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A high throughput profiling method for cutinolytic esterases

Pasi Halonen*, Tapani Reinikainen[1], Antti Nyyssölä, Johanna Buchert

VTT Biotechnology, P.O. Box 1500, FIN-02044 VTT, Finland

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ABSTRACT

A procedure for identifying and profiling cutinolytic esterases was developed by combining traditional plate screen assays with an automated robotic system. In the first phase, the micro-organisms were screened on agar plates with cutin or the model substrate polycaprolactone as the sole carbon sources. In the second phase, *p*-nitrophenyl esters of fatty acids were used as the substrates in an automated activity assay of liquid culture media. The variables used were pH and the carbon chain length of the fatty acid moiety of the *p*-nitrophenyl substrate. Finally, ^{3}H-labelled cutin was used as a specific substrate to verify the positive hits and to validate the screening procedure. With pH as the variable in the automatic screen, esterase production of cutinase positive strains typically proceeded in two stages: first an esterase with neutral activity optimum was produced, after which a strong esterolytic response in the alkaline range was detected. With carbon chain length of the fatty acid as the variable best correlation with cutinase production was obtained with strains showing a high ratio of activities towards *p*-nitrophenyl-butyrate and *p*-nitrophenyl-palmitate.

1. Introduction

The aerial parts of plants such as leaves and fruits are covered by a dense and chemically resistant barrier called the cuticle [1–3]. It protects plants from microbial infections, environmental pollutants and electromagnetic radiation [4,5]. It also prevents desiccation and affects the loss of solutes from the leaf interior as well as the transport of non-volatile chemicals into the leaf [6]. The major structural constituent of the cuticle is cutin, which is composed mostly of hydroxy and epoxy fatty acids with C_{16} and C_{18} carbon chains [7]. The cutin polymer is formed via esterification of primary alcohols, resulting in a complex three-dimensional structure into which waxes are embedded [8].

Many micro-organisms produce extracellular cutinases (EC 3.1.1.74) that hydrolyze the ester bonds of the cutin layer. All of the biochemically well-characterized cutinases are serine esterases, containing the classical Ser-His-Asp triad similar to serine proteases and several lipases [9]. The pH optima reported for the cutinases are typically in the neutral or the alkaline range [10,11]. Cutinase production has been widely reported for phytopathogenic fungi [12–16], but also bacterial producers are known [17–20]. Cutinases may be required for pathogenic infection as they enable the microbe to penetrate through the plant cuticle. Additionally, cutinases have a role in the saprophytic growth of micro-organisms aiding in the decomposition of the organic matter of the soil [21,22].

Due to the heterogeneity of the esterified structures found in cutin, cutinases have been proposed to have a wide variety of applications in processing of different raw materials in different media [9,23,24]. For example, cutinases could be utilised in laundry and dishwashing applications as lipolytic enzymes for fat removal, because cutinases are active in both aqueous and emulgated environments [25,26]. For the use in the textile industry, cutinases have been successfully tested in combination with pectin lyase for cotton bioscouring to improve the wettability of raw cotton fabrics [27]. Cutinases have also been applied in the enzymatic modification of the surface of polyester fibers [28].

In the food industry, cutinases could be used for opening up the cutin structure for cell wall degrading enzymes (e.g. cellulolytic enzymes) or for facilitating the release of bioactive compounds from, e.g. vegetable peels or berries. Hence, cutinases could be used in the production of cutin-derived chemicals from plant waste in the fruit and vegetable processing industry [29]. However, despite their wide potential no cutinase preparations have been offered for large-scale commercial use so far.

The reports on the systematic screening of cutinolytic activities are scarce. In the present work we have developed an efficient screening method for the discovery of cutinolytic esterases that combines both traditional plate assays and automated esterase profiling using a robotic workstation. We show that the current method can be used for easy detection of different isoenzymes and

* Corresponding author. Tel.: +358 40 518 5791; fax: +358 20 722 2840.
E-mail addresses: Pasi.Halonen@vtt.fi (P. Halonen), antti.nyyssola@vtt.fi (A. Nyyssölä).
[1] Current address: Finnish Police, National Bureau of Investigation, Forensic Laboratory, Jokiniemenkuja 4, P.O. Box 285, FI-01370 Vantaa, Finland.

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for studying the expression patterns of the esterases. Finally, the positive hits are verified by employing ^{3}H-labelled cutin.

