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In vitro bi-enzymatic synthesis of benzaldehyde from phenylalanine: practical and mechanistic studies

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Abstract

The oxidation of phenylalanine to benzaldehyde was performed in vitro by a bi-enzymatic system D-amino acid oxidase-peroxidase. We describe attempts to optimise experimental conditions and we propose a probable mechanism for the transformation.

Keywords: Phenylalanine; Bi-enzymatic synthesis; Benzaldehyde; D-amino acid oxidase; Peroxidase

1. Introduction

The biotransformation of phenylalanine by cultures of microorganisms or moulds, and by crude extracts of enzymes has previously been shown to produce variable amounts of benzaldehyde. Researchers have found "natural" benzaldehyde production to be possible through these biotransformations [1–3], as an alternative to extraction from vegetable sources. In these studies, benzaldehyde was a product of complex metabolic pathways that were more or less elucidated. Moreover, different enzymes were involved in these biotransformations, which led to different intermediates of reactions. Less complicated systems were also studied. For example, Porter and Bright described an in vitro bi-enzymatic transformation of L-phenylalanine by purified L-amino acid oxidase in the presence of horseradish peroxidase [4].

Since D-amino acid oxidase from *Trigonopsis variabilis* and peroxidase from *Coprinus cinereus* have become available in large quantities and at low cost, we decided to revisit the mechanism of this transformation, using D-phenylalanine. Although we used D-amino acid as a starting material we believe that our results most probably can be transposed to systems based on L-phenylalanine and less available enzymes L-amino acid oxidase or L-aminotransferase [5].

2. Experimental section

2.1. General

GC was performed on an apparatus equipped with a CP.SIL 19 CB column (Chrompack) using nitrogen as a gas vector. The temperature was increased from 150 to 250 °C, at a rate 5 °C/min. Benzophenone was used as an internal standard, added after the extraction step.

^{1}H NMR (200 or 250 MHz) of the organic extract was performed in $CDCl_3$, after cautious evaporation of the solvent. NMR of the aqueous phase was done in D_2O after complete evaporation. Sodium acetate was used as an internal standard.

2.2. Enzymes and other catalysts

Two commercial enzymes were used: immobilised D-amino acid oxidase from *T. variabilis* (*TvDAO*, EC 1.4.3.3, from Fluka; specific activity: 28 U/g), and peroxidase from *C. cinereus* (*CIP*, EC 1.11.1.7, from Novozymes; specific activity: 115 kPODU/ml; one PODU converts 1 μmol of hydrogen peroxide per min in a system where 2,2′-azinobis-(3-

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ethyl-benzothiazoline-6-sulphonate) is oxidised). SDS polyacrylamide gel electrophoresis of *CIP* indicated that it contains a pure protein. However, directly before use *CIP* was dialysed against 0.5 M NaCl to eliminate stabilizing additives, mainly low molecular weight polyethylene glycol.

In certain cases Microperoxidase-11 (MP-11, an haem-containing undecapeptide derived from cytochrome *c*) from Sigma or Hemin (ferriprotoporphyrine [IX] chloride, the prosthetic group of peroxidase and MP-11) from Fluka was used in place of peroxidase.

2.3. Oxidation of DL-phenylalanine

An amount of 125 mg (3.5 U) of immobilised *TvDAO* were suspended in 20 ml of a buffered (0.15 M pyrophosphate buffer pH = 8.3) aqueous solution of DL-phenylalanine (1 mmol). An amount of 1 mmol of additive [or 200 kU of catalase (specific activity 70 kU/ml, from Fluka)] and 0.5 ml of *CIP* (57.5 kU) were added. The mixture was stirred in an orbital shaker (300 rpm) at RT. All experiments reported in Table 1 were done under an atmosphere of pure oxygen. The main advantage of this system is that a closed vessel (equipped with a balloon) is used, avoiding any loss of volatile product. However, we observed no difference in yield or rate of production of benzaldehyde when air was used. The reaction medium was then extracted with ethyl acetate, and the organic phase was analyzed by GC and NMR.

