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Journal of Molecular Catalysis B: Enzymatic 11 (2001) 199–205

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

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Novel biosynthetic routes to non-proteinogenic amino acids as chiral pharmaceutical intermediates

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Abstract

Transaminases catalyse the reversible transfer of amino and keto groups between an amino acid and keto acid substrate pair. Many bacterial transaminases accept a wide array of keto acids as amino acceptors and are useful as commercial biocatalysts in the preparation of amino acids. Since the reaction equilibrium typically lies close to unity, several approaches have been described to improve upon the 50% product yield, using additional enzymes. The present work describes an efficient means to significantly increase product yield in transamination using the aromatic transaminase of *Escherichia coli* encoded by the *tyrB* gene, with L-aspartate as the amino donor. This is achieved by the introduction of the *alsS* gene encoding the acetolactate synthase of *Bacillus subtilis*, which eliminates pyruvate and alanine produced as a by-product of aspartate transamination. The biosynthesis of the non-proteinogenic amino acid L-2-aminobutyrate is described using a recombinant strain of *E. coli* containing the cloned *tyrB* and *alsS* genes. The strain additionally carries the cloned *ilvA* gene of *E. coli* encoding threonine deaminase to produce the substrate 2-ketobutyrate from L-threonine. An alternate coupled process uses lysine ε-aminotransferase in concert with a transaminase using L-glutamate as the amino donor. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Biotransformation; Transaminase; Amino acid; Acetolactate synthase; Lysine aminotransferase

1. Introduction

In bacteria, transaminases are involved directly or indirectly in the biosynthesis of most proteinogenic amino acids, often as the final step in a biosynthetic pathway [9]. Biochemical and genetic studies on bacterial transaminases (aminotransferases) have shown that there is often considerable redundancy amongst these enzymes. For example, the aromatic aminotransferase of *Escherichia coli* is capable of synthesising aspartate and leucine in addition to the aromatic amino acids phenylalanine and tyrosine. Similarly, the *E. coli* branched chain transaminase can synthesise phenylalanine and methionine in addition to the branched chain amino acids, isoleucine, leucine, and valine [4]. The relaxed substrate specificity of microbial transaminases has been useful in the development of biotransformation approaches for the synthesis of non-proteinogenic amino acids,

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PII: S1381-1177(00)00055-2

which are now in increasing demand as intermediates for the synthesis of peptidomimetic pharmaceuticals and in combinatorial chemistry. Although a number of biocatalytic and chemo-enzymatic methods have been described for the production of unnatural or non-proteinogenic [10,11,17] amino acids, transaminases possess many features appropriate for efficient biocatalysts, including high turnover numbers and no requirement for external cofactor recycling. A number of compounds including L-2-aminobutyric acid and L-tertiary leucine have now been produced using transaminase-based biotransformations [2,8,14].

The commercial feasibility of a biotransformation process also relies heavily upon other criteria such as the availability of inexpensive starting materials, the reaction yield, and the complexity of product recovery. In the case of transaminase processes, the reversible nature of the reaction, as shown in Fig. 1, and the presence of a keto acid by-product are the concerns that limit the overall yield and purity of product, and have led to efforts to increase the conversion beyond the typical 50% yield of product [5,13]. Additionally, there are cost considerations in the large-scale preparation of keto acid substrates such as 2-ketobutyrate, which are not commodity chemicals.

To address these issues, we have engineered synthetic biochemical pathways to enhance the biosynthesis of non-proteinogenic amino acids such as L-2-aminobutyrate using microbial transaminases. In addition to the cloned transaminase gene, the engineered *E. coli* incorporates the cloned *E. coli* K12 *ilvA* gene encoding threonine deaminase to generate 2-ketobutyrate from the commodity amino acid L-threonine, and the cloned *alsS* gene of *Bacillus subtilis* 168 to eliminate pyruvate, the keto acid by-product of the reaction, through the formation of acetolactate. The effect of the concerted action of these enzymes on the overall efficiency of 2-aminobutyrate biosynthesis and the potential broader application of this type of reaction is described.

