 57, 79, 161, 179, 251, 307, 331, 342, 351, 370, 439, 447, 451	14
Q	19, 47, 91, 167, 197, 233, 255, 261, 301, 335, 415, 444, 452	13

A bioinformatics tool, BetaTPred2, was applied to predict β-turn in the D-PhgAT structure from the given amino acid sequence. This program predicts β-turns in proteins from multiple alignment by using neural network with residue accuracy of 75.5%. The results from the prediction are shown in Figure 2.



PREDICTION RESULTS

```
Sequence                MSILNDYKRK TEGSVFWAQR ARSVMPDGVT ADTRVFDPHG LFISDAQGVH KTDVDGNVYL
Secondary Structure     CCCHHHCCCC CHHHHHHHHH HHHHCCCCCC CCCCCCCCCC EEEECCCCCE EEECCCCEHE
Turn Residues           nnnnttttttt nnnnnnnnnn nnnnnntttt tnnnttttttn nnnnttttnn nnnttttnnn

Sequence                DFFGGHGALV LGHGHPRVNA AIAEALSHGV QYAASHPLEV RWAERIVAAF PSIRKLRFTG
Secondary Structure     HHCHCHHCCC CCCCCHHHHH HHHHHHHHCC CCCCCCHHHH HHHHHHHHHC CCCCEEEECC
Turn Residues           nnnnnnnnnn ntttttnnnnn nnnnnnnnnn nttttnnnnn nnnnnnnnnn ttttnnnntt

Sequence                SGTETTLLAL RVARAFTGRR MILRIATHYH GWHDFSASGY NSHFDGQPAP GVLPEIAKNT
Secondary Structure     CCHHHHHHHH HHHHHHCCCC EEEEEECCCC CCCHHHHEEC CCCCCCCCCC CCCCCCCCCC
Turn Residues           ttnnnnnnnn nnnnnnnttt nnnnnnnttt ttnnnnnnnt ttttttttttt ttttttttt

Sequence                LLIRPDDIEG MREVFAQHGS DIAAFIAEPV GSHFGVTPVS DSFLREGAEL ARQYGALFIL
Secondary Structure     CECCCCHHHH HHHHHHHCCC CCEEEEEECC CCCCCCCCCC HHHHHHHHHH HHHCCCEEEE
Turn Residues           ttttttnnnn nnnnnnnttt ttnnnnnnnt ttttttttn nnnnnnnnnn nnnnnnnnnn

Sequence                DEVISGFRVG NHGMQALLDV QPDLTCLAKA SAGGLPGGIL GGREDVMGVL SRGSDRKVLH
Secondary Structure     ECCCCCCECC CCHHHHHCCC CCCEEEEECHC CCCCCCEEEE EECHHHHHHH HCCCCCCEEC
Turn Residues           nnttttttntt ttnnnnnnnt tttnnnnttt ttttttnnnn nnnnnnnnnn nttttt nnnt

Sequence                QGTFTGNPIT AAAAIAAIDT ILEDDVCAKI NDLGQFAREA MNHLFARKGL NWLAYGRFSG
Secondary Structure     CCCCCCCHHH HHHHHHHHHH HHCCCHHHHH HHHHHHHHHH HHHHHHCCC CCCEEEEEEE
Turn Residues           ttttttttnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnttt ttnnnnnnnn

Sequence                FHLMPGLPPN TTDTGSITRA EVARPDVKMI AAMRMALILE GVDIGGRGSV FLSAQHEREH
Secondary Structure     EHEEEECEEC CCCCCCCCHC CHCCCHHHHH HHHHHHHHHC CCEECCCCCE EECCCCCHHH
Turn Residues           nnnnnnnnnn ttttttttnn ttttnnnnnn nnnnnnnnnn nnnnttttnn nntttt nnnn

Sequence                VEHLVTTFDR VLDRLADENL LSWQPTNLSG NQS
Secondary Structure     HHHHHHHHHH HHHHHHCCC CCCCCCCCCC CCC
Turn Residues           nnnnnnnnnn nnnnnnnntt ttttttttt ttt

15/03/2009 BETATPRED2 (JOB NUMBER 71046)
```

Figure 2. β-turns in D-PhgAT as predicted by BetaTPred2. The residues in the query sequence predicted as β-turns are shown as "t" and non-β-turn residues are shown as "n".

GETAREA and ArealMol were applied to predict solvent accessible surface and percentage of solvent expose. Crystal structure of D-PhgAT (PDB: 2CY8) was from the Protein Data Bank and the programs were used to compute the percentage of solvent expose of each residue. Residues are considered to be solvent exposed if the percent value exceeds 50% and to be buried if it is less than 20%. The results, focused only on asparagine (N) and glutamine (Q) residues in the D-PhgAT, are shown in Table 2.

Table 2. Percent solvent exposed of asparagine (N) and glutamine (Q) in D-PhgAT

Position	GETAREA (%)	ASAView (%)	ArealMol (%)	Status
N5	49.17	53.00	43.00	
N57	28.00	22.40	22.00	
N79	48.49	52.20	44.00	
N161	ND	ND	ND	ND
N179	30.74	33.40	28.00	
N251	29.37	33.40	28.00	
N307	26.64	29.10	23.00	
N331	25.95	26.70	23.00	
N342	19.12	20.8	16.00	Buried
N351	46.44	47.30	38.00	
N370	91.52	89.40	80.00	Exposed
N439	88.11	87.50	77.00	Exposed
N447	ND	ND	ND	ND
N451	ND	ND	ND	ND
Q19	67.75	69.60	59.00	Exposed
Q47	85.66	96.1	74.00	Exposed
Q91	58.79	62.40	51.00	Exposed
Q167	ND	ND	ND	ND
Q197	67.19	-	53.00	Exposed
Q233	77.27	64.60	68.00	Exposed
Q255	0.01	0.3	0.01	Buried
Q261	61.59	65.00	56.00	Exposed
Q301	ND	ND	ND	ND
Q335	52.63	63.30	46.00	
Q415	39.19	32.90	33.00	
Q444	83.42	91.70	79.00	Exposed
Q452	ND	ND	ND	ND

ND = “not determine”, because these residues are missing in the current crystal structure data of the enzyme.

The analysis of crystal contacts was performed via CryCo server. The program requires crystal structure of D-PhgAT (PDB: 2CY8) from the Protein Data Bank and analyzes crystal contacts both with and without crystal environment according to contact surfaces. The results, focused only on asparagine (N) and glutamine (Q) residues in the D-PhgAT, are shown in Table 3.

Table 3. Crystal contacts area of asparagines (N) and glutamines (Q) in D-PhgAT as predicted by CryCo server

Position	Crystal contacts area ($A^{\circ 2}$)	Status
N5	0.0	Not crystal contacts residue
N57	1.9	Crystal contacts residue
N79	15.7	Crystal contacts residue
N161	ND	ND
N179	27.9	Crystal contacts residue
N251	0.0	Not crystal contacts residue
N307	16.3	Crystal contacts residue
N331	0.0	Not crystal contacts residue
N342	0.0	Not crystal contacts residue
N351	25.2	Crystal contacts residue
N370	0.0	Not crystal contacts residue
N439	28.8	Crystal contacts residue
N447	ND	ND
N451	ND	ND
Q19	30.1	Crystal contacts residue
Q47	0.0	Not crystal contacts residue
Q91	83.1	Crystal contacts residue
Q167	ND	ND
Q197	-	ND
Q233	26.5	Crystal contacts residue
Q255	0.0	Not crystal contacts residue
Q261	0.0	Not crystal contacts residue
Q301	ND	ND
Q335	0.0	Not crystal contacts residue
Q415	0.0	Not crystal contacts residue
Q444	67.8	Crystal contacts residue
Q452	ND	ND

ND = “not determine”, because these residues are missing in the current crystal structure data of the enzyme.

Only highly solvent exposed N and Q residues predicted to be situated in β-turns and involve in the crystal contacts were chosen for mutation. Residues of the substrate binding-pocket, active site, inter-domain or the inter-chain interactions were excluded from the candidates. From these criteria, there were only 2 residues considered as suitable for mutation and they are shown in Table 4 and their positions in 3D-structure are shown in Figure 3.

Table 4. Characteristics of the amino acid residues candidates for mutation

Amino acid	Domain	β-turn residue	Solvent exposed residue	Crystal contacts residue
N439	C-terminal	Yes	Yes	Yes
Q444	C-terminal	Yes	Yes	Yes

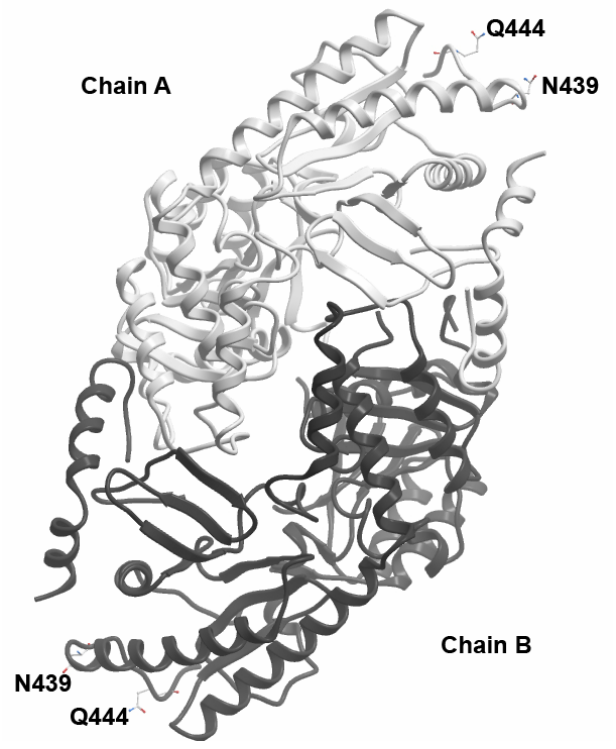


Figure 3. The target residues Asparagine (N) 439 and glutamine (Q) 444 in 3D-structure of D-PhgAT. The figure was generated using the ICM-Browser 3.6 program.

1.2 Site-directed mutagenesis and protein expression

Primers for site-directed mutagenesis were designed using QuikChange® Primer Design Program based on dpgA gene. According to designed strategy, N439 was replaced with aspartic acid (D) using primers N439D and N439D_antisense as forward and reverse primers, respectively. The Q444 was replaced with glutamic acid (E) using Q444E and Q444E_antisense primers, as forward and reverse primers, respectively. The resulting plasmids, pEPL-N439D, pEPL-Q444E, and pEPL-N439D Q444E, were transformed to *E. coli* XL-10 Gold.

For constructing plasmids that express the mutant D-PhgAT having hexa-histidine tag at the C-terminus, the same strategies were employed but using the parent plasmid having the hexa-His sequence. The resulting plasmids, pEPL-N439D-Hc, pEPL-Q444E-Hc, and pEPL-N439D Q444E-Hc were transformed to *E. coli* XL-10 Gold.

Examples of variants D-PhgAT expression in *E. coli* are shown in Figure 4. After purification, the D-PhgAt variants were found to be as active as the wild-type enzyme Figure 5.

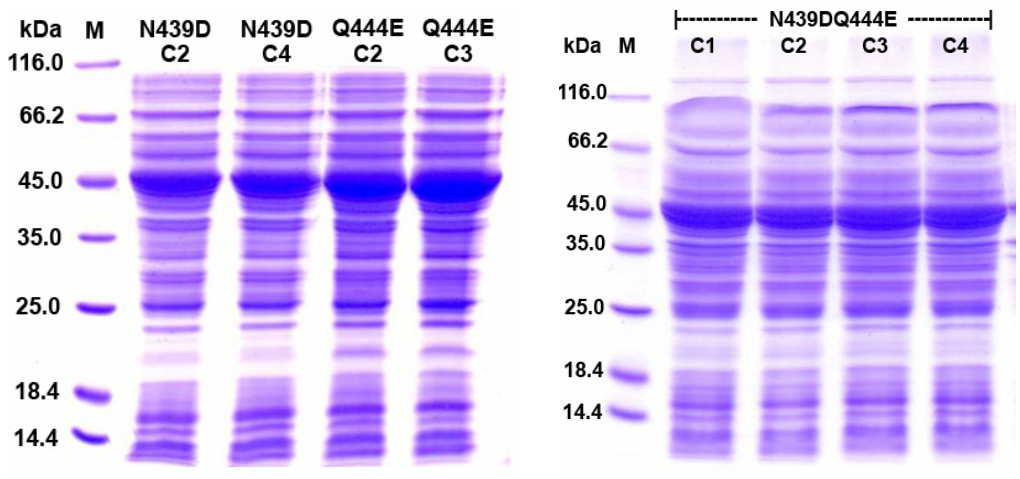


Figure 4. SDS-PAGE analysis of total cellular proteins of *E. coli* expressing D-PhgAT-(N439D)-H_6C and D-PhgAT-(Q444E)-H_6C. The enzyme variants can be seen as thick protein bands at ~ 45 kDa.

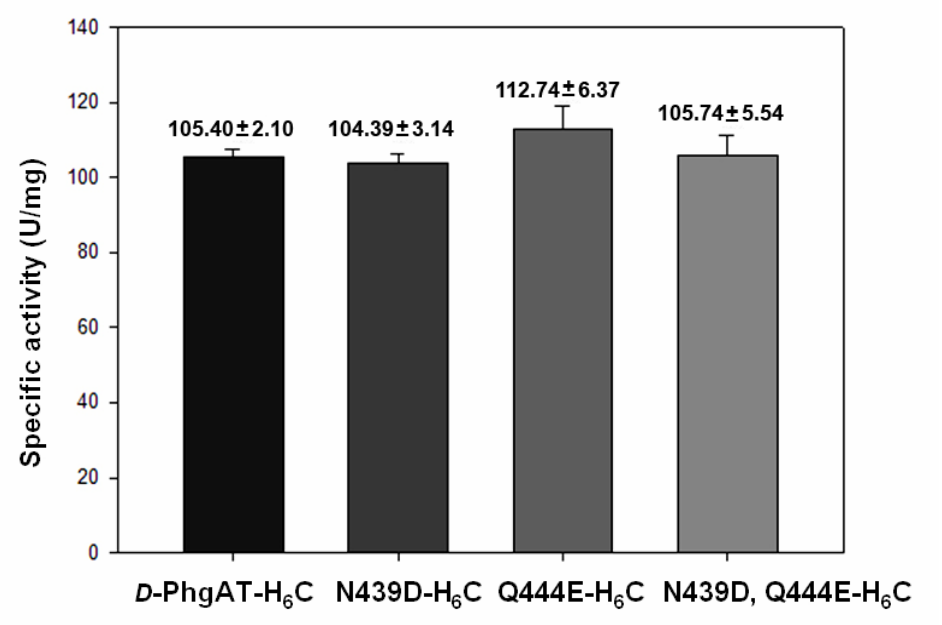


Figure 5. Specific activity of D-PhgAT-H_6C variants which are D-PhgAT-H6C and D-PhgAT-(N439D)-H_6C, D-PhgAT-(Q444E)-H_6C), and D-PhgAT-(N439D, Q444E)-H_6C, respectively. The enzyme activity determinations were performed by spectrophotometric assay in 50 mM CAPSO buffer (pH 9.5).

1.3 *in vitro* protein solubility of D-PhgAT variants

The in vitro solubility measurement was performed at room temperature (25°C) by using concentration method with modification. The solubility assay was measured at the neural pH (pH = 7.6) and the optimal pH of enzyme activity (pH = 9.5) without any salt in the solution. Protein concentration of the soluble phase was determined with the absorption at 280 nm and the molar absorption coefficient of 37,930 $M^{-1}cm^{-1}$. The in vitro solubility values of D-PhgAT with and without H_6C-tagged enzymes at the storage pH and the optimal working pH are shown in Figure 6 and 7, respectively.

It can be clearly seen that single mutation at N439D or Q444E could significantly increase the enzyme solubility with the Q444E mutation having greater effect. Combining the two mutations resulted in further increase in enzyme solubility 5 – 6 folds compared with the wild-type enzyme. However, the solubility enhancing effect of these mutations was negatively affected by the presence of the hex-histidine tag at the C-terminus of the enzyme, especially the doubly mutated variant. These indicated that the positively charged hexa-histidine tag might interact with the negatively charged D439 and E444 thus diminishing solubility enhancing effect of these mutants.

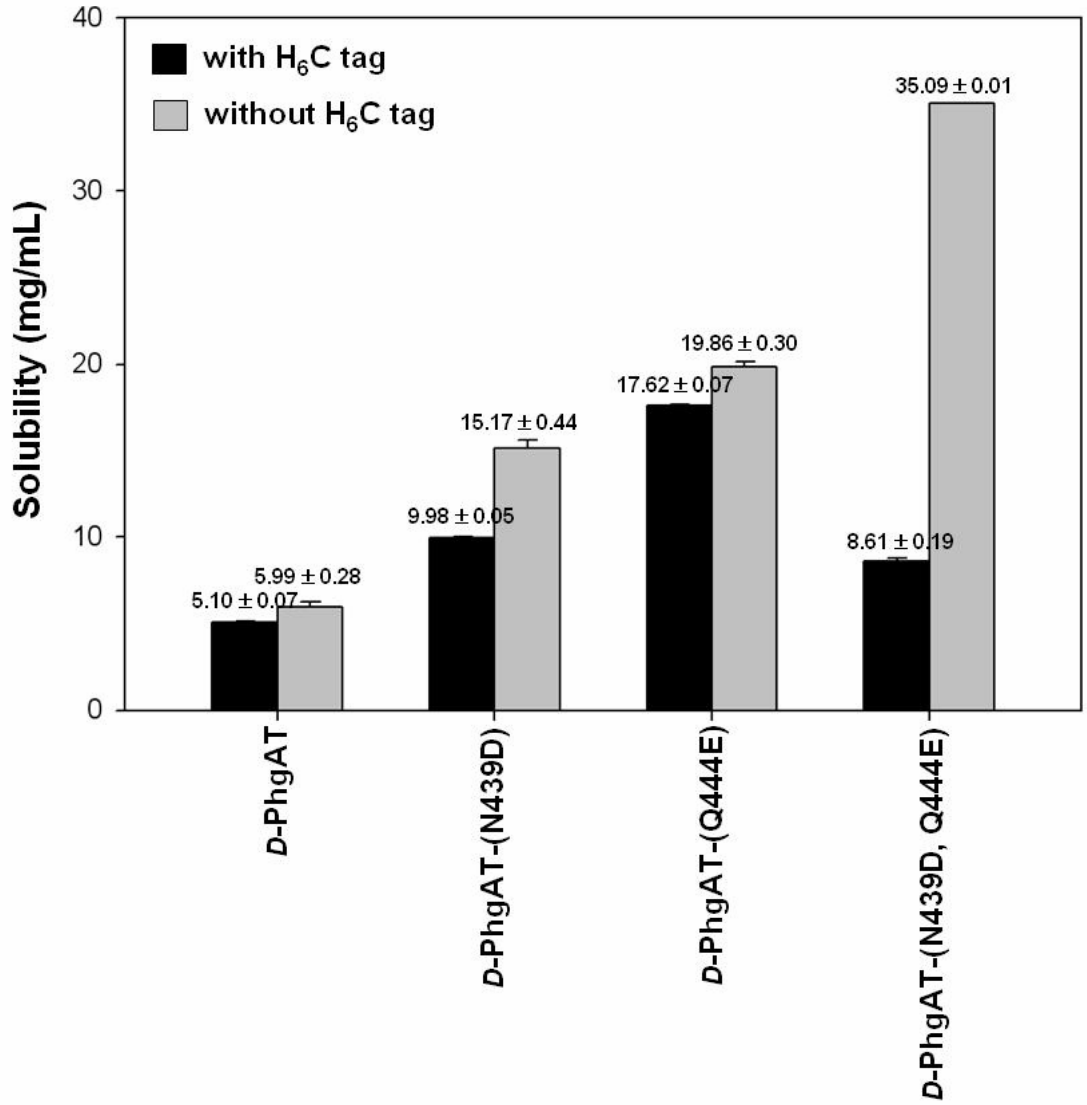


Figure 6. Comparison of *in vitro* solubility of D-PhgAT variants with and without H_6C-tag at pH = 7.6 (the storage pH), 25 °C.