2.4. Oxidation of phenylpyruvate

Sodium phenylpyruvate (0.5 mmol) was dissolved in 20 ml of 0.15 M pyrophosphate buffer pH = 8.3, with 1 mmol of additive and 0.5 ml (57.5 kU) of *CIP* (or the corresponding molar amount of Hemin or MP-11). The mixture was stirred in an orbital shaker (300 rpm) at RT, under air or oxygen during 24 h. The choice of gas did not affect the conversion. The reaction medium was then extracted with ethyl acetate, and the organic phase was analyzed by GC and NMR.

3. Results and discussion

For our investigations we chose immobilised D-amino acid oxidase from *T. variabilis* and peroxidase from *C. cinereus*, because both are industrial enzymes with excellent thermostability and are available in large quantities. Recently D-amino acid oxidase from *T. variabilis* was investigated as a catalyst for industrial applications [6–8].

When we treated a solution of D-phenylalanine with these two enzymes, benzaldehyde was formed but with a conversion of less than 20%. Thus, we decided to find out the conditions for an optimal production. The results of this first step of our work are reported in the below section.

3.1. Practical studies

D-Amino acid oxidase catalyses at the expense of O_2 an oxidative deamination of D-phenylalanine to the ammonium salt of the corresponding α-keto acid, phenylpyruvate. Consequently, we reasoned that phenylpyruvate should be the actual substrate of *CIP*, and we first investigated its conversion to benzaldehyde. This transformation occurs at the expense of molecular oxygen, and we checked that added hydrogen peroxide had no influence on its course.

It has already been mentioned few times in the literature that $MnCl_2$ alone can promote the oxidation of phenylpyruvate by oxygen (Table 1, Entry 3), probably by a mechanism different to that used by a peroxidase [3,9,10]. The presence of a catalytic amount of manganese salts can also accelerate the tautomeric keto-enol equilibrium of phenylpyruvate, thus facilitating its oxidation [3]. With the combination of manganese salts and *CIP*, clearly acting in a synergetic way (Table 1, Entry 5), benzaldehyde was obtained with yields of up to 50%. Hemin (dispersed on Hyflo Super Cel®) and

Table 1
Oxidation of phenylalanine or phenylpyruvate into benzaldehyde catalysed by various haem-containing catalysts

Entry	Substrates	Catalysts	Additives	Yield of benzaldehyde[a]
1	Phenylpyruvate	–	–	1.6
2	Phenylpyruvate	*CIP*	–	5.5
3	Phenylpyruvate	–	$MnCl_2$	3.5
4	Phenylpyruvate	*CIP*	Ascorbic acid	<0.1
5	Phenylpyruvate	*CIP*	$MnCl_2$	50
6	Phenylpyruvate	Hemin[b]	$MnCl_2$	21
7	Phenylpyruvate	MP-11	$MnCl_2$	12
8	DL-Phe	*TvDAO*	–	<0.1
9	DL-Phe	*TvDAO* + *CIP*	Thioanisole, $MnCl_2$	35
10	DL-Phe	*TvDAO* + *CIP*	Catalase (200 kU), $MnCl_2$	38
11	DL-Phe	*TvDAO* + *CIP*	Thioanisole	29
12	DL-Phe	*TvDAO* + *CIP*	–	20

[a] Relative to D-phenylalanine; estimated by GC, with benzophenone as an internal standard.
[b] Hemin due to its low solubility in water was used on mineral support (Hyflo Super Cel®) after immobilisation in ratio 3.25 mg of Hemin to 1 g of Hyflo Super Cel® [12].

MP-11, used instead of *CIP*, also permitted some conversion although less efficiently (Table 1, Entries 6 and 7).

Next we studied the overall transformation of phenylalanine to benzaldehyde. It was rapidly recognised that DL-phenylalanine (either from commercial sources or chemically synthesised from L-phenylalanine by racemisation [11]) could be used as a substrate.

Previous reports on the enzymatic synthesis of α-keto acids from amino acids using D-amino acid oxidase indicate that the formed hydrogen peroxide has a deleterious effect on the enzyme [13–16]. Moreover, the formed phenylpyruvic acid has also been shown to be sensitive to H_2O_2; phenylpyruvate can be decarboxylated to phenylacetate in the presence of H_2O_2 [16]. Thus, hydrogen peroxide must be completely eliminated from the medium for the reaction to be completed. For this to occur, a third enzyme (catalase) should be used. Guisan and co-workers have shown that not less than 31 kU of catalase in combination with 15 U of D-amino acid oxidase must be used to give a 100% yield of phenylpyruvate from D-phenylalanine [16]. Following Guisan's observation we used a very large amount of peroxidase (and catalase) in all our experiments. The best conversion during the oxidation of D-phenylalanine to benzaldehyde was obtained when 200 kU of catalase was present (Table 1, Entry 10). Alternatively, in order to remove H_2O_2, a co-substrate oxidisable by peroxidase could be added to the reaction medium. Our best co-substrate was thioanisole (Table 1, Entry 9), which in this process was oxidised by *CIP* to the corresponding sulphoxide [15]. Other potential oxidisable co-substrates such as tyrosine or iodide ions did not increase the yield significantly (data not shown).

