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Co-oxidation of β-carotene in biphasic media

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Abstract

Co-oxidation of β-carotene is used to originate aroma compounds of the ionone family. This system involves xanthine oxidase-generated free radicals to cleave the carotenoid. However, free radicals also degrade the reaction products decreasing yields. In order to avoid this degradation, we investigated in this study the possibility of carrying out co-oxidation in biphasic media containing hexane, benzene or dichloromethane. Hexane and benzene enabled a good enzymatic activity, whereas, for dichloromethane, no co-oxidation occurred. β-Carotene's bleaching was observed only when carotene was solubilized in the aqueous phase suggesting that free radicals did not enter the organic phase. With hexane and benzene, time courses of product accumulation were different from those obtained in aqueous media as degradation did not occur resulting in conversion yields about 11 times higher. These results show that it is possible to carry out β-carotene in biphasic systems, which enable to extract β-ionone and related molecules before further degradation by free radicals.

Keywords: β-Carotene; Xanthine oxidase; Biphasic media

1. Introduction

Ionones and related molecules are involved in the aroma of many fruits and vegetables where they are probably occurring from carotenoids degradation [1]. The flavor industry is interested by natural processes enabling production of these molecules. We described in previous papers [2,3] a system involving xanthine oxidase generated free radicals degrading β-carotene and giving rise to β-ionone, 5,6-epoxy-β-ionone and dihydroactinidiolide (Fig. 1). This system was interesting as it enabled us to investigate the involvement of free radicals in the degradation and the pathway to dihydroactinidiolide, an important compound in black tea aroma. However, the production yields of these compounds were low as they were oxidized and further degraded by free radicals. Degradation occurring on the products has also been observed by Mordi et al. [4,5] with β-cyclocitral, which should be a major reaction product [6] but was detected only in small amounts [5].

This bioconversion involves a hydrophobic substrate which is converted to hydrophobic products, whereas, the enzyme is active in the aqueous phase. Such a problem is common in organic synthesis and can be resolved by the use of biphasic media [7,8]. The organic solvents used have to be carefully chosen in order to avoid new problems like enzyme inhibition [9,10], or weak extraction of the products. In the co-oxidation reaction, the system is complicated by the fact that two reactions take

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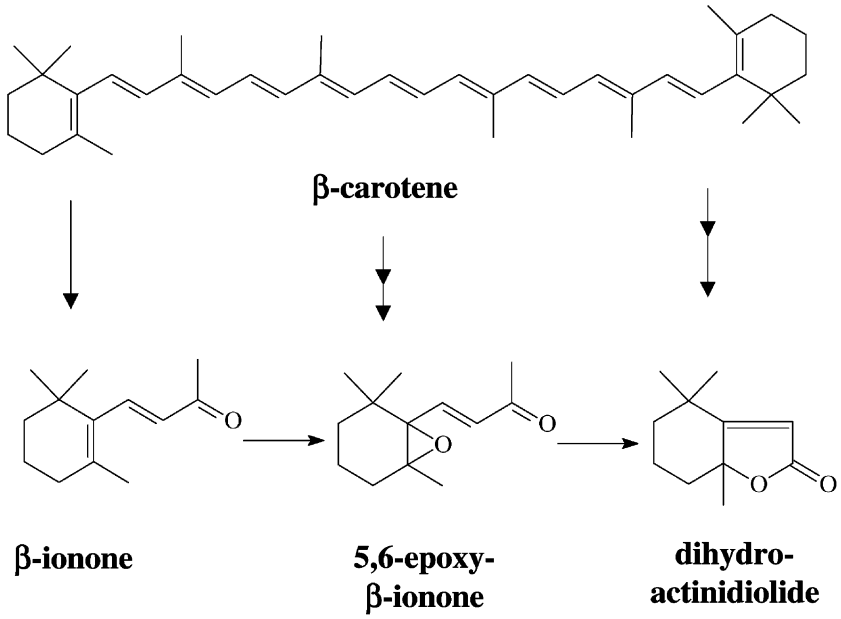

Fig. 1. Structure of β-carotene and of its co-oxidation products: β-ionone, which can be degraded to 5,6-epoxy-β-ionone, itself giving rise to dihydroactinidiolide.

place, the radical-mediated cleavage of β-carotene to aroma compounds and the xanthine oxidase catalyzed oxidation of acetaldehyde generating radical species. The partitioning of the reaction has to be carefully driven to favor the contact between enzyme and substrate (in an environment enabling activity) and between radicals and β-carotene and to avoid any interaction between radical species and aroma compounds.

