AHAS enzyme platform: genesis of C-C building blocks and chiral alpha-hydroxy ketones

**ABSTRACT**

Genetically engineered Acetohydroxy acid synthase (AHAS) enzymes constitute a platform technology which can be used to generate a wide range of Chiral alpha-hydroxy ketones by acyloin condensation involving pyruvic acid and an aromatic aldehyde. The conversion is stoichiometric and without byproducts. The enzymes are also capable of generating intermediates which could end up as petroleum industry intermediates offering a “green” alternative to scarce petroproducts.

**BACKGROUND**

Chirality is a key factor in the efficacy of many drug products and agrochemicals. The production of single enantiomers of chiral intermediates has become increasingly important in the pharmaceutical and fine chemical industry. Enzyme-catalysed reactions have advantages over chemical synthesis because their extraordinary regio- and stereospecificities lead to high yields of desired compounds with minimal formation of by-products (1). Bioactive molecules, such as drugs, usually have only one enantiomer with the desired bioactivity, while the other may be inactive or even deleterious to health.

A number of enzyme catalysed reactions, using aldolases, lipases and lyases in the synthesis of biologically important compounds are already established (Biotransformation in organic chemistry). However, only a few examples are known of practical biotransformations using thiamin diphosphate (ThDP)-dependent enzymes, despite their potential for both breaking and formation of asymmetric carbon-carbon bonds (2). These reactions require no additional driving force or redox agents, so that no regeneration of cofactors such as ATP or NAD is needed for the synthesis.

ThDP-enzymes and their synthetic potential have been reviewed for nonoxidative decarboxylation of α-keto acids (α-ketoacid decarboxylases), oxidative decarboxylation of α-ketoacids (pyruvate oxidase), carboligation (transketolase) and cleavage of C-C bonds (transketolase, benzaldehyde lyase). All these enzymes are involved in the formation of a ThDP-bound — “active aldehyde” intermediate either by decarboxylation of an α-ketoacid or by transfer from corresponding donor. Pyruvate decarboxylase and benzoylformate decarboxylase are capable of performing acyloin-type condensation side-reactions, leading to formation of chiral α-hydroxy ketones (2).

Chiral α-hydroxyketones are versatile building blocks for stereoselective organic and pharmaceutical chemistry (3). One important chiral α-hydroxyketone is R-phenylacetyl carbinol (R-PAC), used as a synthon in the production of various drugs having α and β adrenergic properties, including L-ephedrine, pseudoephedrine, norephedrine, norpseudoephedrine, and phenylpropanolamine (4). These drugs are used as decongestants, antiasthmatics, and so forth (5). R-PAC production was the first commercialized chiral biotransformation process, and presently relies on a fermentative process using the yeast Saccharomyces cerevisiae, glucose and benzaldehyde (6).

The traditional fermentation process using whole yeast is limited by various factors, such as the metabolic status of the yeast, substrate toxicity, various byproducts, end product inhibition and so on, affecting R-PAC production and causing subsequent difficulties in downstream processing (5). There are many strategies applied to overcome these disadvantages. The partially purified PDC from Candida utilis has been found to have more potential in terms of carboligation activity and byproduct formation compared to whole cell biotransformation. When partially purified PDC is used for biotransformation, the overall yield of R-PAC is increased but a significant amount of pyruvate is diverted to forming acetaldehyde which also hampers PDC activity (7).
Pyruvate decarboxylases (PDC) from *S. cerevisiae* (S.c. PDC) and *Zymomonas mobilis* (Z.m. PDC) have been investigated for their ability to increase the carboligation activity for R-PAC formation. These enzymes differ significantly in stability and carboligation ability. S.c. PDC exhibits high carboligase activity, but low stability, whereas Z.m. PDC, although more stable, shows low carboligase activity. Recently, the relevant catalytic properties of Z.m. PDC were improved by means of site-directed mutagenesis which increased the carboligase activity 5–6 fold (8, 9). Another interesting approach is the utilization of the ability of Z.m. PDC to accept acetaldehyde instead of pyruvate as the donor substrate in R-PAC synthesis (10).

Subsequent investigations showed that PDC from *Rhizopus javanicus* has good carboligation potential for the production of R-PAC. A detailed comparison has been made between *C. utilis* and *R. javanicus* PDCs, with optimized buffer and cofactor systems leading to good molar yields based on added benzaldehyde and very low production of byproducts (11, 12).

The carboligase ability of PDC is not restricted to benzaldehyde as the only acceptor substrate. The enzyme can form α-hydroxy ketones using a wide range of aliphatic, aromatic, heterocyclic, and α,β-unsaturated aldehydes (3, 13). Most of the biotransformations have been carried out using fermenting yeast and only a few have used isolated PDC. All of them are quite inefficient processes due to low reaction rates and a low achievable conversion of substrates to the desired products.