In summary, under optimal conditions (thioanisole + $MnCl_2$ or catalase + $MnCl_2$ added), yields of benzaldehyde reached 35–38% relatively to D-phenylalanine.

3.2. Mechanistic studies

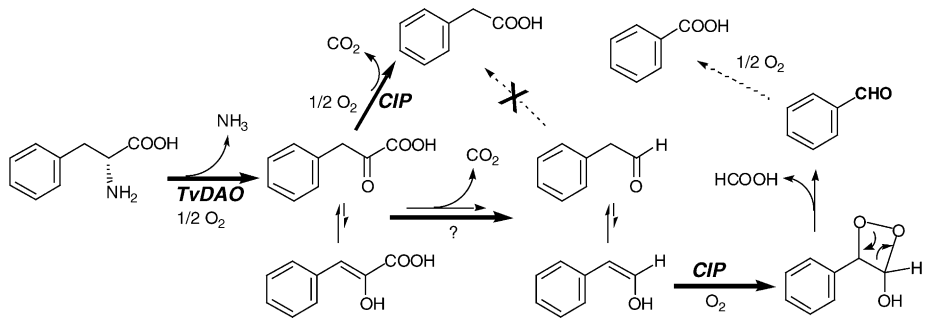
The first goal of mechanistic studies was to solve why the yield of benzaldehyde is so low (50% from phenylpyruvate, 38% from D-phenylalanine). An extraction of the reaction medium at pH 8.3 with ethyl acetate gave only pure benzaldehyde, as shown by GC and NMR. A second extraction from the acidified aqueous solution showed in GC a significant peak of benzoic acid, and large amount of a compound identified as phenylacetic acid. When phenylpyruvic acid was used as a substrate of *CIP*, it was completely oxidised after 24 h under pure oxygen, giving 50% benzaldehyde, 34% phenylacetic acid, and 16% benzoic acid. We have not observed any other by-products by GC or NMR.

Similarly, starting from D-phenylalanine as a substrate of the bi-enzymatic system, the same proportions of products were obtained. However, 18% of phenylalanine still remained after 24 h, as a result of inactivation of *TvDAO*, probably by reactive intermediate species produced by oxidation of phenylpyruvate.

We considered a priori several possible mechanisms for this transformation. Clearly, the pathway of the described bi-enzymatic transformation of D-phenylalanine to benzaldehyde through phenylpyruvate is different from that determined for the metabolic conversion of L-phenylalanine in vivo by moulds and bacteria or by bacterial cell free extracts [1–3].

The possibility of a benzylic oxidation of phenylpyruvate catalysed by *CIP* [17] was ruled out by testing phenylserine as a potential substrate of the bi-enzymatic system. This compound was not transformed to benzaldehyde. Phenylacetic acid was also ineffective as a precursor of benzaldehyde. Although this compound was found in our reaction media, it was not an intermediate but rather a by-product of the transformation. We found in the literature few results regarding the transformation of phenylpyruvate by vegetable systems. Conn and Seki first shortly reported an oxidative conversion to benzaldehyde by mitochondrial preparations [18]. These authors mentioned that CO_2 is the sole by-product. Peroxidase and manganese ions were described for the first time in this paper as effective catalysts. In another study conducted later with partly purified sweet clover peroxidase, the conversion of phenylpyruvate to benzaldehyde was ruled out, but the final products of oxidation were not identified [19]. Villablanca and Cilento reported the horseradish peroxidase catalysed conversion of phenylpyruvic acid to oxalic acid and an "excited form of benzaldehyde". Consequently, they proposed a one step mechanism for the oxidative cleavage of phenylpyruvic acid [9].

