Biogenic Polychlorinated Dioxin and Furan from Trichlorophenol

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2,4,5 trichlorophenol(2,4,5 TrCP) formed significant amounts of hexachlorodibenzodioxin(H6CDD) when treated enzymatically with horseradish peroxidase, culture solution of Phanerochaete chrysosporium or diluted whey. In addition the results using Phanerochaete chrysosporium show considerable formation of chlorinated dioxins at a natural peroxidase tare. Peroxidase activity is a good measure to estimate the formation of dioxins from 2,4,5-TrCP. Competive reactions observed in whey can reduce the formation by orders of magnitude.

Introduction

Peroxidases are common enzymes in nature. They have a low substrate specifity and a high redox-potential. Peroxidase catalysed reactions are characterized by free radicalic substrate intermediates, which may react in a variety of ways.
Peroxidases are involved in biogenic polymerizations reactions, such as the formation of lignin. Also, the initial degradation of the lignin-polymer by white- and brownrotting fungi is peroxidase catalysed *(DILL,KRATHE EPELIN;1988/ HAIDER;1988)*. In animals peroxidases function in antimicrobial defense systems *(SEYMOUR ET AL.;1984)*.
ÖBERG ET AL.(1989) and *SVENSON ET AL.(1989)* demonstrated in vitro the formation of PCDD/F in the presence of peroxidases and 2,4,5 trichlorophenol.

The aim of our research was to demonstrate biogenic formation of PCDD/F, in a complex biological peroxidase producing system acting on 2,4,5 TrCP. Supporting experiments with horseradish peroxidase were conducted to analyse the influence of low enzyme activities, and the effect of substrate concentration on PCDD/F formation.
This paper presents results of well defined experiments similar to Öberg's and Svenson's, but with step by step reduced peroxidase activities, and varied substrate concentrations. Secondly, we describe the results of experiments with two biogenic peroxidase containing media: culture filtrate of Phanerochaete chrysosporium and whey.

Experimental/Methods

All experiments were done with 250ml measuring flasks at room temperature. The 2,4,5 TrCP used was purified until no PCDD/F could be detected (detection limit). Hydrogen peroxide was used to start the reaction either after dissolving 2,4,5 TrCP and horseradish peroxidase in buffer solution, or after the addition of an aqueous 2,4,5 TrCP solution to a biogenic peroxidase containing media. The concentration of 2,4,5 TrCP was determined with HPLC/UV at 222nm before and after the reaction.
All samples were cleaned up on an Alox-coloumn and concentrated to a final volume of 20 - 100ul. The samples were quantified with a labelled ^{13}C PCDD/F standard mixture. The measurements were conducted using a GC/MS (HP5870-ULTRA I;25m/HP5970).

Experiments with horseradish peroxidase

The experiments were performed with 2.0U/ml, 0.2U/ml, 0.02U/ml and 0.005U/ml (U=Pyrogallol Units) peroxidase. The assays were buffered with 0.01 molar 2,2 dimethylsuccinate at pH 4.0.
After the reaction the samples (250ml) were alkalinized (three pellets NaOH) and shaken three times with 80ml n-hexan/dichloromethane (80/20vol.%). The extract was filtered through sodium sulphate and concentrated with a rotary evaporator to a volume of 1 - to 2ml.

Experiments with culture filtrate of Phanerochaete chrysosporium

The culture was cultivated using the methods of *KIRK ET AL.(1978)* and *ZITZELSBERGER(1987)*. The cultures were buffered at pH 4 with 0.01 molar 2,2 dimethylsuccinate. The peroxidase activity of the culture solution was determined using a modified enzyme test *(GALEAZZI ET AL.;1986;1989)* with chloropromazinehydrochloride as the electron donor. The test was calibrated with horseradish peroxidase.
At the point of highest enzyme activity the cultures were harvested and combined. The mycel was seperated from the culture solution with a steel net. For experiments only the filtrate was used.
The chemical analysis followed the procedure above with one modification: The addition of 20ml sodium chloride during the extraction.

