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A novel and efficient immobilised tannase coated by the layer-by-layer technique in the hydrolysis of gallotannins and ellagitannins

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ABSTRACT

Tannase (EC 3.1.1.20) was quantitatively immobilised onto Eupergit® C 250 L and coated by layers of alternatively charged polystyrene sulfonate and polyallylamine hydrochloride, respectively. The layer-by-layer (LbL)-coated immobilised tannase, *i.e.*, LbL-tannase retained its original activity and showed significant resistance to deactivation and maintained 50% of its activity after seven consecutive cycles of hydrolysis reactions, each run for 24 h. The LbL coating did not hinder the substrate access to the active site of the enzyme. Both gallo- and ellagitannins were efficiently hydrolysed upon treatment with LbL-tannase. The formation of gallic acid and the reaction patterns were followed by HPLC and the recently developed ^{31}P NMR analytical method for the characterisation of tannins.

1. Introduction

The term tannins comprises a large, structurally rather heterogeneous family of polyphenols characterised by the common ability to complex and thereby subsequently precipitate (and eventually denaturate) proteins [1–3]. Accounting for teh structural diversity of tannins [4–13], they are historically classified in two main classes, hydrolysable and condensed tannins, depending on characteristic structural motifs. Characteristic structural motifs were recently shown to be delineable on the basis of ^{31}P NMR spectroscopy using phosphitylated derivatives [14,15]. Their structural diversity, their manifold functions in plants, as well as their applications in various aspects of everyday life make them interesting research subjects of currently ever-growing interest. Due to the diverse biological effects exhibited by tannins, their (biological) degradation is of interest as well. Tannin acyl hydrolase, better known as tannase (EC 3.1.1.20), is a hydrolytic enzyme active on tannins [16,17]. This enzyme was accidentally discovered by Tieghem [18] during an experiment aiming at the production of gallic acid; the aqueous solution of tannins used in this experiment was contaminated with two fungal species, as was later discovered. Many more producers of tannase have been identified since then, with the main producers of tannase being fungi, yeasts, bacteria and even a few animals [16]. Most of the research is based on fungal tannase, however, with the main producers of tannase belonging to the family of *Candida* species [19,20].

Structurally, tannase is a globular protein mainly composed of β-sheet structures [21,22], and is often found to be multimeric, comprising two to eight subunits, exhibiting total molecular weights between 50 and 320 kDa, depending on the source of extraction. The subunits are organised in pairs, forming a hetero-octamer with a molecular weight of about 300 kDa in the case in which the maximum of eight subunits is present [23]. Tannase is a glycoprotein: fungal and yeast-originating tannases have carbohydrate contents that range from 5.4 to 64%, respectively [22,24–26].

Tannase was found to be active on both major subclasses of tannins, the class of hydrolysable tannins, gallotannins—characterised by a core sugar moiety esterified with gallic acids, and ellagitannins—characterised by tetrahydrodiephinic acid esters (Fig. 1) [16,27].

In gallotannins, tannase catalyses the hydrolysis of the ester bonds of these hydrolysable tannins to yield gallic acid and glucose (Scheme 1). Tannase was found to hydrolyse both the simple galloyl esters of an alcohol moiety and the galloyl esters of gallic acid. For this reason, Haslam and Stangroom [27] proposed that the tannase activity could be composed of two separate enzymatic activities: a "depsidase" activity, that hydrolyses the depside bonds typical for galloyl esters of gallic acid, and an "esterase" activity that catalyses the cleavage of simple galloyl esters such as methyl gallate, ethyl gallate, *neo*-propyl gallate, and *neo*- and *iso*-amyl gallate [27].

The biochemical mechanism underlying tannase activity on ellagitannin is, however, not completely understood yet due to the

Abbreviations: LbL, layer-by-layer; NMR, nuclear magnetic resonance; HPLC, high pressure liquid chromatography; HHDP, hexahydrodiphenoic.