Our own attempts to characterise the formation of oxalic acid during this process were unsuccessful. The colorimetric method we used for that purpose also excluded the possibility of glyoxilic acid formation [20]. Finally, using another specific colorimetric method [21], we proved the formation of formic acid in the reaction medium (one molar equivalent relatively to benzaldehyde). Thus, our results are in discrepancy with those of Villablanca and Cilento [9]. When the reaction was carried out under argon in a carefully deoxygenated medium, we isolated tiny traces of phenylacetaldehyde, which we unambiguously characterised by GC and MS. Since independently to the above-mentioned studies, a peroxidase catalysed conversion of phenylacetaldehyde to benzaldehyde and formic acid had been demonstrated [22], we investigated this reaction on commercial phenylacetaldehyde under our conditions. Indeed, we found that this compound was efficiently transformed to benzaldehyde by *CIP*. Some benzoic acid was also formed, but no trace of phenylacetic acid could be found. Thus, if benzoic acid probably comes from the over-oxidation of benzaldehyde, phenylacetic acid is not a product of oxidation of phenylacetaldehyde, but comes directly from phenylpyruvic acid. The known oxidative decarboxylation of phenylpyruvate caused by H_2O_2 must be eliminated, because hydrogen peroxide was absent in our conditions. Thus, the formation of phenylacetic acid from phenylpyruvic acid is probably catalysed by *CIP* at the expense of oxygen, through a similar mechanism to that described by Chiou and Que [23] for α-keto acid decarboxylation that occurs in the presence of molecular oxygen and a Fe^{2+}/tripodal ligand catalyst.



Scheme 1. Proposed pathway of the transformation of D-phenylalanine to benzaldehyde via phenylpyruvic acid and phenylacetaldehyde, catalysed by the bi-enzymatic system *TvDAO*/*CIP*. Thin arrows: uncatalysed or auto-catalytic steps; thick arrows: enzyme-catalysed steps; dotted arrows: formation of by-products.

When the oxidative transformation of either phenylpyruvate or phenylacetaldehyde was conducted in deuterated water, up to 50% of the formed benzaldehyde contained one deuterium atom in the formyl group, as determined by GC-MS. This confirms an easy equilibration of the two substrates with their respective enol forms. In the presence of ascorbic acid, the conversion of phenylpyruvate was completely inhibited, what strongly indicates the formation of radical intermediates (Table 1, Entry 4). No inhibition was observed in the presence of mannitol or superoxide dismutase, which rules out the implication of superoxide ion or hydroxyl radical [24] presence during the reaction. The successful replacement of *CIP* with either Hemin or MP-11 (Table 1, Entries 6 and 7) indicates that the reaction takes place on the metalloporphyrin centre, regardless of the role of the apoprotein. These results led us to propose the following mechanistic pathway: a non-oxidative decarboxylation of phenylpyruvate (or of its enol form) into phenylacetaldehyde, and then an oxidative deformylation of the enol form of phenylacetaldehyde leading to benzaldehyde and formic acid, most probably through a dioxetane ring (Scheme 1).

However, the mechanism of decarboxylation of phenylpyruvate is still unclear. When present, *CIP* might play a role as a protein capable to form reversibly an imine by reaction with the keto group, thus catalysing decarboxylation [25]. This hypothesis of course cannot be refereed to when phenylpyruvate is oxidised by isolated Hemin.

4. Conclusions

We have studied a novel bi-enzymatic transformation of D-phenylalanine into benzaldehyde. Porter and Bright [4] closely studied a related system of L-phenylalanine transformation to benzaldehyde by a mixture of L-amino acid oxidase and horseradish peroxidase. They concluded that α-aminocinnamate, a tautomeric form of the imino acid produced by amino acid oxidase, was the actual substrate of peroxidase.

Our studies suggest, rather, that phenylpyruvic acid is the substrate of *CIP*. We successfully used phenylpyruvate and phenylacetaldehyde as substrates for *CIP*-catalysed formation of benzaldehyde. Moreover, we found that oxidation of either phenylpyruvate by *CIP* or phenylalanine by our *TvDAO*/*CIP* bi-enzymatic system led to the same compounds in the same proportions. Consequently, we propose a new pathway for the conversion of phenylalanine to benzaldehyde via phenylpyruvic acid and phenylacetaldehyde.

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