2. Materials and methods

2.1. Micro-organisms

The 38 micro-organisms chosen from the VTT Culture Collection for screening studies were: seven bacteria [*Burkholderia cepacia*, VTT Culture Collection Number E-94512 (ATCC 25416), *Burkholderia gladioli* E-94511, *Pectobacterium carotovorum* E-981115, *Pseudomonas tolasii* E-94518, *Streptomyces lydicus* E-92202, *Xanthomonas campestris* E-81025, *Xanthomonas* sp. E-97888]; eight single-cell yeasts [*Candida apicola* C-87174, *Candida versatilis* C-82065 (ATCC 60131), *Kluveromyces marxianus* C-95234, *Pichia guilliermondii* C-97290, *Pichia jadinii* C-71015 (ATCC 9226) and C-84155, *Rhodosporidium toruloides* C-71034 (ATCC 10788), *Yarrowia lipolytica* C-97297]; and twenty-three fungi [*Alternaria alternata* D-79111, *Aspergillus foetidus* D-71001 (ATCC 10254), *Aspergillus fumigatus* D-82195, *Aspergillus niger* D-81078 (ATCC 6275), *Aspergillus oryzae* D-88354 (ATCC 26850), *Botryotinia fuckeliana* D-76044 (ATCC 12481), *Curvularia inaequalis* D-79116, *Fusarium oxysporum* D-82183, *Fusarium pallidoroseum* D-82184, *Fusarium solani* f. sp. *pisi* D-03918 (ATCC 200576, positive control), *Gibberella baccata* D-82187, *Gibberella intricans* D-82087, *Gliocladium virens* D-83215 (ATCC 9645), *Melanocarpus albomyces* D-96490, *Mycosphaerella tassiana* D-76045, *Nectria haematococca* var. *brevicona* D-82185, *Poria placenta* D-90413, *Rhizomucor miehei* D-82193 (ATCC 16457), *Rhizopus microsporus* D-82192 (ATCC 22959), *Stereum rugosum* D-84235 (ATCC 64657), *Thanatephorus cucumeris* D-79114, *Thermomyces lanuginosus* D-96488, and *Wallemia sebi* D-96478].

2.2. Isolation of apple cutin

Apples (Jona Gold) were peeled and the cutin was isolated from the peels using a modification of a previously described method [30]. The peels were boiled for 3 h and the fruit flesh was removed. The dried and powdered peels were incubated at 40 °C for 2 days in the presence of 50 mM sodium acetate buffer; pH 5.0 supplemented with 0.3 FPU/ml Econase CE (AB Enzymes) and 10 nkat/ml Pectinex Ultra SP (Novozymes) and washed with ethanol. The embedded waxy components were removed by Soxhlet extraction with 70% MeOH, EtOH, and hexane:isopropanol (3:2). The enzyme treatment (with five times higher activities) and the solvent extractions were repeated and the cutin dried.

2.3. Plate screening

The basis of the media for the screening plates was Czapek-Dox agar. Isolated cutin at 1% (w/v) was used as the sole carbon source instead of sucrose in the pH-indicator plates. The pH of the medium was adjusted to 8.1 and Phenol Red (Merck) was used as the indicator at 25 mg l^{-1}. The polycaprolactone (PCL) used as the sole carbon source at 0.05% (w/v) in the PCL plates was pulverized essentially as described previously [31].

2.4. Liquid cultivations and sample preparation for automated screening

The micro-organisms (selected from the cutin and PCL plate screens) were grown under aerobic conditions in shake flasks for the analysis of esterolytic activities secreted in the media. The cultivation medium was Yeast Nitrogen Base (Difco) supplemented with 0.1% (v/v) of acetic acid. The pH of the media was adjusted to pH 6.8 for bacteria, pH 5.6 for fungi, and pH 5.4 for yeasts. The production of cutinolytic enzymes was induced by adding 1% (w/v) apple cutin into the cultivation media. Samples (5 ml) were taken daily for 14 days from the cultivations, frozen in liquid N_2 and stored for activity assays.

The frozen cultivation media samples were thawed and centrifuged (3000 × *g*, 20 min, 4 °C) to sediment the cells and possible polysaccharides. The supernatants were pipetted into Vivaspin 6 concentrators (Vivascience) and the buffer was changed to 10 mM MES (Sigma–Aldrich), pH 6.8 by two concentration cycles. Finally, the concentrated sample was transferred to a parental microtiter plate for the automated activity screening.

2.5. Automated screening of esterase activity

The pH-profiles of the esterases in the cultivation liquid samples were determined with *p*-nitrophenyl-butyrate (Sigma–Aldrich) as the substrate. The *p*-nitrophenyl esters of fatty acids (Sigma–Aldrich) used in the analysis of substrate specificity at pH 6.8 were: *p*-nitrophenyl-acetate (with a two carbon carboxylic acid; designated as pNP-C_2, or C_2), *p*-nitrophenyl-propionate (C_3), *p*-nitrophenyl-butyrate (C_4), *p*-nitrophenyl-valerate (C_5), *p*-nitrophenyl-caproate (C_6), *p*-nitrophenyl-caprylate (C_8), *p*-nitrophenyl-caprate (C_{10}), *p*-nitrophenyl-laurate (C_{12}), *p*-nitrophenyl-myristate (C_{14}), and *p*-nitrophenyl-palmitate (C_{16}).

