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Authors: Xiaomin Xu, Andrada But, Ron Wever, and Frank Hollmann

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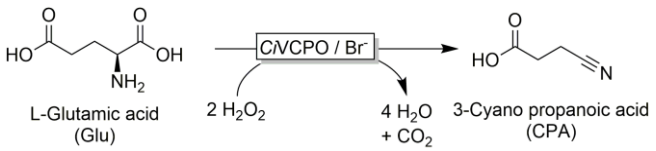
Towards preparative chemoenzymatic oxidative decarboxylation of glutamic acid

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

Abstract. The chemoenzymatic oxidative decarboxylation of glutamic acid to the corresponding nitrile using the vanadium chloroperoxidase from *Curvularia inaequalis* (*Ci*VCPO) as HOBr generation catalysts has been investigated. Product inhibition was identified as major limitation. Nevertheless, 1630000 turnovers and k_{cat} 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] 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[2] 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,[3] or electrochemical methods[4] also an enzymatic approach has been developed (Scheme 1).[5]



Scheme 1. Oxidative decarboxylation of L-glutamic acid yielding 3-cyanopropanoic acid using the vanadium-dependent chloroperoxidase from *Curvularia inaequalis* (*Ci*VCPO) and catalytic amounts of bromide. For reasons of simplicity, the protonation stage of the reagents is ignored.

High selectivity (>99%) and full conversion of L-glutamic acid into 3-cyanopropanoic acid was observed for the enzymatic procedure.[5] The substrate loadings, however, were as low as 5 mM, which is neither economic feasible nor environmentally acceptable.[6] Increasing the substrate concentration is, therefore, an important task to demonstrate that highly selective catalysts like enzymes can be use at preparative scale.[7]

[a] X. Xu, Dr. A. But, Dr. C.E. Paul, Prof. Dr. F. Hollmann
Department of Biotechnology
Delft University of Technology
van der Maasewg 9, 2629 HZ Delft, The Netherlands
E-mail: f.hollmann@tudelft.nl
[b] Prof. Dr. R. Wever
University of Amsterdam, Van't Hoff Institute for Molecular Sciences, Amsterdam, The Netherlands
[†] Both authors contributed equally.

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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* (*Ci*VCPO),[8] was used in this endeavor.

As a starting point we increased the initial L-glutamic acid concentration five-fold higher than in previous experiments,[5] H_2O_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).

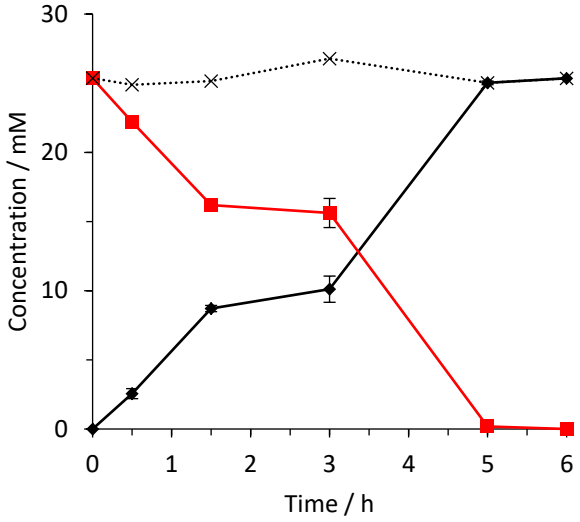

Figure 1. The conversion of L-glutamic acid (■) into 3-cyanopropanoic acid (◆), mass balance (X). Reaction conditions: [glutamic acid] = 25 mM, [NaBr] = 0.5 mM, [*Ci*VCPO] = 55 nM, H_2O_2-dosage: 12 mM h^{-1} (from a 0.5 M stock solution, considering the final volume), 20 mM sodium citrate buffer (pH 5.6), room temperature (22°C). The error bars represent the range of minimum duplicate experiments.

It should be noted here that adding stoichiometric amounts of H_2O_2 from the beginning of the reaction had a rather detrimental effect on the product formation.[5] 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_2O_2 with HOBr yielding singlet oxygen ($^1O_2$, *vide infra*).[9] 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 *Ci*VCPO.[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_2O_2 (Figure 2a) and observed a linear correlation between H_2O_2 dosage rate and overall product accumulation rate up to a H_2O_2 dose rate of 40 mM h^{-1}. Higher dosing rates did not significantly increase the overall productivity. Consequently, the yield of H_2O_2 into the desired product (CPA)

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decreased from 95% at 12 mM h^{-1} to 34% at 100 mM h^{-1} (Figure 2b).

