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Engineering of A Novel Biochemical Pathway for the Biosynthesis of L-2-aminobutyric Acid in *Escherichia coli* K12

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Abstract—L-2-Aminobutyric acid was synthesised in a transamination reaction from L-threonine and L-aspartic acid as substrates in a whole cell biotransformation using recombinant *Escherichia coli* K12. The cells contained the cloned genes *tyrB*, *ilvA* and *alsS* which respectively encode tyrosine aminotransferase of *E. coli*, threonine deaminase of *E. coli* and α-acetolactate synthase of *B. subtilis* 168. The 2-aminobutyric acid was produced by the action of the aminotransferase on 2-ketobutyrate and L-aspartate. The 2-ketobutyrate is generated in situ from L-threonine by the action of the deaminase, and the pyruvate by-product is eliminated by the acetolactate synthase. The concerted action of the three enzymes offers significant yield and purity advantages over the process using the transaminase alone with an eight to tenfold increase in the ratio of product to the major impurity.

Introduction

Enzymatic transamination is involved directly or indirectly in the biosynthesis of most proteinogenic amino acids.[1] Biochemical studies on the transaminases (aminotransferases) of bacteria have revealed that in many cases the major transaminases can each participate in the synthesis of a variety of amino acids with quite divergent side chain structure. For example, the aromatic aminotransferase of *E. 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.[2] The relatively relaxed substrate specificity of microbial transaminases has been useful in the development of biotransformation approaches for the synthesis of non-proteinogenic amino acids, which are now in increasing demand as intermediates for the synthesis of peptidomimetic pharmaceuticals. Transaminases possess additional 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.[3,4]

In addition to the identification of the appropriate biosynthetic enzyme, the feasibility of all biotransformation processes depends 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 Figure 1 and the presence of a keto acid by-product is a concern which limits the overall yield and purity of product, and has led to efforts to increase the conversion beyond the typical 50% yield of product.[5,6] 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 a synthetic biochemical pathway to enhance the biosynthesis of 2-aminobutyrate using a microbial transaminase. 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 *B. subtilis* 168 which eliminates pyruvate, the keto acid by-product of

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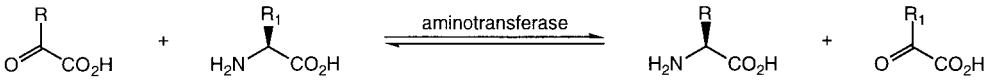

Figure 1. The L-amino acid aminotransferase reaction.

the reaction, through the formation of non-reactive acetolactate. The effect of the concerted action of these three enzymes on the overall biosynthesis of 2-aminobutyrate and the potential broader application of this type of biochemical engineering is described in this article.

Results

Cloning and expression of biocatalyst genes

Plasmid pIF347 carries the cloned *ilvA* gene of *E. coli* K12. The gene encodes threonine deaminase.[7] 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[8] immediately upstream on the vector.

Plasmid pME64 carries the cloned *tyrB* gene.[9] 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.[10] The *tyrB* promoter which is normally subject to transcriptional repression by the *tyrR* repressor protein[11] is deregulated due to the repressor titration effect of the high copy number plasmid vector.

Plasmid pPOT300 carries the *B. subtilis alsS* gene.[12] 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 λP_R promoter region regulated by the temperature sensitive *λ-C1857*. The expression of *alsS* is repressed at 30°C and induced by shifting to 40°C. The reaction sequence is illustrated in Figure 2.

Biosynthesis of L-2-aminobutyrate using W3110/pME64 and W3110/pIF347 with 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 incubation, samples were taken and analysed for amino acid content 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 Figure 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. Figure 3 illustrates the relative levels of amino acids present.

