Examination of the Substrate Scope of Prenylated Flavin Mononucleotide-Dependent Ferulic Acid Decarboxylase from *Aspergillus niger*

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Abstract

Global warming is one of the world's most foremost problems and anthropogenic carbon dioxide (CO_2) emissions are one of the major contributing factors to global warming. Reversible decarboxylases present a novel solution to this problem, possessing the ability to fix CO_2 onto a wide range of molecules. In principle, this technique could be used to produce molecules for various practical uses, such as; medicines, polymers or useful intermediates in chemical synthesis.

This work examines the substrate scope of prenylated flavin mononucleotide (prFMN)-dependant ferulic acid decarboxylase from *Aspergillus niger* on a range of novel bulky and nitrogen substituted heterocycles in an attempt to elucidate the full reactivity of this enzyme.

Key words

Aspergillus niger, decarboxylase, carboxylation, prenylated flavin mononucleotide, biocatalysis

1. Introduction

Decarboxylases are enzymes that are capable of processing the carboxylic acid group of a molecule to generate CO_2 and a resulting C-H bond. Reversible decarboxylases possess the ability to carry out the reverse of this described reaction, instead, fixing CO_2 onto the accepted substrate.[1] This reaction is of importance as there seems to be an opportunity to use CO_2; a greenhouse gas that contributes to global warming, in order to produce molecules with synthetic/medicinal properties. This technique, in principle, could be used to valorise CO_2, providing financial incentive to reduce anthropogenic CO_2 emissions.

A new class of reversible decarboxylase with an interesting co-factor requirement and substrate scope acceptance was discovered recently.[2-3] This enzyme class, prenylated flavin mononucleotide (prFMN)-dependant reversible decarboxylases is relatively unexplored and an interesting class to examine in this study. Specifically, ferulic acid decarboxylase from *Aspergillus niger* (anFDC) was selected due to its unique carboxylation profile, natively carrying out decarboxylation of acid (Figure 1).[4]

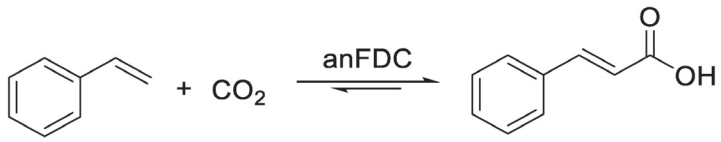

Figure 1. Schematic diagram of the carboxylation of styrene with prFMN dependent anFDC.

This report will examine this enzymes ability to accept bulky phenolic acids, as well as derivatives of ferulic acid to establish some limits as to what kinds of structures may be accepted by the enzyme. The substrates selected for this study were carefully chosen to probe specific positions of the ferulic acid derivates, particularly the unsaturated alpha and beta positions of cinnamic acid.

2.Materials and Methods

2.1 AnFDC_sequence[5]

CAGGTCGACTCTAGAGGATCCATGAGCGCGCAACCTGCTCACCTTTGTTTCCGATCATTCGTCGAA
GCTCTGAAAGTTGACAACGATCTGGTGGAGATTAACACCCCCATTGACCCGAATCTGGAAGCGGC
CGCCATTACGCGCAGGGTTTGTGAGACTAATGACAAGGCCCCACTGTTCAATAACCTGATAGGCAT
GAAAAATGGCCTCTTTCGTATTTTAGGTGCCCCGGGCTCGCTGCGCAAAAGCTCAGCGGATCGTTA
TGGGCGGCTTGCCCGTCACCTCGCGCTACCACCGACGGCGTCTATGCGCGAAATCCTGGATAAGAT
GCTCAGCGCCTCAGATATGCCACCGATTCCGCCAACGATTGTCCCGACCGGTCCTTGCAAAGAAAA
TTCTCTGGATGACTCGGAATTTGATTTAACAGAATTACCGGTGCCCCTGATCCATAAAAGTGACGG
AGGGAAATACATTCAGACATACGGCATGCATATCGTTCAGAGTCCCGATGGCACCTGGACGAACT
GGTCAATAGCTCGTGCGATGGTGCATGACAAGAATCATCTGACCGGTCTTGTCATTCCGCCCCAAC
ATATCTGGCAGATCCATCAGATGTGGAAAAAAGAAGGCCGTTCCGATGTGCCGTGGGCTCTGGCA
TTCGGTGTCCCGCCTGCGGCGATTATGGCGAGCTCGATGCCTATTCCGGATGGGGTAACCGAGGCA
GGCTATGTGGGCGCAATGACCGGGTCCAGTTTGGAGTTAGTGAAGTGCGACACTAATGATCTGTAC
GTCCCGGCCACCTCGGAAATAGTCCTGGAGGGTACATTGAGCATCTCCGAAACTGGCCCAGAAGG
TCCGTTCGGCGAAATGCACGGCTATATCTTTCCCGGCGATACCCACCTCGGTGCCAAATATAAAGT
TAACCGAATTACGTACCGTAACAACGCTATTATGCCTATGAGCTCTTGTGGCCGCCTGACCGATGA
GACTCATACCATGATTGGCAGCCTAGCGGCCGCAGAGATCCGTAAATTGTGCCAGCAAAACGACC
TGCCGATCACAGATGCGTTTGCCCCATTCGAATCGCAGGTGACGTGGGTGGCACTTCGCGTGGATA
CGGAAAAACTGCGCGCAATGAAAACGACTAGTGAAGGGTTTCGGAAACGCGTAGGTGATGTAGTT
TTCAATCACAAAGCAGGTTACACCATCCACCGCTTGGTCCTGGTAGGTGATGACATCGATGTTTAC
GAAGGAAAAGACGTTTTATGGGCATTTTCTACACGTTGCAGACCAGGTATGGATGAAACGCTGTTT
GAGGACGTACGGGGATTTCCGTTGATTCCGTATATGGGTCATGGAAATGGGCCGGCGCATCGCGG
CGGTAAGGTGGTTAGCGATGCGCTTATGCCTACCGAGTATACCACTGGACGTAACTGGGAAGCTG
CAGATTTTAACCAATCCTATCCGGAAGATCTGAAACAGAAAGTGCTGGACAACTGGACCAAGATG
GGCTTTAGTAAGGATCCCCGGGTACCGAGCTC