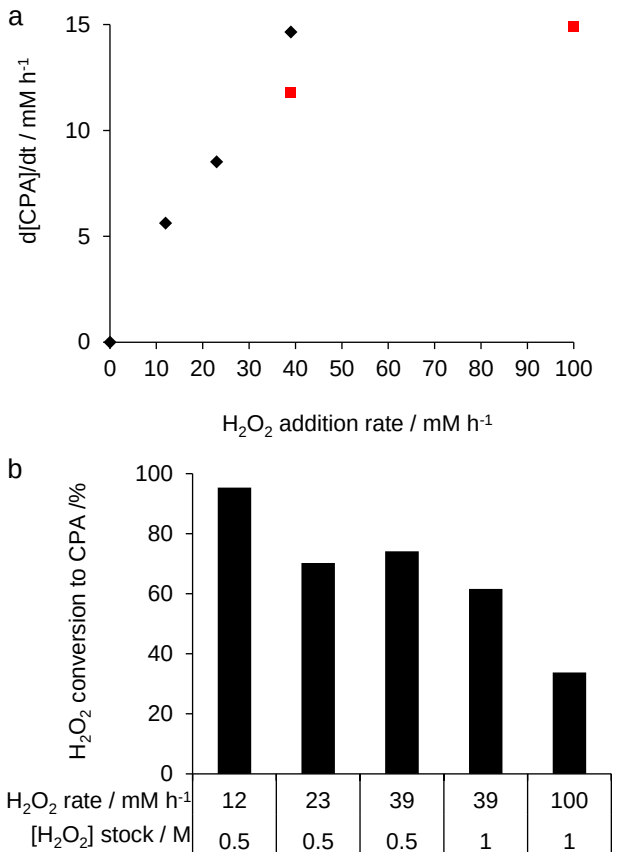

Figure 2. a) The correlation between the addition rate of H_2O_2 and the formation rate of the product (CPA). Reaction conditions: [L-glutamic acid] = 25 mM, [NaBr] = 0.5 mM, [*Ci*VCPO] = 55 nM, H_2O_2-dosage: from a 0.5 M stock solution (◆) and from a 1 M stock solution (■), 20 mM sodium citrate buffer (pH 5.6), room temperature (22°C). **b)** The conversion of H_2O_2 into CPA as a function of the addition rate of H_2O_2.

We attribute the decrease of H_2O_2 conversion with increasing H_2O_2 dose rate to the above-mentioned undesired side reaction (Equation 1).[10]

$$H_2O_2 + OBr^- \xrightarrow{\text{spontaneous}} Br^- + H_2O + {}^1O_2$$

Equation 1. The formation of singlet oxygen by the spontaneous reaction between H_2O_2 and OBr^-.

In fact at a H_2O_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_2O_2 flow rate to 39 mM h^{-1} for further experiments. Under these conditions an average turnover frequency (*Ci*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.

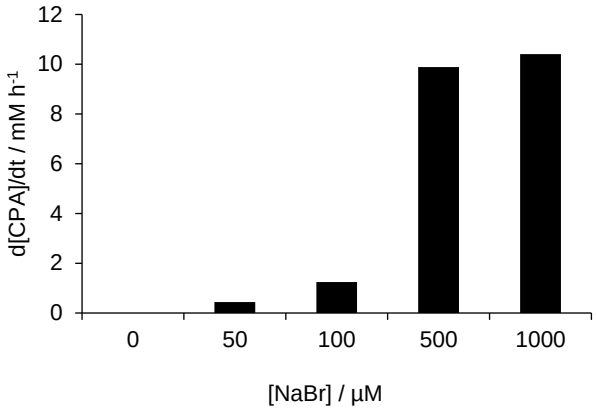

Figure 3. The influence of the concentration of NaBr on the overall CPA formation rate. Reaction conditions: [L-glutamic acid] = 25 mM, [*Ci*VCPO] = 55 nM, H_2O_2-dosage: 39 mM h^{-1} from a 1 M stock solution, 20 mM sodium citrate buffer (pH 5.6), room temperature (22°C).

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).

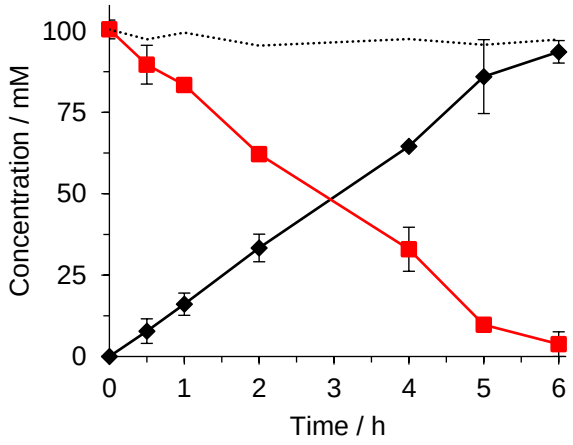

Figure 4. Scale up of the oxidative decarboxylation of sodium glutamate (■) to CPA (◆), (···) mass balance. [sodium glutamate] = 100 mM, [NaBr] = 5 mM, [*Ci*VCPO] = 55 nM, H_2O_2-dosage: 39 mM h^{-1} from a 1 M stock solution, 20 mM sodium citrate buffer (pH 5.6), room temperature (22°C). The error bars represent the range of duplicate experiments.