2. Results

2.1. Cloning and expression of bacterial genes

Plasmid pIF347 carries the cloned *ilvA* gene of *E. coli* K12. The gene encodes threonine deaminase [1]. The enzyme converts L-threonine to 2-ketobutyrate as the first step in the biosynthetic pathway to isoleucine from threonine. On this plasmid, the *ilvA* gene is expressed constitutively from the modified *pheA* promoter region [12] immediately upstream on the vector.

Plasmid pME64 carries the cloned *tyrB* gene [7]. The gene encodes the aromatic aminotransferase of *E. coli*. The gene is expressed from its native promoter on plasmid pAT153, a copy number mutant of pBR322 [15]. The *tyrB* promoter which is normally subjected to transcriptional repression by the *tyrR* repressor protein [16] is deregulated due to the repressor titration effect of the high copy number plasmid vector.

Plasmid pPOT300 carries the *B. subtilis alsS* gene [3]. The *alsS* gene encodes acetolactate synthase, which converts two pyruvate molecules to acetolactate with the loss of CO_2. On this plasmid, the *alsS* gene is expressed from the lP_R promoter region regulated by the temperature sensitive l-C1857. The expression of *alsS* is repressed at 30°C and induced by shifting to 40°C. The reaction sequence is illustrated in Fig. 2.

2.2. Biosynthesis of L-2-aminobutyrate using L-aspartic acid and 2-ketobutyrate as substrates

Two reactions schemes were carried out. In Scheme A, cells of W3110/pME64 (*tyrB*) were reacted with 500 mM 2-ketobutyrate and 500 mM L-aspartic acid. In Scheme B, cells of W3110/pME64 (*tyrB*) and W3110/pPOT300 (*alsS*) were reacted with 500 mM 2-ketobutyrate and 500 mM L-aspartic acid. Following 24 h of incubation, samples were taken and analysed for amino acid content

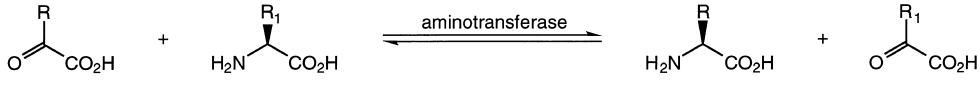

Fig. 1. The L-amino acid aminotransferase reaction.

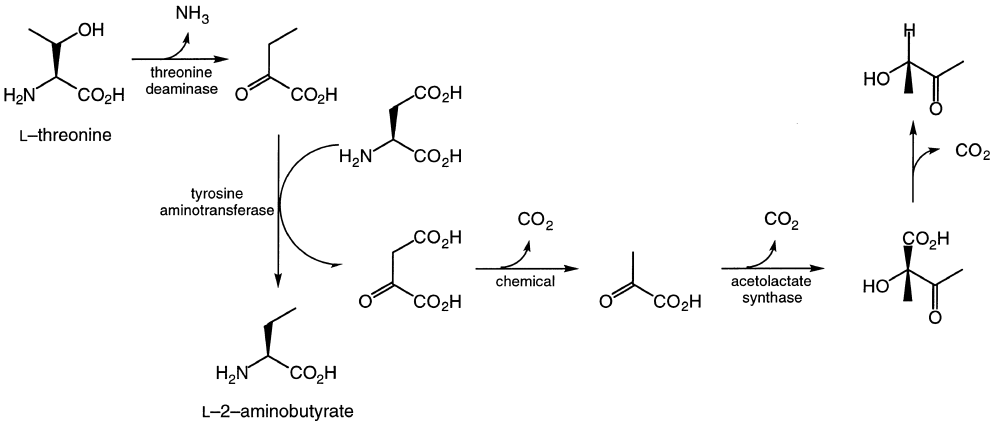

Fig. 2. Engineered biosynthetic pathway to L-2-aminobutyric acid.

by HPLC. The reaction in Scheme A contained 23.89 mg/ml L-2-aminobutyrate, 9.62 mg/ml L-alanine, and 1.16 mg/ml L-aspartate. The reaction in Scheme B contained 30.04 mg/ml L-2-aminobutyrate, 1.65 mg/ml L-alanine, and 1.28 mg/ml L-aspartate.The results are shown in Fig. 3. The aspartate amino donor is almost entirely consumed in the reaction. The oxaloacetate produced from transamination of aspartate spontaneously decarboxylates to produce pyruvate which in turn is a substrate for cellular enzymes, including transaminases, producing L-alanine as a by-product. The final concentration of 2-ketobutyrate was not determined.