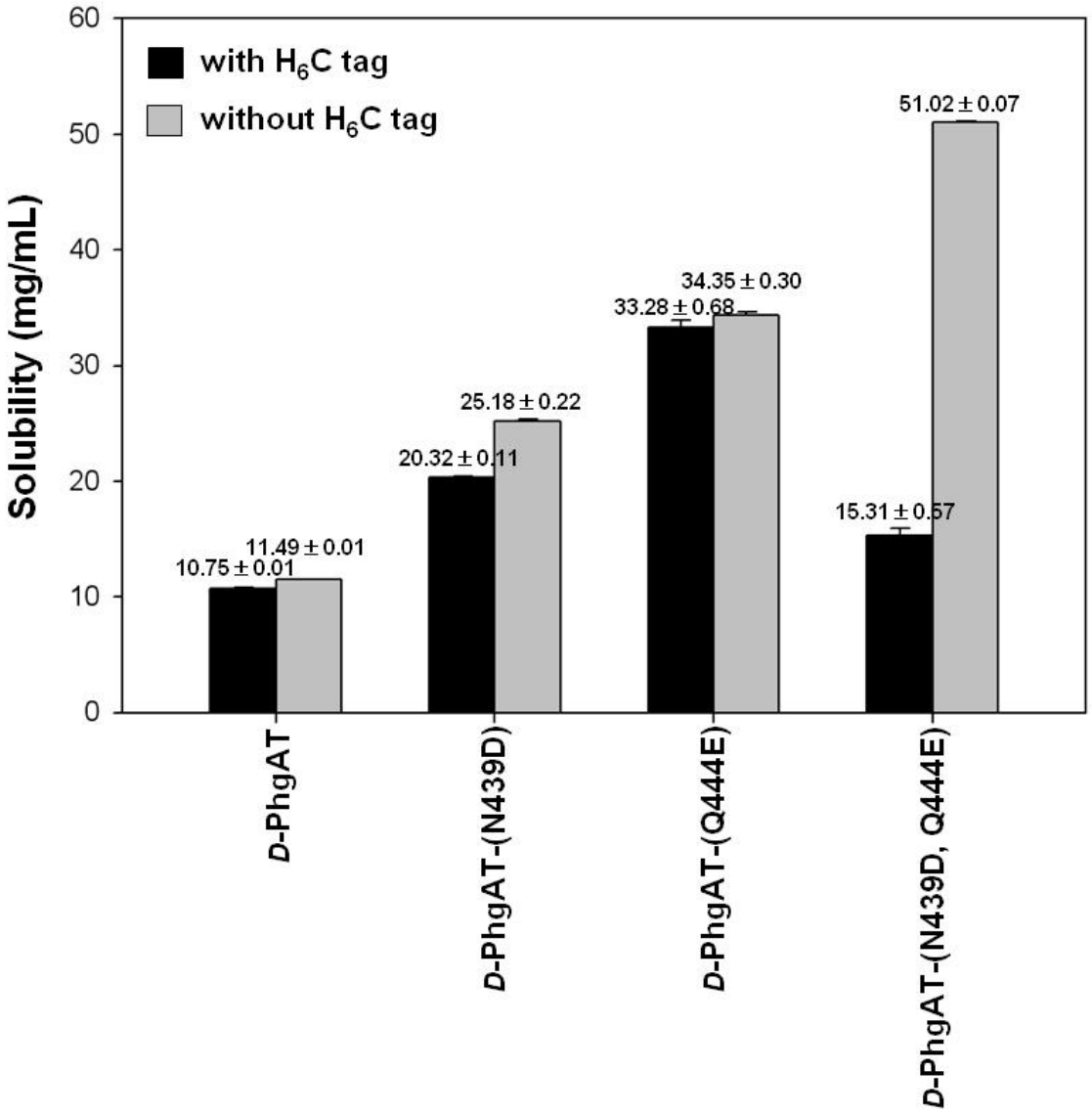


Figure 7. Comparison of *in vitro* solubility of D-PhgAT variants with and without H_6C-tag at pH = 9.5 (the working pH), 25 °C.

Part V

Spectrophotometric enzymatic cycling method using L-glutamate dehydrogenase and D-phenylglycine aminotransferase for determination of L-glutamate in foods.

โปรดดูเอกสารในภาคผนวก

Part VI

Sensitive non-radioactive determination of aminotransferase stereospecificity for C-4' hydrogen transfer on the coenzyme.

โปรดดูเอกสารในภาคผนวก

สรุปและวิจารณ์ผลการทดลอง

งานวิจัยที่ได้ดำเนินการในโครงการครั้งนี้ได้แก่

1. การทำ directed mutagenesis ต่อยีน *dpgA* เพื่อเปลี่ยน serine ที่ปลายสายด้าน C ของ D-PhgAT ทำให้สามารถตรึงเอนไซม์กับ thiol-containing matrix ได้อย่างจำเพาะ และรวดเร็ว โดยที่ยังคงรักษา activity ส่วนใหญ่ของ enzyme ไว้ได้ เนื่องจากการตรึงเอนไซม์เกิดที่ amino acid ที่อยู่ปลายสุดของสายด้าน C-terminus ที่ยื่นออกไปภายนอก ทำให้ไม่เกิดการรบกวนโครงสร้างและการ folding ของเอนไซม์ และการ immobilization ทำให้เอนไซม์มีความคงตัวมากขึ้น

2. การทดลองใช้เอนไซม์ที่ตรึงนี้ ในปฏิกิริยาการสังเคราะห์สาร D-phenylglycine และเนื่องจาก activity ของเอนไซม์ D-PhgAT ถูกยับยั้งได้ด้วย benzoylformate ซึ่งใช้เป็น substrate .ในกรณีนี้ เรียกว่าเกิด substrate inhibition ที่ค่อนข้างแรง จึงได้ใช้ระบบที่ใช้ benzoylformate ในรูปที่ดูดซับอยู่บน Amberlite (IRA400) resin beads ซึ่งจะปลดปล่อยสารตั้งต้นคือ benzoylformate ออกมาทำปฏิกิริยาทีละน้อย ไม่ถึงขั้นยับยั้งเอนไซม์ จึงสามารถทำงานได้ และเมื่อปลดปล่อย benzoylformate ออกมาแล้ว Amberlite (IRA400) ยังสามารถดูดซับ 2-oxoglutarate ที่เกิดจากปฏิกิริยาไว้ได้ด้วย ทำให้ปฏิกิริยาดำเนินไปในทิศทางที่เกิด product คือ D-phenylglycine ได้ดีขึ้น จากการทดลองพบว่าเมื่อปฏิกิริยาสิ้นสุด ได้ สาร D-phenylglycine 20.25 กรัมต่อลิตร หรือคิดเป็นอัตราการเกิดผลิตภัณฑ์ประมาณ 68 %

3. การพัฒนาระบบการแสดงออกของยีนเพื่อการผลิต D-PhgAT โดยใช้แบคทีเรีย *E. coli* เป็นเซลล์เจ้าบ้าน ซึ่งเมื่อใช้ระบบที่มี strong promoter พบว่า เอนไซม์ D-PhgAT ถูกสร้างขึ้นมาในปริมาณมาก แต่ทั้งหมดอยู่ในรูปที่เป็น inclusion body และไม่สามารถทำให้เกิดการละลายและ re-folding ได้ จึงได้ดำเนินการแก้ไขในหลายวิธี และวิธีที่ได้ผลดีมากคือ การใช้ชุดของ *E. coli* chaperones ที่เหมาะสม แสดงออกร่วมด้วย ซึ่งช่วยในเรื่อง folding ของเอนไซม์ D-PhgAT ที่ express ออกมาในปริมาณมาก ให้อยู่ในรูป soluble active form ทำให้สามารถผลิต D-PhgAT ได้เพิ่มขึ้นจาก 0.44 unit.L^{-1}.OD^{-1} เป็น 1,768 unit.L^{-1}.OD^{-1} หรือประมาณ 4,000 เท่า

4. การพัฒนาระบบการแสดงออกของยีนเพื่อการผลิต D-PhgAT โดยใช้ยีสต์ *Pichia pastoris* ในกรณีนี้ต้องสังเคราะห์ยีนของเอนไซม์ D-PhgAT ขึ้นมาใหม่ ให้มี codons เหมาะสมสำหรับการแสดงออกใน *Pichia pastoris* และต้อง integrate recombinant gene นี้เข้าไปใน chromosome ของเซลล์เจ้าบ้าน ในการวิจัยระยะแรก พบว่าระบบนี้สร้างเอนไซม์ D-PhgAT ได้น้อยมาก อย่างไรก็ดีจากการทดลองปรับปรุงต่อ ๆมาจึงพบว่า การใช้ชุดของ *E. coli* chaperones ที่เหมาะสม แสดงออกร่วมด้วย ช่วยให้ระบบนี้สามารถผลิต soluble functional D-PhgAT ได้ และยังพบต่อมาอีกว่า ถ้า integrate ยีนของ *E. coli* chaperones และ D-

PhgAT หลายๆ copies เข้าไปใน chromosome ของ *Pichia pastoris* ที่เป็นเจ้าบ้าน จะช่วยเพิ่มปริมาณการเกิด D-PhgAT ได้อย่างมาก ณ ปัจจุบันสามารถผลิตได้ในระดับ 14,717 unit.L^{-1}.OD^{-1} ซึ่งสูงกว่าระบบที่ใช้ *E. coli* เป็นเซลล์เจ้าบ้าน มากกว่า 8 เท่า

5. เอนไซม์ D-PhgAT มีค่าการละลาย (water solubility) ต่ำ ไม่สามารถทำให้เอนไซม์ในรูปสารละลายความเข้มข้นสูงได้ เป็นปัญหาในช่วงการทำ enzyme purification และการเก็บรักษาเอนไซม์ไว้ใช้งานนานๆ และการมีค่าการละลายต่ำอาจเป็นสาเหตุหนึ่งของการเกิดเป็น inclusion body ได้ง่ายใน expression host การวิจัยครั้งนี้จึงได้แก้ปัญหา ด้วยการทำ structure-guided mutagenesis โดยศึกษาโครงสร้าง 3-มิติ ของเอนไซม์ D-PhgAT เท่าที่มีข้อมูลอยู่ และใช้โปรแกรมทาง Bioinformatics ในการช่วยวิเคราะห์ว่าควรทำ mutation ณ จุดใดบ้าง จากนั้นจึงทำการทดลองจริงในห้องปฏิบัติการ ซึ่งพบว่าการทำ mutation เพียง 2 จุด คือที่ Asparagine (N) ตำแหน่งที่ 439 และ Glutamine (Q) ตำแหน่งที่ 444 โดยเปลี่ยนเป็น Aspartic acid และ Glutamic acid ตามลำดับ พบว่าทำให้เพิ่มการละลายของ D-PhgAT จาก 11.5 เป็น 51 มก/มล หรือประมาณ 5 เท่า
6. ในระหว่างการดำเนินการวิจัยในครั้งนี้ ได้มีหัวข้อวิจัยที่เกิดขึ้นใหม่ ซึ่งไม่ได้นำเสนอไว้ในข้อเสนอโครงการวิจัย ในครั้งแรก แต่เกิดจากการสังเกต เกิดความคิดในกระบวนการแก้ปัญหาระหว่างการวิจัย ได้แก่

   6.1 การประยุกต์ใช้เอนไซม์ D-PhgAT ในการตรวจวัดปริมาณ L-glutamate ด้วยวิธีหมุนเวียนสารตั้งต้น ซึ่งเป็นวิธีการวัดที่รวดเร็ว วัดได้ละเอียด และมีการรบกวนน้อย ซึ่งผู้วิจัยและคณะได้ตีพิมพ์วิธีการนี้ในวารสารวิชาการนานาชาติแล้ว

   6.2 การพัฒนาวิธีการใหม่สำหรับการพิสูจน์ stereospecificity ของการถ่ายทอดโปรตอนบน C-4' ของ coenzyme โดย aminotransferase เป็นวิธีการที่ไม่ใช้สาร radioactive ดังเช่นวิธีที่มีใช้ก่อนหน้านี้ แต่มีความไว (sensitivity) ในระดับเดียวกัน หรือดีกว่า ผู้วิจัยได้ใช้วิธีการใหม่นี้ในการศึกษาคุณสมบัติของ เอนไซม์ D-PhgAT และได้ตีพิมพ์วิธีการใหม่นี้ในวารสารวิชาการนานาชาติแล้ว

ข้อเสนอแนะสำหรับงานวิจัยในอนาคต

การวิจัยต่อเนื่องที่ควรทำในอนาคต ได้แก่

1. การดัดแปลงโครงสร้างโมเลกุลของ D-PhgAT เพื่อลดการยับยั้งจาก substrate
2. การพัฒนากระบวนการตรวจวัดและเติม substrate ที่เหมาะสม และวิธีการดึง product ออกจากระบบ เพื่อให้ปฏิกิริยาการสังเคราะห์ด้วยเอนไซม์ D-PhgAT มีอัตราเร็วเพิ่มขึ้น และทำได้ยาวนานมากขึ้นไปอีก

Output จากโครงการวิจัย

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ จำนวน **3** เรื่อง

1. Rojanarata T, Isarangkul D, **Wiyakrutta S**, Meevootisom V, Woodley JM*. Controlled-release biocatalysis for the synthesis of D-Phenylglycine. Biocatal Biotransfor May 2004; 22(3): 195-201.
2. Khampha W, Meevootisom V, **Wiyakrutta S***. Spectrophotometric enzymatic cycling method using L-glutamate dehydrogenase and D-phenylglycine aminotransferase for determination of L-glutamate in foods. Anal Chim Acta Aug 2004; 520(1-2): 133-139.
3. Jomrit J, Summpunn P, Meevootisom V, **Wiyakrutta S***. Sensitive non-radioactive determination of aminotransferase stereospecificity for C-4' hydrogen transfer on the coenzyme. Biochem Biophys Res Commun 2011 Feb 25; 405(4): 626-31.

ผลงานที่อยู่ระหว่างการเขียน manuscripts จำนวน **3** เรื่อง

1. Chantarasiri A, Meevootisom V, Isarangkul D, **Wiyakrutta S***. Improving *in vitro* solubility of D-phenylglycine aminotransferase by structure-guided mutagenesis. (*manuscript in preparation to be submitted to* New Biotechnology *in July 2011*)
2. Jariyachawalid K, Laowanapiban P, Meevootisom V, **Wiyakrutta S***. Effective enhancement of *Pseudomonas* D-phenylglycine aminotransferase functional expression in *Pichia pastoris* by co-expressing *Esherichia coli* GroEL-GroES. (*manuscript in preparation to be submitted to* Microbial Cell Factories *in July 2011*)
3. Laowanapiban P, Jomrit J, Meevootisom V, **Wiyakrutta S***. *Pseudomonas* stereo-inverting D-phenylglycine aminotransferase is closely related to glutamate-1-semialdehyde 2,1-aminomutase.
(*manuscript in preparation to be submitted for publication in August 2011*)

2. การนำผลงานวิจัยไปใช้ประโยชน์

เชิงพาณิชย์ (มีการนำไปผลิต/ขาย/ก่อให้เกิดรายได้ หรือมีการนำไปประยุกต์ใช้โดย ภาคธุรกิจ/บุคคลทั่วไป)

เชิงนโยบาย (มีการกำหนดนโยบายอิงงานวิจัย/เกิดมาตรการใหม่/เปลี่ยนแปลงระเบียบข้อบังคับหรือวิธีทำงาน)

เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง)

☑ เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)

- สร้างนักวิจัยใหม่

- ผลิตบัณฑิตระดับปริญญาเอก สาขาจุลชีววิทยา จำนวน **2** คน คือ

1. นาย ธีรศักดิ์ โรจนราชา
2. น.ส. วนิดา คำพา

- ผลิตบัณฑิตระดับปริญญาโท สาขาจุลชีววิทยา จำนวน **1** คน คือ

1. นาย มงคล เจียรกุลประเสริฐ

- ผลิตบัณฑิตระดับปริญญาโท สาขาจุลชีววิทยา จำนวน **1** คน คือ

1. น.ส. จันทราทิพย์ โจมฤทธิ์

3. อื่น ๆ

☑ การเสนอผลงานในที่ประชุมวิชาการ

1. Chantarasiri A, Meevootisom V, Laowanapiban P, Isarangkul D, **Wiyakrutta S***. Increasing solubility of D-phenylglycine aminotransferase. Commission on Higher Education Congress II, University Staff Development Consortium (CHE – USDC Congress II). Dusit Thani Pattaya Hotel, Chonburi, Thailand. August 27th – 29th, 2009.
2. Jomrit J, Hongsukapun P, **Wiyakrutta S***. Improving functional D-phenylglycine aminotransferase production by co-expression with chaperones in *E. coli*. The 35th Congress on Science and Technology of Thailand. The Tide Resort (Bangsaen Beach), Chonburi, Thailand. October 15th – 17th, 2009.
3. Jomrit J, Summpunn P, Meevootisom V, **Wiyakrutta S***. High production of functional D-phenylglycine aminotransferase in *Escherichia coli* by addition of benzyl alcohol, pyridoxine, and co-expression with chaperones. The 20th National Grad Research Conference. Mahidol University, Salaya, Nakornprathom, Thailand. February 2nd – 3rd, 2011.
4. Jomrit J, Summpunn P, Meevootisom V, **Wiyakrutta S***. A sensitive non-radioactive method for determination of stereospecificity aminotransferases for C-4' hydrogen transfer of the coenzyme. IX European Symposium of The Protein Society. Stockholm, Sweden. May 22nd – 26th, 2011.

ภาคผนวก

1. สำเนา ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ จำนวน 3 เรื่อง
2. สำเนา ผลงานเสนอในที่ประชุมวิชาการระดับชาติ จำนวน 3 เรื่อง
3. สำเนา ผลงานเสนอในที่ประชุมวิชาการนานาชาติ จำนวน 1 เรื่อง

Biocatalysis and Biotransformation, 2004 VOL. 22 (3). pp. 195–201

Taylor & Francis
Taylor & Francis Group

Controlled-release Biocatalysis for the Synthesis of D-Phenylglycine

T. ROJANARATA[1,2], D. ISARANGKUL[3], S. WIYAKRUTTA[2], V. MEEVOOTISOM[2] and J.M. WOODLEY[1]*

[1] *Department of Biochemical Engineering, University College London, Torrington Place, London WC1E 7JE, UK;* [2] *Department of Microbiology, Faculty of Science, Mahidol University, Rama VI Rd., Bangkok 10400, Thailand;* [3] *Center for Biotechnology, Institute of Science and Technology for Research and Development, Mahidol University, Salaya, Nakorn Pathom 73170, Thailand*

(Received for publication 1 March 2004; Revised received 10 May 2004)

An isolated and immobilised aminotransferase cloned from *Pseudomonas stutzeri* ST-201 into *Escherichia coli* was used to synthesise D-phenylglycine. The reaction was characterised by an unfavourable equilibrium constant and substrate inhibition. The use of a controlled-release system via the use of Amberlite (IRA 400)-adsorbed benzoylformate proved a useful technique to circumvent these issues. This resulted in a four-fold improvement in product concentration achievable to yield a final D-phenylglycine concentration of 10.25 mg/ml.