In these experiments *Ci*VCPO performed excellent 1630000 turnovers at an average turnover frequency of 75 s^{-1}. Noteworthy, also the H_2O_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_2O_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.

10.1002/cctc.201902194

We suspected substrate inhibition to account for this and therefore determined the *Ci*VCPO 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 *Ci*VCPO; even in the presence of 500 mM glutamate, its activity in the MCD assay was reduced by only 23%.

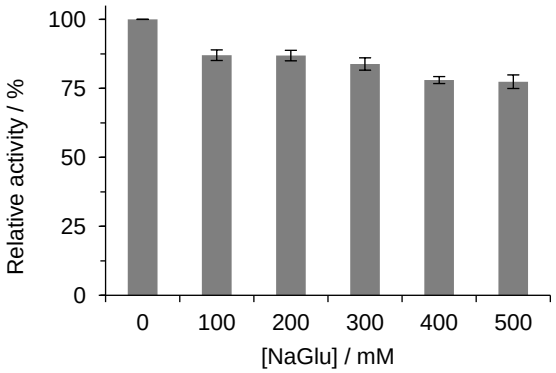

Figure 5. Influence of sodium glutamate (NaGlu) on the activity of *Ci*VCPO. Assay conditions: [MCD]= 50 µM, [H_2O_2]= 1 mM, [NaBr]= 0.5 mM, [Na_3VO_4]= 100 µM, 50 mM sodium citrate buffer (pH 5), T= 25 °C, 290 nm. The reaction was started with addition of 0.8 nM *Ci*VCPO that was pre-incubated for 5 min with different amounts of NaGlu. The error bars represent the standard deviation of triplicate experiments.

Next, the possibility of *Ci*VCPO inhibition by the product, 3-cyanopropanoic acid, was investigated (Figure 6). With increasing CPA concentration, the observed activity of *Ci*VCPO 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 *Ci*VCPO. Possibly, CPA coordinates to the prosthetic vanadate thereby preventing the coordination of H_2O_2 to initiate the catalytic cycle but further studies will be necessary to fully elucidate the inhibitory mechanism.

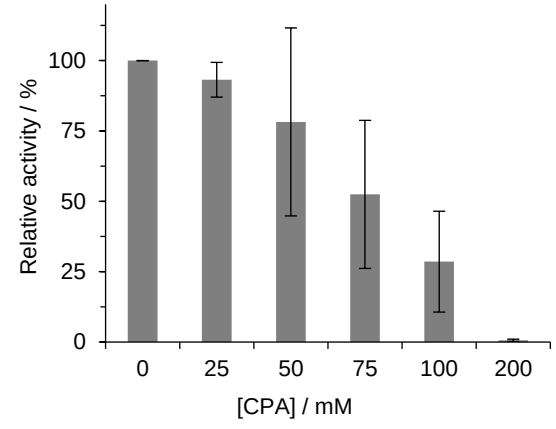

Figure 6. Inhibition of *Ci*VCPO by 3-cyanopropanoic acid (CPA). Assay conditions: [MCD]= 50 µM, [H_2O_2]= 1 mM, [NaBr]= 0.5 mM, [Na_3VO_4]= 100 µM, 50 mM sodium citrate buffer (pH 5), T= 25 °C, 290 nm. The reaction was started with addition of 0.8 nM *Ci*VCPO that was pre-incubated for 5 min with different amounts of CPA. The error bars represent the standard deviation of triplicate experiments.

Lastly, the oxidative decarboxylation of Glu by *Ci*VCPO 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 *Ci*VCPO. 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%),[11] but higher selectivity towards the nitrile was obtained by using *Ci*VCPO. Derivatisation to the corresponding ester or amide would certainly increase the efficiency of the extraction as demonstrated previously. [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 H_2SO_4).

In conclusion, we demonstrate that the chemoenzymatic oxidative decarboxylation of glutamate is indeed a possible alternative to the established chemical and the new catalytic methods. 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 reactor with 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 nitriles.

Experimental Section

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

Enzymatic reaction conditions. In a 4 mL gals vials a solution (2 mL starting volume) containing 0.5 mM NaBr, 55 nM *Ci*VCPO, 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 H_2O_2, which was added with a continuous flow rate (see captions of figures) at room temperature (about 22°C). The reaction was quenched by adding $Na_2S_2O_3$. 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 *Ci*VCPO activity, a standardised assay reported previously was used.[13] In short: in a disposable UV plastic cuvette a solution (1 mL) containing 50 µM monochlorodimedone (MCD), 1 mM H_2O_2, 0.5 mM NaBr, 100 µM Na_3VO_4 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 *Ci*VCPO. The enzyme activity was calculated using a molar extinction coefficient for MCD of 20 $(mM \cdot cm)^{-1}$. 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 H_2SO_4 solution. Next, 100 nM *Ci*VCPO was added and the reaction was started by addition of H_2O_2 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

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

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Entry for the Table of Contents

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*Author(s), Corresponding Author(s)**

Page No. – Page No.
Towards preparative chemoenzymatic oxidative decarboxylation of glutamic acid.