In Scheme A, where only the transaminase activity is present, the molar yield of L-2-aminobutyrate is 46.4% (232 mM) from the 500 mM 2-ketobutyrate substrate. In Scheme B, with the acetolactate synthase present, the yield of L-2-aminobutyrate is slightly increased to 58% (290 mM). The ratio of L-2-aminobutyrate to L-alanine, the major amino acid impurity in g/l, is 2.5:1 in Scheme A. With the acetolactate synthase activity present, this ratio is increased to 18.2:1, thus simplifying product isolation. Fig. 3 illustrates the relative levels of amino acids present.

2.3. Biosynthesis of L-2-aminobutyrate using L-aspartic acid and L-threonine as substrates

Two reaction schemes were carried out. In Scheme C, cells of W3110/pME64 (*tyrB*) and W3110/pIF347 (*ilvA*) were reacted with 500 mM L-threonine and 500 mM L-aspartic acid. In Scheme D, cells of W3110/pME64 (*tyrB*), W3110/pIF347 (*ilvA*). and W3110/pPOT300 (*alsS*) were reacted with 500 mM L-threonine and 500 mM L-aspartic acid. Following 24 h of incubation, samples were taken and analysed for amino acid content by HPLC. The reaction in

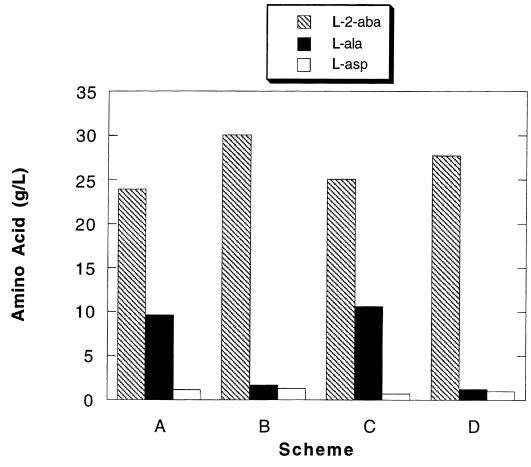

Fig. 3. Final amino acid concentrations following the biosynthesis of L-2-aminobutyrate using recombinant *E. coli* cells. In Schemes A and B, the cells did not carry the *ilvA* gene encoding threonine deaminase since 2-ketobutyrate rather than L-threonine was used as the keto acid substrate. Scheme A: W3110/pME64 (*tyrB*). Scheme B: W3110/pME64 (*tyrB*)/W3110/pPOT300 (*alsS*). Scheme C: W3110/pME64 (*tyrB*)/W3110/pIF347 (*ilvA*). Scheme D: W3110/pME64 (*tyrB*)/W3110/pIF347 (*ilvA*)/W3110/pPOT300 (*alsS*).

Scheme C contained 25.05 mg/ml L-2-aminobutyrate, 10.6 mg/ml L-alanine, and 0.69 mg/ml L-aspartate. The reaction in Scheme D contained 27.71 mg/ml L-2-aminobutyrate, 1.23 mg/ml L-alanine, and 1.01 mg/ml L-aspartate. In each case, the L-threonine and L-aspartic acid substrates were almost entirely consumed. Residual 2-ketobutyrate was determined to be less than 1 g/l (data not shown). In Scheme C, where threonine deaminase and transaminase activities are present, the molar yield of L-2-aminobutyrate is 46.4% (243 mM) from the 500 mM 2-ketobutyrate substrate. In Scheme D with the acetolactate synthase present, the yield of L-2-aminobutyrate is increased to 54% (270 mM). The ratio of L-2-aminobutyrate to L-alanine, the major amino acid impurity in g/l, is 2.4:1 in Scheme C. With the additional acetolactate synthase activity present, this ratio is increased to 22.5:1. This is shown in Fig. 3. Acetolactate levels were not accurately quantified as it is relatively unstable under the reaction conditions and decomposes to acetoin.

