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Biocatalytic large-scale production of 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid from hydrolysed safflower oil by a crude soybean-flour extract as lipoxygenase source

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Abstract. Lipoxygenase-1 extracted from crude soybean-flour was incubated with hydrolysed safflower oil as a substrate in a 7-l fermentor. Optimized reaction conditions included pH 10.0, 5°C and air saturation. Under these conditions production on a 40-g/l scale can easily be carried out yielding up to 80% 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid with a stereo-configuration of essentially (> 95%) *S*.

Introduction [a]

Fatty-acid hydroperoxides are interesting compounds for certain industrial and pharmaceutical research areas. For example, hydroperoxides of arachidonic acid may serve as precursors for prostaglandin and leukotriene synthesis while hydroperoxides of linoleic acid and linolenic acid may act as natural fungicides in agriculture[1–3]. Another interesting application is the production of precursors of natural flavour compounds[4].

Enzymes can play a pivotal role in the natural synthesis of regio- and enantio-selective hydroperoxides and their derivatives. A possible route to these compounds uses the enzyme lipoxygenase because of its high selectivity and specificity. Lipoxygenase (EC 1.13.11.12) is a nonheme iron-containing dioxygenase which catalyses the incorporation of molecular dioxygen into polyunsaturated fatty acids possessing a (1*Z*,4*Z*)-pentadienyl moiety. Lipoxygenase-1 from soybean is one of the most studied lipoxygenases. The lipoxygenase reaction starts with the abstraction of a hydrogen atom from the methylene group between the two double bonds (Scheme 1). The substrate thereby reduces the iron of the enzyme to Fe^{II} and a pentadiene radical is formed. Under aerobic conditions[5], this radical reacts antarafacially with dioxygen to a hydroperoxy radical. By oxidation of the iron to Fe^{III} and protonation of the peroxide anion, the hydroperoxy fatty acid is formed which is then released from the enzyme. At lower oxygen concentration the pentadiene radical may dissociate from the enzyme. Subsequently, the Fe^{II} enzyme may react with the hydroperoxide, ultimately leading to the formation of dimers and additional secondary products, such as oxodienoic acids (OXOD).

Regio- and stereo-selectivities have been found to vary with the source of the lipoxygenase and the reaction conditions. For example, the incubation of linoleic acid with soybean lipoxygenase at pH 9.0 may lead to almost 95% production[6] of an *E,Z*-conjugated diene hydroperoxide 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid [13(*S*)-HPOD], whereas barley lipoxygenase yields more than 96% 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid [9(*S*)-HPOD][7]. In this study, we report the optimization of the production of 13(*S*)-HPOD by using soybean lipoxygenase as a biocatalyst under industrially interesting conditions. Other recent studies on the application of lipoxygenase as a biocatalyst typically used laboratory conditions, including surfactants[8–9] or high oxygen pressures[10]. In the conversion of linoleic acid to 13(*S*)-HPOD, the solubility of substrate and oxygen play important roles. Stability of the enzyme and minimization of foam production are also important parameters. Instead of pure linoleic acid, a substrate of an industrially more relevant nature was used, namely, hydrolysed safflower oil containing about 76% linoleic acid. For the same reason a crude lipoxygenase preparation from defatted soybean flour was applied instead of purified lipoxygenase-1. As a source of dioxygen, air was used instead of pure oxygen gas. In this study we investigated the stability of the crude lipoxygenase preparation and the effect of pH on its activity. *Galliard* et al.[11] showed that for purified lipoxygenase and substrate, the activity and product specificity are strongly influenced by pH. We have optimized the reaction conditions for the production of 13(*S*)-HPOD with hydrolysed safflower oil as the substrate and crude lipoxygenase-1 as the enzyme. To that end, pH, temperature and oxygen concentrations were varied, and the resulting product profiles were established and evaluated.

