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Plants as a source of butyrylcholinesterase variants designed for enhanced cocaine hydrolase activity

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ABSTRACT

Cocaine addiction affects millions of people with disastrous personal and social consequences. Cocaine is one of the most reinforcing of all drugs of abuse, and even those who undergo rehabilitation and experience long periods of abstinence have more than 80% chance of relapse. Yet there is no FDA-approved treatment to decrease the likelihood of relapse in rehabilitated addicts. Recent studies, however, have demonstrated a promising potential treatment option with the help of the serum enzyme butyrylcholinesterase (BChE), which is capable of breaking down naturally occurring (−)-cocaine before the drug can influence the reward centers of the brain or affect other areas of the body. This activity of wild-type (WT) BChE, however, is relatively low. This prompted the design of variants of BChE which exhibit significantly improved catalytic activity against (−)-cocaine. Plants are a promising means to produce large amounts of these cocaine hydrolase variants of BChE, cheaply, safely with no concerns regarding human pathogens and functionally equivalent to enzymes derived from other sources. Here, in expressing cocaine-hydrolyzing mutants of BChE in *Nicotiana benthamiana* using the MagnICON virus-assisted transient expression system, and in reporting their initial biochemical analysis, we provide proof-of-principle that plants can express engineered BChE proteins with desired properties.

1. Introduction

Cocaine is the second most widely abused recreational drug in the United States after marijuana [20]. Cocaine addiction is a chronic disorder addressed through prolonged, often ineffective, behavioral intervention and for which there is no approved pharmacological treatment. Similarly, acute intoxication (i.e. overdose) by cocaine is also only symptomatically treated [17,19].

The serum enzyme butyrylcholinesterase (BChE) is a bioscavenger capable of binding several plant alkaloids [3,11,13,16,23]. BChE is likewise capable of hydrolyzing several plant secondary metabolites and their synthetic derivatives such as succinylcholine, acetylsalicylic acid (aspirin) and cocaine [12,14]. Cocaine is hydrolyzed by serum BChE into the inactive metabolite ecgonine methyl ester and the inactive side product, benzoic acid, unlike the hepatic pathway through which the drug is converted into the bioactive metabolite norcocaine. However, due to its relatively low catalytic efficiency against the relevant enantiomer of (−)-cocaine, and despite its strategic disposition in the circulation, in situations of exposure to acutely toxic concentrations of cocaine (as in the case of cocaine overdose), the endogenous BChE is expected to be easily overwhelmed.

Several groups have created site-directed mutant variants of BChE to improve catalytic efficiency against (−)-cocaine [1,4,5,10,22,24,25]. In order to utilize these enzymes as a possible anti-cocaine treatment, a sustainable, cost effective supply of the protein must be established. Here we report expression of cocaine-hydrolyzing mutants of BChE in the dicotyledonous plant *Nicotiana benthamiana* using the MagnICON virus-assisted transient expression system and their initial biochemical analysis. This work provides the proof-of-principle that plants may be an attractive means of producing cocaine-hydrolyzing variants of BChE in quantities relevant for clinical use.

2. Materials and methods

2.1. Cloning of plant-expression optimized synthetic genes encoding BChE variants and their expression in plants

The plant-expression optimized gene encoding the WT form of human BChE, pBChE [6,7] with C-terminal His_tag (H_6) was used as template for introduction of site-directed mutations (QuickChange kit, Stratagene) to create the following sited-directed mutations:

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F227A/S287G/A328W/Y332A, A199S/S287G/A328W/Y332G [25], A199S/F227A/S287G/A328W/Y332G, and F227A/S287G/A328W/Y332G [27]. The genes were transiently expressed in wild-type (WT) *N. benthamiana* plants using the MagnICON vector system based on deconstructed tobacco mosaic virus [TMV, [18]].

2.2. Enrichment preparation of BChE variants and biochemical analyses

The proteins were partially purified following a protocol similar to one used for WT pBChE [6,7] based on concanavalin A (ConA) chromatography.

Estimation of concentration of BChE and variants thereof was conducted using quantitative immunoblot assay with highly purified samples of plasma-derived and plant-derived BChE, whose molar concentrations were previously determined [6,7], serving as standards. To this end, standards were resolved by SDS–PAGE on 8% polyacrylamide gels, transferred to nitrocellulose membranes, immunodecorated with rabbit polyclonal anti-hBChE antibodies (kind gift of Dr. Oksana Lockridge), and detected by anti-rabbit IgG-Horse Radish Peroxidase (HRP) antibodies followed by chemiluminescence assay. High resolution (at least 600dpi) grey-scale images were used for densitometry analysis with Image J Software and data was used to plot standard curves fitted by linear-regression (GraphPad Prism). Samples of variants with unknown concentrations were resolved alongside the standards and densitometry results together with the regression equations were used to obtain concentration of the BChE variants. Several dilutions of samples were applied to make sure samples were well within the linear range of the standard curve. Results showed excellent correlation with butyrylthiocholine (BTC) hydrolysis assays (see below) by the mutants and individual specific activities could thus be calculated. In all subsequent experiments we have used these specific activities to estimate BChE variant concentration.