A control strain of W3110 which carried only plasmid pIF347 and therefore lacked the cloned transaminase was similarly reacted with 500 mM L-threonine and 500 mM L-aspartic acid. Following 20 h of incubation, this reaction produced only 6 mM L-2-aminobutyrate and 4 mM L-alanine. This is approximately a 1% reaction yield, presumably due to a low level of background transaminase activity from the host cell. The aspartic acid concentration at the end of the reaction was reduced by 22% to 390 mM.

In each reaction, the yield of amino acid product is inconsistent with the complete consumption of the aspartate substrate. We have also determined the final level of 2-ketobutyrate to be less than 1 g/l. We consider the discrepancy to be due to partial catabolism of both aspartic acid and 2-ketobutyrate by the host cells, which retain a degree of background metabolic activity. In *E. coli*, aspartic acid is catabolised by the activity of aspartate ammonia lyase (aspartase), encoded by the *aspA* gene, which converts aspartate to fumarate, a TCA cycle intermediate. To determine the potential of aspartase to consume aspartic acid, strain HW1452, a W3110 derivative mutated in *aspA*, was incubated with 800 mM sodium aspartate and compared to wild type W3110 cells incubated under similar conditions. Following incubation at 37°C for 20 h, the reaction containing W3110 cells was sampled and found to contain 536 mM L-aspartic acid, a reduction of 33%. The reaction containing HW1452 contained 712 mM L-aspartic acid, a reduction of 11%. It therefore appears likely that the action of aspartase is at least partly responsible for the catabolism of aspartic acid in this process.

3. Discussion

Enantiomerically pure L-2-aminobutyric acid was produced from L-threonine and L-aspartic acid in a whole cell biotransformation using recombinant *E. coli* cells, expressing cloned genes encoding threonine deaminase, aromatic aminotransferase, and acetolactate synthase. The concerted action of the three enzymes increased the overall yield of product and more importantly, reduced the accumulation of L-alanine as a by-product, such that L-2-aminobutyric acid represented over 92% of the total amino acids present at the end of the reaction. In the absence of acetolactate synthase, L-2-aminobutyric acid represented only 68% of the total amino acids present.

The results demonstrate the value of coupled reactions to enhance the overall performance and increase the commercial feasibility of a biotransformation process. This is particularly important in a transamination reaction where the reaction equilibrium, typically lying close to one, results in equimolar concentrations of substrates and products. The synthetic biochemical pathway operates in a single recombinant strain of *E. coli* and has been successfully operated at commercial scale. In a further development of the process, the introduction of lysine ε-aminotransferase, as shown in Fig. 4, allows for the complete elimination of aspartate and thus alanine in the bioprocess, replacing acetoin by Δ1-piperidine-6-carboxylic acid as the by-product (data not shown). This pathway is also being developed for large-scale operation. The increasing availability of microbial transaminases from diverse gene banks and genomic databases enables these synthetic pathways to be applied to the biosynthesis of an increasing range of non-proteinogenic or unnatural amino acids. The alternate pathways using lysine aminotransferase or acetolactate synthase provide a degree