[a] **Nomenclature and abbreviations**
arachidonic acid = (all-*Z*)-eicosa-5,8,11,14-tetraenoic acid; GC = gas chromatography; HOD = hydroxy fatty acid; HPOD = hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid; linoleic acid = 9(*Z*),12(*Z*)-octadecadienoic acid; α-linolenic acid = 9(*Z*),12(*Z*),15(*Z*)-octadecatrienoic acid; LOX-1 = lipoxygenase-1; MS = mass spectrometry; OXOD = oxodienoic acid; RP-HPLC = reversed-phase high-performance liquid chromatography

Experimental

Preparation and activity measurement of crude LOX-1

Defatted soybean flour (gift from Unilever Research Laboratorium, Vlaardingen) was stirred for 2 h at 4°C in 0.1M sodium acetate buffer (pH 4.5, 1/10 w/v). The extract was centrifuged (17,000× *g* for 60 min

at 4°C), filtered and stored at 4°C. The activity of the crude extract was determined by measuring the increase in absorbance at 234 nm with a spectrophotometer (Hewlett Packard HP 8452A diode array spectrophotometer). Sodium borate buffer (980 μl, 0.1 M pH 9.0) was added to a cuvette followed by the addition of 40 nmol linoleic acid (99%, Fluka A.G., Buchs, Switzerland). The reaction was started by addition of 10 μl enzyme. The activity was calculated with a molar absorbance of 25,000 M^{-1} cm^{-1} and the activity calculated in units (1 unit = 1 μmol per min).The activity of this crude extract was measured before each large-scale experiment.

Incubation of hydrolysed safflower oil with crude LOX-1

Hydrolysed safflower oil (gift from Unilever Research Laboratorium, Vlaardingen) was incubated with crude LOX-1 in a 2-l working volume in a 7-l stirred tank bioreactor (Applikon Dependable Instruments, Schiedam, The Netherlands). The bioreactor was equipped with a thermometer, an oxygen electrode (Ingold dissolved oxygen probe), and a pH electrode (Ingold gel-filled electrode) which were connected to a console (ADI 1035 BIO Console, Applikon) and a biocontroller (Applikon Bio Controller ADI 1030), that automatically adjusts the parameters by P,I,D, (proportional gain, integral time, derivative time) regulation. The pH value was automatically maintained, either by the addition of 3M NaOH or 1M H_3BO_3. The oxygen concentration was kept constant, either by adjusting the speed of agitation between 400 to 1250 rpm or letting some extra air or N_2 into the bioreactor. The bioreactor was equipped with three baffles and a marine impeller to increase the oxygen transfer. The bioreactor further contained a sampling system, a manometer, a tuning valve and a pressure-relief valve to maintain a slight overpressure to reduce foam formation if necessary. Borate buffer (0.1M, 2 l) was allowed to reach the set temperature, then safflower oil was added and the pH was allowed to settle. Typically, safflower oil was added to a final concentration of 1 g/l linoleic acid, unless indicated otherwise. The reaction was started with the addition of the enzyme with a pump to yield a final concentration of up to 100 U/l. During the reaction, duplicate samples were taken and diluted with methanol to stop the reaction and the absorbance at 234 nm was measured. After 2 h the reaction was stopped by acidification with 2M HCl to pH 3.5.

Extraction and analysis of the reaction mixture

From the acidified reaction mixture, a 30-ml sample was taken and extracted with diethyl ether (2×30 ml). The solvent was removed from the combined fractions and the residue was dissolved in 1 ml methanol. The reaction products were analysed by reversed-phase high-performance liquid chromatography (RP-HPLC) on a CP-Spher C18 column (5 μm, 4.6×250 mm, Chrompack) using a HP1040A diode array UV detector and HP 7994A analytical workstation (Hewlett Packard). Isocratic elution was performed with THF/MeOH/H_2O/HOAc (25/30/45/0.1, v/v)[13] at a flow rate of 1.0 ml/min. Using this method, a complete separation is achieved between HPODs, hydroxy fatty acids (HODs) and OXODs. HPODs and HODs were detected at 234 nm, and OXODs at 280 nm with a molar absorbance of 22,000 M^{-1} cm^{-1}(Ref. 14).

Enantiomeric composition of the reaction mixture

The reduction of hydroperoxide fatty acids to the corresponding hydroxy fatty acids was carried out on ice under a stream of nitrogen for 30 min with a 200-μl sample which was diluted to 3 ml with methanol using a 10-fold excess of $NaBH_4$. The mixture was then diluted to 50 ml with water and acidified to pH 3.5 with 2M HCl to remove the excess $NaBH_4$. The acidified mixture was then extracted with an octadecyl reversed-phase extraction column (J.T. Baker) which was pre-treated with 10 ml methanol followed by 10 ml water. After extraction, the column was washed with water to remove any salts and eluted with 3 ml methanol. Subsequently, the sample was treated with an excess of diazomethane to form methyl esters. After 1 h, the excess of diazomethane was removed by evaporation and the residue dissolved in n-hexane. Individual isomers were isolated by SP-HPLC with a CP-Spher Si column (5 μm, 4.6x250 mm, Chrompack) and *n*-hexane/diethyl ether/HOAc (90/10/0.1, v/v) at a flow rate of 2 ml/ min. The solvent was removed and the product was dissolved in 1 ml methanol. The *R*- and *S*-enantiomers of the products were separated on a Chiralcel OD-R column (J.T. Baker) with MeOH/H_2O/HOAc (85/15/0.1, v/v) at a flow rate of 0.5 ml/min.GC analysis of the reaction products

