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A colorimetric assay for the determination of acetyl xylan esterase or cephalosporin C acetyl esterase activities using 7-amino cephalosporanic acid, cephalosporin C, or acetylated xylan as substrate

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Abstract

A bromothymol blue-based colorimetric assay has been devised to screen for acetyl xylan esterase or cephalosporin C (CPC) deacetylase activities using 7-amino cephalosporanic acid (7-ACA), CPC, or acetylated xylan as substrate. These enzymes are not screened with their natural substrates because of the tedious procedures available previously. Acetyl xylan esterase from *Bacillus pumilus* CECT 5072 was cloned, expressed in *Escherichia coli* Rosetta (DE3), and characterized using this assay. Similar K_M values for 7-ACA and CPC were obtained when compared with those described using HPLC methods. The assay is easy to perform and can be carried out in robotic high-throughput colorimetric devices normally used in directed evolution experiments. The assay allowed us to detect improvements in activity at a minimum of twofold with a very low coefficient of variance in 96-well plates. This method is significantly faster and more convenient to use than are known HPLC and pH-stat procedures.

Keywords: Colorimetric assay; pH indicator; 7-Aminocephalosporanic acid; Cephalosporin C; Acetylated xylan

Deacetyl cephalosporins are a highly valuable starting material for producing semisynthetic β-lactam antibiotics. These compounds are derived from cephalosporin C (CPC)[1] or from 7-amino cephalosporanic acid (7-ACA) by chemical or enzymatic processes [1], although the enzymatic process has the advantages of mild reaction conditions of pH, temperature, and high yield [2]. Many esterases are active on these compounds, and most belong to the carbohydrate esterase family 7 [3]. Among them, some acetyl xylan esterases (AXEs) show high specific activity over these substrates [4]. Hence, these enzymes have great potential application for the chemoenzymatic deacetylation of cephalosporins for antibiotic synthesis. The reaction that is carried out by AXE is based on the removal of an acetyl group of the 3-position from cephalosporins (Fig. 1A). In addition, AXE hydrolyzes the ester linkages of the acetyl groups of acetylated xylan (Fig. 1B) [5,6]. This substrate is the major constituent of hemicellulose and is the most abundant renewable polysaccharide in plants after cellulose. Xylan is predominantly a 1,4-β-D-xylose polymer and is commonly substituted to various degrees with acetyl, arabinosyl, and glucuronyl residues [7]. More than 60% of xylose residues are esterified with acetic acid in position 2 and/or position 3 of the xylose moieties in hardwood xylans [8]. Therefore, AXE is one of the accessory enzymes that are part of the xylanolytic

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[1] *Abbreviations used:* CPC, cephalosporin C; 7-ACA, 7-amino cephalosporanic acid; AXE, acetyl xylan esterase; 7-DACA, 7-amino-3-deacetylcephalosporanic acid; DCPC, deacetylcephalosporin C; *p*-NPA, *p*-nitrophenyl acetate; HTS, high-throughput screening; BTB, bromothymol blue; LB, Luria–Bertani; TB, Terrific broth; IPTG, isopropyl β-D-1-thiogalactopyranoside; OD, optical density; CV, coefficient of variance; FPLC, fast protein liquid chromatography; tRNA, transfer RNA; CCD, CPC deacetylase.

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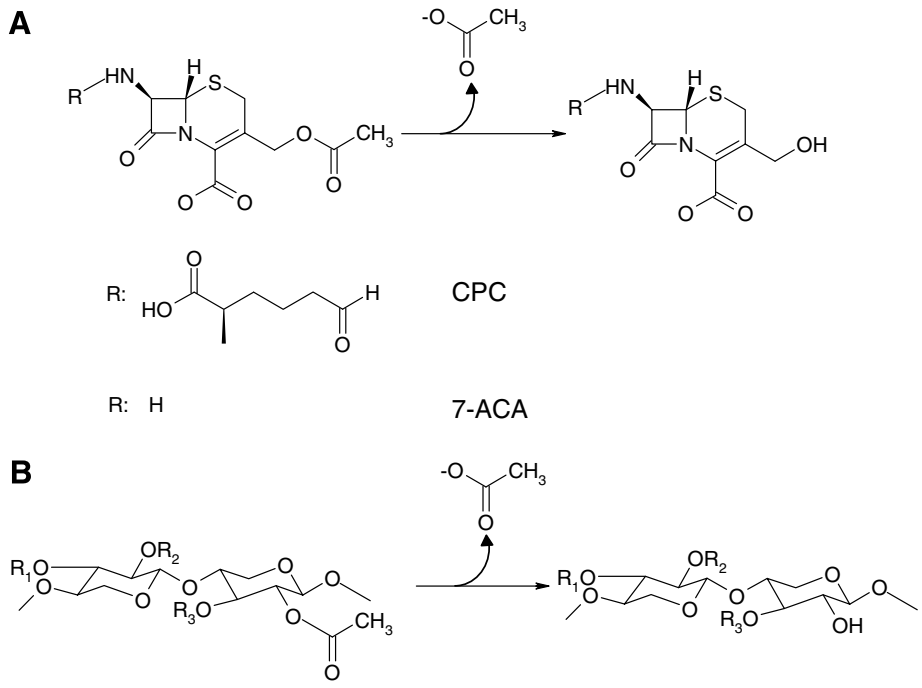