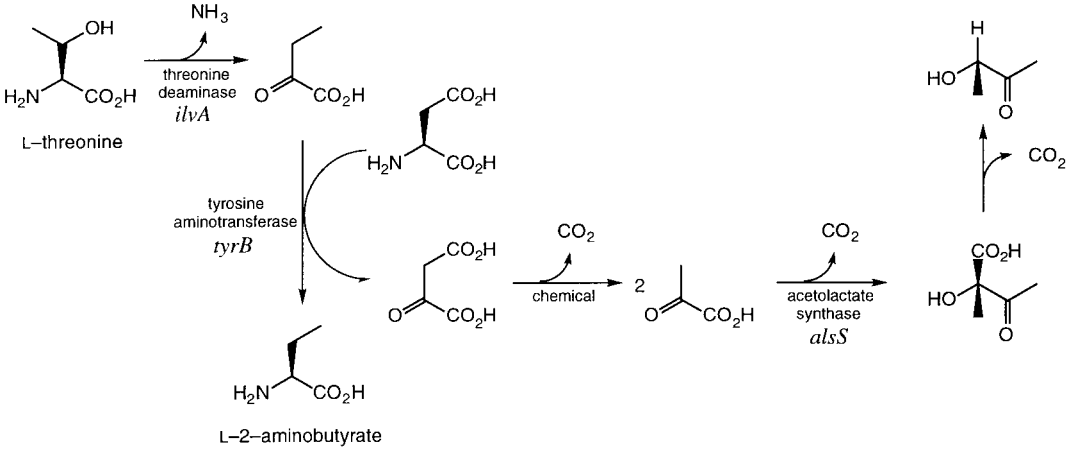

Figure 2. Engineered biosynthetic pathway to L-2-aminobutyric acid.

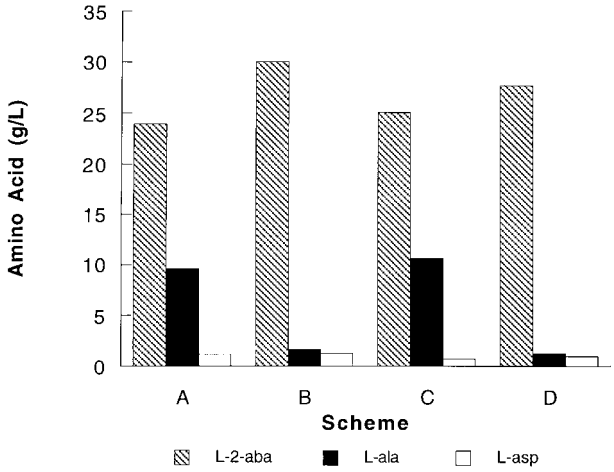

Figure 3. Biosynthesis of L-2-aminobutyrate using recombinant *E. coli* cells. Scheme A: W3110/pME64 (*tyrB*). Scheme B: W3110/pME64 (*tyrB*)/W3110/pPOT300 (*alsS*). Scheme C: W3110/pME64 (*tyrB*)/W3110/pIF347 (*ilvA*). Scheme D: W3110/pME64 (*tyrB*)/W3110/pIF347 (*ilvA*)/W3110/pPOT300 (*alsS*).

Biosynthesis of L-2-aminobutyrate using W3110/pME64 and W3110/pIF347 with 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 incubation, samples were taken and analysed for amino acid content by HPLC. The reaction in 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 the 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 in 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 Figure 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 carred 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 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 that the final level of 2-ketobutyrate is less than 1 g/L. We considered 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.

Discussion

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 for 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 of the transamination 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 final amino acids present when either L-threonine or 2-ketobutyric acid were used as the substrate. In the absence of threonine deaminase the yield and purity are comparable to the three gene system but the commercial feasibility of the process is significantly diminished by the high cost and unavailability of 2-ketobutyrate.

The results demonstrate the value of reaction coupling to enhance the overall performance of a biotransformation process. This is particularly important in a transamination reaction where the reaction equilibrium, typically lying close to 1, results in residual equimolar concentrations of substrates and products. This is apparent in the reaction reported by Tokarski et al.[13] where the *E. coli* K12 alanine-valine aminotransferase was used in conjunction with threonine deaminase to produce L-2-aminobutyrate. In that case the amino donor used was alanine rather than aspartate, with the reaction reaching an equilibrium point where the

L-alanine concentration exceeded that of the L-2-aminobutyrate. Since these two amino acids are extremely similar in structure and chemical properties, this can introduce significant difficulties in the isolation and recovery of the L-2-aminobutyrate.

