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DOI
10.1002/cctc.201902194

Publication date
2020

Document Version
Final published version

Published in
ChemCatChem

Citation (APA)
Xu, X., But, A., Wever, R., & Hollmann, F. (2020). Towards Preparative Chemoenzymatic Oxidative Decarboxylation of Glutamic Acid. ChemCatChem, 12(8), 2180-2183. https://doi.org/10.1002/cctc.201902194

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Towards Preparative Chemoenzymatic Oxidative Decarboxylation of Glutamic Acid

Xiaomin Xu,[a] Andrada But,[a] Ron Wever,[b] and Frank Hollmann*[a]

The chemoenzymatic oxidative decarboxylation of glutamic acid to the corresponding nitrile using the vanadium chloroperoxidase from Curvularia inaequalis (CVCPO) as HOBr generation catalysts has been investigated. Product inhibition was identified as major limitation. Nevertheless, 1630000 turnovers and kcat of 75 s\(^{-1}\) were achieved using 100 mM glutamate. The semi-preparative enzymatic oxidative decarboxylation of glutamate was also demonstrated.

The production of biobased chemicals often requires the removal of (oxygen) functionalities from biomass-derived starting materials,[1-4] as in case of the oxidative decarboxylation of amino acids in the production of biobased nitriles. The oxidative decarboxylation of L-glutamic acid (Glu) the most abundant amino acid in biomass,[5] generates the corresponding nitrile, 3-cyanopropanoic acid (CPA). CPA is a potential starting material for a range of products such as acrylonitrile, succinonitrile or pharmaceuticals.

The oxidative decarboxylation of amino acids can be mediated by hypobromite (HOBr). In order to minimise undesired oxidative side-reactions, using HOBr in low concentrations is advisable. For this, next to some chemocatalytic,[6-8] or electrochemical methods[9] also an enzymatic approach has been developed (Scheme 1).[10]

High selectivity (> 99%) and full conversion of L-glutamic acid into 3-cyanopropanoic acid was observed for the enzymatic procedure.[10] The substrate loadings, however, were as low as 5 mM, which is neither economically feasible nor environmentally acceptable.[11] Increasing the substrate concentration is, therefore, an important task to demonstrate that highly selective catalysts like enzymes can be use at preparative scale.[12]

The aim of this research was to scale up the conversion of L-glutamic acid into 3-cyanopropanoic acid by increasing the substrate loadings. The highly active and robust enzyme vanadium chloroperoxidase from Curvularia inaequalis (CVCPO),[10] was used in this endeavor.

As a starting point we increased the initial L-glutamic acid concentration five-fold higher than in previous experiments.[3] H\(_2\)O\(_2\) was added over time to the reaction mixture using a syringe pump. Pleasingly, we observed full conversion of the starting material into the desired CPA within approximately 5 hours reaction time (Figure 1).

It should be noted here that adding stoichiometric amounts of H\(_2\)O\(_2\) from the beginning of the reaction had a rather detrimental effect on the product formation.[10] Under otherwise identical conditions only half of the product was formed (Figure S1). In contrast to heme-dependent haloperoxidases, this phenomenon is not due to an inactivation of the biocatalyst but rather the result of an undesired reaction of H\(_2\)O\(_2\) with HOBr yielding singlet oxygen (\('O_2\), vide infra).[13] The biocatalyst performed 450000 catalytic cycles corresponding to an average turnover frequency over 5 h of 25 s\(^{-1}\). Even though these numbers are convincing, they still somewhat fall back behind the catalytic potential of CVCPO.[7] We therefore systematically investigated some reaction parameters influencing the overall rate of the oxidative decarboxylation reaction.

First, we varied the flow rate of H\(_2\)O\(_2\) (Figure 2a) and observed a linear correlation between H\(_2\)O\(_2\) dosage rate and overall product accumulation rate up to a H\(_2\)O\(_2\) dose rate of 40 mMh\(^{-1}\).

