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Biochemie/Biochemistry

Biocatalytic hydroxylation of linoleic acid in a double-fed batch system with lipoxygenase and cysteine

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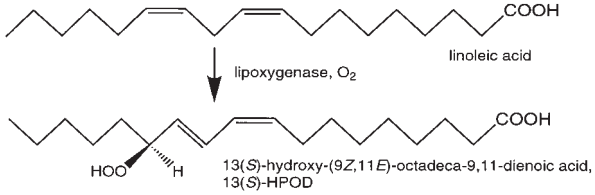
The enzymatic large-scale preparation of unsaturated fatty acid hydroperoxides is the first step in the preparation of the corresponding fatty acid hydroxides. Since hydroxides are more suitable than hydroperoxides as precursors of fine chemicals like certain flavour compounds, a convenient and effective reduction step of hydroperoxides is required. In this paper a double-fed batch procedure for the enzymatic preparation of fatty acid hydroperoxides including the application of cysteine as a mild reducing agent is described. A crude extract from defatted soybean flour was used as the source of lipoxygenase, while the primary substrate was linoleic acid from hydrolyzed safflower oil. Enzyme and substrate were fed synchronously into the bioreactor that already contained buffer solution and cysteine. Typically, reactions with 40 g/l substrate in a 7 l reactor were done on a 2 l scale. Without any intermediate work-up, a final product yield of 90% could be achieved consisting for nearly 96% of 13(*S*)-hydroxy-(9*Z*,11*E*)-octadeca-9,11-dienoic acid.

Biokatalytische Hydroxylierung von Linolsäure in einem doppelt gespeisten Chargensystem mit Lipoxygenase und Cystein. Die enzymatische Aufarbeitung von ungesättigten Fettsäure-Hydroperoxiden im größeren Maßstab ist der erste Schritt in der Präparation der entsprechenden Fettsäure-Hydroxide. Da Hydroxide als Vorstufen hochwertiger Chemikalien, wie z. B. bestimmte Aromastoffe, besser geeignet sind als Hydroperoxide, ist ein bequemer und effizienter Reduktionsschritt notwendig.
In dieser Arbeit wird ein doppelt gespeistes Chargenverfahren zur enzymatischen Herstellung von Fettsäure-Peroxiden, welches den Einsatz von Cystein als milde reduzierende Komponente berücksichtigt, beschrieben. Ein Rohextrakt aus entfettetem Sojabohnenmehl wurde als Quelle für die Lipoxygenase verwendet, wobei das erste Substrat Linolsäure aus hydrolysiertem Safloröl stammt. Enzym und Substrat wurden gemeinsam in einen Bioreaktor gegeben, der bereits Pufferlösung und Cystein enthält. Gewöhnlicherweise wurden die Reaktionen in einer Größenordnung von 2 l und einer Substratkonzentration von 40 g/l in einem 7 l Reaktor durchgeführt. Ohne jede zwischenzeitliche Aufarbeitung konnte eine Endproduktausbeute von 90% erzielt werden, die nahezu aus 96% 13(*S*)-Hydroxy-(9*Z*,11*E*)-octadeca-9,11-diensäure bestand.

1 Introduction

Lipoxygenases (LOX) (EC 1.13.11.12) catalyze the dioxygenation of fatty acids that contain one or more (1*Z*, 4*Z*)-pentadiene systems, yielding chiral, *E,Z*-conjugated hydroperoxy fatty acids [1], eq. (1).



