lized pH- and glutaraldehyde-treated *P. dacunhae* cells. The substrate solution (adjusted to pH 8.5 with ammonia) consisting of 1.5 *M* ammonium fumarate and 1 m*M* Mg^{2+} is applied to the column containing immobilized *E. coli* cells at a flow rate of space velocity = 1.0/hr. After addition of PLP and pyruvic acid (these concentrations are 0.1 and 1 m*M*, respectively) into the effluent, pH of the solution is adjusted to 6.0 by addition of acetic acid. The solution is passed through the immobilized *P. dacunhae* closed column at a flow rate of space velocity = 0.06/hr and a pressure of about 8 kg/cm^2 achieved by plunger pump.

Crystallization of L-*Alanine from Column Effluent*. The effluent of appropriate volume is concentrated to about one-fourth of its original volume and cooled to 15°. L-Alanine crystallized is collected by centrifugation or by filtration and washed with 80% aqueous ethanol. The yield of L-alanine from ammonium fumarate is about 90% (theoretical). $[\alpha]_D^{20}$ = +14.8 (c = 10 in 6 *N* HCl).

Conclusion

In 1982, Tanabe Seiyaku Co. Ltd. successfully industrialized a continuous production system of L-alanine from ammonium fumarate, using a column reactor containing immobilized pH-treated *E. coli* cells and a closed column reactor containing immobilized pH- and glutaraldehyde-treated *P. dacunhae* cells.

By this system, L-alanine has been produced at low cost. This is considered to be the first industrial application of sequential enzyme reactions using two immobilized microbial cells.

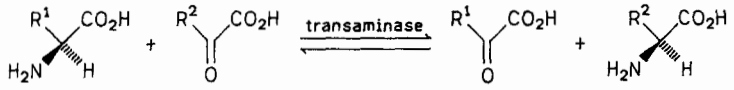
[44] Immobilized Aminotransferases for Amino Acid Production

By J. DAVID ROZZELL

Aminotransferases

Background

Aminotransferases (more commonly called transaminases, EC 2.6.1._) are a widely distributed class of enzymes. These enzymes catalyze the synthesis and breakdown of amino acids in microorganisms,



SCHEME 1.

plants, and animals by the transfer of an amino group from an α-amino acid to a 2-ketoacid as shown in Scheme 1.

The first evidence for aminotransferases was published by Needham[1] and Szent-Györgyi and co-workers[2] who noticed a relationship between the L-glutamic acid, L-aspartic acid, and oxaloacetic acid levels in pigeon breast muscle. Banga and Szent-Györgyi[3] demonstrated the reversibility of glutamic-pyruvic transaminase (EC 2.6.1.2, alanine aminotransferase) by chemically isolating the amino acid products L-glutamate and L-alanine. Since that time, a large number of aminotransferases have been discovered and characterized. One feature of aminotransferases is the requirement for the small molecule, pyridoxal 5′-phosphate, for catalytic activity, this cofactor being bound through a Schiff base linkage to the ε-amino group of an active-site lysine. Although the binding of pyridoxal 5′-phosphate to the enzyme is reversible, most aminotransferases show maximal catalytic activity at cofactor concentrations of 100 μM or less. Such low saturating concentrations of pyridoxal phosphate are an important property of aminotransferases; at concentrations of 100 μM or less, the cost of the cofactor in biocatalytic transamination processes is a relatively minor component of the total cost.

The mechanism of transamination is well known, and has been reviewed previously.[4] The reaction catalyzed by aminotransferases occurs as the result of two distinct half-reactions: the first involves transfer of the amino group of the L-amino acid donor to pyridoxal 5′-phosphate to yield a 2-ketoacid product which is released from the enzyme and an enzyme-bound pyridoxamine 5′-phosphate; the second is the binding of the 2-ketoacid to be transaminated to the enzyme and the transfer of the amino group from pyridoxamine 5′-phosphate to this 2-ketoacid to produce the desired L-amino acid and regenerate the pyridoxal 5′-phosphate. As a result, aminotransferases characteristically exhibit Ping-Pong kinetics.