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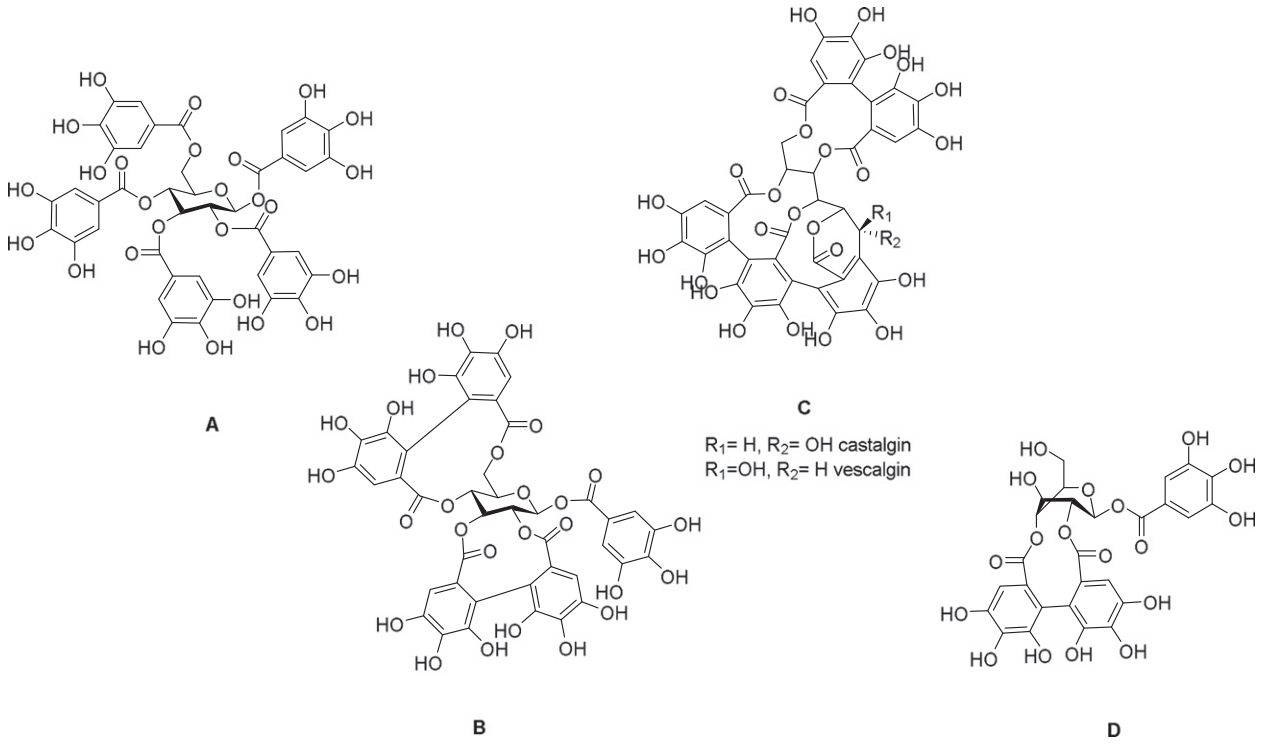


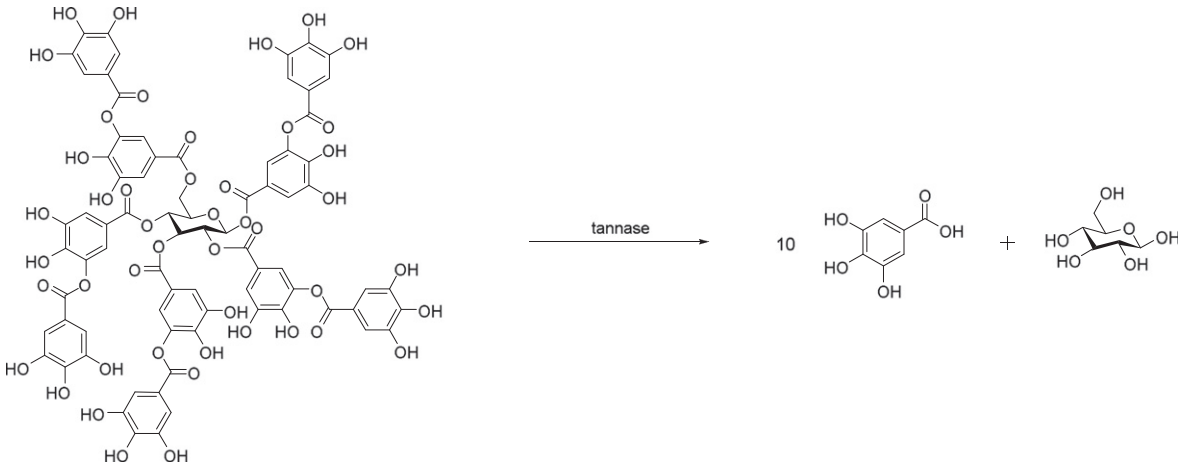
Fig. 1. Typical structure for gallotannins (A, pentagalloyl D-glucose) and ellagitannins (B, Casuarictin; C, R1 = H, R2 = OH—Castalgin, R1 = OH, R2 = H—Vescalgin; D, Phyllanemblinins).

chemical complexity and diversity of ellagitannins [3]. Nevertheless, it is known that the selective hydrolysis of galloyl groups of the ellagitannin phyllanemblinin (Fig. 1d) is catalysed by tannase [12].

To date, tannase is used in food and beverage industries, as well as in chemical and pharmaceutical applications [1,2]. In the food and beverage industries, tannase is employed as clarifying agent against the turbidity of beverages (wine, beer and coffee flavoured soft drinks) and as debittering agent in fruit juices [28], since tannins are responsible for the turbidity of wines and beers, etc. due to their capability to complex and precipitate proteins, while they confer bitterness and astringency to fruit juices due to their interaction with the corresponding receptors [29,30].

Pharmaceutical and chemical companies employ tannase for the production of gallic acid whose synthesis is very expensive and not always selective [16,19]. Gallic acid is used as an intermediate for chemical and enzymatic synthesis of pyrogallols and gallic acid esters, *e.g.*, propyl gallate, which finds application as antioxidant additive in greases and oils and beverages [31,32]. Gallic acid is also employed in the manufacturing of trimethoprim, an antibacterial drug [33].

Enzymes in general exhibit a number of features that make their use advantageous when compared to conventional catalysts, namely their chemo-, regio- and stereospecificity, and the possibility to operate under mild reaction conditions, which is extremely beneficial in the context of developing environmentally friendly processes [34]. There are, however, also several constraints connected to the industrial use of enzymes: they are often sensitive or structurally unstable; they have to be preferentially used in aqueous solutions [34]. Several approaches



Scheme 1. Hydrolysis of tannic acid, as example for the hydrolysis of hydrolysable tannins by tannase.