(1)

These enzymes are widely distributed in nature and occur in both plants and animals. From soybeans, lipoxygenase can relatively easily be obtained in large amounts. It produces almost exclusively 13(*S*)-hydroxy-(9*Z*,11*E*)-octadeca-9,11-dienoic acid (13(*S*)-HPOD) from its natural substrate linoleic acid (9*Z*,12*Z*)-octadeca-9,12-dienoic acid) at pH values between 9 and 11 [2]. Reduction of this hydroperoxide leads to the corresponding hydroxy fatty acid, which is interesting as a precursor for certain flavour compounds [3]. Reduction can easily be done with sodium borohydride,

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hydrazine or triphenylphosphine, but these chemicals are less suitable in a biocatalytic process. Other options to reduce hydroperoxides include the combined actions of glutathione and glutathione peroxidase [4]. Although elegant and effective, this method is economically less attractive. We therefore investigated the possibility of using the mild reducing agent cysteine. In previous work, *Elshof* et al. [5] described optimized reaction conditions for a large-scale production of 13(*S*)-HPOD from hydrolyzed safflower oil with a crude soybean-flour extract as a source of lipoxygenase. In this study, the application of cysteine in a double-fed batch of substrate and enzyme is described.

2 Materials and Methods

Incubations on 21 scale were performed in a 71 fermenter (*Applikon Dependable Instruments*, Schiedam, The Netherlands) using a concentration of 40 g/l linoleic acid from hydrolyzed safflower oil (*Unilever* Research Laboratory, Vlaardingen). This hydrolyzed oil consisted for 76% of free linoleic acid and will be further referred to as substrate A. A crude extract of defatted soybean flour (*Unilever* Research Laboratory, Vlaardingen) was used as the source of lipoxygenase. The extract was prepared by stirring the defatted soybean flour for 2 h at 4° C in a 0.1 M sodium acetate buffer (pH 4.5, 1/10 w/v), followed by centrifuging and filtering over cheesecloth. The activity was determined by measuring the change of the absorbance at 234 nm (eq. (1)). One unit (U) of enzyme activity is defined as the formation of HPOD at a rate of 1 μmol/min. In preparative reactions, the final enzyme concentration in the reactor was 2230 U/l. This corresponded to approximately 250 to 300 ml of extract. The incubation mixture further consisted of 0.1 M borate buffer (pH 10.0, total volume of 2 l). The pH was measured continuously during the experiment with a gel-filled *Ingold* pH electrode, and adjusted by allowing the addition of 3 M NaOH. The oxygen concentration was measured with an *Ingold* dissolved-oxygen probe, and kept as high as possible by bubbling air through the solution and agitating the mixture at variable speeds between 400 and 1250 rpm. The bubbling of air through the solution caused foam formation that could be kept within reasonable limits by applying some pressure in the reactor (upper limit 0.5 bar). An advantageous side effect here was an enhanced oxygen transfer. Activity measurements and RP-HPLC analysis were performed as described by *Elshof* et al. [5].

2.1 Varying the mode of substrate addition

In studying the effect of various modes of adding substrate A, the mode of enzyme addition was the same in all cases. It was done at a rate of 2 ml/min, which corresponded to approximately 30 U/min. The following variations were tried:

- Addition of substrate A at a pump speed of 2 ml/min, which corresponded to an amount of substrate A of 4.85 mmol/min or 1.36 g/min. It took 59 min to feed in all the substrate A, while the total amount of enzyme was added in 150 min.
- Addition of substrate A at a pump speed of 1 ml/min, which corresponded to an amount of substrate A of 2.43 mmol/min or 0.68 g/min. It took 118 min to completely feed in all the substrate A, while the total amount of enzyme was added in 144 min.
- Addition of all substrate A, batchwise, followed by adding the enzyme in 136 min. During these reactions, samples of a few ml were taken at regular time intervals and diluted in MeOH to stop the reaction. Subsequently, the conversion was determined by measuring the absorbance at 234 nm. Product analysis was done with HPLC as described by *Elshof* et al. [5].

2.2 Varying the mode of enzyme addition

In studying the effect of various modes of adding enzyme, the mode of substrate A addition was the same in all cases. It was done during 59 min at a rate of 2 ml/min, which corresponded to 4.85 mmol/min or 1.36 g/min. The following variations were tried:

- Adding enzyme at a pump speed of 2 ml/min, corresponding to 30 U/min in a total time of 150 min.
- Adding enzyme at a pump speed of 1 ml/min, corresponding to 15 U/min in a total time of 270 min.
- Addition of all enzyme, batchwise, followed by adding the substrate.