Keywords: D-Phenylglycine; Resins; Controlled-release biocatalysis

INTRODUCTION

Biocatalysis is becoming widely accepted as a powerful means to complement conventional catalysis in the synthesis of complex molecules. However, while biocatalysis offers the ability to carry out reactions in a highly selective way, it is frequently the case that operation in an industrial context may be limited by constraints. In particular the effective supply of substrate is difficult. Recently a number of isolated reports have demonstrated the potential for the use of substrate-containing resins as controlled-release agents to the biocatalytic reaction (D'Arrigo *et al.*, 1997, 1998; Houng and Liau, 2003; Vicenzi *et al.*, 1997). This has analogies with work in two-liquid phase biocatalysis where, under particular conditions, big productivity improvements have previously been obtained (e.g. Woodley and Lilly, 1992). The principle is to use the resin as a reservoir for substrate, which is gradually released (via mass transfer) into solution. The driving force for this transfer is dependent upon the rate of reaction and the consequent deviation of the substrate concentration in solution and on the resin, from equilibrium. To date, the approach has been used to enhance asymmetric bioreductions using various types of yeast (e.g. D'Arrigo *et al.*, 1997; Vicenzi *et al.*, 1997) and still more recently used with *Escherichia coli* (Simpson *et al.*, 2001) for Baeyer-Villiger monooxygenase catalysed synthesis of an optically pure lactone. These reports are preliminary but it is clear that there are a number of advantages to such an approach as a means to operate at high concentrations of substrate in the reactor while maintaining a low concentration in the region of the biocatalyst. In particular this could have application to assist those reactions where there is a need to operate at high substrate concentration to drive the equilibrium, while these concentrations are also inhibitory to the biocatalyst. An excellent illustrative example is the synthesis of the unnatural amino acid, D-phenylglycine which is used as a side chain for the anti-infective drugs ampicillin and cephalexin. Industrial syntheses use classical resolution of a racemate, which is readily available from the Strecker reaction on benzaldehyde (Sheldon, 1996) or resolution of DL-hydantoin, also prepared from benzaldehyde (Aida *et al.*, 1986). Other totally chemical (Calmes *et al.*, 1996; Voyer *et al.*, 1997) and chemoenzymatic methods (Basso *et al.*, 2000; Beard and Page, 1998; Garcia and Azerad, 1997; Gokhale *et al.*, 1996) have also been reported. More recently a D-phenylglycine aminotransferase (D-phgAT) was discovered in *Pseudomonas stutzeri* ST-201 (Wiyakrutta and Meevootisom, 1997) and this has now been cloned and overexpressed in *E. coli* (Laowanapiban, 2001).

* Corresponding author. Tel.: + 44-20-7679-3778. Fax: + 44-20-7916-3943. E-mail: j.woodley@ucl.ac.uk

ISSN 1024-2422 print/ISSN 1029-2446 online
DOI: 10.1080/10242420410001727355

Both wild type and recombinant enzymes are pyridoxal-5′-phosphate (PLP) dependent and selectively catalyse the reversible transamination of D-phenylglycine (or D-4-hydroxyphenylglycine) with 2-oxoglutarate (as the exclusive amino acceptor) giving L-glutamate. Due to the ''stereo-inverting'' activity of the enzyme, L-glutamate, which is a cheap amino donor, together with benzoylformate can be used to synthesise enantiomerically pure D-phenylglycine in a single reaction (Fig. 1). This route needs no amino acid racemases to isomerise the L-amino acid amino donor, as required with typical D-aminotransferases that accept only a D-amino acid substrate. Although D-phgAT has appropriate characteristics to function as a promising biocatalyst, with a rapid reaction rate and no requirement for external cofactor regeneration, the reaction has a low equilibrium constant (0.08), which favours D-phenylglycine degradation rather than synthesis, and substrate inhibition. In this paper we report findings on the characterisation and potential application of controlled-release biocatalysis to circumvent some of the constraints in this reaction. The approach using a resin for this reaction is schematically illustrated in Fig. 2. In addition to control of the substrate concentration the resin also affords a sink for the α-ketoglutarate byproduct and, since high levels of product can now potentially be produced, above saturation concentration the D-phenylglycine will precipitate out of solution.

MATERIALS AND METHODS

Materials

Thiopropyl Sepharose 6B used for enzyme immobilisation was obtained from Amersham Biosciences (Uppsala, Sweden). Amberlite IRA-400(Cl) (trialkylbenzyl ammonium) strong anion exchange resin was purchased from Sigma Aldrich (Poole, Dorset, UK). All other chemicals were obtained from Fluka Chemie (Buchs, Switzerland).

Enzyme Modification and Immobilisation

D-phgAT modification was previously carried out. Mutagenic primers were designed to replace a serine 453 with cysteine (S453C). This modification was proven to facilitate immobilisation on a thiol-containing matrix without any deleterious effect on enzyme activity (Unpublished data). The gene was cloned into the pET-17b expression vector and overexpressed in *E. coli* BL21(DE3) obtained from Novagen (Madison, WI, USA). The enzyme purification was carried out following the previously described protocol for recombinant D-phgAT (Laowanapiban, 2001).

To immobilise D-phgAT, the commercially available Thiopropyl Sepharose 6B was reswollen according to the manufacturer's instructions and washed three times with binding buffer (100 mM Tris buffer (pH 7.5), 100 mM NaCl and 1 mM EDTA). Subsequently, 3 ml of binding buffer containing 3 mg of freshly reduced (using immobilised TCEP disulfide reducing gel from Pierce (Rockford, IL, USA)) modified D-phgAT was incubated with 3 ml of gel. The reaction was carried out at 4°C for 3 h with gentle shaking. The suspension was then centrifuged and the beads were rinsed with the binding buffer until no enzyme activity was detectable in the supernatant. Finally, the beads were resuspended in the binding buffer with 25% glycerol and stored at 4°C.

HPLC Analysis

The concentrations of substrates and products were analyzed by HPLC using System Gold™ The Personal™ Chromatograph (Beckman Instrument, Inc., Fullerton, CA, USA). 20 μl of sample were injected onto a Spherisorb ODS2 column (250 × 46 mm) (Waters, Milford, MA, USA) operating at 25°C. Assay conditions were: mobile phase, isocratic 50 mM potassium phosphate buffer (pH 7.0) at a flow rate of 1.0 ml/min; UV detection at 254 nm.

Determination of Enzyme Activity

Enzyme activity was determined by two methods. In both cases the initial enzyme activity was determined based on a linear progress curve. A spectrophometric assay was used to determine D-phgAT activity in the direction of benzoylformate and L-glutamate synthesis. The assay was carried out in a 1 ml stirred reaction mixture containing 10 μl immobilised enzyme, 100 mM PIPES buffer (pH 8.0), 1 mM D-phenylglycine, 1 mM α-ketoglutarate, 25 μM

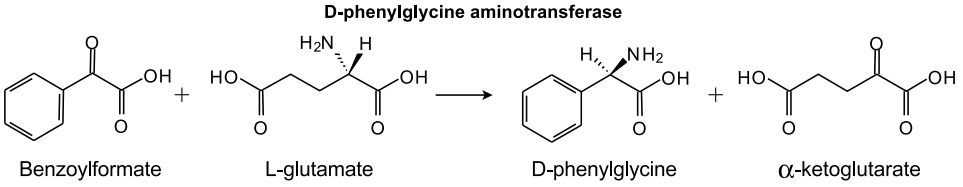


FIGURE 1 Synthesis of D-phenylglycine from benzoylformate and L-glutamate, catalysed by the stereoinverting D-phenylglycine aminotransferase.

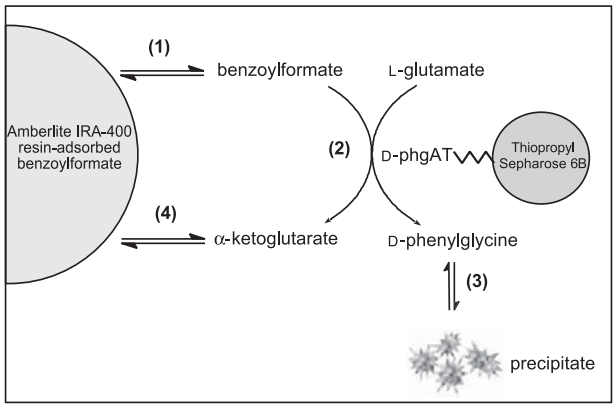


FIGURE 2 Schematic for the synthesis of D-phenylglycine from L-glutamate (fed as solution) and released benzoylformate from Amberlite IRA-400 resin-adsorbed substrate (1). The reaction is catalysed by immobilised D-phenylglycine aminotransferase on Thiopropyl Sepharose 6B (2) to yield the desired product which is spontaneously precipitated (3), and the by-product α-ketoglutarate which is adsorbed onto the resin (4) to prevent the accumulation.

PLP and 25 mM EDTA. The rate of benzoylformate formation was measured as a function of time by monitoring the increase in absorbance (254 nm) using a Kontron Uvikon 922 spectrophotometer (Kontron Instruments, Watford, Bucks, UK). A second method was used for determining D-phgAT activity in the direction of D-phenylglycine synthesis. 100 mM PIPES buffer (pH 8.0), benzoylformate, L-glutamate, 25 μM PLP and 25 mM EDTA were incubated with 100 μl of immobilised enzyme in a 1 ml reaction mixture at 37°C. Aliquots were taken at intervals, heated in boiling water for 2 min and filtered through a membrane (0.45 μm). The clear filtrate was subsequently assayed for D-phenylglycine by HPLC.

Determination of pH Effect on Enzyme Activity and Stability

The effect of pH on the activity of immobilised enzyme was examined using the spectrophotometric method of activity determination. Initial enzyme activity was measured at 37°C at a range of pH values between 5 and 12 using 100 mM of the following buffers: citrate buffer (pH 5, 6), PIPES buffer (pH 6–8), Tris buffer (pH 7–9) and CAPS buffer (pH 9–12). Activity measurements were made over a 1.5 min time period to ensure a linear product-time profile.

The effect of pH on the stability of the immobilised enzyme was measured by incubating immobilised enzyme at a range of pH values between 7.5 and 9.5, 37°C for 6 h. At intervals, aliquots of enzyme were assayed for residual activity using the standard spectrophometric method.

Determination of Temperature Effect on Enzyme Activity and Stability

The effect of temperature on enzyme activity was examined by the standard spectrophotometric assay in the range of 20–80°C at pH 8.0. Activity measurements were made over a 1.5 min time period to ensure a linear product-time profile. Thermal stability was studied by incubating the enzyme in 100 mM PIPES buffer (pH 7.5) at 37, 40, 50 and 60°C, for 6 h. At intervals, aliquots of enzyme were assayed for residual activity using the standard spectrophometric method.

Determination of the Effect of Substrate Concentration on Enzyme Activity

The effect of substrate concentration on the reaction rate was determined by incubating 100 μl immobilised enzyme in 100 mM PIPES buffer (pH 7.5) containing benzoylformate and L-glutamate at a range of initial concentrations between 50 and 1500 mM at 37°C. The rate of D-phenylglycine formation was measured over a 30 min reaction.

Study of Adsorption of the Substrates and Products onto the Resin in Dilute Solution

Prior to use, the anion exchange resin Amberlite IRA-400 was washed sequentially with 1 M HCl solution, deionised water, 1 M NaOH solution, deionised water, 1 M HCl solution, deionised water (until the pH equaled 7) and dried at 60°C for 24 h. One gram of dried resin was added to 10 ml of 30 mM D-phenylglycine, benzoylformate, L-glutamate or α-ketoglutarate at pH 5, 7, 7.5, 8 and 9. Subsequently, all the samples were incubated in a shaking incubator at 200 rpm, 37°C for 12 h. The residual (unadsorbed) concentration was measured using HPLC, from which the amount of adsorbed compounds (per gram dried resin) was calculated.

Use of the Amberlite IRA-400 Resin-adsorbed Benzoylformate as a Contolled-release Substrate

In order to prepare resin adsorbed-benzoylformate, 20 g of previously washed and dried Amberlite IRA-400 resin was added to 40 ml 2 M benzoylformate (pH 7.5) at 37°C under agitation of 200 rpm. After 6 h, the adsorbed resin was filtered and dried at 60°C for 24 h. The amount of benzoylformate released from the resin was investigated by shaking 0.5 g resin adsorbed-benzoylformate in 25 ml 100 mM PIPES buffer (pH 7.5). Periodically, the supernatant sample was taken and subjected to spectrophometric measurement of benzoylformate. The desorption was repeated using fresh buffer until no further release was observed and the results used to

calculate the cumulative amount of benzoylformate released.

The effect of the amount of resin adsorbed-benzoylformate on D-phenylglycine synthesis was studied by adding varying amounts of resin adsorbed-benzoylformate to a 1 ml reaction containing 100 mM Tris buffer (pH 7.5), 1 M L-glutamate, 25 μM PLP and 100 μl immobilised enzyme. The reaction proceeded at 37°C under agitation at 200 rpm for 12 h. A control reaction with no resin adsorbed-benzoylformate was conducted using benzoylformate solution alone at a final concentration of 100 mM. Upon sampling, sufficient water was added to the reaction to dissolve the product. Immobilised enzyme and Amberlite IRA-400 resin were separated using a 0.45 μm-syringe filter and the filtrate was diluted with water prior to analysis of D-phenylglycine and benzoylformate concentrations by HPLC.

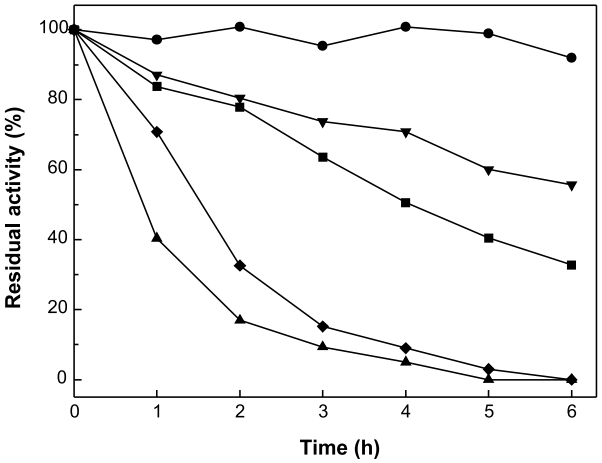


FIGURE 4 Effect of pH on the stability of immobilised enzyme. Residual activities were determined periodically after incubation of the enzyme at 37°C at pH 7.5 (●) and 8.0 (▼) in 100 mM PIPES buffer, 8.5 (■) and 9.0 (◆) in 100 mM Tris buffer and 9.5 (▲) in 100 mM CAPS buffer.

RESULTS AND DISCUSSION

pH Effect on Enzyme Activity and Stability

The pH-activity profile (Fig. 3) shows that the immobilised enzyme was highly active in alkali conditions with the highest activity at pH 9 in Tris or CAPS buffer. Initial activity was measured over a brief period to negate the effects of any instability, especially at extremes of pH. Some differences were observed between the types of buffer used and, in particular, higher enzyme activity was observed in PIPES buffer than that in Tris buffer at the same pH. When the pH was increased from 7 to 9, although the enzyme activity increased, the stability was significantly decreased (Fig. 4). Enzyme which had been incubated at a pH above 9 lost all activity with in 5–6 h. Incubation of the enzyme at pH 7.5 maintained 90% of its initial activity over a period of 6 h. Consequently, PIPES buffer at pH 7.5 was chosen for further D-phenylglycine synthesis experiments.

Effect of Temperature on Enzyme Activity and Stability

Figure 5 shows the effect of temperature on enzyme activity. Initial activity was measured over a brief period in order to negate the effects of any instability. Above 20°C, enzyme activity increased with increasing temperature reaching a maximum at 50–65°C. It then declined severely as the temperature was raised above 70°C. A range of 37–60°C where the enzyme was most active was chosen for the study of thermal stability over a period of 6 h and the result is illustrated in Fig. 6. Higher temperatures led to enzyme inactivation. At 60°C

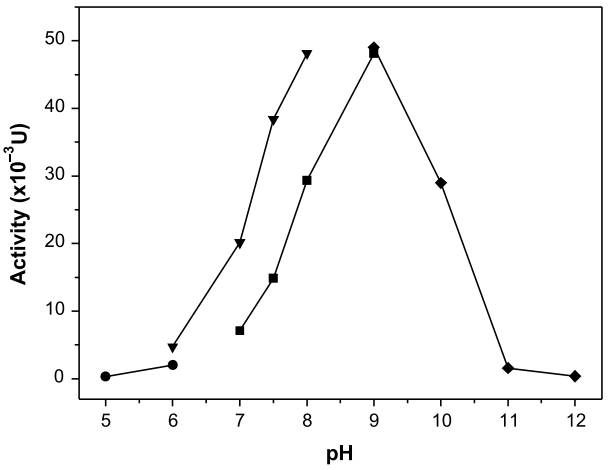


FIGURE 3 Effect of pH on the activity of immobilised enzyme determined at 37°C in 100 mM of citrate (●), PIPES (▼), Tris (■) and CAPS (◆) buffer.

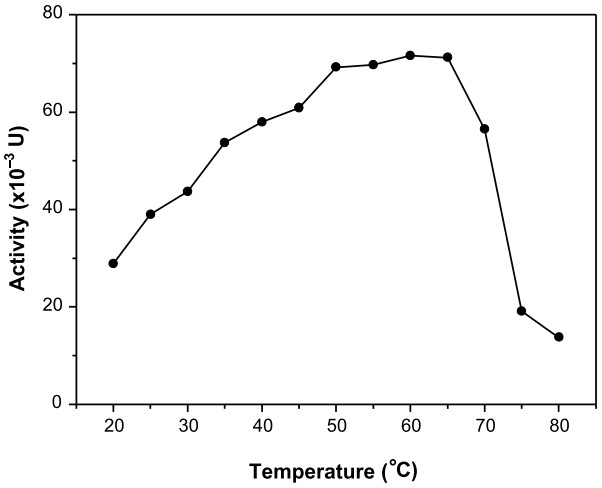


FIGURE 5 Effect of temperature on the activity of immobilised enzyme determined in 100 mM PIPES buffer (pH 8.0) containing 1 mM D-phenylglycine and 1 mM α-ketoglutarate.

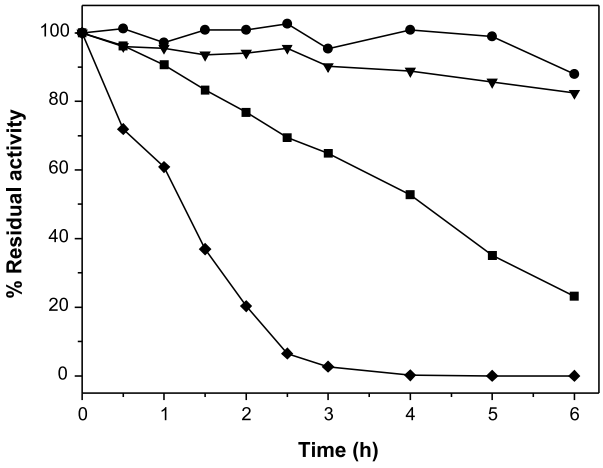


FIGURE 6 Effect of temperature on the stability of immobilised enzyme. Residual activities were determined periodically after the incubation of enzyme in 100 mM PIPES buffer, pH 8.0 at 37 (●), 40 (▼), 50 (■) and 60°C (◆).

where the enzyme was most active, no residual activity was found after incubation for 4 h. Therefore, the optimal temperature to carry out the reaction is 37°C, and this was used for all subsequent experiments.

Effect of Substrate Concentration on Enzyme Activity

In order to select a suitable substrate supply method, the effect of substrate concentration on enzyme activity was studied. At low concentrations, benzoylformate showed a notable inhibitory effect towards the enzyme activity, while L-glutamate showed an increase in reaction rate up to 1.25 M (Fig. 7). At higher L-glutamate concentrations, the reaction rate fell sharply indicating inhibition.