2.2 Protein expression and analysis

The expression plasmid was transformed into *E. coli* strain BL21 (DE3). The transformants from the same gene were cultured in 3 ml of Luria-Bertani (LB) medium supplemented with kanamycin (50 μg/ml) or ampicillin (100 μg/ml) at 30°C for OD_{600} = 0.4～0.8. IPTG was then added to 0.2 mM and the cells were continuously cultured at 20°C for 18 hr. The cells were harvested by centrifugation, and cell disruption using sonication (Bioruptor UCD-250, TOSHO DENKI CO., LTD, Japan). Each expression of soluble protein or total cell protein fraction (soluble plus insoluble) was detected by the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.3 Biotranformation set up

Substrate (10 mM, 10 μl from 1000 mM DMSO stock), DMSO (100μl, 10% v/v total reaction volume) and Kpi buffer 100 mM pH 7.5 (890 μl). Finally, lyphollised cell extract (30 mg) was added to the reaction mixture and the gas tight vial crimped closed. Reactions were left for 16 hours at 30°C in a shaking incubator. For carboxylation reactions $KHCO_3$ (300 mg) was added as a dry powder to the reaction mixture before quickly crimping shut.

Upon completion, a 100 μl aliquot of the reaction mixture was added to an acetonitrile: H2O mixture (1:1 with 3% TFA, 900 μl), left for 15 minutes at room temperature, centrifuged and the resulting clarified liquid used directly for HPLC analysis using Cosmosil C18 (5C18-MS-II, 4.6 ID x 150 mm) column, isocratic 70% acetonitrile in H_2O (3% TFA) in 10- or 15-minute runs.

3. Results and discussion

Initially, anFDCs ability to carry out the thermodynamically disfavoured carboxylation reaction on styrene to produce cinnamic acid was examined. While technically feasible, the carboxylation direction of this reaction is heavily thermodynamically disfavoured, largely due to the high gain in entropy upon decarboxylation. This, combined with the complex proposed reaction mechanism is thought to be the reason why carboxylation was found to be non-existent for this enzyme in this study.[6]

Moving on from this, a carefully selected panel of substrates was chosen based on their substitution pattern in order to establish if derivatised phenolic acids could be accepted by anFDC (Figure 2). These were to be tested in the thermodynamically favoured decarboxylation direction for the reasons mentioned in the previous paragraph.

