Structure, Function, and Application of Microbial Lactonases

Sakayu Shimizu* and Michihiko Kataoka

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 proteophormiae*. 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 mM NH₄OH. 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

*Correspondence: Prof. Dr. S. Shimizu
Department of Agricultural Chemistry
Kyoto University
Kitashirakawa, Sakyoku
Kyoto 606-01, Japan
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°C 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.

**Development of a Fermentation Process for the Manufacture of Riboflavin**

**Adolphus P.G.M. van Loon**, Hans-Peter Hohmann, Werner Bretzel, Markus Hümbelin, and Magdalena Pfister

**Introduction**

Vitamin B<sub>2</sub> (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.

**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 gossypii* is a naturally occurring over-