Adsorption of the Substrates and Products onto the Resin

Various ionic and non-ionic resins were examined for their capability to adsorb the substrates and products, as a potential means of controlled-release biocatalysis and/or potentially *in-situ* product removal (Lye and Woodley, 1999). Of those resins tested, Amberlite IRA-400 showed promising characteristics and was the most suitable for the system. At the pH at which the synthesis reaction was conducted (pH 7.5), the adsorption of L-glutamate as well as the desired product D-phenylglycine onto Amberlite IRA-400 was very low even after 12 h of incubation (Fig. 8). By contrast, the resin can efficiently adsorb benzoylformate, the substrate whose concentration should be kept low in the reaction to minimise the substrate inhibitory effect. In addition, Amberlite IRA-400 adsorbs α-ketoglutarate well, which may thus simultaneously help remove this by-product while the reaction is in progress (as a form of *in-situ* product removal).

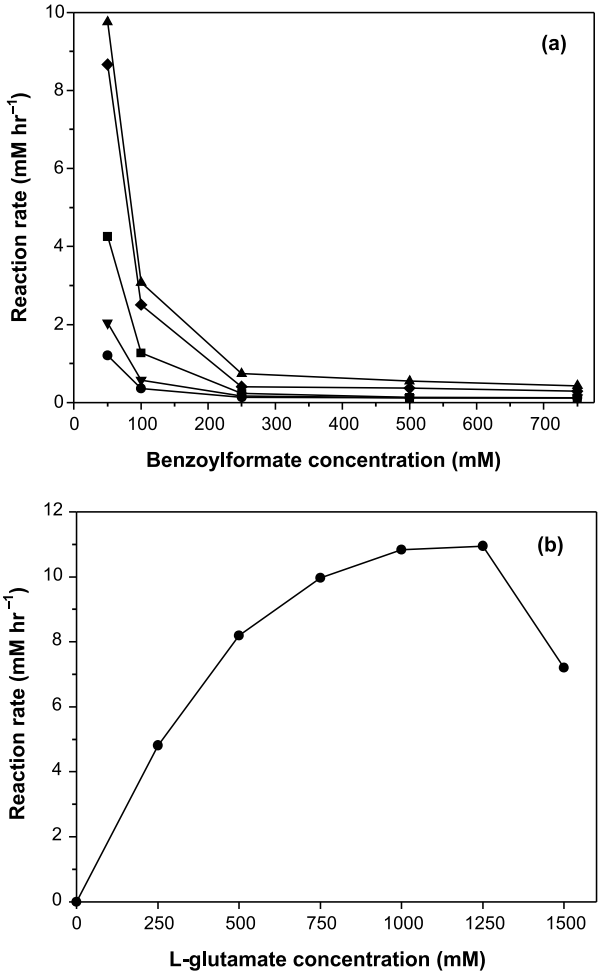


FIGURE 7 Effect of substrate concentration on the D-phenylglycine synthesis rate. (a) Effect of benzoylformate at 50 (●), 100 (▼), 250 (■), 500 (◆) and 750 mM (▲) L-glutamate concentrations. (b) Effect of L-glutamate concentration at 50 mM benzoylformate.

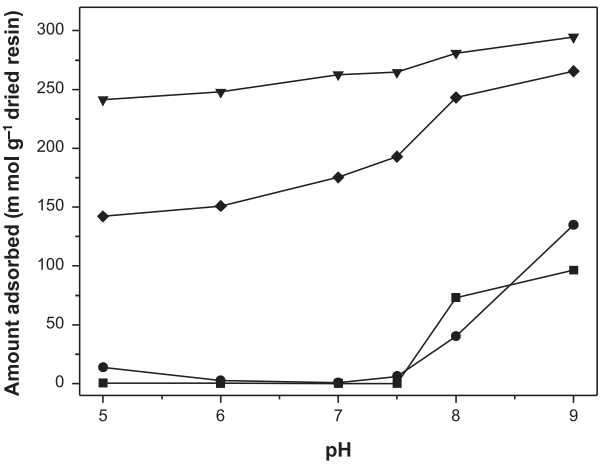


FIGURE 8 Adsorption to Amberlite IRA-400 under agitation at 200 rpm, 37°C with 30 mM solution of D-phenylglycine (■), L-glutamate (●), α-ketoglutarate (◆) and benzoylformate (▼).

Use of the Amberlite IRA-400 Resin-adsorbed Benzoylformate as a Controlled-release Substrate

Amberlite IRA-400 resin-adsorbed benzoylformate was prepared in order to supply substrate via a controlled-release mechanism to the enzyme in the synthesis. Using the previously described methods, one gram of adsorbed resin releases approximately one mmole of benzoylformate at pH 7.5. The effect on D-phenylglycine synthesis was studied when different amounts of adsorbed resin were used. Clearly the addition of 0.05–0.4 g benzoylformate-adsorbed resin in a 1 ml reaction enhanced both the D-phenylglycine synthesis rate and yield, compared to the reaction without the resin (Fig. 9). When smaller amounts of adsorbed resin were used, the initial rate of reaction was faster but D-phenylglycine synthesis ceased earlier, possibly due to the low residual benzoylformate which was needed to drive the reaction. The rate of release is such that the benzoylformate concentration in solution is controlled. This does not necessarily imply 'slow-release' of the substrate as observed by some other workers (e.g. Houng and Liau, 2003). The amount of benzoylformate in solution is entirely dependent on the intrinsic rate of release from the resin and the rate of reaction. This is clearly seen in Fig. 9b where adjustments to the rate of release (via resin loading per unit volume) at constant enzyme concentration affect the amount of benzoylformate in solution and hence reaction rate (Fig. 9a). Hence the rate is a function of the amount of resin added passing through an optimum at 0.05 g. The yield is also a function of the amount of resin added and passes through an optimum at 0.2 g. At higher values the rate is reduced and therefore the reaction may not have reached the endpoint in the 12h of experimental measurement. Similarly results have been observed for yeast reductions in the presence of varying amounts of resin-adsorbed substrate (D'Arrigo *et al.*, 1998). Whenever D-phenylglycine was formed at a concentration greater than 5 mg/ml (approximate aqueous solubility at pH 7.5, 37°C), solid product conspicuously precipitated in the reaction mixture, acting as a spontaneous *in-situ* product removal. After 6 h, the reaction with 0.1 g resin-adsorbed benzoylformate gave the highest D-phenylglycine amount of 10.25 mg/ml (68% yield) while the reaction in the absence of resin yielded only 2.73 mg/ml (16% yield). Thus, the addition of adsorbed resin can be used to reduce the inhibitory effect of benzoylformate. Similar reaction profiles were obtained from the reaction with 0.5 g-resin added and without resin, although the initial concentrations of benzoylformate in both reactions were 158 and 110 mM, respectively.

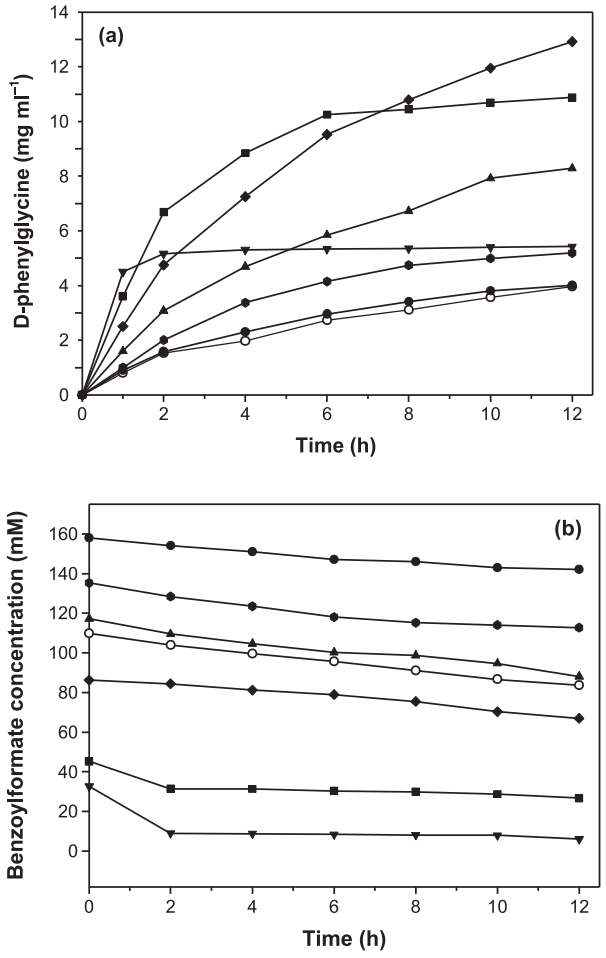


FIGURE 9 Effect of the amount of resin-adsorbed benzoylformate on the reaction. (a) Effect on D-phenylglycine synthesis in 1 ml-reaction and (b) effect on benzoylformate concentration in the supernatant. Amounts of resin used: 0 (○), 0.05 (▼), 0.1 (■), 0.2 (◆), 0.3 (▲), 0.4 (●) and 0.5 g (●).

CONCLUSIONS

A method has been proposed to overcome the severe substrate inhibition of benzoylformate on a recombinant D-phenylglycine aminotransferase. Without optimisation, using a resin-adsorbed substrate, a four-fold improvement in product concentration obtainable was achieved. This confirms previous reports of the usefulness of resins in the controlled release method of supplying substrates to improve biocatalytic productivity.

Acknowledgements

The authors are grateful for financial support from the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (Grant No. PHD/00222/2541) to one of us (T.R.) to enable this work to be undertaken at the Department of Biochemical Engineering, University College London.

References

Aida, K., Chibata, I., Nakayama, K. and Yamada, H. (1986) Microbial production of D-p-hydroxyphenylglycine, *Prog. Ind. Microbiol.* **24**, 269–279.

Basso, A., Braiuca, P., De Martin, L., Ebert, C., Gardossi, L. and Linda, P. (2000) D-phenylglycine and D-4-hydroxyphenylglycine methyl esters via penicillin G acylase catalysed resolution in organic solvents, *Tetrahedron: Asymmetry* **11**, 1789–1796.

Beard, T.M. and Page, M.I. (1998) Enantioselective biotransformation using Rhodococci, *Antonie van Leeuwenhoek* **74**, 99–106.

Calmes, M., Danunis, J., Mai, N. and Natt, F. (1996) A convenient synthesis of optically active phenylglycine, *Tetrahedron Lett.* **37**, 379–380.

D'Arrigo, P., Fantoni, G.P., Servi, S. and Strini, A. (1997) The effect of absorbing resins on substrate concentration and enantiomeric excess in yeast reduction, *Tetrahedron: Asymmetry* **8**, 2375–2379.

D'Arrigo, P., Fuganti, C., Fantoni, G.P. and Servi, S. (1998) Extractive biocatalysis: a powerful tool in selective control in yeast biotransformations, *Tetrahedron* **54**, 15017–15026.

Garcia, M.J. and Azerad, R. (1997) Production of ring-substituted D-phenylglycines by microbial or enzymatic hydrolysis/deracemisation of the corresponding DL-hydantoins, *Tetrahedron: Asymmetry* **8**, 85–92.

Gokhale, D.V., Bastawde, K.B., Patil, S.G., Kalkote, R.R., Joshi, R.R., Joshi, R.A., Ravidranathan, T., Gaikwad, B.G., Jagdand, U.V. and Nene, S. (1996) Chemoenzymatic synthesis of D(-)phenylglycine using hydantoinase of *Pseudomonas desmolyticum resting cells*, *Enzyme Microb. Technol.* **18**, 353–357.

Houng, J.Y. and Liau, J.S. (2003) Applying slow-release biocatalysis to asymmetric reduction of ethyl 4-chloroacetoacetate, *Biotechnol. Lett.* **25**, 17–21.

Laowanapiban, P. (2001) D-phenylglycine aminotransferase from *Pseudomonas stutzeri* ST-201: gene cloning, sequence analysis, comparative structural modeling and over-expression in *Escherichia coli* Ph.D. Thesis, Mahidol University (Bangkok, Thailand).

Lye, G.J. and Woodley, J.M. (1999) Application of in situ product-removal techniques to biocatalytic processes, *Trends Biotechnol.* **17**, 395–402.

Sheldon, R.A. (1996) Chirotechnology: designing economic chiral syntheses, *J. Chem. Tech. Biotechnol.* **67**, 1–14.

Simpson, H.D., Alphand, V. and Furstoss, R. (2001) Microbiological transformations 49. Asymmetric biocatalysed Baeyer-Villiger oxidation: biocatalyst in the presence of adsorbent resin, *J. Mol. Catal. B: Enzymatic* **16**, 101–108.

Vicenzi, J.T., Zmijewski, M.J., Reinhard, M.R., Landen, B.E., Muth, W.L. and Marler, P.G. (1997) Large scale stereoselective enzymatic ketone reduction with in-situ product removal via polymeric adsorbent resins, Enzyme Microb, *Technol.* **20**, 494–499.

Voyer, N., Roby, J., Chenard, S. and Barberis, C. (1997) Enantioselective synthesis of phenylglycines using (-)sparteines-BuLi complex, *Tetrahedron Lett.* **38**, 6505–6508.

Wiyakrutta, S. and Meevootisom, V. (1997) A stereo-inverting D-phenylglycine aminotransferase from Pseudomonas stutzeri ST-201: purification, characterization and application for D-phenylglycine synthesis, *J. Biotechnol.* **55**, 193–203.

Woodley, J.M. and Lilly, M.D. (1992) Process engineering of two-liquid phase biocatalysis, *Prog. Biotechnol.* **8**, 147–154.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Analytica Chimica Acta 520 (2004) 133–139
ANALYTICA CHIMICA ACTA
www.elsevier.com/locate/aca

Spectrophotometric enzymatic cycling method using L-glutamate dehydrogenase and D-phenylglycine aminotransferase for determination of L-glutamate in foods

Wanida Khampha, Vithaya Meevootisom, Suthep Wiyakrutta*

Department of Microbiology, Faculty of Science, Mahidol University, Rama VI Road, Bangkok 10400, Thailand

Received 10 December 2003; received in revised form 6 May 2004; accepted 7 May 2004

Available online 13 July 2004

Abstract

This report describes a new spectrophotometric method capable of determining low levels of L-glutamate. The assay is based on substrate cycling between L-glutamate dehydrogenase (GlDH) and the novel enzyme D-phenylglycine aminotransferase (D-PhgAT). In this system, GlDH converts L-glutamate to 2-oxoglutarate with concomitant reduction of NAD^+ to NADH. The 2-oxoglutarate is recycled to L-glutamate in a transamination reaction catalyzed by D-PhgAT using D-4-hydroxyphenylglycine as an amino donor, which is converted to 4-hydroxybenzoylformate. Both NADH and 4-hydroxybenzoylformate strongly absorb UV light at 340 nm ($\varepsilon_{340\,nm} = 6.22 \times 10^3$ and $8.90 \times 10^3\,l\,mol^{-1}\,cm^{-1}$, respectively). The signal amplification effect of the cycling reactions is thus further enhanced by the combined absorption of the two accumulating reaction products. The standard calibration curve for L-glutamate was linear from 0.2 to 20 μM, with a detection limit of 0.14 μM. Food samples can be significantly diluted before subjected to the assay, thus reducing the effects of interfering substances. Because of the unique substrate specificity of D-PhgAT, L-glutamate could be selectively determined in the presence of other common amino acids at relatively high concentrations. The assay was satisfactorily applied to measure L-glutamate in various kinds of food products. The procedure is simple, rapid, accurate, and should be easily automated.

Keywords: D-Phenylglycine aminotransferase; Enzymatic substrate cycling assay; Food; Glutamate

1. Introduction

Seasonings that are rich in free L-glutamate have, due to their appetite-enhancing effects, a long history of use in foods since the time of Greek and Roman civilizations and ancient Asia. At present, the purified monosodium glutamate (MSG), a sodium salt form of L-glutamic acid, is used worldwide as an additive to enhance the flavour of many foodstuffs. L-glutamic acid as a component of a mixture of amino acids and small peptides obtained from acid- or enzymatichydrolysis of proteins is also used for the same purpose. Nevertheless, there is an ongoing debate whether MSG is responsible for a complex of symptoms initially described as Chinese restaurant syndrome [1] (CRS). More recently it is referred to as MSG symptom complex as it is known to occur in certain groups of people after ingestion of food containing high amount of MSG. In addition to the MSG symptom complex, ingestion of MSG has been alleged to cause or exacerbate numerous conditions, including asthma, urticaria, atopic dermatitis, ventricular arrhythmia, neuropathy and abdominal discomfort [2]. However, the US Food and Drug Administration (FDA) has classified MSG as generally recognized as safe (GRAS) and the acceptable daily intake (ADI) for MSG is not specified.

In Thailand and other Southeast Asian countries, large volumes of fish sauces are produced for local consumption and for export each year. Accurate determination of L-glutamate to monitor the fermentation process and as an essential part of the quality control of fish sauce products has proved to be difficult. The presence of relatively large amounts of other amino acids and the high salt concentration usually interfere with L-glutamate assay methods currently commercially available. Thus, the development of an improved selective, sensitive, and simple assay for measuring

* Corresponding author. Tel.: +66 0 2201 5536; fax: +66 0 2644 5411.
E-mail address: scsvy@mahidol.ac.th (S. Wiyakrutta).

0003-2670/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2004.05.044

L-glutamate is of importance to the fish sauce industry. It will as well benefit other food industries where the free L-glutamate content in the products needs to be quantified.

Among several methods for the determination of L-glutamate that have been published, those based on the enzymatic recycling of the substrate (L-glutamate) have been shown to be superior in terms of enhanced sensitivity [3,4]. These assays have been performed in different measurement modes and assay formats, e.g. spectrophotometeric methods [3], fluorometric methods [4], enzyme based electrode [5,6], and flow injection analysis (FIA) [7–9]. Substrate cycling assays have also been applied for the determination of other amino acids such as L-phenylalanine where the sensitivity was reported to be improved 50-fold [10]. It should however be noted that in these methods there is only one accumulating reaction product (usually NADH) that yields the analytical signal, which is measured either directly or coupled to an auxiliary enzyme reaction in order to generate a measurable product [3,4].

In the present paper, we report the development of a new L-glutamate assay method that operates on the substrate cycling principle and generates two accumulating products, which strongly absorb UV light at the same wavelength. The signal amplification effect of the cycling reactions is thus further enhanced.

2. Experimental

2.1. Chemicals and reagents

The following reagents were of the highest grade from Sigma (St. Louis, MO): L-glutamic acid, monosodium salt; β-NAD^+; pyridoxal-5′-phosphate (PLP); D-phenylglycine; D-4-hydroxyphenylglycine; 2-oxoglutaric acid; tris(hydroxymethyl) aminomethane (Tris); benzoic acid; and potassium sorbate. Methyl-*p*-hydroxzybenzoate and L-glutamate dehydrogenase (GlDH) (EC 1.4.1.3) from bovine liver were from Fluka. The L-glutamic acid assay kits were from Boehringer Mannheim. D- and L-amino acids used in the interference studies were from Sigma. D-phenylglycine aminotransferase (EC 2.6.1.72) was purified from a recombinant *E. coli* expressing the cloned gene encoding the D-PhgAT from *Pseudomonas stutzeri* ST201 as previously described [12]. Molar absorption coefficients at 340 nm ($\varepsilon_{340\,nm}$) of NADH and 4-hydroxybenzoylformate in 0.1 M Tris–HCl pH 7.5, determined from the appropriately diluted solutions of each compound, were 6.22×10^3 and $8.90 \times 10^3\,l\,mol^{-1}\,cm^{-1}$, respectively.