The substrate solution for the substrate specificity assays containing 3 mM of the *p*-nitrophenyl fatty acid ester and 3% (w/v) Triton X-100 was prepared as described previously [32]. For the pH-profile analysis with *p*-nitrophenyl-butyrate as the substrate the Triton X-100 concentration of the solution was lowered to 0.3%. In order to avoid the formation of precipitates fresh substrate solutions were prepared prior to the measurements.

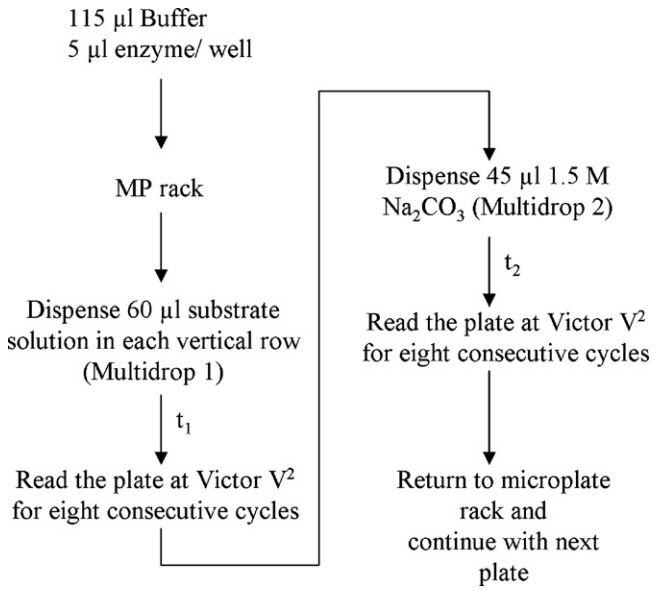

Fig. 1. The automated procedure used for esterase fingerprinting. Buffered samples of the concentrated cultivation media in a 96-well microplate were transferred to the substrate dispensing station (Multidrop1), after which the reaction was followed by spectroscopy (max 10 min). With pHs below 6.2, the reaction was stopped by adding alkali to the wells and the end-points of the reactions were determined.

The buffers used in the assays were 0.1 M phosphate-citric acid (pH 2.8–5.6), MES (pH 5.6–7.2), Tris (pH 7.2–9.0) and CAPS (pH 9.0–9.8). The final Triton X-100 concentration was 1% in the substrate specificity assays and 0.1% in the pH-profile assays. The final substrate concentration in the reactions was 1 mM.

A procedure for fast activity screening using ORCA (Optimized Robot for Chemical Analysis; Beckman Coulter) and 96-well microtiter plates was developed (Fig. 1). A set of progeny plates was first prepared from the parental plate by manual pipetting and placed on a microtiter plate rack. During the automated cycle the robotic arm of ORCA picked the first progeny plate from the microtiter plate rack to the substrate dispensing station (Multidrop 1; Labsystems). After substrate addition the plate was transferred to the reading station (Victor V^2; Wallac) and the reaction in each well was followed by measuring the release of *p*-nitrophenol at 405 nm for several minutes. Since *p*-nitrophenol is not ionized at acidic pH, single point measurements were used for assays at pHs below 6.2. For these measurements the plate was transferred to a second suspensing station after data collection (Multidrop 2; Labsystems), where stopping solution (1.5 M Na_2CO_3) was added to each well. After the addition, the plate was transferred to the reading station and each well was followed for an additional period. The amount of released *p*-nitrophenol was quantified by preparing a standard curve with *p*-nitrophenol (Sigma–Aldrich) at each pH and upon Na_2CO_3 addition.

Esterase activity was calculated by linear regression from the data points (typically eight) collected during the absorbance measurement. In cases where the change in absorbance was clearly non-linear, only a lower limit for the activity could be estimated. The autodegradation of the substrate at various pHs was followed as above with buffer replacing the enzyme. For calculation of the specific activities, the amounts of protein were quantified by the Bradford method with bovine serum albumin as the standard.

2.6. Cutinase assay

Tritiated cutin was prepared as previously described [33] with minor modifications. The labelled cutin (specific activity 4×10^6 dpm/mg) was mixed with unlabelled cutin to gain a suitable specific activity for radioactivity measurements. The labelled substrate (8 mg) was incubated with 100 µl of cultivation media for 24 h (total volume 2 ml), and 100 µl portions were taken from the reaction mixture at regular intervals. The amount of released radioactivity was quantified by liquid scintillation counting after the extraction of cutin hydrolysis products from the reaction mixture with ethyl acetate. A purified steryl esterase from *M. albomyces* [34] and a commercial cellulase preparation, Econase (AB Enzymes) were used as the control enzymes.

3. Results

A three-step procedure was developed for screening cutinase activities of several micro-organisms from the VTT Culture Collec-