2.3 Addition of cysteine to the reaction mixture at the end of the reaction

In this experiment the conversion was determined of 40 g/l substrate A by the enzyme preparation, which was added at a pump speed of 2 ml/min to a total volume of 2 l. After 200 min, a twofold molar amount of cysteine over the starting amount of substrate A was pumped into the fermenter. Cysteine was dissolved in 0.1 M borate buffer at pH 10.0 and pumped into the fermenter at a speed of 4 ml/min to a final volume of 2.5 l. Although cysteine was dissolved in buffer, the pH of the reaction mixture was lowered and addition of 3 M NaOH was necessary to keep the buffer at pH 10.0. In calculating the conversion level, a correction was made for the volume of buffer, wherein cysteine was dissolved.

2.4 Simultaneous addition of substrate and enzyme to a cysteine solution

In these experiments, a molar amount of cysteine 1.5 to 2 times the total amount of substrate A was brought into the reactor before substrate or enzyme were added. Both, the enzyme and substrate A solutions, were then fed in at a speed of 2 ml/min. The presence of cysteine in the reaction medium caused more foam formation, which made the addition of antifoam A necessary. Antifoam A was diluted 1:1 with demineralized water and added with a pump after a reaction time of 30 min (40 ml), and a second amount after 70 min (20 ml). Samples were taken from the reaction mixture and the conversion level and product compositions were determined.

3 Results

3.1 Mode of substrate addition

The effect of varying the mode of substrate addition was investigated while the rate of enzyme addition was kept constant at approximately 30 U/min. In Fig. 1 the results of the conversion of substrate A by lipoxygenase are shown for adding substrate A at a speed of 2.43 and 4.85 mmol/min, respectively. Also the results are shown of the experiment, wherein the total amount of substrate A was already in the fermenter at the beginning of the experiment.

In all cases, the conversion rate was the same in the very beginning of the reactions, due to the excess of substrate A. After about 30 min, the conversion rate began to decline for the batchwise experiment. The availability of the total

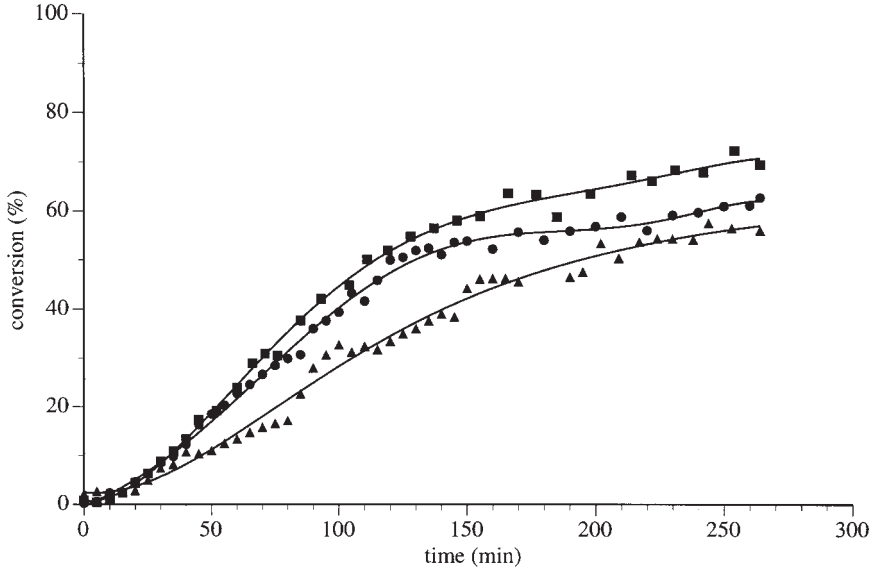

Fig. 1. Addition of substrate at different speeds at a fixed enzyme addition rate of 30 U/min. Substrate A addition: (■) 4.85 mmol/min; (●) 2.43 mmol/min; (▲) batchwise addition.