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cctc.201902194

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Scheme 1. Oxidative decarboxylation of L-glutamic acid yielding 3-cyanopropanoic acid using the vanadium-dependent chloroperoxidase from Curvularia inaequalis (CVCPO) and catalytic amounts of bromide. For reasons of simplicity, the protonation stage of the reagents is ignored.
We attribute the decrease of H$_2$O$_2$ conversion with increasing H$_2$O$_2$ dose rate to the above-mentioned undesired side reaction [Eq. (1)].

$$\text{H}_2\text{O}_2 + \text{OBr} \rightarrow \text{Br}^- + \text{H}_2\text{O} + ^1\text{O}_2 \quad (1)$$

In fact, at a H$_2$O$_2$ flow rate of 100 mM, this reaction was so dominant that bubble formation was observed in the reaction vessel. Therefore, we limited the H$_2$O$_2$ flow rate to 39 mM h$^{-1}$ for further experiments. Under these conditions an average turnover frequency ($\text{Ci}_{\text{VCPO}}$) of more than 63 s$^{-1}$ was calculated.

Next, we varied the concentration of the Br$^-$ co-catalyst (Figure 3). Interestingly, it turned out that the initially chosen 0.5 mM was already the optimal value as previously reported.$^{[5]}$ Lower concentrations resulted in reduced product formation rates while higher Br$^-$ concentration seemingly did not influence the reaction rate.

Next, the concentration of the glutamate in the reaction mixture was further increased to 100 mM, which gave excellent reaction rates and almost complete conversion (96%) of the starting material into the desired product (Figure 4).

In these experiments CVCP0 performed excellent 1630000 turnovers at an average turnover frequency of 75 s$^{-1}$. Noteworthy, also the H$_2$O$_2$ yield was on average 80%. The latter observation may be attributed to an increased rate of the (desired) reaction between OBr$^-$ and glutamate over the (undesired) reaction with H$_2$O$_2$. Further increase of the glutamate concentration, however, did not lead to the anticipated improvements (Figure S3). On the contrary, lower amounts of product (after prolonged reaction times) were obtained.
compared to the amounts shown in Figure 4. For example, using 500 mM glutamate resulted in only 31 mM of CPA after 24 h reaction.

We suspected substrate inhibition to account for this and therefore determined the CiVCPO activity in the presence of different concentrations of glutamate (Figure 5). Very much to our surprise, increasing glutamate concentrations showed limited influence on the activity of CiVCPO; even in the presence of 500 mM glutamate, its activity in the MCD assay was reduced by only 23%.

Next, the possibility of CiVCPO inhibition by the product, 3-cyanopropanoic acid, was investigated (Figure 6). With increasing CPA concentration, the observed activity of CiVCPO decreased. In the presence of 75 mM CPA the enzyme activity was reduced by 50%, whereas in the presence of 200 mM the enzyme lost almost completely its activity in the MCD assay. It can be concluded that CPA, the product of oxidative decarboxylation, significantly inhibits CiVCPO. Possibly, CPA coordinates to the prosthetic vanadate thereby preventing the coordination of $\text{H}_2\text{O}$ to initiate the catalytic cycle but further studies will be necessary to fully elucidate the inhibitory mechanism.

Lastly, the oxidative decarboxylation of Glu by CiVCPO was performed at semi-preparative scale. From a 200 mL reaction scale (100 mM Glu), 0.827 g CPA (42% isolated yield, 96% pure) was obtained after 5 h reaction with 100 nM CiVCPO. CPA was isolated by extraction in organic solvents, however, the extraction efficiency was low (see Experimental section). Based on the isolated yield, 420000 turnovers were performed which is less than in the small scale (Figure 4), however, CPA remained in the aqueous phase even after the second extraction. The isolated yield is in agreement with previously reported chemical reaction with NaOCl/NaBr (43%),\textsuperscript{[11]} but higher selectivity towards the nitrile was obtained by using CiVCPO. Derivatization to the corresponding ester or amide would certainly increase the efficiency of the extraction as demonstrated previously.\textsuperscript{[12]} Also continuous liquid-liquid extraction appears
to be a promising method to increase the isolated yield. It is 
worth to mention that the semi-preparative reaction was 
performed without additional buffer (therefore less waste) and 
instead the substrate, sodium glutamate, was used as a buffer 
(where the pH was adjusted to pH 5.6 with H2SO4).