The goal of our study was to investigate whether the use of biphasic media was appropriate for this co-oxidation reaction. We studied the degradation of β-carotene in various biphasic media in order both to solubilize β-carotene and to extract reaction products from the action of free radicals. Organic phases were more or less efficient to solubilize β-carotene but protected this substrate from the free radicals attack. Pseudosolubilization in micelles included in the aqueous phase was thus a requisite for β-carotene degradation. However, solvents were useful for products extraction and concentrations with 10% hexane or benzene were about 11 times higher than in the simple aqueous system.

2. Materials and methods

2.1. Reactions

Reactions were carried out as previously described [2] at 37 °C in a 2-l enzymatic reactor with a 250 rpm stirring. The reaction volume was 300 ml. The aqueous phase was composed of 27×10^{-3} U/ml xanthine oxidase (grade III from buttermilk), acetaldehyde, 48 mM, phosphate buffer, 50 mM, pH 8.0. For experiments in the presence of solvents, 10% (v/v) organic solvent (acetone, hexane, benzene or dichloromethane) was added.

β-Carotene was solubilized either in the aqueous phase with Tween 80 according to Ben Aziz et al. [11] as previously described [2] or in the organic phase. Initial β-carotene concentration was comprised between 10 and 90 mg/l in the reactor volume.

2.2. Analyses

β-Carotene was quantified throughout the reaction with a Merck HPLC system with a Merck Lichrocart

250-4, Lichrospher 100RP18, 5 μm column. Solvents were acetonitrile (90–35%), chloroform (10–45%) and acetic acid (10%) in THF (10%). The flow rate was 0.8 ml/min, the injected volume, 10 ml and the detection was carried out with a photodiode Merck L-3000 in the absorbance mode at $\lambda = 450$ nm, as detailed previously [2]. In aqueous phase experiments, samples (20 ml) were harvested at six different times, methyl isoeugenol was added as internal standard and samples were extracted with CH_2Cl_2 and concentrated under N_2 to a final volume of 3 ml. For biphasic experiments, 1 ml of the organic solvent was removed from the reactor and after addition of the internal standard, used without concentration for analysis. Analyses were carried out in a Varian 3400 gas chromatograph [2].

3. Results and discussion

3.1. Production of β-ionone from solutions with different initial concentration of β-carotene

To investigate whether yields of bioconversion were related to the substrate concentration, we first carried out reactions with different initial concentrations of β-carotene. Degradation of β-carotene (Fig. 2A) and accumulation of β-ionone (Fig. 2B) were investigated in aqueous systems containing 10, 45 or 90 mg/l initial concentration of β-carotene. In the three cases, more than half of the initial β-carotene was degraded in the first 2 h and then, the rate of degradation decreased (Fig. 2A). For 10 mg/l, β-carotene was even undetectable after 3 h whereas, for 45 and 90 mg/l, about 7 mg/l remained after 24 h.

Concentrations of β-ionone increased for 3 or 4 h and then decreased (Fig. 2B). These concentrations were in the same order of magnitude for the extreme initial concentrations (10 and 90 mg/l) yielding 250 μg/l. However, the medium β-carotene concentration investigated (45 mg/l) was yielding more β-ionone (450 μg/l). This optimal substrate concentration can be explained by the light inhibition of xanthine oxidase by β-carotene micelles at higher concentrations [12]. There might also be an optimal free radicals/β-carotene ratio as β-carotene can be an efficient radical scavenger [13,14] and, with high amounts of β-carotene, the free radical generation system would not be efficient enough.