Tab. 1. Effect of variable substrate A addition on the product profile (% composition*).

substrate A [mmol/min]	13-OXOD	13-HOD	9-HOD	13-*ZE*-HPOD	13-*EE*-HPOD	9-*EZ*-HPOD	9-*EE*-HPOD	yield
2.43	0.4	2.5	0.4	93.2	0.3	2.6	0.6	63
4.85	0.5	1.2	0.2	92.7	0.3	3.9	1.2	71
batchwise	0.5	1.9	0.4	90.9	0.5	4.8	1.0	57

* The figures were truncated to their last significant digit. Values are the mean of three determinations. Errors have been omitted from the table for clarity.

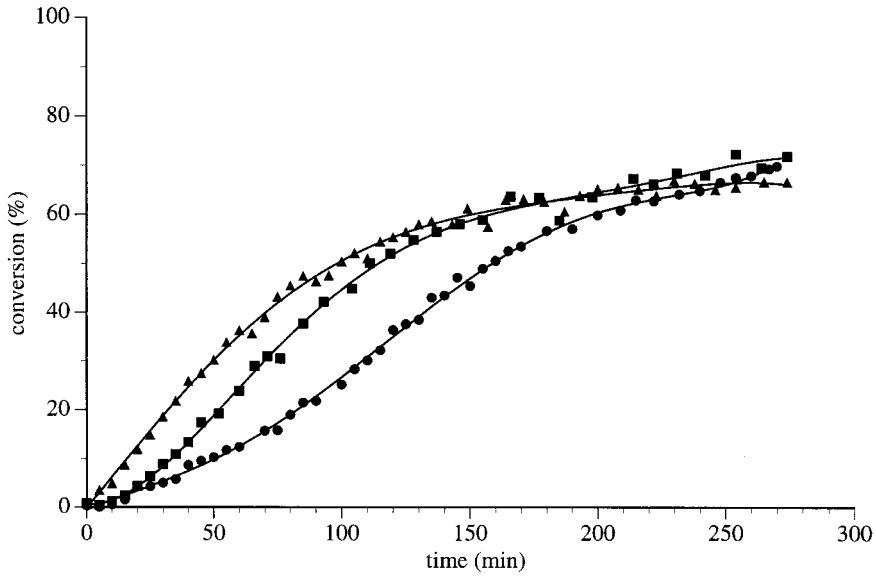

Fig. 2. Addition of enzyme at different speeds with a fixed substrate A addition rate of 4.85 mmol/min. Enzyme addition: (■) 30 U/min; (●) 16 U/min; (▲) batchwise addition.

Tab. 2. Effect of variable enzyme addition on the product profile (% composition*).

enzyme [U/min]	13-OXOD	13-HOD	9-HOD	13-*ZE*-HPOD	13-*EE*-HPOD	9-*EZ*-HPOD	9-*EE*-HPOD	yield
30	0.5	1.2	0.2	93.7	0.3	2.9	1.2	72
16	0.4	0.9	0.2	94.0	0.3	3.2	1.0	70
batchwise	1.3	1.3	0.2	93.6	0.3	2.6	0.7	67

* The figures were truncated to their last significant digit. Values are the mean of three determinations. Errors have been omitted from the table for clarity.

amount of substrate A caused a higher oxygen consumption in the beginning of the reaction compared with the experiments, wherein the substrate A was added with a pump (data not shown). Addition of substrate A with a pump gave higher yields (Tab. 1). In the batchwise addition of substrate A only 57% could be obtained after about 270 min, while adding substrate A at 2.43 mmol/min or 4.85 mmol/min, yielded conversions of 63% or 71%, respectively after 270 min.

3.2 Mode of enzyme addition

The effect of varying the mode of enzyme addition was investigated while the rate of substrate A addition was kept constant at approximately 4.85 mmol/min. Substrate A was added in 60 min. In Fig. 2 the results of the conversion of substrate A with lipoxygenase are shown for adding the enzyme at a rate of either 16 or 30 U/min and for the batchwise addition. In all cases the total amount of enzyme added was 2230 U/l. At 30 U/min the enzyme was added in 150 min, while at 16 U/min this took 270 min.