In conclusion, we demonstrate that the chemoenzymatic 
oxidative decarboxylation of glutamate is indeed a possible 
alternative to the established chemical and the new catalytic 
products. The product inhibition and the isolation of the 
product are currently the main bottlenecks of this reaction 
which could be solved by selective in situ solid phase extraction 
or by using a packed bed immobilised VCPO. 
Product isolation could be circumvented by direct conversion 
of CPA to a more hydrophobic product. Furthermore, this 
preparative scale opens the route towards the oxidative 
decarboxylation of other amino acids with different side chain 
functionalities and their corresponding nitrites.

Experimental Section

Enzyme preparation. Vanadium chloroperoxidase from Curvularia 
inaequalis (CVCPO) was obtained from heterologous expression in 
recombinant Escherichia coli and purified by heat treatment (see 
Supplementary information).

Enzymatic reaction conditions. In a 4 mL glass vials a solution (2 mL 
starting volume) containing 0.5 mM NaBr, 55 nM CVCPO, different 
concentration of glutamic acid or sodium glutamate monohydrate 
in 20 mM sodium citrate buffer (pH 5.6) was prepared. The reaction 
was started by addition of H2O2, which was added with a 
continuous flow rate (see captions of figures) at room temperature 
(about 22°C). The reaction was quenched by adding Na2SO4. For 
each time point a separate reaction vial was prepared. The 
conversion of Glu and formation of CPA was analysed by two 
different HPLC methods (see Supplementary information).

Enzyme activity assay. To assess CVCPO activity, a standardised 
assay reported previously was used. In short: in a disposable UV 
plastic cuvette a solution (1 mL) containing 50 μM monochloro-
medone (MCD), 1 mM H2O2, 0.5 mM NaBr, 100 μM Na2VO4 in 50 mM 
sodium citrate (pH 5.6) was prepared. The absorbance of MCD 
solution was followed at 290 nm, 25°C. The reaction was started 
with the addition of CVCPO. The enzyme activity was calculated 
using a molar extinction coefficient for MCD of 20 (mM·cm)−1. For 
the inhibition tests, the enzyme was incubated before the assay 
with different concentrations of inhibitor, for 5 min, at room 
temperature.

Semi-preparative reaction conditions. In a 500 mL round-bottom 
flask an aqueous solution (200 mL deionised water) containing 
100 mM monosodium glutamate monohydrate (3.78 g, 20 mmol) 
and 0.5 mM NaBr, was adjusted at pH 5.6 with a 2 M H2SO4 solution. 
Next, 100 nM CVCPO was added and the reaction was started by 
addition of H2O2 50 mM·h−1 (10 mL of 1 M stock/h) by a syringe 
pump at room temperature (about 22°C). After 5 h the product was 
isolated by extraction in ethyl acetate (2×100 mL) and diethyl ether 
(3×70 mL) (see Supplementary information).

Acknowledgements

This work was financially supported by the European Research 
Commission (ERC consolidator grant, No. 648026), the European 
Union (H2020-BBI-PPP-2015-1-720297), and the Netherlands 
Organization for Scientific Research (VICI grant, No. 724.014.003) 
and the Guangzhou Elite Project. We thank Lloyd Malée for 
technical support and Dr. Sabry Younes for useful discussions.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: glutamic acid · biocatalysis · vanadium 
chloroperoxidase · nitriles · oxidative decarboxylation

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Manuscript received: November 23, 2019
Revised manuscript received: December 22, 2019
Accepted manuscript online: December 24, 2019
Version of record online: January 22, 2020