For GC analysis the reaction products were reduced as described before. The hydroxyl function was subsequently derivatized into the trimethylsilyl ether by reaction with 1 ml of a silylating mixture of dry pyridine, hexamethyldisilazane and trimethylchlorosilane (5/1/1, v/v). The resulting TMS derivatives were analyzed on a CP-sil5CB column using a GC (Varian Model 3700) or a GC/MS (Fisons Instruments GC8000 series/MD800) under electron impact ionization with an ionization energy of 70 eV, an injection temperature of 250°C, and an ion source of 200°C. The program started at 140°C with an increase of 4°C/min to 280°C. At 280°C, the temperature was kept constant for 10 min.

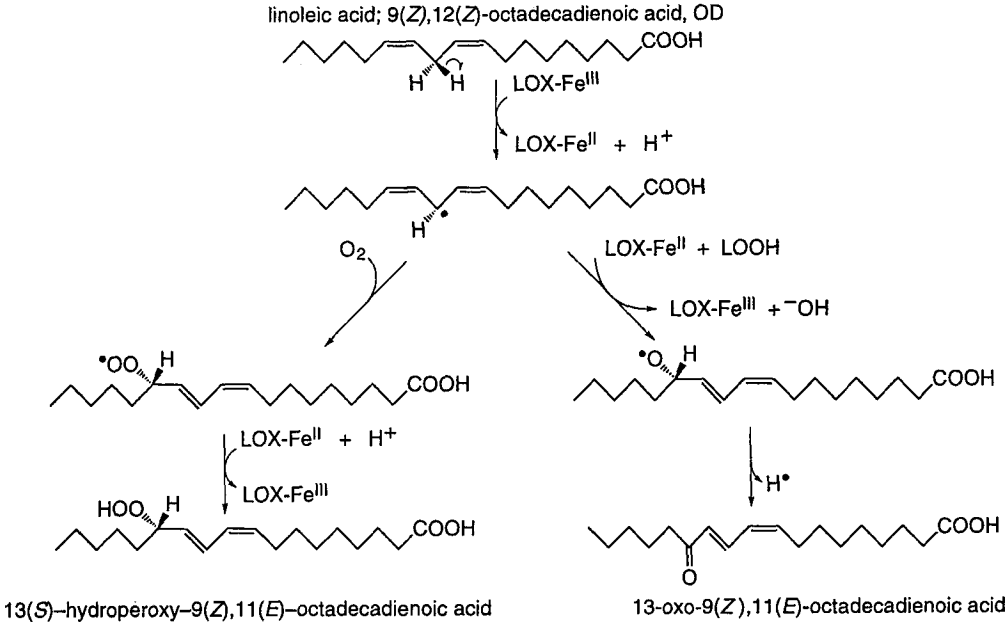
Results and discussion

Stability of LOX-1

To investigate the stability of crude LOX-1, two different enzyme extracts were prepared, stored at 4°C, and the activity measured (see Figure 1). The decrease in activity was most pronounced during the first few days, after which it tended to level off. It was necessary to measure the activity of the used LOX-1 extract just before an experiment and add some extra enzyme during the reaction in order to compensate for this loss of activity.

Effect of pH on product formation

The activity was measured spectrophotometrically by adding 10 μl LOX-1 extract to 980 μl phosphate buffer at different



Scheme 1. Mechanism for the lipoxygenase-catalysed dioxygenation of linoleic acid.