2.3. Enzymatic assays

Two enzyme assays were performed. The spectrophotometric Ellman assay was used to assess basic BChE activity with BTC (Sigma) as the substrate (1 mM). Assays were run at 30 °C in a Spectramax 190 spectrophotometer (Molecular Devices) as previously described [8]. To evaluate cocaine hydrolysis, a previously described radiometric assay was used with ^{3}H cocaine as substrate over a wide range of concentrations [2]. Data were subjected to non-linear regression analysis (Sigma-Plot), and estimates of V_{MAX} and K_M were derived along with their standard errors. Turnover numbers (K_{CAT}) could be derived, in turn, from these V_{MAX} values and the assay's molar concentrations of BChE variants obtained as described above.

3. Results and discussion

We have previously described the production of a double mutant of BChE A328W/Y332A in transgenic plants [9]. This mutant has enhanced hydrolytic activity toward (−)-cocaine [15,21,22]. The catalytic prowess of this mutant, (which we call Variant 1) was subsequently improved by introducing additional or different site-directed changes to create Variants 2 (F227A/S287G/A328W/Y332A), 3 (A199S/S287G/A328W/Y332G), 4 (A199S/F227A/S287G/A328W/Y332G), and 5 (F227A/S287G/A328W/Y332G) [25,27].

Using the MagnICON expression system [18], deconstructed-TMV-based vectors were introduced into WT tobacco plants by infiltration either by using needle-less syringe injection or by application of vacuum on whole plants submerged in agrobacterial suspensions (Fig. 1).

Leaf samples were harvested at the indicated time points and assayed by the Ellman-assay and immunoassay to determine the expression level of the BChE enzyme variants (Fig. 2). Multiple 0.2 g leaf samples from different plants were assayed per time point for BChE activity. Peak expression time was around 14 days but with some variation among the variant forms (14–17 days). Accumulation levels varied considerably between the various mutants and ranged from 16 to 100 mg per kg fresh weight leaf material (Fig. 2).

Partial purification was achieved by ConA affinity chromatography as exemplified for Variant 4 (Fig. 3), and Variants 3–5 were tested for cocaine hydrolysis activity in a radiometric assay [2]. Michaelis–Menten constant (K_M) values for Variants 3–5 were (mean ± SEM, respectively) 2.6 ± 0.1, 2.7 ± 0.1, and 12.4 ± 1.2 μM compared to the reported WT BChE K_M of 4.5 μM [22]. The turnover number was determined for one variant thus far (Variant 4) and was 5200 ± 63 min^{-1} (mean ± SEM), similar to the established value of 5700 min^{-1} determined for the variant derived from mammalian cell system [26]. The efficiency of catalysis (K_{CAT}/K_M) was determined for one mutant thus far (Variant 4) and was (1.91 ± 0.09) × 10^9 M min^{-1}, a ~1500-fold increase over the established value of 1.3 × 10^6 M min^{-1} for WT BChE [4]. This outcome is very similar to that reported for the original version of the same mutant expressed in mammalian cell culture [26]. However, a caveat to bear in mind is that the present experiments did not bring the isolated protein to the level of purity at which active site titrations could be performed to establish beyond all possible doubt the precise abundance of catalytic units. These findings therefore do not prove that catalytic efficiency in the plant-derived enzyme is exactly identical to that which the mutant BChE would exhibit if isolated from mammalian, or especially human cells. Nonetheless they represent powerful encouragement for a plan to produce human BChE-based cocaine hydrolases for testing in higher animals, including non-human primates. Moreover, they strongly suggest that such enzymes could exhibit the properties expected and needed for eventual therapeutic applications in humans.

Fig. 1. Transient plant expression of cocaine-hydrolase variants of BChE. *Agrobacterium tumefaciens* cells harboring the deconstructed TMV-vectors containing the recombinant BChE variant genes (A) were infiltrated by applying vacuum to whole-submerged *N. benthamiana* plants (B1) or by leaf injection with needle-less syringe into leaves (B2). Plants were harvested at 14–17 days post-infiltration when peak expression is reached (C).