Obviously, the rate of conversion at the beginning of the reaction was considerably lower when the enzyme was added with a pump compared to the batchwise addition. The difference in the conversion rates between gradual and batchwise addition of the enzyme began to vanish between 55 and 110 min. Maximum conversions were 72% and 70% corresponding to adding the enzyme at 30 and 16 U/min, respectively. The maximum yield with the batchwise addition of enzyme was found to be 67%. The product specificities (Tab. 2) for 13-*ZE*-HPOD were better than 93% in all cases.

3.3 Adding cysteine to the reaction mixture at the end of the reaction

In this experiment the enzyme preparation and substrate A were pumped into the fermenter at a speed of 30 U/min and 4.85 mmol/min, respectively, to a total volume of 2 l. After 200 min, a start was made with the dropwise addition of a 480 ml solution of cysteine in buffer. This solution was added over a 2 h time range in a total amount of twice the total molar amount of the original substrate A. The results of the reactions with and without adding cysteine were compared (Fig. 3).

With addition of cysteine a slightly higher conversion was observed compared to the experiment without cysteine. The maximum yields were 76% and 72% with and without cysteine, respectively. The differences between the two reactions started to appear after 200 min, when the addition of cysteine was started. Probably, the reduction of the hydroperoxides caused a lower product inhibition resulting in a higher yield. The differences were minor because the addition of cysteine was only started when already a reduced enzyme activity was found and the formation of product was already declining.

The effect of cysteine on the formation of regio- and stereoisomers vs. time is shown in Tab. 3. Before addition of cysteine, the product consisted for 92% of 13-*ZE*-HPOD and for 2.4% of 13-HOD and after addition of cysteine for 93.8% of 13-HOD, while only 1.8% 13-*ZE*-HPOD was left.

3.4 Addition of cysteine to the incubation mixture before addition of substrate A and enzyme

In these experiments, cysteine was dissolved in the reaction buffer in the fermenter before substrate A and enzyme were added. During the reaction the presence of cysteine caused considerable foam formation, and addition of antifoam agent during the reaction was necessary. After 30 min reaction time, approximately 40 ml 1:1 (v/v) diluted antifoam-A was pumped into the fermenter during 10 min, and another 20 ml were added after a reaction time of 70 min. The reaction was performed twice: first with the addition of cysteine and substrate A in a molar ratio of 1.5 and then another one with a molar ratio of 2. The results of these reactions are shown in Fig. 4, including the control experiment without cysteine.

The progress curves in Fig. 4 clearly demonstrate the difference between the addition of cysteine at the start of the reaction and the control experiment without cysteine. The effect of cysteine can be explained by the reduction of hydroperoxy fatty acids, which partly prevented the maximum conversion of native lipoxygenase Fe(II) into the active Fe(III) form resulting in a lower initial rate. High concentrations of hydroperoxide have an irreversibly inhibitory effect on the lipoxygenase reaction as described by *Smith* and *Lands* [6], and *Emken* and *Dutton* [7]. Reduction of the hydroperoxide by cysteine thus had a favourable effect on the level of conversion as the reaction proceeded beyond 100 min. Ultimately, the extent of the reaction in the presence of cysteine was almost 90%, and 69% in its absence. The addition of cysteine in a molar ratio of 1.5 with respect to the substrate afforded a product consisting for 83.2% of 13-*ZE*-HOD and for 14.8% of 13-*ZE*-HPOD after a reaction time of 4 h (Tab. 4). By increasing the amount of cysteine to twice the molar amount of substrate A, only 2.8% of the 13-*ZE*-HPOD was left and less byproducts were formed.

4 Concluding Remarks

The influence of a fed-batch system was investigated whereby fresh enzyme and substrate were added with a pump to a borate buffer of pH 10.0. 2 l reactions were performed on a 40 g/l scale using substrate A and a lipoxygenase preparation under conditions as described by *Elshof* et al. [5]. Also the influence was studied of the addition of cysteine as reducing agent during and before the reaction.