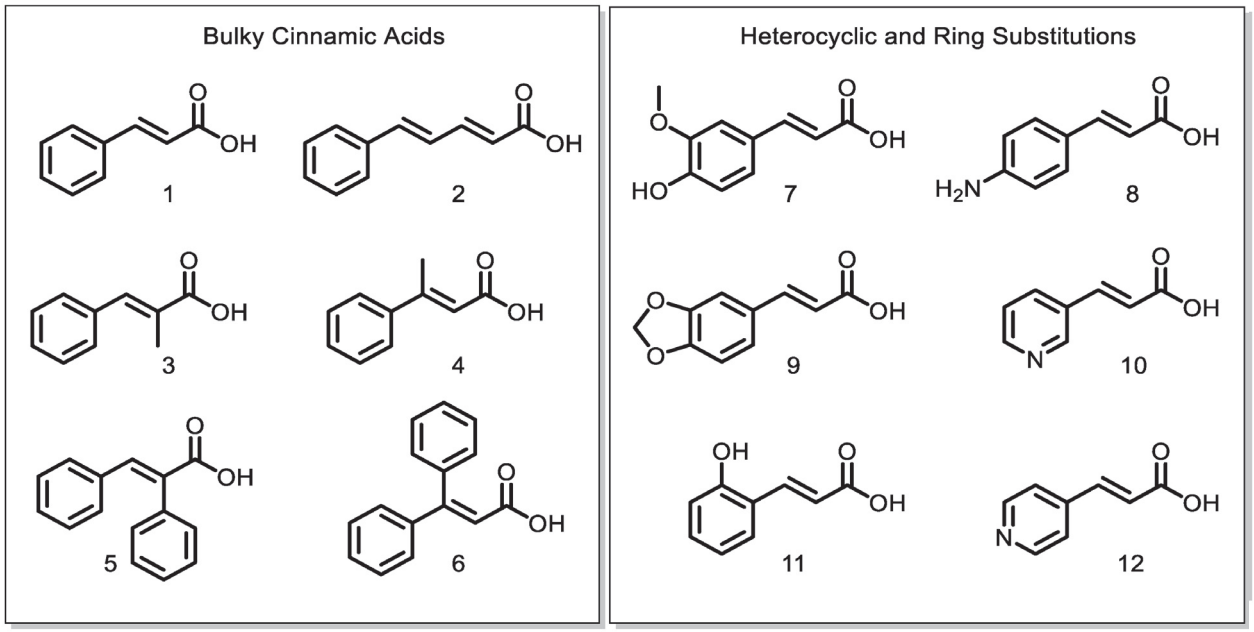

Figure 2 – Substrates tested with anFDC in order to examine the boundaries of substrate acceptance The Left-hand side showcases some of the bulky cinnamic acid derivatives, while the right-hand side is more akin to derivatives of ferulic acid.

The anFDC decarboxylase gene was established from the literature, expression vector assembled and heterologous expression performed as described in the methods. The lysed crude cell extract was used directly in biotransformations using gas tight, crimped lid glass vials to control gas leakage.[7]

As can be seen in Table 1, overall, the enzyme exhibited some level of promiscuity, however the extent to which conversion progressed was not significant in most cases. Interestingly, the enzyme was able to accept substrate 2, a structural analogue of cinnamic acid with and extended conjugated system.

Additionally, substrate 3 was the only molecule that featured a substitution at the α, β – unsaturated carbonyl and was still accepted for carboxylation. This result is corroborated by the published crystal structure (4ZAB.pdb), which indicates a small amount of space available at the alpha position, while no space at the beta position.

As expected, high conversion was observed for the native substrates, however the molecule that featured the highest conversion of the non-native substrates was substrate 9. Again, when compared with the published crystal structure, this observation appeared to makes sense, with an apparent abundance of space in the aromatic 3 and 4 positions of the bound phenolic acids in the anFDC active site.

Table 1 – Table of results for anFDC conversion for the substrate scope examination study (substrates in Figure 2). Reaction conditions – 10 mM substrate, 30 mg lyophilised whole cell extract in Kp_i Buffer (50 mM, pH 7.5), DMSO (10% v/v), 24h, 30°C 150 rpm. Reactions were performed in triplicate and the presence of decarboxylation conversion recorded in Table 1.

Substrate	Yield (%)	Substrate	Yield (%)
1	>99	7	> 99
2	16	8	15
3	19	9	30
4	< 0	10	< 0
5	< 0	11	10
6	< 0	12	< 0

In summary, this work has examined anFDCs ability to carry out decarboxylation on a small selection of structurally diverse phenolic acid substrates. The results of which show a relatively promiscuous substrate acceptance into anFDC, and appears to agree well with a published crystal structure. Further work should be carried out in the future comprising of a more robust and varied substrate acceptance panel as well as directed evolution studies to further enhance substrate acceptance, and increase the carboxylation propensity.

4. Acknowledgements

This work was supported by FY2021 Japan Society for Promotion of Sciences (JSPS) Postdoctoral Fellowship for Research in Japan, and by a grant-in-aid for Scientific Research (S) from JSPS (Grant No. 17H06169) to Y. Asano.

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Aspergillus niger 由来のプレニル化フラビンモノヌクレオチド依存性フェルラ酸脱炭酸酵素の基質特異性の検討

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要旨

地球温暖化は世界で最も重要な問題の一つであり、人為的な二酸化炭素（CO_2）の排出は地球温暖化の主な要因となっている。可逆的に作用する脱炭酸酵素は、広範囲の分子を基質として、CO_2を固定する能力を備えており、この問題に対する新しい解決策の一つを提示している。本酵素を触媒として用いて、医薬品、ポリマー、または化学合成における有用な中間体等、様々な実用的な用途の化合物の合成に使用できる可能性がある。本論文では、*Aspergillus niger* 由来のプレニル化フラビンモノヌクレオチド（prFMN）依存性フェルラ酸脱炭酸酵素について、一連の新規な、かさ高い窒素置換複素環化合物を基質として用いて、本酵素の基質特異性を解明した。

Key words

Aspergillus niger、脱炭酸酵素、炭酸固定、プレニル化フラビンモノヌクレオチド、生体触媒