Acetohydroxyacid Synthase (AHAS or acetolactate synthase, EC 2.2.1.6; formerly EC 4.1.3.18) found in microorganisms catalyses two homologous reactions by condensing an active acetaldehyde group derived from pyruvate by decarboxylation, with either pyruvate to form acetolactate or alpha-ketobutyrate to form acetohydroxybutyrate. These compounds are intermediates in the biosynthesis of valine and isoleucine respectively (14). The carboligase activity of AHAS is an intrinsic feature of this enzyme. The active site of the enzyme is naturally designed to accommodate the second substrate, an α-keto acid, for the condensation reaction, and the ThDP-bound — “active aldehyde” intermediate is apparently protected against protonation, preventing acetaldehyde formation. AHAS enzymes are also capable of catalyzing the condensation reaction of pyruvate with nonphysiological substrates such aldehydes to form alpha hydroxy ketones (Figure 1 (15)).

<table>
<thead>
<tr>
<th>Aldehydes</th>
<th>Reaction</th>
<th>Alpha Hydroxy Ketones</th>
<th>Conversion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rrit</td>
<td>Position 2, R+ Me</td>
<td>50%</td>
<td></td>
</tr>
<tr>
<td>H3C₂H₅</td>
<td>Position 2</td>
<td>31-81%</td>
<td>3-59%</td>
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<tr>
<td>H₂C₂H₆</td>
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<tr>
<td>H₃CaCn</td>
<td>Position 4</td>
<td>3-64%</td>
<td>3-64%</td>
</tr>
</tbody>
</table>

Figure 3. Range of substrates reactions by AHAS system (16)
AHAS isozyme I has the ability to accept a variety of aldehyde compounds in an enantiospecific condensation with pyruvate to form various chiral aryl-acylcarbinols with high efficiency (16). An International Patent Application has been filed for this process (17) (PCT/IL03/00057 (2003)) by the inventors and granted.

THE POSSIBILITIES

The AHAS enzyme system offers the possibility of generating:
1. Acetolactate from Pyruvate (Figure 2)

The enzyme is capable of coupling together two pyruvate molecules to give acetolactate, which could be a good building block to transform further into petroleum intermediates. Acetolactate can be converted to acetoin, which in turn could be converted to 2,3 butanediol and methylhydroxyketone. These are currently petroleum products. Fermentative production of butanediol became unviable when petroleum sources became available (18). In the future, with rising petroleum prices, there could be a revival of non-petroleum routes to this intermediate.

2. Alpha-hydroxy ketones (Figure 3)

The production of chiral alpha-hydroxy ketones with near stoichiometric yields has been well studied and patented (15-17). These intermediates could be the chiral building blocks of existing drugs and could also generate a library of compounds by further conversion to aminoalcohols, many of which have pharmacological activity. This library could be scanned for potential pharmacological activity by in-silico means and then shortlisted for screening as potential lead molecules.

The alpha-hydroxy ketones could be the further integrated with other enzyme systems to generate useful compounds. For example, with the help of transaminases, aminoalcohols could be formed. With the help of oxynitrilases, chiral cyanhydrin derivatives could be formed. This complementarity could extend the range of substrates possible.

CURRENT PERSPECTIVE ON AHAS (PRESENT SCENARIO)

Under license, the AHAS technology is being explored for possible applications and techno commercial viability. The enzyme has been immobilized to facilitate the use of batch or packed column bed reactors. Various approaches have been used to bring about techno commercial viability. The enzyme has been genetically engineered by the patenting group to create variants which have specific properties. The enzymes have been made more robust by joining together the two subunits by a linkage. The enzyme has been made more tolerant to some amino acids like Valine in the feed stocks. The cost of production of the alpha-hydroxy ketone or acetolactate holds the key to the use of AHAS as a competitive alternative to synthetic approaches. Work on the optimization in the following areas is underway:

1. Substrate cost: Pyruvic acid, the acyloen donor, has to be externally added which adds to the cost. In case of yeast whole cell biotransformation it is endogenously produced. This process is a less efficient but cheaper alternative.

2. Enzyme cost: The cost of production of enzyme including adjuvants like buffers, etc, is a major area for conservation.

3. Enzyme life: Various factors like aldehyde toxicity, bioburden, etc. need to be addressed to ensure longer life.

Various approaches have been tried to overcome the above areas of concern. Genetic engineering of yeast to express the AHAS enzyme has been tried. Whole cell biotransformation has been tried as an alternative. Proof of concept of whole cell biotransformation, using fermented broth rich in pyruvate added to E. coli cells having AHAS expressed intracellularly, has been demonstrated in our laboratories.

CONCLUSION

To conclude, the AHAS enzyme platform offers the potential to be exploited in the following areas: A) Generation of Acetolactate, which holds a potential for being enlisted as an alternative route to some petrochemical intermediates. B) Generation of Chiral alpha-hydroxy ketones by acyloan condensation to generate a library of compounds which could be important intermediates to produce aminoalcohols. The aminoalcohols produced could themselves be active API's or comprise a library of compounds which could be screened for physiological activity, as new drug entities.

REFERENCES

18. M. Volosh, N. B. Jansen, M. R. Ladisch, G. T. Tsao, R. Narayan and V. W. Radwell, Purdue University, West Lafayette, IN, USA, Industrial Chemicals, Biochemicals and Fuels

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