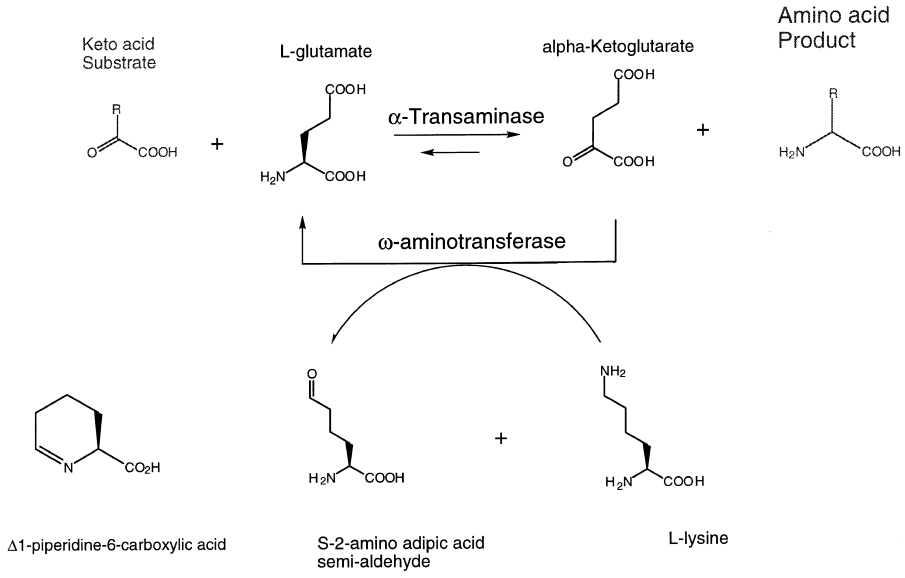

Fig. 4. Coupled α/ω (lysine) aminotransferase reaction.

of flexibility important to the final isolation and recovery of the product, also a key element in determining the success of biocatalytic processes. The use of whole metabolically active cells allows all the necessary enzymes to be produced from cloned genes in a single microorganism, simplifying biocatalyst preparation but potentially introducing undesirable side reactions through the normal metabolic activity of the cell. This was apparent in this case, as catabolism of the aspartate and 2-ketobutyrate substrates leads to a loss of product. Further engineering of the host strain can remove the undesired side reactions through deletion of appropriate chromosomal genes. This is a realistic prospect due the high degree of understanding of *E. coli* metabolism. The potential to engineer and optimise novel and useful synthetic pathways will continue to expand as the biochemical pathways of *E. coli* and other microorganisms become ever more clearly defined.

4. Experimental

4.1. DNA manipulation

General DNA handling including PCR, restriction analysis, and recovery, chromosomal and plasmid DNA preparations, and *E. coli* transformation was carried out using standard methods as previously described [6].

4.2. Preparation of cell cultures for biotransformation

Cell cultures of W3110 carrying pME64, pPOT300, or pIF347 were prepared by inoculating 50 ml of LB medium with a single colony from an LB agar plate and culturing overnight at 37°C in a 500-ml shake flask in a shaking incubator. Antibiotics, where appropriate, were added at the concentrations of 100 ug/ml ampicillin, 40 ug/ml kanamycin, and 10 ug/ml chloramphenicol. Overnight cultures were then used to inoculate 1 l of LB plus antibiotics to an initial OD600 of 0.05. These were grown at 37°C in a 4-l flask with agitation at 300 rpm until the OD600 reached 1.0. The cells were then recovered by centrifugation at 10,000 $\times g$ for 5 min, washed in 50 mM Tris–HCl buffer and similarly pelleted. The required weight of pelleted wet cells was then added to biotransformation mixes.

4.3. Construction of pME64

Plasmid pME64 was constructed as described in Ref. [7].

4.4. Construction of pIF347

To construct pIF347, the *ilvA* gene encoding threonine deaminase was amplified from the chromosome of *E. coli* K12 by PCR. The *ilvA* gene was specifically amplified by PCR using the oligonucleotide primers:

5′ CGC GGA TCC ATC ATG GCT GAC TCG CAA CCC C 3′

and

5′ CTC GCA TGC CAG GCA TTT TTC CCT AAC CCG CC 3′.

The PCR product was cleaved using the restriction enzymes *Bam*HI and *Sph*I and 1.57 kB fragment thus generated was ligated to 4.1 kB fragment of pIF312 (Ian Fotheringham) which had been similarly cleaved with *Bam*HI and *Sph*I. The resulting plasmid was named pIF347.

4.5. Construction of pPOT300

The *alsS* gene was then amplified from the *B. subtilis* chromosome using PCR. *B. subtilis* chromosomal DNA was prepared using the same procedure as for *E. coli* K12. The *alsS* gene was specifically amplified by PCR using the following oligonucleotide primers.

5′ TTT GGA TCC ATC ACA AGA TAT TTA AAA TTT 3′

5′ TTT AGC GTC GAC GCA TGC TCC TTT TAT TTA GTG CTG TTC 3′.