2.2. D-Phenylglycine aminotransferase (D-PhgAT) activity assay

D-PhgAT activity of the purified enzyme preparation was determined using a spectrophotometric enzyme kinetic assay by measuring the rate of benzoylformate formation upon transamination of D-phenylglycine with 2-oxoglutarate as an amino acceptor. A reaction mixture containing 0.1 M Tris–HCl pH 7.5, 10 mM D-phenylglycine, 25 mM 2-oxoglutarate, 5 μM PLP, and 5 μM EDTA was pre-incubated at 30 °C in a water bath. A 980 μl aliquot of the mixture was added into a standard 10 mm quartz cuvette equilibrated at 30 °C in a temperature controlled cell compartment of a spectrophotometer (BioSpec-1601, Shimadzu). The reaction was started by adding 20 μl of an appropriately diluted D-PhgAT solution and rapidly mixed with the reaction mixture. The rate of benzoylformate formation was determined by monitoring the increment of UV absorption at 254 nm for 180 s and the initial linear rate of the reaction was calculated. One unit of the D-PhgAT activity was defined as the amount of enzyme capable of producing one micromole of benzoylformate per minute under the defined condition.

2.3. Determination of L-glutamate by the enzymatic cycling assay

GlDH and D-PhgAT working solutions were freshly prepared and their activities were determined shortly before use. The reagent mixture solution for the cycling reaction was composed of 0.1 M Tris–HCl pH 7.5, 8 mM D-4-hydroxyphenylglycine, 2 mM NAD^+, $2\,U\,ml^{-1}$ GlDH, $0.1\,U\,ml^{-1}$ D-PhgAT, and L-glutamate (standard or sample). The solution containing all the components except NAD^+ was equilibrated at 30 °C in a standard 10 mm quartz cuvette in a temperature controlled spectrophotometer (BioSpec-1601, Shimadzu). The reaction was started by adding NAD^+ to the pre-warmed reaction solution with rapid mixing, and this also brought all the components of the solution to their final concentration as stated above. The time course of the increment in the UV absorbance at 340 nm due to the continuous accumulation of the reaction products, NADH and 4-hydroxybenzoylformate, was recorded. The slope of the progress curve was estimated by the least square linear regression analysis of 21 data points using the kinetics software program of the spectrophotometer. The overall reaction velocity was calculated as the rate of absorbance change per unit time ($\Delta A\,min^{-1}$). The L-glutamate concentration range suitable for preparing a standard calibration curve was determined.

2.4. Measurement of L-glutamate in food samples

All food products tested in this report were obtained from grocery stores in Bangkok. Fish sauce and soy sauce samples were diluted with sterile distilled water before subjected to analysis. Ready-made seasoning cubes, which are in solid form and are composed of fats, were pre-treated to completely dissolve L-glutamate from the matrix. One gram of the samples was accurately weighed and dissolved in a measured volume of distilled water, then heated at ∼70 °C for 10 min, cool to room temperature and the

solution was clarified by filtering through a filter paper (Whatman no. 1).

For the determination of L-glutamic acid content using the Boehringer Mannheim L-glutamic acid assay kit which is based on a spectrophotometric end-point analysis [13], samples were diluted to contain estimated amount of 70 mg L-glutamic acid per liter (~0.48 mM) before submitting to the assay according to the manufacturer's instructions. The same set of the samples was further diluted 10-fold to contain an estimated amount of 7 mg L-glutamic acid per liter (~48 μM) and submitted to the determination of L-glutamic acid content using the GlDH/D-PhgAT substrate cycling assay method. The assay was carried out as described in Section 2.3. The cycling reaction solution was freshly prepared on a daily basis and the same batch was used for both preparation of a standard calibration curve and assay of the samples. Results obtained from the two methods were compared.

3. Results and discussion

3.1. The GlDH/D-PhgAT substrate cycling system

The GlDH/D-PhgAT substrate cycling system for the determination of L-glutamate is shown in Fig. 1. The analyte L-glutamate is first converted to 2-oxoglutarate and ammonia by the action of GlDH with a concomitant reduction of NAD^+ to NADH. The 2-oxoglutarate is recycled back into L-glutamate in the transamination reaction catalyzed by D-PhgAT using D-4-hydroxyphenylglycine as an amino donor, which itself is converted to 4-hydroxybenzoylformate. Both NADH and 4-hydroxybenzoylformate strongly absorb UV light at 340 nm. By this method, the signal amplification effect of the cycling reactions is further enhanced by the combined absorption of the two continuously accumulating reaction products. Additionally, in each round of the cycle, one molecule of ammonium ion produced in the GlDH reaction was neutralized by one molecule of 4-hydroxybenzoylformate formed in the D-PhgAT reaction thus keeping the pH of the reaction medium relatively constant.

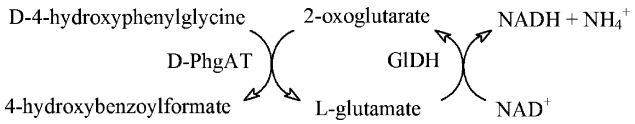


Fig. 1. Reaction scheme of the GlDH/D-PhgAT substrate cycling system for the determination of low levels of L-glutamate. Each turn of the cycle generates one molecule each of NADH and 4-hydroxybenzoylformate. These two continuously accumulating products strongly absorb UV light at 340 nm.

To demonstrate the degree of signal amplification of the system, the absorbance–time courses obtained from the determination of 5 μM level of L-glutamate using the cycling and the non-cycling systems were compared. The cycling assay was performed as described in Section 2.3. The non-cycling assay was carried out similarly but in the absence of D-4-hydroxyphenylglycine, thus only the GlDH reaction was in effect. The latter assay system was essentially

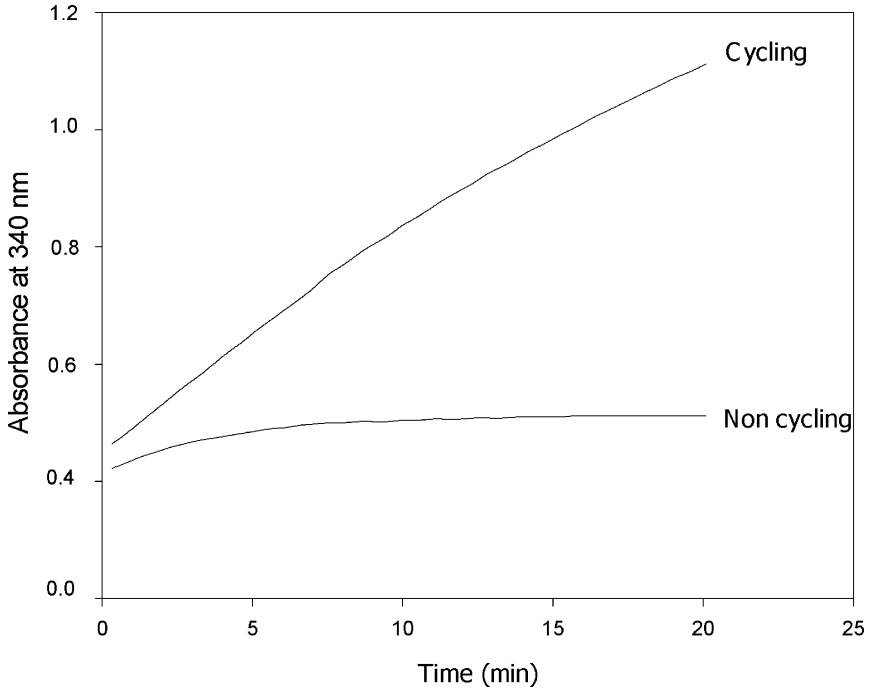


Fig. 2. Comparison between cycling and non-cycling reactions for the L-glutamate assay. The cycling assay was performed using the standard cycling reaction mixture (for details, see text). In the non-cycling system, D-4-hydroxyphenylglycine was omitted.

the end-point method in which the reaction was allowed to proceed until all the L-glutamate was consumed and an equal amount of NADH that gave rise to the analytical signal was produced. As shown in Fig. 2, the absorbance in the non-cycling system increased slowly, reaching a plateau after 20 min where almost all the L-glutamate was consumed and a limited total absorbance change could be measured. By contrast, the cycling system gave much higher signal in a much shorter period of time. The system quickly attained a steady state where the GlDH and D-PhgAT reactions proceeded at a constant rate (in the opposite direction). This was demonstrated by the linear absorbance–time curve that began promptly after mixing in the last substrate and it persisted until the absorbance change (ΔA) reached 0.3 AU. The slope of the curve during this period was proportional to the reaction rate at the steady state and thus to the amount of L-glutamate in the system. For the L-glutamate concentration of 5 μM the linear period persisted for 7 min before the ΔA of 0.3 AU was reached. Because the slope of the curve, and not the absorbance end-point, was taken for calculation, we found that monitoring the absorbance for 3 min gave satisfactory assay results for L-glutamate concentration at this level.

3.2. Optimization of the cycling reaction

The enzymatic cycling assay method was first optimized to establish the suitable substrate concentrations for the system. Varied concentrations of D-4-hydroxyphenylglycine from 0 to 25 mM were tested, with the NAD^+ and L-glutamate concentrations fixed at 2 mM and 5 μM, respectively. The result is shown in Fig. 3A. Increasing the D-4-hydroxyphenylglycine concentration in the sub-mM region resulted in a sharp rise in the cycling reaction velocity reaching a maximum at the D-4-hydroxyphenylglycine of 1 mM. At higher D-4-hydroxyphenylglycine concentrations the reaction rate slowly decreased, possibly due to substrate inhibition effect on the D-PhgAT. D-4-hydroxyphenylglycine at the concentration of 8 mM was selected because fluctuation of concentration around this value did not affect the cycling reaction rate. For NAD^+, its concentration was varied from 0 to 8 mM, with the D-4-hydroxyphenylglycine and L-glutamate fixed at 8 mM and 10 μM, respectively. The result is shown in Fig. 3B. The concentration of 2 mM NAD^+ was chosen since it gave a relatively high reaction velocity while the background absorption of the reaction solution was not too high (<0.5 AU).

Optimal ratio between the GlDH and D-PhgAT as well as optimal amounts of the two enzymes for the reaction were investigated using the reaction mixture that contained 8 mM D-4-hydroxyphenylglycine, 2 mM NAD^+ and 5 μM L-glutamate. Under this condition, the GlDH:D-PhgAT activity ratio of 20:1 was found to be the combination that gave the maximum cycling reaction rate (data not shown). Using GlDH and D-PhgAT at the concentrations of 2 and 0.1 U ml^{-1}, respectively, resulted in a satisfactorily rapid reaction rate, and a large absorbance change could be obtained within 3 min.

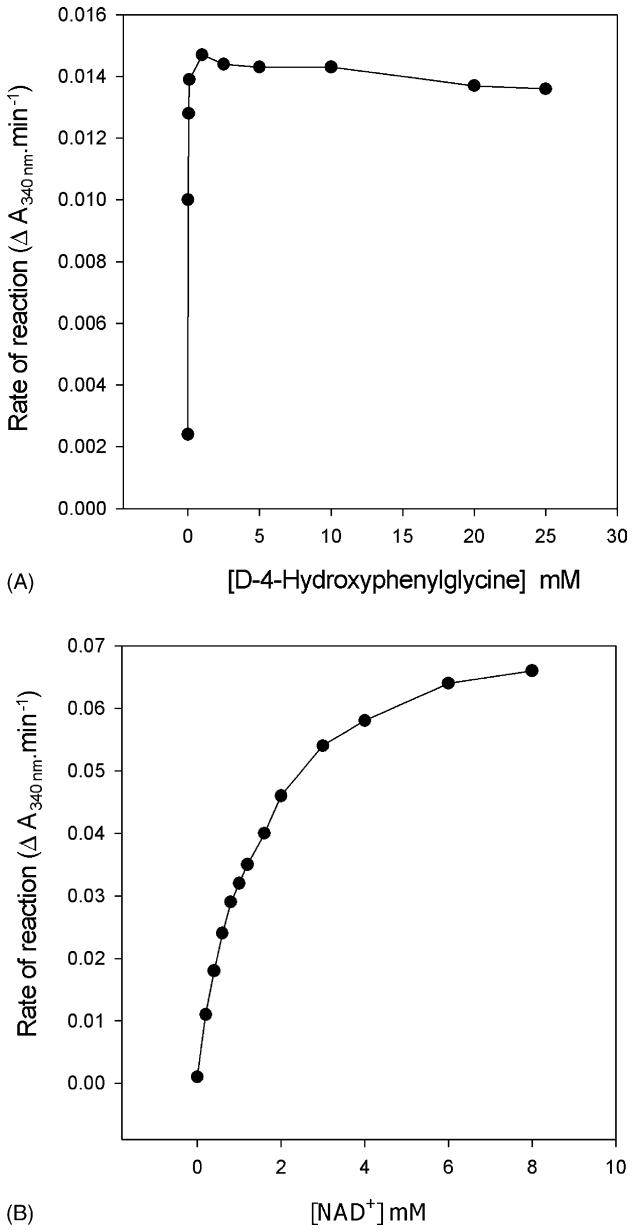


Fig. 3. Effect of substrate concentration on the rate of the cycling reaction: (A) D-4-hydroxyphenylglycine, (B) NAD^+ (see Section 3.2 for details).

The GlDH/D-PhgAT L-glutamate cycling activity was favoured by alkaline pH. However, at pH 8.0 or higher, the background UV absorption at 340 nm of the reaction mixture was undesirably high. At pH 7.5, the background absorption of the solution decreased significantly to an acceptable limit while the reaction rate was still high. A pH of 7.5 was thus chosen as optimal for this assay method.

3.3. Linear range and sensitivity of the assay

Fig. 4 shows the sensitivity and linearity of this enzymatic cycling system for the determination of low levels of

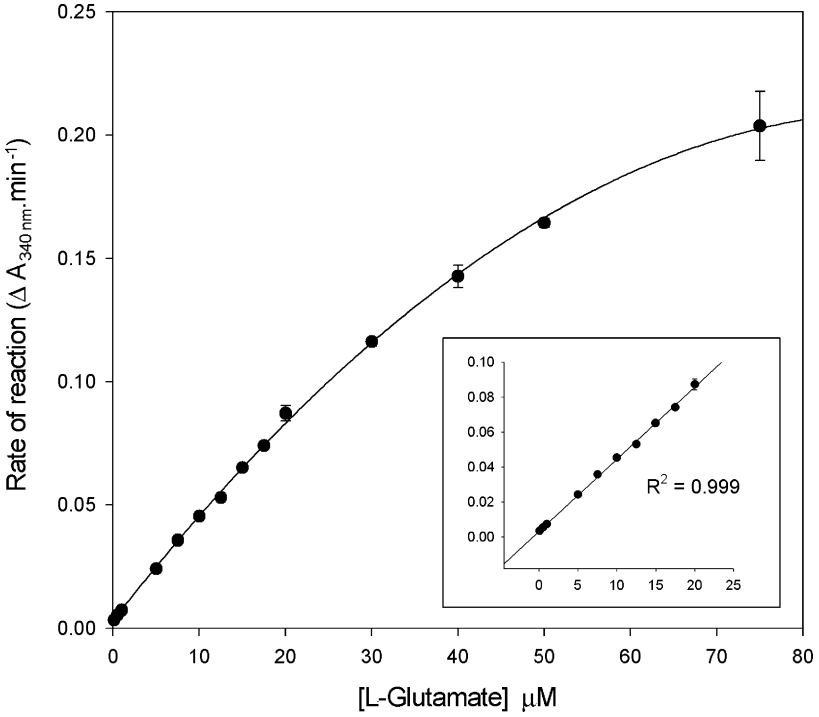


Fig. 4. Cycling assay standard calibration curve for L-glutamate; conditions as described in Section 2.3. Each point is the mean value from triplicate assays; the error bar represents the standard deviation.

L-glutamate. Using the cycling reaction condition as mentioned in Section 3.2, excellent linear relationship (r^2 = 0.999) between the reaction rate and the concentration of L-glutamate over the range of 0.2–20 μM was obtained. The lower limit of detection for L-glutamate, calculated as a signal-to-noise ratio of 3, was found to be 0.14 μM with a relative standard deviation of 5.1 (10 replicates). By contrast, the L-glutamic acid assay kit from Boehringer Mannheim is useful for measuring L-glutamate in the range of 1.36–48 μM and the detection limit is 1.36 μM [13]. Thus the GlDH/D-PhgAT cycling assay is ∼10 times more sensitive than the Boehringer Mannheim method. The lower limit of detection and sensitivity of this cycling assay can still be further improved by using higher amounts of the cycling enzymes in the reaction or by extending the reaction time, or both. The reaction mixture of the present cycling assay contained 2 U GlDH and 0.1 U D-PhgAT per ml, and the progress of the reaction was monitored for 3 min. In comparison, 1 ml of the Boehringer Mannheim assay reaction contained 9 U GlDH and 0.1 U diaphorase while the reaction time was approximately 15 min. Theoretically, if the amounts of GlDH and the D-PhgAT in the cycling assay are increased to 9 and 0.45 U, respectively, and the reaction time is extended to 15 min, we would expect 22.5 times further improvement in the sensitivity of this method, making it 225 times more sensitive than the Boehringer Mannheim method. The present cycling system can hence be flexibly adjusted to obtain the required degree of sensitivity suitable for particular applications, providing that the progress curve during the course of measurement is within the linear range. Thus, this method should be readily adaptable for measuring L-glutamate at sub-micromolar levels such as in biological and clinical samples [4]. Using more enzymes or extending the reaction time will not improve the lower limit of detection or sensitivity of the Boehringer Mannheim assay method since it is based on the end-point measurement.

The reproducibility of the assay method was evaluated by 15 replicate measurements of solutions containing 10 and 2 μM L-glutamate. The relative standard deviations were found to be 1.8 and 2.7%, respectively.

3.4. Specificity and interference

Recovery analysis was carried out in order to study the specificity of the assay and the possible interference from other L- and D-amino acids commonly present together with L-glutamic acid in foods. A solution containing 10 μM L-glutamate and 10 μM of the amino acid in question was subjected to the cycling assay for L-glutamate. The analytical signal obtained was compared with the signal obtained when the solution containing only 10 μM L-glutamate was assayed. As shown in Table 1, very little interference from all the D- and L-amino acids tested was observed indicating a high degree of specificity of the method. Regarding interference from other amino acids, the GlDH/D-PhgAT cycling assay for L-glutamate has a better specificity than the cycling methods using glutamate oxidase with glutamic-oxalacetic transaminase [14], glutamate oxidase with glutamic-pyruvic transaminase [3,4], and GlDH with glutamic-pyruvic transaminase [4]. The

Table 1
Effects of D- and L-amino acids on the GlDH/D-PhgAT cycling assay for L-glutamate

Amino acid	Recovery (%)[a]	Amino acid	Recovery (%)[a]
Glycine	97.94 ± 1.47	D-Glutamate	98.61 ± 0.63
L-Alanine	99.80 ± 1.92	D-Alanine	95.21 ± 0
L-Arginine	101.69 ± 2.21	D-Arginine	98.98 ± 0.62
L-Aspartate	101.77 ± 0.72	D-Aspartate	98.75 ± 2.32
L-Histidine	97.94 ± 1.47	D-Histidine	100.29 ± 1.97
L-Isoleucine	101.45 ± 2.96	D-Isoleucine	95.965 ± 0.21
L-Leucine	96.66 ± 1.45	D-Leucine	97.49 ± 3.33
L-Lysine	96.66 ± 0.53	D-Lysine	97.98 ± 1.30
L-Methionine	105.45 ± 0.76	D-Methionine	96.91 ± 0.91
L-Phenylalanine	102.06 ± 0.96	D-Phenylalanine	98.83 ± 2.46
L-Serine	99.80 ± 1.72	D-Serine	99.62 ± 1.92
L-Threonine	98.50 ± 1.56	D-Threonine	99.91 ± 3.53
L-Tyrosine	100.12 ± 2.55	D-Tyrosine	101.81 ± 0.90
L-Valine	98.98 ± 1.57	D-Valine	97.40 ± 2.39
L-Proline	102.54 ± 2.77	D-Asparagine	99.06 ± 1.97
L-Cysteine	98.67 ± 1.68	D-Glutamine	103.11 ± 2.30
		D-Tryptophan	99.56 ± 2.39

[a] Analytical signals from the assay of 10 μM L-glutamate in the absence (S_0) and presence (S) of 10 μM of the tested amino acid were measured. Recovery was calculated from $(S/S_0) \times 100$. The values given are from triplicate determinations presented as mean ±SD.