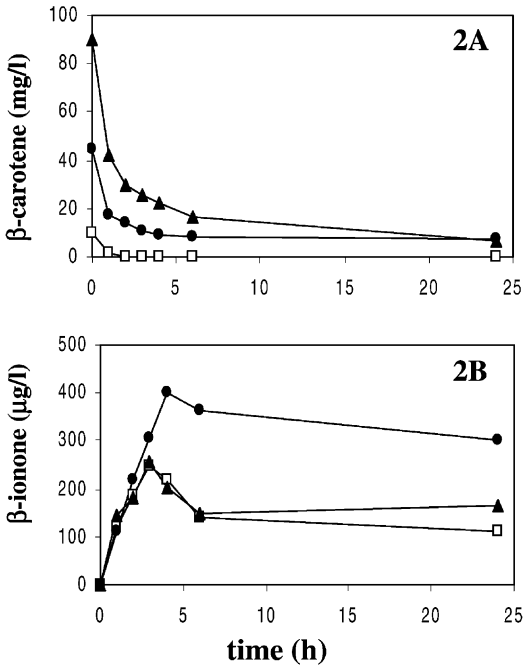

Fig. 2. Degradation of β-carotene in co-oxidation in aqueous phase containing various initial concentrations of β-carotene. (A) Concentration of β-carotene in mg/l; (B) concentration of β-ionone in μg/l. Initial concentrations are: (▲) 90 mg/l, (●) 45 mg/l and (□) 10 mg/l.

The accumulation of β-ionone results from its production and concomitant degradation. It is therefore difficult to analyze degradation separately. However, the decrease in the β-ionone concentration occurred only until 6 h (Fig. 2B), and after that time, β-carotene was only slightly degraded (Fig. 2A). The degradation of β-ionone seems thus to occur mainly at the beginning of the reaction when the radical-generating system is more sufficient [2].

In order to decrease this product degradation, we modified the system to extract and protect β-ionone and related compounds all along the reaction in an organic phase.

3.2. Degradation of β-carotene in presence of solvents

From the four solvents tested, β-carotene degradation occurred only in the presence of hexane or

benzene. More polar solvents like dichloromethane or acetone inhibited the enzyme. In this latter case, the reaction occurred in a one-phase system due to the good miscibility of acetone in the aqueous phase. This reaction had been carried out based on results presented by Fridovich [15] who observed an increased xanthine oxidase activity in the presence of water miscible solvents. Our results suggest on the contrary that less polar solvents are more appropriate. Chaplin et al. [7] have already observed a better conversion in more hydrophobic solvents for amine oxidase. Two main reasons can explain this: first, less polar solvents can better extract reaction products decreasing thus enzyme inhibition but also organic solvents with high log *P* are known to be less harmful to biocatalysts in biphasic systems [16] especially in low water activity environments [17]. For xanthine oxidase, data concerning the activity in biphasic media are lacking. This enzyme is usually studied or utilized in aqueous systems although in its original medium, milk, interfaces play a major role. It would, thus, be of interest to study the activity of xanthine oxidase in the presence of various solvents with different log *P*.

In the presence of hexane or benzene, degradation required the "pseudosolubilization" of carotene in Tween 80 micelles in the water core. When solubilized in the hexane or benzene phase, no bleaching occurred suggesting that radicals were not entering the organic phase or were inactive in this phase or at the interface. It was also possible that the turnover of carotene at the interface was not sufficient to observe a detectable bleaching. We can notice, however that, although the amount used should have been soluble, β-carotene was not completely solubilized in hexane and particles were still visible in this phase. However, this observation alone cannot explain the lack of bleaching in the organic phase.