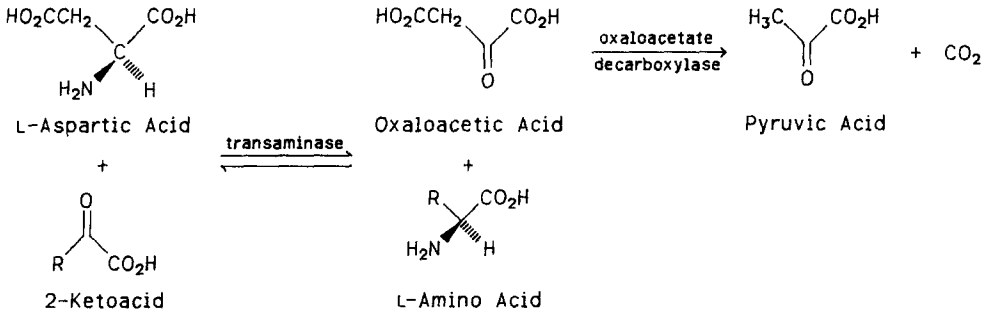
[1] D. M. Needham, *Biochem. J.* **24,** 208 (1930).
[2] E. Annau, I. Banga, A. Blazo, V. Bruckner, K. Laki, F. B. Staub, and A. Szent-Györgyi, *Z. Physiol. Chem.* **224,** 105 (1936).
[3] I. Banga and A. Szent-Györgyi, *Z. Physiol. Chem.* **245,** 118 (1937).
[4] A. E. Braunstein, "The Enzymes IX" (P. D. Boyer, ed.), Part B, pp. 379–481. Academic Press, New York, 1973.

Advantages and Disadvantages for Use in Biocatalysis

Although aminotransferases have been known for decades, these enzymes have seen little use as biocatalysts until now. However, since the normal function of aminotransferases is the biosynthesis and metabolism of amino acids, it is natural to look to these enzymes as potentially useful catalysts for the production of amino acids. In principle, almost any desired amino acid can be produced from the appropriate 2-ketoacid using an inexpensive amino acid as the amino donor. There are a number of advantages to the use of this kind of technology. (1) The aminotransferase enzymes catalyze the stereoselective synthesis of only L-amino acids from their corresponding 2-ketoacids. No D isomer is produced, and no resolution is required. (2) Aminotransferases have uniformly high catalytic rates, capable of converting up to 400 μmol of substrate/min per milligram of protein. (3) Many of the required 2-ketoacid precursors can be conveniently prepared by chemical synthesis at low cost. (4) The capital investment for an immobilized enzyme process using aminotransferases is much lower than for a fermentation process, and the productivity of the bioreactor is more than an order of magnitude higher. (5) The technology is generally applicable to a broad range of L-amino acids because aminotransferases exist with varying specificities. For example, there are enzymes specific for the transamination of amino acids with acidic side chains, aromatic side chains, branched alkyl side chains, etc. Such broad scope allows a number of different L-amino acids to be produced with the same equipment and often the same biocatalyst.

We have demonstrated laboratory-scale processes for the production of a variety of L-amino acids including L-alanine, L-phenylalanine, L-tyrosine, L-tryptophan, and several others. As an example, we have immobilized the commercially available glutamic-pyruvic aminotransferase from porcine heart on porous glass by covalent attachment, and obtained a stable biocatalyst with an activity of 400 International Units per gram. A column packed with 500 mg of this immobilized enzyme was operated continuously for 6 months and produced 160 mg L-alanine/hr from pyruvic acid as a starting material. This example illustrates the potential of immobilized aminotransferases applied to the production of L-amino acids.

There is one inherent disadvantage to the practice of this technology as described so far; as a catalyst, the aminotransferase can only accelerate the approach to equilibrium between the L-amino acid and 2-ketoacid precursors on one side of the equation and the 2-ketoacid and L-amino acid products on the other. Thus, the equilibrium constant for the generic transamination reaction as written in Scheme 1 is near unity, and the



SCHEME 2.

conversion of a 2-ketoacid feedstock to a desired L-amino acid will not proceed to completion in most cases. The key to the development of a general and commercially successful transamination process for the production of L-amino acids lies in overcoming this problem of incomplete conversion of a 2-ketoacid to the desired L-amino acid.