Table 2
Effects of commonly used food additives on the GlDH/D-PhgAT cycling assay for L-glutamate

Substance	Recovery (%)[a]
100 μM NaCl	101.30 ± 1.49
10 μM Benzoate	101.18 ± 1.41
10 μM Sorbate	98.03 ± 0.93
10 μM L-Ascorbic acid	103.35 ± 2.41
10 μM Succinate	99.42 ± 1.53
10 μM Citrate	97.38 ± 0.62
10 μM Acetate	96.07 ± 2.47
10 μM Methyl paraben	99.13 ± 0

[a] Analytical signals from the assay of 10 μM L-glutamate in the absence (S_0) and presence (S) of the tested substance at the stated concentration were measured. Recovery was calculated from $(S/S_0) \times 100$. The values given are from triplicate determinations presented as mean ±SD.

improved specificity of the present method is due mainly to the highly substrate specific property of the D-PhgAT [11] whose substrate is D-4-hydroxyphenylglycine, the D-amino acid rarely encountered in nature. The method should be useful for determination of L-glutamate in samples that contain other amino acids in comparatively high concentrations such as protein hydrolysates and particularly the fish sauces produced in Southeast and East Asian countries [15].

Effects of the commonly used food additives and preservatives on the assay method were also investigated. As shown in Table 2, no significant interference was observed.

3.5. Application to analysis of food samples

Samples of fish sauces, soy sauces, and ready-made seasonings were subjected to the assay for L-glutamate content by both the present GlDH/D-PhgAT cycling method and the

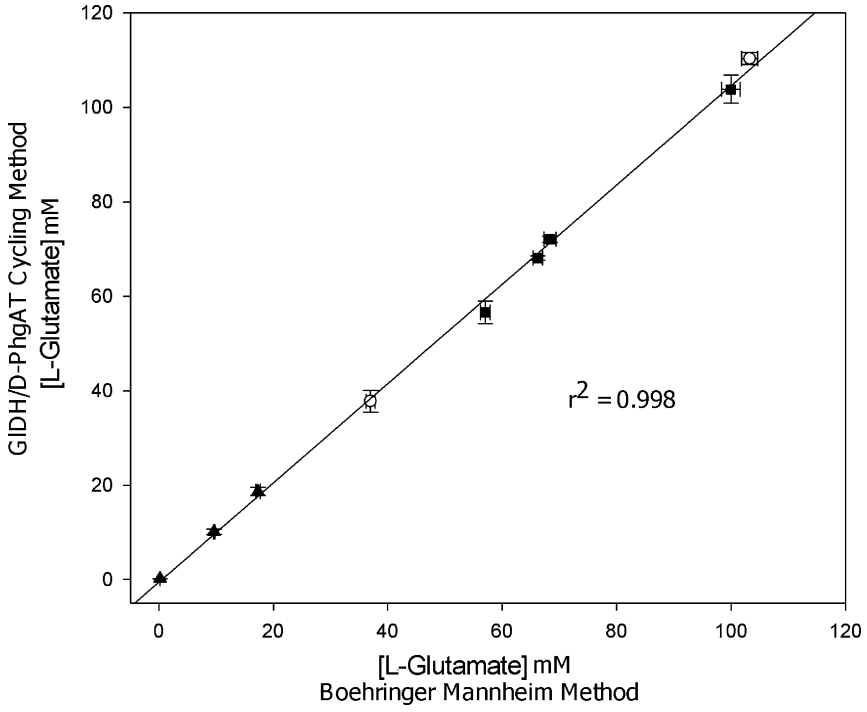


Fig. 5. Comparison of L-glutamate levels in nine food samples as determined by the GlDH/D-PhgAT cycling method and the Boehringer Mannheim L-glutamic acid assay kit. Each point is the mean value from triplicate assays; the error bar represents the standard deviation. (▲) Ready-made seasonings; (○) soy sauces; (■) fish sauces.

Boehringer Mannheim L-glutamic acid assay method. As shown in Fig. 5, the results obtained from the two methods were in good agreement ($r^2 = 0.998$).

Because the GlDH/D-PhgAT cycling method has a working range at 0.2–20 μM of L-glutamate concentration, average food samples can be diluted several folds before subjected to the assay. In the case of Thai fish sauces that contain around 14.89 g l^{-1} [15] or 80 mM of free glutamate, the sample could be diluted 1000-fold. This has an advantage since the substances that contribute to the background absorption or potentially interfere with the assay reaction were significantly diluted out thus minimizing their interfering effects.

4. Conclusions

The convenient utilization of the novel enzyme D-PhgAT in combination with GlDH gave rise to a substrate cycling assay for L-glutamate with improved performance. The proposed assay provided an alternative mean for L-glutamate determination with some advantages as compared with the previous substrate cycling methods [3,4,14]. Firstly, the sensitivity of the method was enhanced because two instead of normally one accumulating products of the cycling reaction, NADH and 4-hydroxybenzoylformate, strongly absorb UV light at 340 nm. This means that less amount of the cycling enzymes and/or less measurement time is required to obtain the desired level of analytical signal. Secondly, the GlDH/D-PhgAT cycling assay was shown to be more specific than in the previous methods with respect to interference from other amino acids commonly present in foods. This could be achieved because of the high substrate selectivity of the D-PhgAT [11]. Lastly, this method is simple using only two cycling enzymes without the need for a third coupled enzyme and the chromogenic or fluorogenic substrates to generate the measurable analytical signal from the product of the cycling reaction like in the previous methods [3,4,14]. Consequently, the first together with the last advantage mentioned above might have significant effect in reducing the cost of manufacturing of an assay kit based on this proposed method.

The GlDH/D-PhgAT cycling assay was successfully applied to analyze L-glutamate in food products yielding the results in good agreement with the current commercially available assay kit. The method should be particularly useful for determination of L-glutamate in samples that contain large amounts of other amino acids. Adaptations of this method into automated assay formats such as flow injection analysis and enzyme chips are under investigation.

Acknowledgements

The authors wish to thank Associate Professor Jenny Emnéus, Lund University, and Professor T.W. Flegel, Mahidol University, for critical reading of the manuscript. Financial support from the Thailand Research Fund (TRF) through the Royal Golden Jubilee Ph.D. program (grant no. PHD/0144/2542) is gratefully acknowledged.

References

[1] H.H. Schaumburg, R. Byck, R. Gerstl, J.H. Mashman, Science 163 (1969) 826.
[2] R.S. Geha, A. Beiser, C. Ren, R. Patterson, P.A. Greenberger, L.C. Grammer, A.M. Ditto, K.E. Harris, M.A. Shaughnessy, P.R. Yarnold, J. Corren, A. Saxon, J. Nutr. 130 (2000) 1058S.
[3] E. Valero, F. Garcia-Carmona, Anal. Biochem. 259 (1998) 265.
[4] J. Chapman, M.J. Zhou, Anal. Chim. Acta 402 (1999) 47.
[5] T. Yao, H. Yamamoto, T. Wasa, Anal. Chim. Acta 236 (1990) 437.
[6] T. Yao, Y. Nanjyo, H. Nishino, Anal. Sci. 17 (2001) 703.
[7] F. Schubert, D. Kirstein, F. Scheller, R. Appelqvist, L. Gorton, G. Johansson, Anal. Lett. 19 (1986) 1273.
[8] T. Yao, S. Suzuki, T. Nakahara, H. Nishino, Talanta 45 (1998) 917.
[9] C. Mayer, A. Frauer, T. Schalkhammer, F. Pittner, Anal. Biochem. 268 (1999) 110.
[10] K. Nakamura, T. Fujii, Y. Kato, Y. Asano, A.J.L. Cooper, Anal. Biochem. 234 (1996) 19.
[11] S. Wiyakrutta, V. Meevootisom, J. Biotechnol. 55 (1997) 193.
[12] P. Kongsaeree, C. Samanchart, P. Laowanapiban, S. Wiyakrutta, V. Meevootisom, Acta Cryst. D59 (2003) 953.
[13] H.O. Beautler, in: H.U. Bergmeyer (Ed.), Methods of Enzymatic Analysis, vol. VII, 3rd ed., Verlag Chemie, Weinheim, 1985, pp. 369–376.
[14] J.H.T. Luong, A. Mulchandani, K.B. Male, Enzyme Microb. Technol. 13 (1991) 116.
[15] J.-N. Park, Y. Fukumoto, E. Fujita, T. Tanaka, T. Washio, S. Otsuka, T. Shimizu, K. Watanabe, H. Abe, J. Food Comp. Anal. 14 (2001) 113.

Biochemical and Biophysical Research Communications 405 (2011) 626–631

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Sensitive non-radioactive determination of aminotransferase stereospecificity for C-4′ hydrogen transfer on the coenzyme

Juntratip Jomrit [a,c], Pijug Summpunn [a], Vithaya Meevootisom [b,c], Suthep Wiyakrutta [b,c,*]

[a] Department of Biotechnology, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand
[b] Department of Microbiology, Faculty of Science, Mahidol University, Rama 6 Road, Bangkok 10400, Thailand
[c] Center of Excellence for Agricultural Biotechnology: (AG-BIO/PERDO-CHE), Bangkok, Thailand

ARTICLE INFO

Article history:
Received 6 January 2011
Available online 25 January 2011

Keywords:
Aminotransferase
Hydrogen transfer
LC–MS/MS
Non-radioactive
Pyridoxal phosphate
Stereospecificity

ABSTRACT

A sensitive non-radioactive method for determination of the stereospecificity of the C-4′ hydrogen transfer on the coenzymes (pyridoxal phosphate, PLP; and pyridoxamine phosphate, PMP) of aminotransferases has been developed. Aminotransferase of unknown stereospecificity in its PLP form was incubated in ^2H_2O with a substrate amino acid resulted in PMP labeled with deuterium at C-4′ in the pro-*S* or pro-*R* configuration according to the stereospecificity of the aminotransferase tested. The [4′-^{2}H]PMP was isolated from the enzyme protein and divided into two portions. The first portion was incubated in aqueous buffer with apo-aspartate aminotransferase (a reference *si*-face specific enzyme), and the other was incubated with apo-branched-chain amino acid aminotransferase (a reference *re*-face specific enzyme) in the presence of a substrate 2-oxo acid. The ^{2}H at C-4′ is retained with the PLP if the aminotransferase in question transfers C-4′ hydrogen on the opposite face of the coenzyme compared with the reference aminotransferase, but the ^{2}H is removed if the test and reference aminotransferases catalyze hydrogen transfer on the same face. PLP formed in the final reactions was analyzed by LC–MS/MS for the presence or absence of ^{2}H. The method was highly sensitive that for the aminotransferase with ca. 50 kDa subunit molecular weight, only 2 mg of the enzyme was sufficient for the whole test. With this method, the use of radioactive substances could be avoided without compromising the sensitivity of the assay.

1. Introduction

Aminotransferases are ubiquitous in nature and play important roles in amino acid metabolism in all living organisms. They are pyridoxal-5′-phosphate (PLP) dependent enzymes that catalyze the reversible amino group transfer from amino acids to oxo acids. In the key step of the transamination reaction, the α-hydrogen is abstracted from the substrate amino acid and transferred to C-4′ of the bound coenzyme. This process is characteristic of enzymatic transamination and the stereochemistry of the hydrogen transfer to or from C-4′ of the coenzyme has been determined for many aminotransferases [1]. Most of the L-amino acid aminotransferases transfer the hydrogen stereospecifically on the *si*-face at C-4′ of the coenzyme moiety of the external Schiff base while D-amino acid aminotransferase and branched-chain amino acid aminotransferase transfer the hydrogen on the *re*-face (Fig. 1). PLP-dependent enzymes play important roles in various metabolic pathways in all living organisms. To date, more than 300 PLP-dependent enzymes have been described [1] and stereochemistry of the reactions they catalyze has been a subject of much interest. The availability of sensitive and effective methods for stereospecificity characterization will facilitate the study to understand the mechanisms of catalysis of these enzymes.

Originally, the method for determining the stereospecificity of the C-4′ hydrogen transfer of aminotransferases involved the use deuteriopyridoxamine by subjecting it to an enzyme catalyzed transamination and detecting the loss or the retention of the ^{2}H with the coenzyme by nuclear magnetic resonance (NMR) spectroscopy [2]. This method is time consuming and requires a large quantity of the aminotransferase. A more sensitive method was developed which use the PLP specifically labeled with ^{3}H at the C-4′. The labeled PLP is incubated with the apoenzyme and its substrates. The PMP formed is isolated, dephosphorylated with an alkaline phosphatase to become pyridoxamine (PM). The PM is subjected to transamination with pyruvate and pyridoxamine-pyruvate transaminase which specifically removes the *pro*-S proton of the PM. The radioactivity retained in the pyridoxal formed indicates that the enzyme catalyzes hydrogen transfer on the same (*si*) face as the pyridoxamine-pyruvate transaminase does [3]. This method is lengthy and generates radioactive waste. More recently, a simple method based on aspartate aminotransferase and D-amino

* Corresponding author at: Department of Microbiology, Faculty of Science, Mahidol University, Rama 6 Road, Ratchathewi, Bangkok 10400, Thailand. Fax: +66 0 2644 5411.
E-mail address: scsvy@mahidol.ac.th (S. Wiyakrutta).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.01.080

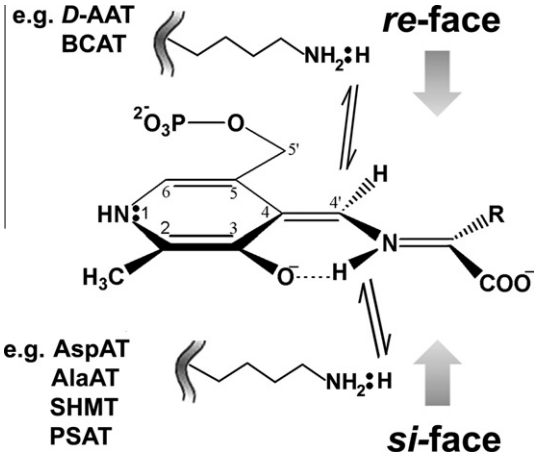


Fig. 1. A planar intermediate external Schiff base complex formed between the coenzyme and the substrate amino acid. The *re*-face is above the plane, the *si*-face is below the plane. Examples of enzymes that catalyze the C-4′ hydrogen transfer on either the *re*-or *si*-face are given with their active site lysine positioned on the *re*-or *si*-face, respectively. (AlaAT, alanine aminotransferase; AspAT, aspartate aminotransferase; BCAT, branched-chain amino acid aminotransferase; D-AAT, D-amino acid aminotransferase; PSAT, phosphoserine aminotransferase; SHMT, serine hydroxymethyltransferase).

acid aminotransferase which are known to catalyze the proton transfer at C-4′ on the *si*-and *re*-face of the coenzyme, respectively, was described [4]. However this method utilizes ^3H_2O which requires special laboratory and facility for handling radioactive substances. We report here the development a highly sensitive method for determining the stereospecificity of the hydrogen transfer at C-4′ of the coenzyme in aminotransferase catalyzed reactions by using ^2H_2O as a labeling agent and detect the release or retention of deuterium on the cofactor by means of LC–MS/MS spectrometry.

2. Materials and methods

2.1. Materials

Branched-chain amino acid aminotransferase (BCAT, EC 2.6.1.42) and D-amino acid aminotransferase (D-AAT, EC 2.6.1.21) with hexahistidine tagged at the C-terminus (BCAT-H6 and D-AAT-H6) were prepared from the *ilvE* gene [5] and the *dat* gene [6], respectively, cloned into pET-11a vector (Novagen), expressed in *E. coli* Tuner (DE3) pLysS and purified from the cell extracts by immobilized Co^{2+} affinity column (BD Talon®, BD Biosciences) chromatography. D-phenylglycine aminotransferase (D-PhgAT) of *Pseudomonas stutzeri* ST-201 [7] was purified from *E. coli* BL 21(DE3) harboring the recombinant plasmid pEPL carrying the *dpgA* gene encoding D-PhgAT as previously described [8]. Porcine heart aspartate aminotransferase (AspAT, EC 2.6.1.1), porcine heart L-alanine aminotransferase (AlaAT, EC 2.6.1.2), malate dehydrogenase (MDH, EC 1.1.1.37), lactate dehydrogenase (LDH, EC 1.1.1.27), L-aspartic acid monopotassium, L-cysteinesulfinic acid, 2-oxoglutaric acid disodium, L-glutamic acid monosodium, sodium 4-methyl-2-oxovalerate, pyridoxal-5′-phosphate, NADH, oxaloacetic acid, D-phenylglycine, deuterium oxide (99.9 atom% ^2H), sodium deuteroxide (40 wt.% solution in ^2H_2O, 99.5 atom% ^2H) were obtained from Sigma Chemical Co. (St. Louis, USA). Enzymes were purified to electrophoretic homogeneity prior to use.

2.2. Aminotransferase activity assays

AlaAT activity was assayed spectrophotometrically by the method of Segal and Matsuzawa [9]. AspAT activity was assayed using the MDH-coupled reaction as described by Karmen [10]. Activity of BCAT was measured by the coupled enzymatic assay of Schadewaldt [11]. D-AAT activity was assayed using a coupled reaction with LDH [12]. D-PhgAT activity was determined using the spectrophotometric enzyme kinetic assay previously reported [13].

2.3. Preparation of apo-AspAT and apo-BCAT-H6

AspAT or BCAT-H6 was incubated with 10 mM L-cysteinesulfinate [14] in 20 mM Tris–HCl (pH 8.5) at 30 °C for 24 h. The reaction mixture was applied into an Amicon Ultra-15 10 K filter device and centrifuged at 3500 rpm, 4 °C for 15 min (Hettich Rotina 380R Centrifuge, UK). The retentate was washed 3 times with 10 mL of 20 mM NaH_2PO_4, pH 5.0 (for AspAT) or pH 6.5 (for BCAT-H6), then with 50 mM HEPES, pH 8.2 and transferred into a fresh tube. The resulting apo-AspAT or apo-BCAT-H6 was verified by checking for the absence of UV absorption peak of the coenzyme, as well as the devoid of their transaminase activities which could be restored upon addition of 10 μM PLP coenzyme.

2.4. Preparation of fully PLP-bound AlaAT, D-AAT, and D-PhgAT

Two milligrams of purified AlaAT, D-AAT, or D-PhgAT was incubated with 100 μM PLP and 10 mM 2-oxoglutarate in 50 mM HEPES buffer pH 7.4, 50 mM HEPES buffer pH 8.2, or CAPSO buffer pH 9.5, respectively, at room temperature for 30 min. Excess substrate, coenzyme and product were removed by using a HiTrap™ desalting column (Pharmacia, Sweden). High molecular mass fractions containing the desired PLP-enzyme eluted at the void volume of the column were pooled and dehydrated by lyophilization.