Fig. 1. Reactions catalyzed by AXE. (A) Acetyl xylan esterase or cephalosporin C deacetylase-catalyzed reaction to generate 7-amino-3-deacetylcephalosporanic acid (7-DACA) or deacetylcephalosporin C (DCPC). (B) Deacetylation of acetylated xylan.

system together with xylanases, β-xylosidases, α-arabinofuranosidases, and methylglucuronidases. All of these are required for the complete hydrolysis of xylan [5].

The usual assay to determine this esterase activity is performed by using *p*-nitrophenyl esters (especially *p*-nitrophenyl acetate [*p*-NPA]) with spectrophotometric measurements at 405–410 nm [9]. However, there is no correlation between activity toward *p*-NPA and activity toward CPC, 7-ACA [2], or xylan. The ratio of activity with different substrates for a single purified enzyme is constant, but when different enzymes are compared or a mutant enzyme is compared with the wild type, the substrate specificity is different, so an improvement in the activity toward *p*-NPA does not imply the same improvement with all of the substrates. Therefore, a more tedious procedure needs to be carried out; an example is the pH-stat assay, in which the hydrolysis of the β-lactam into acetic acid is continuously titrated with sodium hydroxide or ammonia solution [2]. Alternatively, the reaction toward CPC, 7-ACA, or xylan can be monitored by HPLC [1]. The last two methods cannot be used for high-throughput screening (HTS) of those enzymes coming from metagenomic experiments or directed evolution libraries (10^4–10^6 clones) because it involves checking 96 or 384 clones from several microplates, a time-consuming process (>6 h for every plate) [9]. On the other hand, the acetic acid kit can be easily applied following a slightly modified manufacturer's protocol [10]. The main problem is that it is quite expensive and requires the addition of four reagents to each sample. All of these methods are useful when few enzymes are going to be analyzed, but they cannot be applied to the HTS of enzyme libraries. Furthermore, the interest in such substrates has led to the search for new spectrophotometric assays that allow a direct quantitative determination of the reaction for the production of these highly valuable products [11].

In this article, we describe a new colorimetric method based on the color changes produced in a pH indicator occurring during the deacetylating reaction of CPC, 7-ACA, or acetylated xylan caused by AXE from *Bacillus pumilus* CECT 5072. This method is a complement to the known pH-sensitive methods described previously for other hydrolytic enzymes such as lipases [12], esterases [13], nitrilases [14], and alkane dehalogenases [15].

Materials and methods

Materials

Pfu Turbo DNA polymerase and its 10× reaction buffer were obtained from Stratagene (La Jolla, CA, USA). Restriction enzymes were obtained from New England Biolabs (Ipswich, MA, USA). A GeneMorph kit was purchased from Stratagene. T4 DNA ligase was purchased from Roche Diagnostics (Indianapolis, IN, USA). pET28a plasmid and *Escherichia coli* Rosetta (DE3) cells were obtained from Novagen (Madison, WI, USA). A DNeasy system for genomic DNA isolation, QIAprep spin plasmid miniprep kit, QIAquick PCR purification kit, and QIAEX-II gel extraction kit were purchased from Qiagen (Valencia, CA, USA). Bromothymol blue (BTB), 7-ACA, CPC, and antibiotics were purchased from Sigma (Madrid, Spain). Acetylated xylan was prepared following the method described previously by Johnson and coworkers [16]. An