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Structure, Function, and Application of Microbial Lactonases

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1. Novel Microbial Lactonases [1]

Lactonase is a novel enzyme recently discovered by us. The enzyme catalyzes reversible hydrolysis of aldonate lactones to the corresponding aldonate. We isolated this enzyme from a fungus *Fusarium oxysporum*. The enzyme was characterized in some details and shown to be promising as a practical catalyst for optical resolution of racemic pantoyl lactone (PL), a chiral building block for the commercial production of D-pantothenate.

The relative molecular mass of the fungal lactonase is 125 kDa, and the subunit molecular mass is 60 kDa. The enzyme hydrolyzes aldonate lactones, such as D-galactono-γ-lactone and L-mannono-γ-lactone, stereospecifically. D-PL is also a good substrate of the enzyme. All the substrate lactones, which can be hydrolyzed by the enzyme, have a downward OH group at the 2-position, when the lactone rings are drawn in the *Haworth* projection, but the corresponding enantiomers are not hydrolyzed at all. The enzyme also irreversibly hydrolyzes several aromatic lactones, such as dihydrocoumarin and homogentisic-acid lactone. The enzyme contains about 1 mol of calcium per subunit and is highly glycosylated. Calcium seems to be necessary for both enzyme activity and stability.

A similar kind of lactonase was found in *Brevibacterium protophormiae*. The bacterial enzyme hydrolyzes only L-PL; D-PL is not a substrate. Various kinds of aromatic lactones are also hydrolyzed, but aldonate lactones are not hydrolyzed.

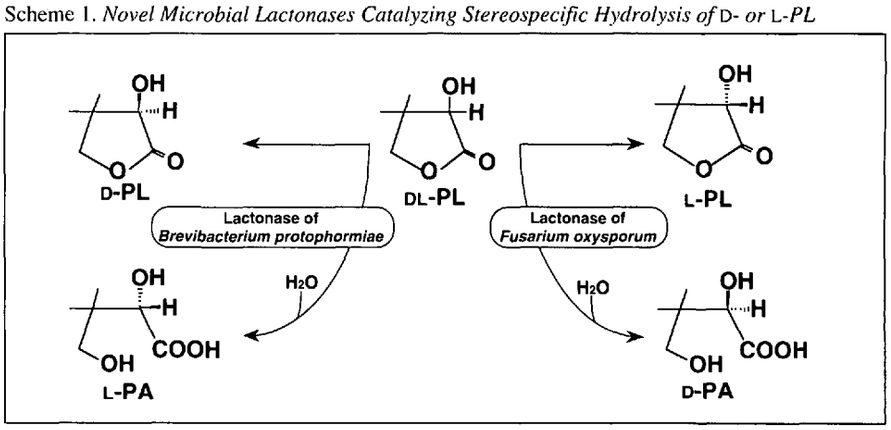
2. Optical Resolution of Racemic PL with Lactonase of *F. oxysporum* [2–4]

If racemic PL is used as a substrate for the hydrolysis reaction by the stereospecific lactonase, only the D- or L-PL might be converted to D- or L-PA and the L- or D-enantiomer might remain intact, respectively. Consequently, the racemic mixture could be resolved into D-PA and L-PL, or D-PL and L-PA as shown in *Scheme 1*. In the case of L-PL-specific lactonase, the optical purity of the remaining D-PL might be low, except when the hydrolysis of L-PL is complete. On the other hand, using the D-PL-specific lactonase, D-PA with high optical purity could be constantly obtained independently of hydrolysis yield. Therefore, we investigated the enzymatic resolution of racemic PL with D-PL-specific lactonase of *F. oxysporum*.

When *F. oxysporum* cells were incubated with DL-PL, only D-PL was hydrolyzed to D-PA; suggesting that *F. oxysporum* cells as well as the purified lactonase catalyze the enantioselective hydrolysis of PL.

Under the optimized conditions, D-PL in a racemic mixture of 5380 mM PL (700 mg/ml) was stereospecifically hydrolyzed to D-PA by the fungal cells with automatically controlling the pH of the reaction mixture at 7.0 with 15 M NH_4OH. The formation of L-PA was barely detected. After 24 h, the concentration of PL hydrolyzed in the reaction mixture reached 2480 mM (322 mg/ml) with an optical purity of 96% ee for D-PA.