Driving the Reaction to Completion

Solving the problem of incomplete conversion of 2-ketoacid starting material to a desired L-amino acid required one important observation regarding the substrate specificity of aminotransferases. Although L-glutamic acid is generally considered to be the amino donor for aminotransferases that catalyze the transamination of a broad range of 2-ketoacids to L-amino acids, we have found that L-aspartic acid can also function competently as a general donor of an amino group with certain enzymes. We have worked extensively with an aminotransferase capable of using L-aspartic acid isolated from *Escherichia coli*.[5]

When L-aspartic acid is used as the amino group donor for the transamination of a given 2-ketoacid, oxaloacetic acid is coproduced along with the desired L-amino acid. Oxaloacetate, unlike 2-ketoglutarate, is a β-ketoacid, and as such can facilely be converted to pyruvic acid via an essentially irreversible decarboxylation step. This may be accomplished chemically by the use of certain metal ions or amines, thermally, or most preferably, enzymatically using the enzyme oxaloacetate decarboxylase. The coupled two-enzyme reaction is illustrated in Scheme 2.

The important feature of this process is the decarboxylation of oxaloacetate to pyruvate. It is this essentially irreversible decarboxylation that drives the entire process to completion to produce L-amino acids in quantitative yields from the appropriate 2-ketoacid precursors. The

[5] C. Mavrides and W. Orr, *Biochim. Biophys. Acta* **336,** 70 (1974).

pyruvic acid by-product is easily separated from the product mixture by crystallization of the L-amino acid or by ion-exchange methods.

We have investigated several methods for decarboxylating oxaloacetate, including catalysis by primary amines and divalent metal ions such as Mg^{2+}, Mn^{2+}, and Zn^{2+} and the enzymatic decarboxylation by oxaloacetate decarboxylase (OAD, EC 4.1.1.3). This chapter will focus on driving the overall reaction by the OAD-catalyzed decarboxylation of oxaloacetate to produce pyruvate and the desired L-amino acid.

Methodology

Sources and Production of Enzymes

Aminotransferases can be isolated from virtually any microbial, plant, or animal source. The most easily obtained enzymes are from porcine heart, yeast, and *E. coli*. However, the usefulness of the individual aminotransferases for amino acid production varies. The glutamic-oxaloacetic aminotransferase from porcine heart (EC 2.6.1.1, aspartate aminotransferase), although very stable and commercially available, is of limited utility for the production of amino acids because of its high specificity for L-glutamic acid, L-aspartic acid, and the corresponding 2-ketoacids as substrates.[6] Other substrates are not transaminated at reasonable rates.

Similarly, the commercially available porcine glutamic-pyruvic aminotransferase (EC 2.6.1.2, alanine aminotransferase) also exhibits the desirable properties of high stability, high specific activity, and lack of severe inhibition even at substrate concentrations up to 0.4 *M*, but the enzyme cannot use L-aspartic acid as the amino group donor. Thus, a highly productive immobilized biocatalyst can be prepared using this aminotransferase, and it can be used for the production of L-alanine from pyruvic acid and L-glutamate, but the reaction cannot be driven to completion by the coupling of oxaloacetate decarboxylase. These readily available enzymes have nonetheless been useful as model aminotransferases in the development and design of biocatalytic transamination processes, and data for the immobilization and use of these enzymes in bioreactors will be presented.

The microorganism *E coli*. is one of the most useful sources of aminotransferases. Of the four aminotransferases from this microorganism characterized to date,[7] we have found the so-called glutamic-oxaloacetic

[6] I. W. Sizer and W. T. Jenkins, this series, Vol. 5, p. 677.
[7] J. T. Powell and J. F. Morrison, *Eur. J. Biochem.* **87,** 391 (1978).

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