2.5. ^2H transfer to PLP catalyzed by the test aminotransferases

The lyophilized fully PLP-bound enzymes were incubated with 10 mM of their respective amino group donor substrates in buffered ^2H_2O. The AlaAT was incubated with L-alanine in 50 mM HEPES p^2H 7.4 at 37 °C for 10 h [15]. The D-AAT was incubated with D-alanine in 50 mM HEPES p^2H 8.2 at 37 °C for 10 h. The D-PhgAT was incubated with D-phenylglycine in 50 mM CAPSO p^2H 9.5 at 35 °C for 3 h. The [4′-^{2}H]PMP formed was released from the enzyme by heat denaturation of the protein (70 °C, 5 min) and separated by ultrafiltration (Amicon Ultra-15 10 K centrifugal filter device). The ultrafiltrate containing [4′-^{2}H]PMP was subjected to anion-exchange chromatography (UNO™Q6R 12 × 53 mm, Bio-Rad; Agilent LC 1100 HPLC system) at ambient temperature. The column was pre-equilibrated with water at 1 mL min^{-1} flow rate. After injecting the sample, the column was washed with water for 6 min then an ascending gradient of 0.2 M CH_3COOH from 0% to 50% was applied at 2 mL min^{-1} during the following 3 min, 50–100% 0.2 M CH_3COOH in 6 min, and isocratic 100% 0.2 M CH_3COOH until the desired peak was eluted. A UV–Visible photodiode array detector was set to monitor the absorbance at the wavelength of 290 and 330 nm with the online absorption spectra recorded to facilitate the identification and purity evaluation of the separated compounds. Fractions containing [4′-^{2}H]PMP were pooled and a 50 μL aliquot was submitted for analysis by liquid chromatography–tandem mass spectrometry (LC–MS/MS, Esquire HCT, Bruker Daltonics) operated in the positive ion mode, $[M + H]^+$.

2.6. Stereochemistry of the C-4′ hydrogen abstraction from PMP during half reactions by the reference enzymes AspAT and BCAT-H6

The reaction mixture (5 mL) contained 2 μM [4′-^{2}H]PMP, 1 mM 2-oxoglutarate, 4 μM of apoenzyme, and 50 mM HEPES pH 8.2. The reactions were carried out at 37 °C (for apo-AspAT) or 30 °C (for

apo-BCAT-H6), for 10 h. Either [4′-^{2}H]PLP or [4′-H]PLP was formed after the C-4′ hydrogen or deuterium was abstracted from the [4′-^{2}H]PMP during half reaction by AspAT and BCAT-H6. The enzyme-bound PLP was isolated from the excess substrate and product by ultrafiltration (Amicon Ultra-15 10K centrifugal filter device). The retentate (250 μL) contained only the enzyme-bound PLP was treated with 0.1 N NaOH to release PLP from the enzyme protein [16]. The PLP released was separated from the denatured protein by ultrafiltration as the above. The filtrate containing PLP was neutralized by adding 0.3 N HCl, and a 50 μL aliquot was submitted for analysis by LC–MS/MS (Esquire HCT, Bruker Daltonics).

2.7. Liquid chromatography–tandem mass spectrometry (LC–MS/MS)

A series 1100 HPLC system (Agilent Technologies) equipped with an autosampler was used for solvent delivery and sample introduction. The HPLC system was coupled to an Esquire HCT, Bruker Daltonics mass spectrometer outfitted with an electrospray ion source. The HyStar 3.2 and DataAnalysis 3.4 softwares (Bruker Daltonics) were used for data acquisition and analysis, respectively. The neutralized PLP solution (20 μL) obtained in the previous section was loaded by means of an autosampler onto a Hypersil GOLD C18 HPLC column (particle size: 3 μm; 150 × 4.6 mm) equipped on the HPLC system. The mobile phase used was 650 mM acetic acid at a flow rate of 0.5 mL min^{-1}. The analytes eluted from the column were subjected to electrospray ionization with source parameters were as follows: −2.5 kV of capillary volt, 25 psi of nebulizer gas, 7 L min^{-1} of dry gas, and 300 °C of dry temperature. The generated ions passed through the glass capillary to the transport and focus region to go into the ion trap mass analyzer which were controlled by typical setting through the Esquire Control software (109.6 V of cap exit, 40 V of skimmer, 12 V of octopole1 DC, 1.7 V of octopole2 DC, 142.1 V of octopole RF, −5 V of lens 1, −60 V of lens 2, and 33.4 trap drive). For efficient trapping and cooling of the ions generated, helium gas was introduced into the ion trap. Analyte ions were analyzed in positive ions mode, $[M + H]^+$. Multiple reaction monitor (MRM) was applied to analyze simultaneously the $[M + H]^+$ of both [4′-H]PLP and [4′-^{2}H]PLP which might be present in the mixture, and to determine the fragmentation pattern of parent masses. The instrument was thus set for parent m/z of 248 and 249, with the isolation width of 1 mass unit to detect the $[M + H]^+$ of [4′-H]PLP and [4′-^{2}H]PLP, respectively. Fragmentation amplitude was 0.5, and fragmentation cutoff was set to 27% of the m/z of the precursor ion.

3. Results

3.1. ^{2}H transfer to PLP catalyzed by the test aminotransferases

AlaAT and D-AAT, the known *si*-face and *re*-face specific aminotransferases, respectively, were subjected to the test to validate the method developed in the present study. First, the two aminotransferases were prepared in the fully PLP-bound form. Next, the PLP-enzymes were incubated in ^2H_2O with one of their respective substrate amino acid. Upon binding to PLP-enzyme, the amino acid causes a transaldimination forming an external Schiff base. The enzyme then abstracts the α-hydrogen from the amino acid moiety of the external Schiff base complex. The abstracted hydrogen is exchanged rapidly with the solvent ^{2}H. Thus with ^2H_2O as a solvent, instead of the hydrogen abstracted from the substrate amino acid, ^{2}H from the solvent ^2H_2O is transferred to the PLP in the subsequent step and finally yielding the PMP labeled with one ^{2}H at the C-4′. The stereochemical configuration of this ^{2}H on the C-4′ of the resulted [4′-^{2}H]PMP is governed by the stereospecific nature of the aminotransferase that catalyzes the reaction. The extent of

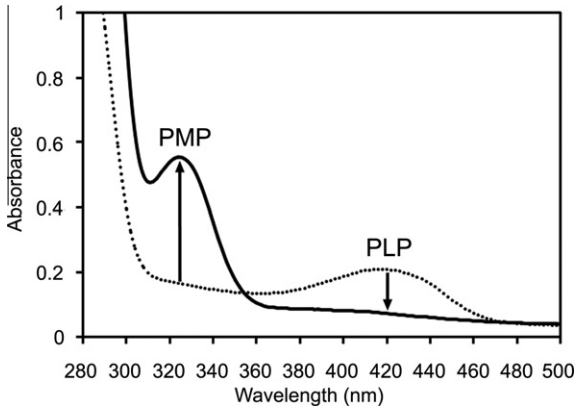


Fig. 2. Absorption spectrum change used for monitoring the extent of the conversion of PLP to PMP in an enzyme-catalyzed transamination reaction.

PMP formation could be estimated by monitoring spectrophotometrically the increase in UV absorption at 325 nm and the concomitant decreased at 418 nm (Fig. 2).

The [4′-^{2}H]PMP coenzyme released into the solution was isolated from the enzyme protein by ultrafiltration and was purified by anion-exchange chromatography. The identity, purity, and quantity of the of the [4′-^{2}H]PMP could be determined using MS/MS. Mass spectra of the [4′-^{2}H]PMP formed by labeling the PLP with ^{2}H in the transamination half reaction catalyzed by AlaAT and D-AAT are shown in Fig. 3A and B, respectively. Only the parent ion (m/z = 250) and the product ion (m/z = 233) corresponding to the PMP labeled with one ^{2}H were evidence in both cases indicated that the [4′-^{2}H]PMP obtained was in a pure form free from the unlabeled PLP, the excess substrate amino acid and the oxo acid product which, if present, could interfere with its use in the next step.

3.2. Abstraction by the reference aminotransferases of the C-4′ hydrogen from the [4′-^{2}H]PMP formed by the aminotransferase under investigation

The AspAT and the BCAT were used as the reference *si*-face and *re*-face specific aminotransferases, respectively. The [4′-^{2}H]PMP generated by the aminotransferase under investigation (AlaAT and D-AAT) was incubated in aqueous solution with apo-AspAT and with apo-BCAT in the presence of their amino acceptor oxo acid, 2-oxoglutaric acid. The reconstituted AspAT catalyzed the transfer of the pro-*S* hydrogen from the C-4′ of the PMP whereas the BCAT abstracted the hydrogen from the pro-*R* position. The PLP generated from these half reactions imparted yellow color to the solution. The completeness of the conversion of PMP to PLP could be assessed by monitoring the disappearance of the absorbance at 325 nm and the increase in the absorbance at 418 nm. Most of the PLP formed remained enzyme-bound and could be released by hydrolysis with 0.1 N NaOH. The PLP isolated was subjected to mass analysis by LC–MS/MS. With the system used in this study, the PLP was eluted from the LC column with a retention time of ca. 5 min. The PLP in which the labeled ^{2}H at the C-4′ was not retained gave rise to a parent ion and a product ion with the m/z values of 248 and 150, respectively. The PLP the ^{2}H of which was retained yielded a parent ion and a product ion with the m/z values of 249 and 151, respectively. Parent ion-product ion mass spectra of the coenzymes analyzed by LC–ESI-MS/MS in this study are shown in Fig. 3.

When the [4′-^{2}H]PMP generated from the AlaAT catalyzed reaction was subjected to C-4′ hydrogen abstraction by the reference *si*-face specific enzyme AspAT, the resulted PLP mass spectrum indicated the loss of the ^{2}H from the coenzyme (Fig. 3A-1). When this same [4′-^{2}H]PMP was subjected to C-4′ hydrogen abstraction

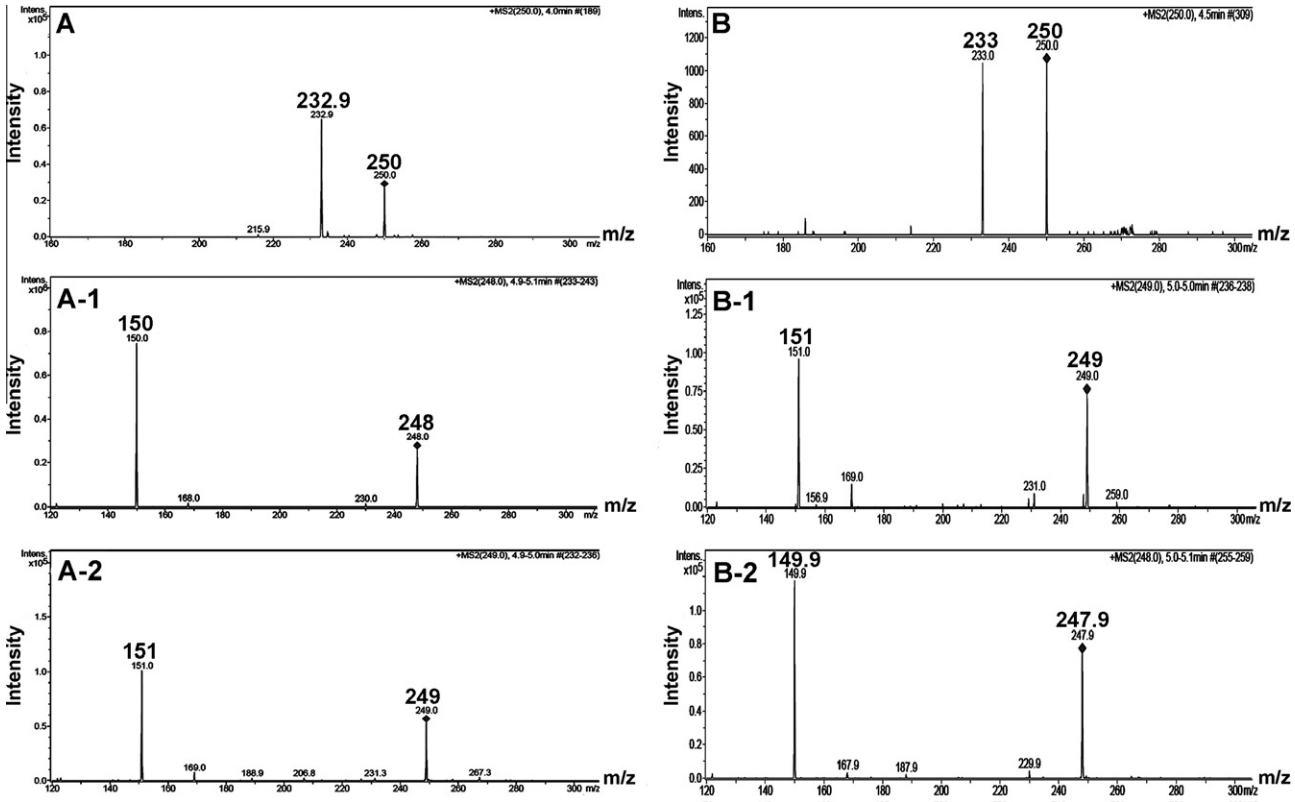


Fig. 3. Positive ion ($[M + H]^+$) ESI-MS/MS MRM mass spectra of the [4′-^{2}H]PMP purified after the coenzyme was labeled with ^{2}H in the transamination reaction catalyzed by AlaAT (A) and by D-AAT (B). Both spectra show a parent ion with m/z = 250 and a product ion with m/z = 233 indicating that the PMP was labeled with one ^{2}H. Positive ion LC–ESI-MS/MS MRM mass spectra of the PLP coenzyme generated after the [4′-^{2}H]PMP from the AlaAT reaction was subjected to transamination by AspAT (A-1) and by BCAT (A-2). Positive ion LC–ESI-MS/MS MRM mass spectra of the PLP coenzyme generated after the [4′-^{2}H]PMP from the D-AAT reaction was subjected to transamination by AspAT (B-1) and by BCAT (B-2). The respective parent and product ions with m/z of 151 and 249 indicating retention of the ^{2}H with the PLP while the parent and product ions with m/z of 150 and 248 indicating removal of the ^{2}H from the coenzyme.

by the reference *re*-face specific enzyme BCAT, the resulted PLP mass spectrum corresponded with the retention of the ^{2}H (Fig. 3A-2). These results confirmed each other that AlaAT catalyzed the C-4′ proton transfer on the same face as that of the AspAT which is the *si*-face, but on the opposite face to that of the BCAT.

In the case of [4′-^{2}H]PMP generated from D-AAT reaction, upon subjecting it to the transaminase half reaction catalyzed by the reference *si*-face enzyme AspAT, mass spectrometric analysis showed that the ^{2}H was retained (Fig. 3B-1). But when the D-AAT generated [4′-^{2}H]PMP was incubated with the reference *re*-face enzyme BCAT, the mass spectrum of the resulted PLP showed that the ^{2}H was removed from the coenzyme (Fig. 3B-2). These results indicated that D-AAT catalyzed the C-4′ proton transfer on the opposite face to that of the AspAT but on the same face as that of the BCAT which is the *re*-face.

This method thus correctly determined the AlaAT as a *si*-face specific enzyme and the D-AAT as a *re*-face specific enzyme. It was applied to study the stereo-inverting D-PhgAT [7] an aminotransferase of unknown stereospecificity for C-4′ proton transfer. With D-phenylglycine as the amino donor substrate, mass spectra of the coenzymes obtained from the reactions (Supplementary Fig. S1) suggested that C-4′ proton transfer in the transamination half reaction of the D-PhgAT on D-phenylglycine took place on the *si*-face of the coenzyme.

4. Discussion

Since the coenzyme constitutes a minute part of the holoenzyme (ca. 1 in 200, for an aminotransferase with 50 kDa subunit), relatively large amount of the enzyme protein is required in order to obtain a sufficient quantity of the coenzyme from the experimental process for analysis. To be able to measure and analyze the coenzyme present in low quantity, the methods for determining stereospecificity of the aminotransferase usually involve the use of radioactive substances to label the coenzymes or the substrate amino acids [1–4]. Methods using non-radioactive labeling with ^{2}H were also employed. The labeled or de-labeled coenzymes resulted from the transamination reactions need to be isolated and analyzed by proton NMR spectrometry. In the latter methods though the use of radioactive chemicals could be avoided, the sensitivities were comparatively lower than the radioactive-based methods and thus larger amounts of the enzyme were required for the experiments.

The method reported in this communication is based on labeling the PLP coenzyme with ^{2}H by the aminotransferase under test, de-labeling with the reference *si*-face and *re*-face specific aminotransferases, and the LC–MS/MS analysis for the presence or absence of the labeled ^{2}H in the coenzyme. Stereochemistry path of the coenzyme during the test follows scheme A or B in Fig. 4 if the aminotransferase in question is a *si*-face or *re*-face specific enzyme, respectively. Only one experimental scheme (either A or B) consisting of three reactions is involved for testing each subject enzyme. By this method, there is no need to prepare the enzyme under test in the apo form. This is particularly suitable for the enzyme whose apo form is difficult to prepare or the apo form is unstable.

Incorporation of the LC–MS/MS to this test gave rise to several advantages. First, the LC–MS/MS instruments available at present are highly sensitive being able to analyze compounds down to

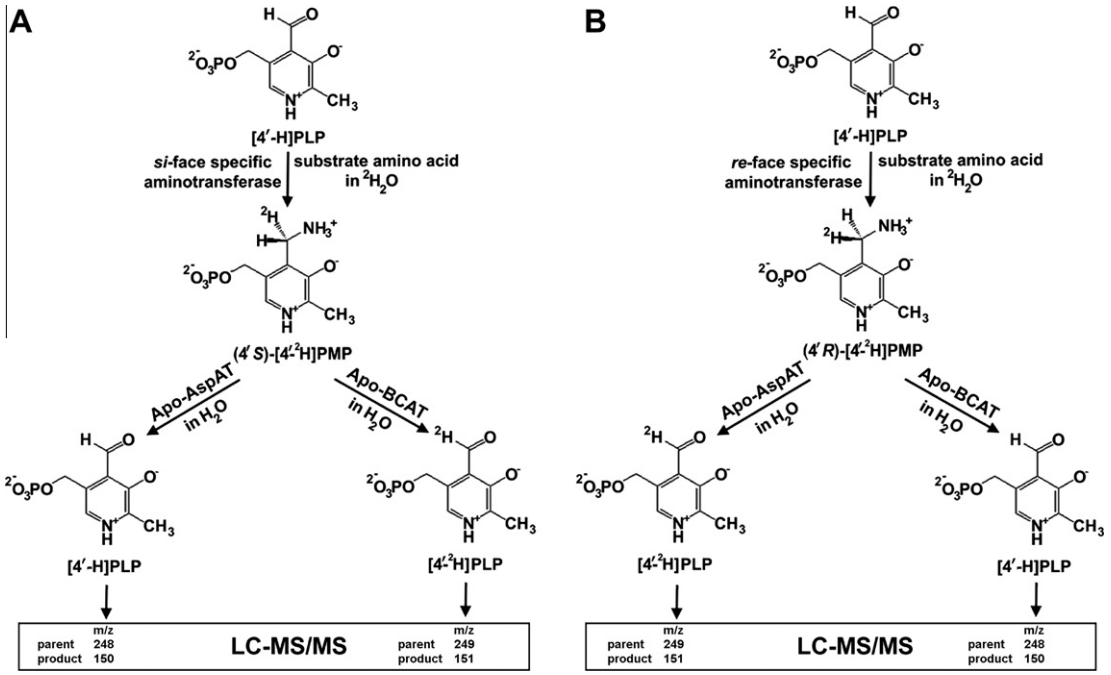


Fig. 4. Stereochemistry and path of the coenzyme in the test scheme when the aminotransferase under investigation is a *si*-face specific enzyme (A) or a *re*-face specific enzyme (B).

the femtogram level. This translates to the requirement of only a small amount of the aminotransferase sample for the test. Around 2 mg sample of an aminotransferase with a subunit molecular weight of ca. 50 kDa is sufficient for the test to give reliable results. This amount of the enzyme is similar to that required by the radioactive-based method [15]. Second, with the use of LC–MS/MS operated in the multiple reaction monitoring mode, the analyte coenzyme can be positively identified from the signals of both the parent ion and its characteristic product ion which are shown together in the mass spectrum (Fig. 3). Additionally, if contamination between PLP and PMP occur e.g. from the incomplete conversion during the reaction with the reference enzyme, this will be readily detected. Third, with the LC–MS/MS the samples of PLP from the transaminase reaction need not be highly purified since the LC– part of the system will separate the coenzyme in a peak of pure compound before passing it to the MS/MS for mass analysis. Fourth, the time required to analyze the coenzyme sample from the reaction by LC–MS/MS was 15 min per sample. This is much shorter than the time for purifying the coenzyme from the reaction mixture and analyzing it with ^{1}H NMR spectrometry as required by the NMR-based methods, and the time to complete the radioactivity counting in the radioactive-based assay [4,15].