In bioprocesses the complexity of the recovery process can often be the major factor in determining the overall process economics. Since the transaminase reaction can use the same amino donor for many different amino acid syntheses the use of acetolactate synthase as a coupled enzyme can be used as a general method to eliminate the occurrence of alanine in the reaction mixture and improve the economics of transaminase bioprocesses.

When considering the overall reaction scheme it is important to consider the particular characteristics of the enzymes chosen. In this case these enzymes were selected not only for their general accessibility through PCR amplification of the known DNA sequence of the host gene but also because of their known substrate preference and allosteric inhibition characteristics. The *E. coli* aromatic aminotransferase possesses sufficiently low stringency to accept 2-ketobutyrate as a substrate whereas the *B. subtilis* acetolactate synthase discriminates effectively between 2-ketobutyrate and pyruvic acid as substrates.[12] This is in contrast to the acetohydroxyacid synthases of *E. coli* K12 which efficiently accept pyruvate and 2-ketobutyrate as substrates, generating acetolactate and α-aceto-β-hydroxybutyrate as intermediates in the biosynthetic pathways of valine and isoleucine, respectively.[1] This is important in order to optimise the availability of the 2-ketobutyrate for transamination.

Nevertheless, it is apparent from the results that despite complete consumption of the substrates, not all of the aspartate and threonine are used in the biosynthesis of 2-aminobutyrate. This is an inherent drawback to the use of fresh, whole cells in the biotransformation. We attribute the loss of substrate to alternate enzymatic conversions occurring due to the high loading of whole cells in the reaction. Aspartate can be catabolised by aspartate ammonia lyase (aspartase) in *E. coli*. We have observed that an *aspA* mutant deficient in aspartase incubated with L-aspartate consumes significantly less aspartate under similar conditions to the wild type strain. Similarly, 2-ketobutyrate is an intermediate in isoleucine biosynthesis in a biosynthetic pathway which is not subject to subsequent regulation. It is also an intermediate in propionyl-coenzyme A biosynthesis. To further optimise the process reported here we are examining the combined effect of mutations in the genes controlling these enzymatic activities to reduce undesired side reactions of the substrates. The ability to engineer the host cell to incrementally improve the efficiency and economics of whole cell biotransformations is an attractive feature of this type of process. Similarly the increasing availability of genomic DNA sequence from diverse microbes is rapidly expanding the potential for coupling biosynthetic activities in industrial processes.

Experimental

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.[14]

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 μg/mL ampicillin, 40 μg/mL kanamycin and 10 μg/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 *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.

Construction of pME64

Plasmid pME64 was constructed as described in ref 9.

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 *BamHI* and *SphI* and the 1.57 kB fragment thus generated was ligated to the 4.1 kB fragment of pIF312 (Ian Fotheringham) which had been similarly cleaved with *BamHI* and *SphI*. The resulting plasmid was named pIF347.

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 *BamHI* and *SalI* and the resulting 1.9 kB fragment was ligated to the 4.76 kB fragment of pPOT3 (Nigel Grinter) which was isolated by agarose gel electrophoresis following similar cleavage by *BamHI* and *SalI*. The resulting plasmid was named pPOT300.

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.

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

Reaction A. Two hundred milligrams 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 μL sample was taken and the cells removed by centrifugation. The sample was then diluted 100 fold and subjected to amino acid analysis by HPLC.

Reaction B. Two hundred milligrams 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 μL sample was taken and the cells removed by centrifugation. The sample was then diluted 100 fold and subjected to amino acid analysis by HPLC.

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

Reaction C. Two hundred milligrams 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 μL sample was taken and the cells removed by centrifugation. The sample was then diluted 100-fold and subjected to amino acid analysis by HPLC.

Reaction D. Two hundred milligrams 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 μL sample was taken and the cells removed by centrifugation. The sample was then diluted 100-fold and subjected to amino acid analysis by HPLC.

L-aspartic acid catabolism. One hundred miligrams 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 μL sample was taken and the cells removed by centrifugation. The sample was then diluted 100-fold and subjected to amino acid analysis by HPLC.

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% 0.05M TEAP buffer pH = 7. Pump B = H_2O. Times: Pump B, 32% at 0 min, and at 6 min. By 8 min, 5% pump B. At 14.1 min revert to starting conditions. Column heater @ 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 @ 338 nm, injection volume 10 L.

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