The PCR product was then cleaved with the enzymes *Bam*HI and *Sal*I and the resulting 1.9 kB fragment was ligated to 4.76 kB fragment of pPOT3 (Nigel Grinter) which was isolated by agarose gel electrophoresis following similar cleavage by *Bam*HI and *Sal*I. The resulting plasmid was named pPOT300.

4.6. HPLC analysis of amino acids

The amino acids were quantified by HPLC using OPA/BOC-Cys derivatization. The mobile phase used a gradient of Pump A = 60% MeOH, 40% 0.05 M TEAP buffer, pH = 7.0. Pump B = H_20. Times: Pump B, 32% at 0 and 6 min. By 8 min, 5% Pump B. At 14.1 min, revert to starting conditions. Column heater at 40°C. The column was a Supelcosil LC-18DB, 3 m, 150 × 4.6 mm. The flow rate was 1.0 ml/min, Detection UV at 338 nm, injection volume was 10 l.

4.7. Biosynthesis of L-2-aminobutyric acid

The effect of acetolactate synthase upon the efficiency of 2-aminobutyrate biosynthesis was investigated using L-aspartic acid and 2-ketobutyrate or L-aspartic acid and L-threonine as substrates.

4.8. Biosynthesis of L-2-aminobutyric acid using 2-ketobutyrate and L-aspartic acid as substrates

4.8.1. Reaction A

A total of 200 mg wet cell weight of W3110/pME64 was added to a solution containing 100 mM Tris–HCl, pH 7.5, 500 mM 2-ketobutyrate, and 500 mM L-aspartic acid, adjusted to pH 7.5 with NaOH in a 2-ml reaction volume. The reaction was sealed and shaken at 37°C for 24 h. Following incubation for 24 h, a 200-ul sample was taken and the cells were removed by centrifugation. The sample was then diluted 100-fold and subjected to amino acid analysis by HPLC.

4.8.2. Reaction B

A total of 200 mg wet cell weight of W3110/pME64 and 100 mg wet cell weight of W3110/pPOT300 were added to a solution containing 100 mM Tris–HCl, pH 7.5, 500 mM 2-ketobutyrate, and 500 mM L-aspartic acid, adjusted to pH 7.5 with NaOH in a 2-ml reaction volume. The reaction was sealed and shaken at 37°C for 24 h.

Following incubation for 24 h, a 200-ul sample was taken and the cells were removed by centrifugation. The sample was then diluted 100-fold and subjected to amino acid analysis by HPLC.

4.9. Biosynthesis of L-2-aminobutyrate using L-threonine and L-aspartate as substrates

4.9.1. Reaction C

A total of 200 mg wet cell weight of W3110/pME64 and 100 mg wet cell weight of W3110/

pIF347 were added to a solution containing 500 mM L-threonine and 500 mM L-aspartic acid, adjusted to pH 8 with NaOH in a 2-ml reaction volume. The reaction was sealed and shaken at 37°C for 24 h.

Following incubation for 24 h, a 200-ul sample was taken and the cells were removed by centrifugation. The sample was then diluted 100-fold and subjected to amino acid analysis by HPLC.

4.9.2. Reaction D

A total of 200 mg wet cell weight of W3110/pME64, 100 mg wet cell weight of W3110/pIF347, and 100 mg/ml W3110/pPOT300 were added to a solution containing 500 mM L-threonine and 500 mM L-aspartic acid, adjusted to pH 8 with NaOH in a 2-ml reaction volume. The reaction was sealed and shaken at 37°C for 24 h.

Following incubation for 24 h, a 200-ul sample was taken and the cells were removed by centrifugation. The sample was then diluted 100-fold and subjected to amino acid analysis by HPLC.

4.10. L-Aspartic acid catabolism

A total of 100 mg wet cell weight of W3110 and 100 mg wet cell weight of HW1452 were added to a solution containing 800 mM L-aspartic acid, adjusted to pH 7.5 with NaOH in a 2-ml reaction volume. The reaction was sealed and shaken at 37°C for 20 h.

Following incubation for 24 h, a 200-ul sample was taken and the cells were removed by centrifugation. The sample was then diluted 100-fold and subjected to amino acid analysis by HPLC.

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