In summary, the LC–MS/MS method for determining the stereospecificity of the coenzyme C-4′ hydrogen transfer of the aminotransferases is as sensitive as the previously reported radioactive-based methods [15] and with additional advantages. The method was satisfactorily verified with a known *si*-face specific and a known *re*-face specific aminotransferases. It was successfully applied to determine the stereochemical mechanism of a novel enzyme D-PhgAT which accepts both L-amino acid and D-amino acid with different R-group as substrates [7]. The D-PhgAT was found to catalyze transamination of D-phenylglycine by transferring the C-4′ hydrogen on the *si*-face of the coenzyme. This is in contrast with D-AATs which catalyze the *re*-face specific hydrogen transfer upon acting on their substrate D-amino acids. The method developed in this study should be useful for characterization of stereospecific nature of not only the aminotransferase group of enzymes as demonstrated in the present study but also other pyridoxal-5′-phosphate dependent enzymes that catalyze various biologically significant reactions such as aldol condensation, decarboxylation, α,β-elimination, β,γ-elimination of amino acids, and oxidation of amines [1].

Acknowledgments

SW acknowledges financial support from the Thailand Research Fund Grant No. RSA4780014. JJ is grateful for the support of a graduate scholarship from the Center of Excellence for Agricultural Biotechnology, Science and Technology, Postgraduate Education and Research Development Office (PERDO), Commission on Higher Education, Ministry of Education, Thailand.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.01.080.

References

[1] K. Soda, T. Yoshimura, N. Esaki, Stereospecificity for the hydrogen transfer of pyridoxal enzyme reactions, Chem. Rec. 1 (2001) 373–384.
[2] H.C. Dunathan, L. Davis, P.G. Kury, M. Kaplan, The stereochemistry of enzymatic transamination, Biochemistry 7 (1968) 4532–4537.
[3] J.G. Voet, D.M. Hindenlang, T.J. Blanck, R.J. Ulevitch, R.G. Kallen, H.C. Dunathan, The stereochemistry of pyridoxal phosphate enzymes. The absolute stereochemistry of cofactor C' 4 protonation in the transamination of holoserine hydroxymethylase by D-alanine, J. Biol. Chem. 248 (1973) 841–842.
[4] K. Nishimura, J. Ito, T. Yoshimura, N. Esaki, K. Soda, A simple method for determination of stereospecificity of aminotransferases for C-4' hydrogen transfer of the coenzyme, Bioorg. Med. Chem. 2 (1994) 605–607.
[5] S. Kuramitsu, T. Ogawa, H. Ogawa, H. Kagamiyama, Branched-chain amino acid aminotransferase of *Escherichia coli*: nucleotide sequence of the *ilvE* gene and the deduced amino acid sequence, J. Biochem. 97 (1985) 993–999.
[6] I.G. Fotheringham, S.A. Bledig, P.P. Taylor, Characterization of the genes encoding D-amino acid transaminase and glutamate racemase, two D-glutamate biosynthetic enzymes of *Bacillus sphaericus* ATCC 10208, J. Bacteriol. 180 (1998) 4319–4323.
[7] S. Wiyakrutta, V. Meevootisom, A stereo-inverting D-phenylglycine aminotransferase from *Pseudomonas stutzeri* ST-201: purification,

characterization and application for D-phenylglycine synthesis, J. Biotechnol. 55 (1997) 193–203.
[8] P. Kongsaeree, C. Samanchart, P. Laowanapiban, S. Wiyakrutta, V. Meevootisom, Crystallization and preliminary X-ray crystallographic analysis of D-phenylglycine aminotransferase from *Pseudomonas stutzeri* ST201, Acta Crystallogr. D Biol. Crystallogr. 59 (2003) 953–954.
[9] H.L. Segal, T. Matsuzawa, L-alanine aminotransferase (rat liver), Methods Enzymol. 17 (1970) 153–159.
[10] A. Karmen, A note on the spectrometric assay of glutamic-oxalacetic transaminase in human blood serum, J. Clin. Invest. 34 (1955) 131–133.
[11] P. Schadewaldt, F. Adelmeyer, Coupled enzymatic assay for estimation of branched-chain L-amino acid aminotransferase activity with 2-Oxo acid substrates, Anal. Biochem. 238 (1996) 65–71.
[12] W.M. Jones, T.S. Soper, H. Ueno, J.M. Manning, D-Glutamate-D-amino acid transaminase from bacteria, Methods Enzymol. 113 (1985) 108–113.
[13] W. Khampha, S. Wiyakrutta, V. Meevootisom, Spectrophotometric enzymatic cycling method using L-glutamate dehydrogenase and D-phenylglycine aminotransferase for determination of L-glutamate in foods, Anal. Chim. Acta 520 (2004) 133–139.
[14] T. Yagi, H. Kagamiyama, M. Nozaki, Cysteine sulfinate transamination activity of aspartate aminotransferases, Biochem. Biophys. Res. Commun. 90 (1979) 447–452.
[15] T. Yoshimura, K. Nishimura, J. Ito, N. Esakik, H. Kagamiyama, J.M. Manning, K. Soda, Unique stereospecificity of D-amino acid aminotransferase and branched-chain L-amino acid aminotransferase for C-4' hydrogen transfer of the coenzyme, J. Am. Chem. Soc. 115 (1993) 3897–3900.
[16] S. D'Aguanno, I.N. Gonzales, M. Simmaco, R. Contestabile, R.A. John, Stereochemistry of the reactions of glutamate-1-semialdehyde aminomutase with 4,5-diaminovalerate, J. Biol. Chem. 278 (2003) 40521–40526.

Program & Abstracts

Commission on Higher Education Congress II

University Staff Development Consortium

(CHE - USDC Congress II)

27 - 29 August, 2009

Dusit Thani Pattaya HoTel

PC1 – 65

INCREASING SOLUBILITY OF *D*-PHENYLGLYCINE AMINOTRANSFERASE

Aiya Chantarasiri, Vithaya Meevootisom, Poramaet Laowanapiban, Duangnate Isarangkul Na Ayudhaya, and Suthep Wiyakruta

Department of Microbiology, Faculty of Science, Mahidol University,Rama VI Rd., Bangkok 10400, Thailand

Objectives:

To genetically engineer *D*-phenylglycine aminotransferase (*D*-PhgAT) to increase its *in vitro* solubility

To determine solubility and catalytic properties of *D*-PhgAT variants compared with those of the wild-type enzyme.

Methods: BetaTPred2, COUDES, GETAREA, and ArealMol were applied to predict β-turn and solvent accessible surface area in the *D*-PhgAT structure. Solvent exposed hydrophilic amino acid residues situated at the β-turns were identified and were used as targets for mutagenesis.Genetic modifications were done on gene encoding C-terminal hexahistidine tagged *D*-PhgAT (*D*-PhgAT-H_6) using QuikChange® Lightning Site-Directed Mutagenesis Kit from Stratagene. Wild-type *D*-PhgAT was expressed using pET-17b plasmid and *E. coli* BL21 (DE3) host. *D*-PhgAT-H_6 variants were expressed using pET-11a plasmid and *E. coli* Tuner (DE3) pLysS host. Wild-type *D*-PhgAT was purified by stepwise ammonium sulfate precipitation, hydrophobic interaction chromatography (Phenyl-Agarose CL-4B), and anion exchange chromatography (DEAE Sepharose™ Fast Flow), respectively. *D*-PhgAT-H_6 variants were purified by immobilized metal ion affinity chromatography (BD Talon™ charged with Co^{2+}). *In vitro* solubility of the wild-type *D*-PhgAT and all its mutants were determined by centrifuging the protein sample in a microconcentrator with 50 kDa cut-off. Catalytic properties of *D*-PhgAT variants were characterized using published methods.

Results: Analysis by bioinformatics tools identified Asn349 and Gln444 as solvent exposed hydrophilic amino acid residues at β-turns in C-terminal domain of the enzyme. The Asn349 and Gln444 were genetically replaced with Asp and Glu, respectively, thus *D*-PhgAT(Asn349Asp)-H_6,*D*-PhgAT(Gln444Glu)-H_6,and *D*-PhgAT(Asn349Asp,Gln444Glu)-H_6 were constructed. *In vitro* solubility and catalytic properties of *D*-PhgAT variants are under investigation.

Discussion: *D*-PhgATis a novel enzyme having a characteristic stereo-inverting transamination activity which can be use to synthesisize enantiomerically pure *D*-phenylglycine and its 4-OH derivative which are important side-chains of semisynthetic penicillins and cephalosporins. However, low *in vitro* solubility of this enzyme is a major problem during preparation, shipping, storage and application. Replacing solvent exposed hydrophilic amino acids at β-turns with charged residues of similar size is expected to increase solubility of the enzyme with minor (if any) unfavorable effect on the catalytic performance.


BURAPHA UNIVERSITY
15-17 October 2009
วทท STT 35th
FACULTY OF SCIENCE
Abstracts
THE 35th CONGRESS
on SCIENCE and TECHNOLOGY
of THAILAND (STT 35)
การประชุมวิชาการวิทยาศาสตร์และเทคโนโลยีแห่งประเทศไทย
ครั้งที่ 35 (วทท 35)
วิทยาศาสตร์และเทคโนโลยีเพื่ออนาคตที่ดีขึ้น
SCIENCE AND TECHNOLOGY FOR A BETTER FUTURE
To Celebrate the 35th Anniversary of Faculty of Science, Burapha University
To Celebrate the 30th Anniversary of Ministry of Science and Technology
October 15 - 17, 2009
Venue : The Tide Resort (Bangsaen Beach), Chonburi, Thailand.
15-17 ตุลาคม 2552 ณ เดอะ ไทด์ รีสอร์ท (หาดบางแสน) จังหวัดชลบุรี
WWW.STT35.SCISOC.OR.TH

B4_B0037 IMPROVING FUNCTIONAL *D*-PHENYLGLYCINE AMINOTRANSFERASE PRODUCTION BY CO-EXPRESSION WITH CHAPERONES IN *E. COLI*

Juntratip Jomrit[1], Pitipoom Hongsukapun[1], Poramaet Laowanapiban[2], Suthep Waiyakrutta[2]
[1]Department of Biotechnology, [2]Department of Microbiology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand

Abstract: *D*-Phenylglycine aminotransferase (*D*-PhgAT) from *Pseudomonase stutzeri* ST-201 catalyzes a reversible stereo-inverting transamination of *D*-phenylglycine and α-ketoglutarate to yield *L*-glutamate and benzoylformate. The *D*-PhgAT makes possible a single step enzymatic process using *L*-glutamate as a low cost amino donor for synthesis of enantiopure *D*-phenylglycine and *D*-4OH-phenylglycine which are important side chains for semisynthetic penicillin and cephalosporin antibiotics. *D*-PhgAT is a relatively hydrophobic protein that tends to form aggregate of inactive enzyme upon over-expression in *E. coli* such as BL21(DE3)/pET17b system. To improve the yield of functional *D*-PhgAT, the encoding gene *dpgA* was co-expressed with different sets of *E. coli* chaperone genes (*dnaK-dnaJ-grpE*, *groEL-groES*, *tig*). The level of active *D*-PhgAT production increased from 10.11 unit/ml (no chaperone co-expression) to 19,887 .64, 12,134.83, 101.12, 3.71, 20.22 unit/ml with the co-expression of GroEL-GroES - DnaK-DnaJ-GrpE set, GroEL-GroES set, DnaK-DnaJ-GrpE set, GroEL-GroES - Tf set, and Tf chaperones, respectively.

B4_B0038 MAINTENANCE OF THE MOUSE EMBRYONIC STEM CELLS, HACM-450, UNDER THE SPECIAL FEEDER FREE CONDITION

Kewalin Inthanon[1] and Weerah Wongkham[1*]
[1]Human and Animal Cell Technology Research Laboratory, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200. Thailand
Email: Kelly_Inthanon@hotmail.com

Abstract: Mouse embryonic stem cells (mesc), hacm-4, have been derived from the pre-implantation blastocysts. The normal 10 days mouse embryonic fibroblasts (mef) were employed as the feeder layer for the mesc in any routine standard maintenance. On the mef-conditioned medium (mef-cm), the mesc expressed technically limited capacity of cellular number expansion. In this study, hacm-4 under the feeder free conditions with metrigel-stempro® were shown with large scale expansion and potentially replaceable to the tumor-cell derived matrices. metrigel-stempro® consists of a basal medium, bsa, and a growth supplement with small molecule additives, trace elements, and growth factors. The stemness and pluripotency of the mesc were evaluated by the immunocytochemistry on the metrigel-stempro® in comparison to the mef-cm. similar results have been shown on positive markers, nanog, tra-1-60, ssea-4, oct-4, sox-2 and nestin, but with negative markers, n-cadherin, ssea-1, sox-1 gata-4, brachyury and β3-tubulin. The cells may be grown indefinitely in vitro while maintaining their original karyotyping and epigenetic status, but these needs to be confirmed from time to time in the long-term cultures.

B4_B0044 *In silico* ANALYSIS OF TESTES EST LIBRARY OF THE BLACK TIGER SHRIMP (*Penaeus monodon*)

Thidathip Wongsurawat[1], Rungnapa Leelatanawit[1], Natechanok Thamniemdee[2], Nitsara Karoonuthaisiri[1], Sirawut Klinbunga[1, 2]
[1]National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), 113 Phaholyothin Rd., Klong 1, Klong Luang, Pathumthani 12120.
[2]Center of Excellence for Marine Biotechnology, Faculty of Science, Chulalongkorn University, Bangkok 10330.

Abstract: Facing the difficulty in reproductive maturation in captivity, the black tiger shrimp farming industry has been deteriorated. To revive the industry, reproduction research program of this economically important animal attempts to understand fundamental biology of both male and female development. In this study, an expressed tag sequence (EST) library was explored to reveal testes-specific genes for further characterization. From 4,803 ESTs sequences, we assembled these sequences and found 2,702 sequences (424 contigs and 2,278 singlets). To identify novel testis specific genes, these sequences were compared by using BLASTn with EST from 2 databases, *Penaeus monodon* ESTs database of BIOTEC and Penaeidae ESTs from NCBI. After compared with two databases, a total of 702 ESTs were shown to be novel testis specific sequences. To explore the functions of these testis specific genes, we used gene ontology classification and found some testis-related gene such as *mago nashi, insulin-degrading enzyme, profilin and seryl-trna synthetase*. Some of these genes are being further characterized for their relevance in testicular development using cDNA microarray technique.

20th National Grad Research Conference

2-3 Feb 2011 Mahidol University Salaya

การประชุมเสนอผลงานวิจัย ระดับบัณฑิตศึกษาแห่งชาติ

ครั้งที่ 20

2-3 กุมภาพันธ์ 2554

บัณฑิตวิทยาลัย มหาวิทยาลัยมหิดล ศาลายา

P-BS019

High production of functional *D*-phenylglycine aminotransferase in *Escherichia coli* by addition of benzyl alcohol, pyridoxine, and co-expression with chaperones

Juntratip Jomrit

Advisors: Pijug Summpunn/ Vithaya Meevootisom/ Suthep Wiyakrutta

Department of Biotechnology, Faculty of Science, Mahidol University

Abstract

D-Phenylglycine aminotransferase (*D*-PhgAT) from *Pseudomonas stutzeri* ST-201 catalyzes a reversible stereo-inverting transamination of *D*-phenylglycine and α-ketoglutarate to yield benzoylformate and *L*-glutamate. Due to the ''stereo-inverting'' transamination activity of this enzyme, *D*-PhgAT is useful as a biocatalyst for the enzymatic synthesis of *D*- phenylglycine and *D*-4OH-phenylglycine in a single step using *L*-glutamate as a cheap amino-group donor, which are important side-chains for industrial production of many penicillin and cephalosporin antibiotics. The enzyme is a relatively hydrophobic protein that tends to form aggregate upon over-expression in *E. coli* host such as the BL21(DE3)/pET17b system. To improve the yield of functional *D*-PhgAT, a number of approaches were done including addition of benzyl alcohol to induce endogenous chaperones production in the host; addition of the coenzyme precursor, pyridoxine hydrochloride, to assist in protein folding and in gaining enzyme activity; and co-expression of the encoding gene, *dpgA*, with *E. coli* chaperone genes (*dnaK-dnaJ-grpE*, *groEL-groES*). Among these, the only successful approach was the co-expressions with molecular chaperones. The level of active *D*-PhgAT production could be increased from 0.44 units.L^{-1}.OD_{600}^{-1} (no chaperone co-expression) to 1,768 units.L^{-1}.OD_{600}^{-1} with the GroEL-GroES - DnaK-DnaJ-GrpE co-expressions.

Keywords: *D*-phenylglycine aminotransferase, coenzyme, co-expression, chaperones

IX European Symposium of The Protein Society

Wonders and Disasters of the Protein World

May 22-26 2011 Stockholm Sweden

Stockholm University

UPPSALA UNIVERSITET

Linköping University

14019

A sensitive non-radioactive method for determination of stereospecificity of aminotransferases for C-4' hydrogen transfer of the coenzyme

Juntratip Jomrit[1], Pijug Summpunn[1], Vithaya Meevootisom[2] and Suthep Wiyakrutta[2]

[1]. Mahidol University, Biotechnology, Bangkok, Thailand
[2]. Mahidol University, Microbiology, Bangkok, Thailand

mizuho_2u@hotmail.com

Abstract

A sensitive non-radioactive method for determination of the stereospecificity of the C-4' hydrogen transfer on the coenzymes (pyridoxal phosphate, PLP and pyridoxamine phosphate, PMP) of aminotransferases has been developed. In this method, the aminotransfrase of unknown stereospecificity in its double PLP form was reacted in ^2H_2O with a substrate amino acid. The resultant PMP was thus labeled with deuterium at C-4' in the *pro-S* or *pro-R* configuration according to the stereospecificity of the aminotransfrase tested. The deuterium labeled PMP was isolated from the protein part of the enzyme and divided into two portions. One portion of the deuterated PMP was incubated in aqueous buffer with apo form of aspartate aminotransferase (as a reference *si*-face specific enzyme) and the other part was incubated with apo-branched-chain L-amino acid aminotransferase (as a reference *re*-face specific enzyme) in the presence of a respective 2-oxo acid substrate in each case. If the aminotransferase in question transfer hydrogen on the opposite face of the coenzyme compared with the reference aminotransferase, the deuterium at C-4' would then be retained resulted in the PLP having 1 mass unit higher than that from the reactions in which the hydrogen transfer catalyzed by the two enzymes occurred on the same face. Molecular mass of the PLP formed in the reactions was analyzed by high-performance liquid chromatography-tandem mass spectrometry. The method was highly sensitive that for the aminotransferase with ca. 50 kDa subunit molecular weight, only 2 mg of the enzyme was sufficient for the whole test. With this method, the use of radioactive substances could be avoided without compromising the sensitivity of assay. The method was satisfactorily verified with an aminotransferase of known stereospecificity, and was successfully applied to determine the stereospecific nature of a novel enzyme, D-phenylglycine aminotransferase.