1. With gradual addition of substrate A to the reaction mixture, higher conversions were obtained than with a batchwise addition of substrate A. Probably, substrate inhibition accounted for the lower yield. The product specificity was good in all cases, being over 90% for 13-*ZE*-HPOD. It was much easier to get sufficient oxygen into the reaction mixture when the substrate was added gradually.
2. The gradual addition of enzyme showed that higher conversions could be obtained than with a batchwise addition. Hydroperoxides inhibit the activity of lipoxygenase and therefore adding the enzyme gradually afforded higher yields. Also, positive effects were observed in the oxygen concentration, which could better be controlled when the enzyme was added gradually. The product specificity was good, yielding more than 92% of 13-*ZE*-HPOD in all cases.
3. The optimal conditions for the addition of substrate A and enzyme were found to be 4.85 mmol/min and 30 U/min, respectively.
4. The addition of cysteine after a reaction time of 200 min showed a maximum yield of 76% compared to 72% without cysteine. By adding cysteine in a twofold molar excess to the substrate an almost complete reduction of hydroperoxide was obtained.

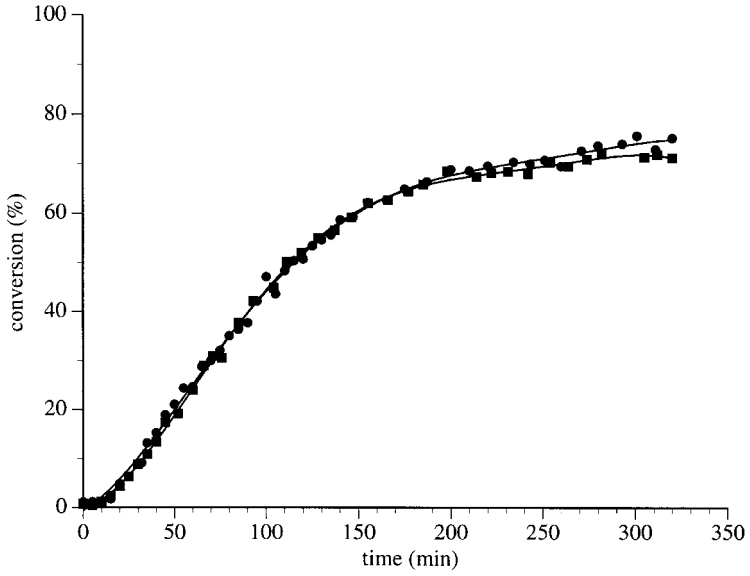

Fig. 3. Conversion of substrate A into hydroperoxides by addition at 4.85 mmol/min and the lipoxygenase preparation at 30 U/min; (■) without cysteine addition; (●) with cysteine addition after 200 min.

Tab. 3. Effect of adding cysteine on the product profile (% composition *) vs. time.

time [min]	13-OXOD	13-HOD	9-HOD	13-*ZE*-HPOD	13-*EE*-HPOD	9-*EZ*-HPOD	9-*EE*-HPOD
200	0.5	2.4	0.3	91.7	0.7	3.3	1.1
234	1.1	32.8	1.9	59.3	1.5	2.4	1.0
260	0.9	57.2	3.4	35.7	1.1	1.1	0.6
280	0.7	73.3	3.3	20.7	0.4	1.2	0.4
320	0.4	93.7	4.1	1.8	–	–	–

* The figures were truncated to their last significant digit. Values are the mean of three determinations. Errors have been omitted from the table for clarity.