Experiments with whey
Fresh milk was precipated with concentrated hydrochloric acid at ph 4.5. After centrifuging the supernatant liquid was filtered with a folded filter. The pH was set to pH 5.5 with 15% sodium hydroxide solution. Three aqueous dilutions were used (80, 20 and 4 vol.%) for the experiments. The peroxidase activity was determined with a test using guaiacol(Pütter;1974). This test was calibrated with horseradish peroxidase.
For extraction saturated sodium oxalate solution (100ml) was added to the whey (250ml). This solution was shaken three times with 300ml ether/n-hexan (66/33vol.%). After that the organic fraction was shaken three times with 100ml of 1% sodium hydroxide solution, filtered through sodium sulphate and concentrated to a volume of 1 - 2ml. The clean-up and measurement was conducted as described above.

Results

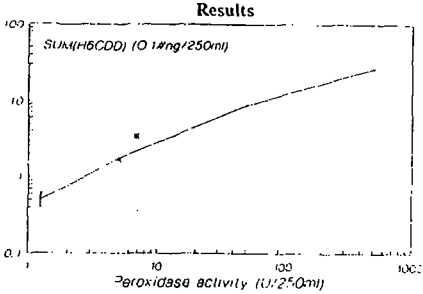

Figure 1: *Correlation between peroxidase activity and formed Dioxins*
POINTSYMBOL: horseradish peroxidase.*STARSYMBOL*: filtrate Phanerochaete chrysosporium.*SCALE*:double logarithic
ABSCISSA: peroxidase activity(U/250ml).*ORDINATE*: (SUM(H6CDD))sum hexachlorinated dioxins(ng/250ml).
[2,4,5 TrCP]: 20ppm.[H_2O_2]: 70ppm(28ppm at 5U/250ml)Volume: 250ml.pH: 4.0.

Figure 1 shows that the amount of hexachlorinated dioxin(H6CDD) formed, correlated with the squareroot of the peroxidase activity. Biogenic samples with culture filtrate of Phanerochaete chrysosporium formed H(6)CDD at the same level as experiments with pure enzyme.

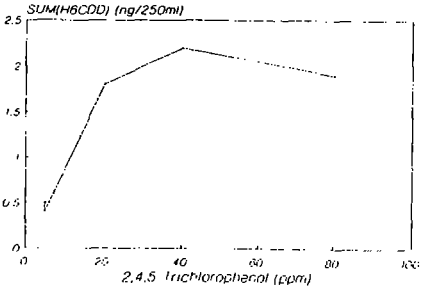

Figure 2: *Correlation between 2,4,5 TrCP concentration and SUM(H6CDD).*
SCALE: linear.*ABSCISSA*: [2,4,5 TrCP](ppm).*ORDINATE*: SUM(H6CDD)(ng/250ml).
Peroxidase activity: 5U/250ml.[H_2O_2]: 70ppm (28ppm at 20ppm 2,4,5 TrCP).Volume: 250ml.pH:4.0.

Figure 2 shows the influence of the concentration of 2,4,5 TrCP on the formation of H6CDD at a peroxidase activity of 5 U/250ml. The formation of H6CDD is limited by the supply of 2,4,5 TrCP up to a concentration of 20 - 40ppm. The reduction of substrate in this experiment was smaller than 20%.

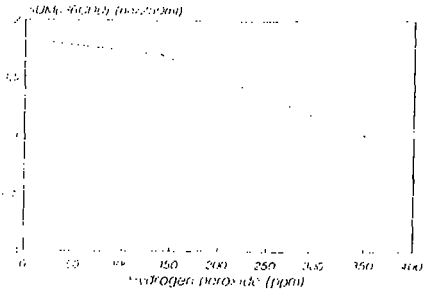

Figure 3: *Correlation between hydrogenperoxide concentration and SUM(H6CDD).*
SCALE: linear *ABSCISSA:* $[H_2O_2]$(ppm).*ORDINATE:* SUM(H6CDD)(ng/250ml).Peroxidase activity: 5U/250ml. [2,4,5 TrCP]:20ppm.Volume: 250ml.pH: 4.0.