Scheme 1. *Novel Microbial Lactonases Catalyzing Stereospecific Hydrolysis of* D- *or* L-*PL*



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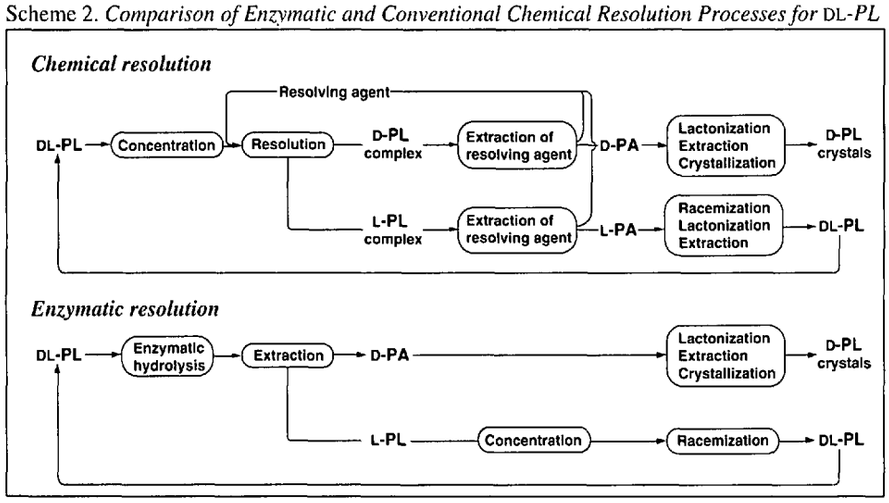
Scheme 2. *Comparison of Enzymatic and Conventional Chemical Resolution Processes for* DL-*PL*



Practical hydrolysis of the D-PL in a racemic mixture is carried out using immobilized cells of *F. oxysporum* as the catalyst. Stable catalyst with high hydrolytic activity can be prepared by entrapping the fungal cells into calcium alginate gels. When the immobilized cells were incubated in a reaction mixture containing 350 g/l DL-PL for 21 h at 30° under the conditions of automatic pH control (pH 6.8–7.2), 90–95% of D-PL was hydrolyzed (optical purity, 90–97% ee). After repeated reactions for 180 times (*i.e.*, 180 d), the immobilized mycelia retained more than 90% of their initial activity.

The overall process for the present enzymatic resolution is compared with the conventional chemical process in *Scheme 2*. The enzymatic process can skip several tedious steps which are necessary in chemical resolution and is highly advantageous for the practical purpose.

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Development of a Fermentation Process for the Manufacture of Riboflavin

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Introduction

Vitamin B_2 (riboflavin) is produced for large-scale commercial applications by chemical synthesis or by a combined fermentative/chemical process. In the last years, novel microbiological production processes for synthesis of riboflavin were developed, some of which represent attractive alternatives to the current production processes. The novel *Roche* process uses a recombinant *Bacillus subtilis* strain. Here, we describe the development of this strain, its use in fermentation, product purification, and analytics. In addition, we discuss other aspects relevant for the introduction of a novel biotechnology-derived product for application in animal feed and human nutrition, such as safety evaluation and environmental aspects. The *Roche* process offers the possibility to obtain a product of superior quality by using more environmentally friendly production technology. Riboflavin, thus, represents a good example to illustrate the contribution of modern biotechnology to replace traditional mineral-oil-based chemical processes by more natural 'green' production processes based on renewable resources like starch or vegetable oil.

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Commercial Application

Riboflavin is mainly used in animal feed and human food. In feed, riboflavin is important as a vitamin for live stock. In food, riboflavin like other vitamins is added mainly to compensate for the loss of vitamins resulting from industrial processing. In addition, riboflavin is used as a colorant for, *e.g.* soft drinks. Only a small amount of the riboflavin sold is used in human pharmaceutical applications to compensate for vitamin deficiencies. Each year, several thousand tons of riboflavin are produced worldwide, mainly by *F. Hoffmann-La Roche Ltd.* (Switzerland) and *BASF* (Germany).

Current Production Process

Currently, riboflavin is produced mainly by chemical synthesis starting from ribose, which is obtained fermentatively using *Bacillus* strains. This process has been developed and continuously improved in the last decades [1]. Development of biotechnological processes for the commercial production of riboflavin has been a focus for research for many years [2–4], however, until recently, these processes were not economically competitive. Riboflavin production strains were developed on the basis of eukaryotic fungi or prokaryotic bacteria. The fungus *Ashbya goshypii* is a naturally occurring over-