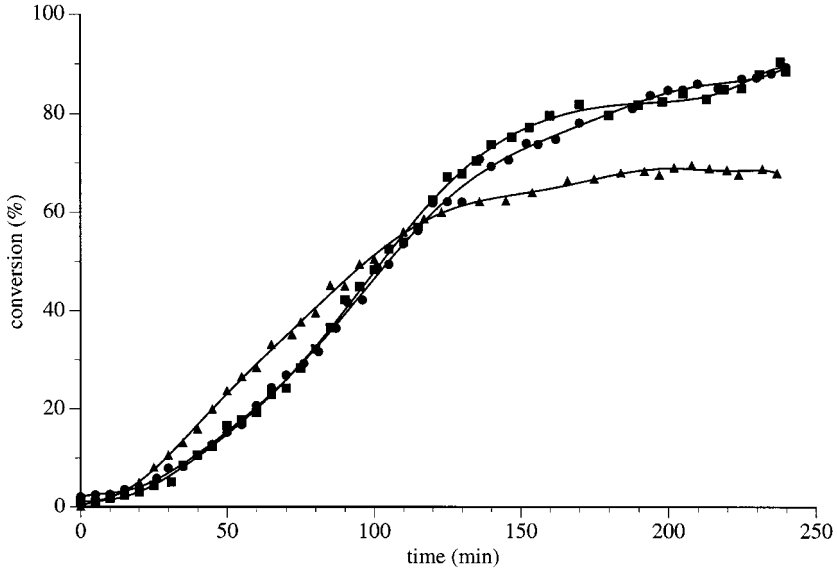

Fig. 4. Conversion into hydroperoxides by adding substrate A at 4.85 mmol/min and lipoxygenase at 30 U/min; (■) with 1.5 times the molar amount of cysteine compared with substrate A; (●) with 2 times the molar amount of cysteine compared with substrate A; (▲) without addition of cysteine.

Tab. 4. Effect of adding cysteine to the reaction mixture before the start of the reaction with lipoxygenase on the product profile (% composition*).

cysteine [mol. amt]	13-OXOD	13-HOD	9-HOD	13-*ZE*-HPOD	13-*EE*-HPOD	9-*EZ*-HPOD	9-*EE*-HPOD	yield
0	0.2	0.7	0.1	95.4	0.5	2.1	1.0	69
1.5	0.8	83.2	1.2	14.8	–	–	–	90
2	0.2	95.8	1.2	2.8	–	–	–	89

* The figures were truncated to their last significant digit. Values are the mean of three determinations. Errors have been omitted from the table for clarity.

5. By adding cysteine at the start of the reaction the foam formation increased, and addition of antifoam was necessary. With cysteine the conversion rate was lower at the beginning of the reaction. The reduction to hydroxy fatty acids caused the start of the reaction to be slower, because a small amount of hydroperoxide is necessary to initially activate the enzyme. However, when the reaction proceeded the reduction probably caused lower product inhibition and finally a higher yield could be obtained. The differences between the maximum yields were considerable being approximately 90% with cysteine and almost 69% without cysteine.

By this method of adding the mild reducing agent cysteine in a double-fed batch with a crude lipoxygenase extract and linoleic acid from hydrolyzed safflower oil, excellent results were obtained in the formation of hydroxy fatty acids in one experimental step.

Abbreviations

OD:	linoleic acid; (9*Z*,12*Z*)-octadeca-9,12-dienoic acid
9-*EE*-:	9-(10*E*,12*E*)- (as prefix to HOD, HPOD and OXOD)
9-*EZ*-:	9-(10*E*,12*Z*)- (as prefix to HOD, HPOD and OXOD)
13-*EE*-:	13-(9*E*,11*E*)- (as prefix to HOD, HPOD and OXOD)
13-*ZE*-:	13-(9*Z*,11*E*)- (as prefix to HOD, HPOD and OXOD)
HOD:	hydroxyoctadecadienoic acid
HPOD:	hydroperoxyoctadecadienoic acid
9-HPOD:	9(*S*)-hydroperoxy-(10*E*,12*Z*)-octadeca-10,12-dienoic acid
13-HPOD:	13(*S*)-hydroperoxy-(9*Z*,11*E*)-octadeca-9,11-dienoic acid
17-HPOD:	17(*S*)-hydroperoxy-(13*Z*,15*E*)-octadeca-13,15-dienoic acid
OXOD:	oxooctadecadienoic acid
LOX-1:	lipoxygenase-1
LOX-2:	lipoxygenase-2
RP-HPLC:	reversed-phase/high performance liquid chromatography

Acknowledgements

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