The enzyme was active as confirmed by the bleaching monitored when β-carotene was in micelles in the aqueous phase (Fig. 3A). The bleaching of β-carotene is shown in Fig. 3A with (co-oxidation) or without (autoxidation) enzyme in aqueous phase or with 10% hexane. Surprisingly, hexane had almost no effect on the degradation of β-carotene especially in co-oxidation systems for which curves were very similar: in both cases, autoxidation happened slowly all along the reaction, whereas, with xanthine oxidase, the reaction occurred rapidly, degrading two-third of

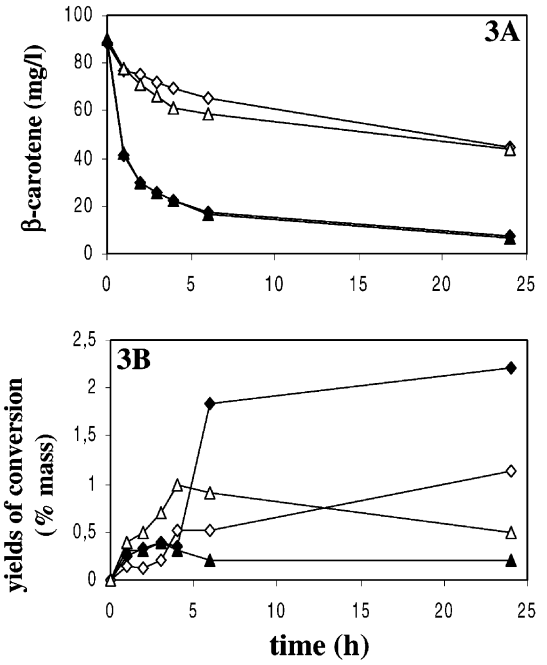

Fig. 3. (A) Degradation of β-carotene in the presence of 10% hexane: (◇) autoxidation (without xanthine oxidase) and (◆) co-oxidation (with the enzyme) or in the aqueous system: (△) autoxidation and (▲) co-oxidation. (B) Yields of conversion of β-carotene to β-ionone ((◆): with 10% hexane, (▲): in the aqueous system) or 5,6-epoxy-β-ionone ((◇): with 10% hexane, (△): in the aqueous system) in mass%.

the β-carotene in the first 2 h. The slight difference between conditions with or without hexane could be explained by the small amount of carotene from Tween micelles reaching the organic phase. Only conditions with 10% solvent were tested as with higher amounts, interactions between micelles and the organic phase were likely to increase favoring the leakage of β-carotene from the aqueous phase to the solvent.

3.3. Accumulation of volatiles during co-oxidation

Accumulation of β-ionone and 5,6-epoxy-β-ionone are shown in Fig. 3B. Yields of conversion to β-ionone in the presence of hexane were very similar to results obtained without the solvent during the first 4 h but, afterwards, β-ionone accumulated in the hexane droplets of the biphasic medium whereas it was degraded in the monophasic aqueous medium. After 24 h, the difference in the yield of conversion

was significant: 2.2% with hexane and 0.2% without. For 5,6-epoxy-β-ionone, results were very different: its concentration was higher than β-ionone in aqueous systems and far lower with hexane. As 5,6-epoxy-β-ionone can be a product of oxidation of β-ionone, we can hypothesize that β-ionone was less modified by the oxygen derived radical species because it was protected in solvent droplets. The oxygen itself, which is important in autoxidation, could be less present in the organic phase than in the aqueous phase, resulting in a higher stability of β-ionone in the solvent. However, the epoxy eventually accumulated also in the solvent droplets even if it reached smaller amounts than β-ionone.

4. Conclusion

The action of free radicals on β-carotene leads to its degradation yielding small molecules like β-ionone or related species. However, in aqueous systems, concentrations of volatiles remain very small, independent of the initial concentration of β-carotene. These products appear to be degraded by the radical species. To avoid this degradation, it was possible to extract β-ionone in an organic phase as long as this phase did not inhibit the enzyme. This was possible with hexane where β-ionone and related molecules accumulated all along the reaction whereas β-carotene, solubilized in Tween micelles, was remaining and was degraded in the aqueous phase. These results have been obtained in the model system with low concentration of β-carotene and low yields of conversion but they show that biphasic media can also be used in biocatalysis involving free radicals. The next step currently investigated in the laboratory consists of investigating the relationship between the activity and the hydrophobicity of the solvent and in optimizing the ratio of solvent.

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