Normally the second peroxidase substrate is hydrogen peroxide. The figure shows that the amount of H6CDD formed is independent of the amount of hydrogen peroxide in the concentration range used in this study.

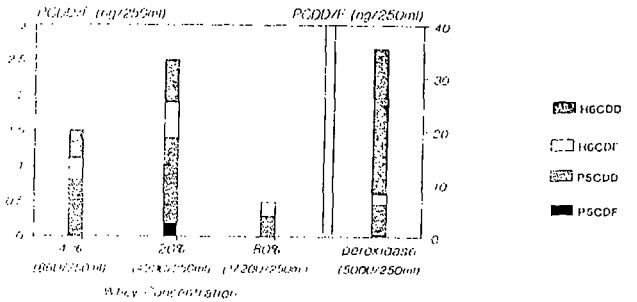

Figure 4: *Relation between whey concentration and formation of PCDD/F, and comparison of the formation of PCDD/F between whey and horseradish peroxidase.*
SCALE: discrete/linear *ABSCISSA:* whey concentration(vol.%),in brackets: peroxidase activity(U/250ml), and activity of horseradish peroxidase(U/250ml).*ORDINATES:* from the basis to the top: concentration pentachlorinated furans and dioxins, and hexachlorinated furans and dioxins(ng/250ml). Volume: 250ml.*HORSERADISH PEROXIDASE:* $[H_2O_2]$: 70ppm.[2,4,5 TrCP]: 20ppm.pH: 4.0.
WHEY: [2,4,5 TrCP]: 40ppm.$[H_2O_2]$: 70ppm.pH: 5.5.

Whey is also a peroxidase containing medium which formed PCDD/F in the presence of 2,4,5 TrCP and hydrogen peroxide, but the yield based on the peroxidase activity was much less than with pure enzyme experiments. Competive reactions were likely responsible for these results: The concentration of 2,4,5 TrCP was held constant while the whey was diluted. The amount of PCDD/F increased from 80% whey to 20% whey.
Also there were great differences with regard to the share between pentachlorinated and hexachlorinated isomers. The experiments at pH 4 showed that H6CDD/F are the predominant PCDD/F formed. However, in whey at a pH of 5.5 dechlorinated penta isomers were predominant. Heptachlorinated or octachlorinated substitution was not found.

Discussion

This paper shows that peroxidase containing biogenic systems are able to combine suitable chlorinated compounds to PCDD/F. On the one hand low levels of enzyme activity*(-FIGURE 1)* were able to dimerize 2,4,5 TrCP, but on the other hand high peroxidase activities*(-FIGURE 4)* did not necessarily produce high amounts of PCDD/F if competive reactions were significant.
In both cases the formation of biogenic dioxins could be explained with peroxidase activity alone.
Peroxidases need hydrogen peroxide as a second substrate. In these experiments we added enough so that it was present in excess. In order for the peroxidase reaction to proceed in nature, the biogenic systems have naturally to produce a sufficient level of hydrogen peroxide.
To estimate the importance of biogenic dioxin sources it is necessary to find relevant peroxidase systems and relevant substrates. It is also important to improve the methods describing the peroxidase activity of non-liquid media.
It was shown that xenobiotics can be degraded by Phanerochaete chrysosporium *(BUMPUS ET AL.; 1985;1989/MILESKI ET AL.;1989)*. Examinations by *BUMPUS ET AL.(1985)* demonstrated the degradation of 2,3,7,8-TCDD in a culture of Phanerochaete chrysosporium. Many examinations accentuate the importance of this fungus. For possible commercial decontamination applications *(MESSNER ET AL.;1988)* the possibility of biosynthesis of toxic compounds should be considered before employing this technique.

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