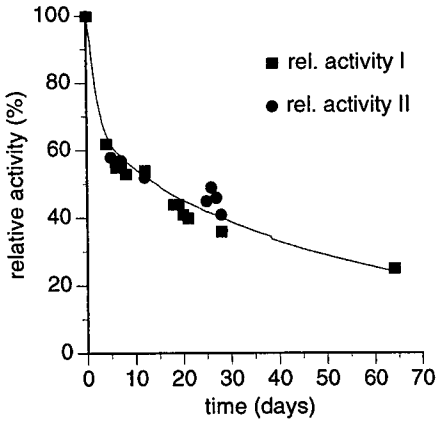


Figure 1. Activity in time of a crude lipoxygenase preparation.

pH values with 10 μl safflower oil. Starting from pH 5.5, the activity was low until pH 7.0 was reached (Figure 2) and then a sharp increase was seen with a maximum at pH 8.0 and only a small decrease at higher values. Almost the same behaviour has been found with purified reactants[11]. The better solubility of the linoleic acid (pK_a 7.9) in the latter medium is also advantageous.

At pH values of 8, 9, 10, and 11, incubations on a 2-l scale were done. Hydrolysed safflower oil was incubated with a crude LOX-1 extract at 10°C and with air saturation. All parameters were kept constant at the adjusted level during these experiments. The decrease in pH due to the addition of the hydrolysed safflower oil, was compensated for by pumping NaOH into the bioreactor. The conversion into HPOD was maximal at pH 8.0 (see Figure 3); at higher pH, a small decrease in yield was observed. The conversion rates at pH 10.0 and 11.0 were higher at the beginning of the reaction than at pH 8.0 and 9.0, probably because of the better solubility of the substrate but a slightly lower yield was observed. In addition to the extent of the conversion, the product composition is also a very important parameter. Figure 4 shows the RP-HPLC chromatogram of the incubation at pH 10.0. The results at different pHs are given in Table I. The main product (peak 4) was identified as 13-(9*Z*,11*E*)-HPOD, while other products that could be detected, were: its reduced derivative 13-HOD, as peak 2; 9-(10*E*,12*Z*)-HPOD as peak 5; 9-HOD as peak 3. Minor products were identified as: 13-(9*E*,11*E*)-HPOD as peak 6; 9-(10*E*,12*E*)-HPOD as peak 7; a small amount of 13-oxooctadecadienoic acid as peak 1. From co-elution with standard samples, and analysis with GC/MS and NMR the structures were fully confirmed. From the peak areas of the different products the composition could be calculated (Table I). After the incubation mixture was acidified, it was extracted immediately, or stored at −20°C because at higher temperatures some product instability was seen, accompanied by an increase in oxooctadecadienoic acid. Analysis of the samples with GC did not reveal any residual linoleic acid.

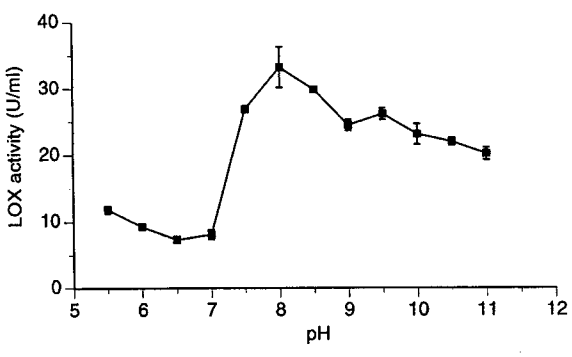


Figure 2. Activity of a crude lipoxygenase extract at different pH values.

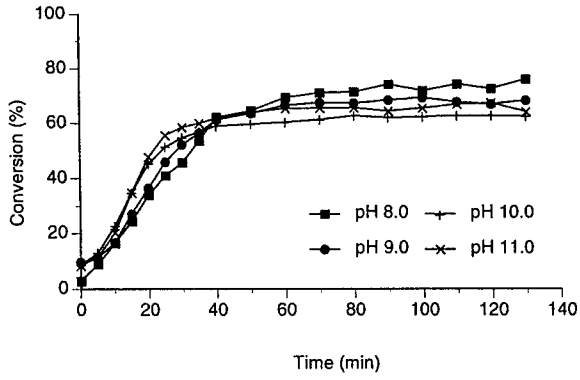


Figure 3. Substrate conversion into hydroperoxides at different pH values.

The amount of 9-HPOD decreased with increasing pH. At the lower pHs some lipoxygenase-2 could be active[14], which produces about 70% 9-HPOD and 30% 13-HPOD[15] at 25°C. At higher pH, lipoxygenase-2 has a negligible activity. The reduction of 13-HPOD to 13-HOD probably occurred because of the presence of some reducing substances in the crude-enzyme preparation. By adding more crude enzyme the amount of 13-HOD also increased (data not shown). By increasing the pH, some more 13-HOD could be detected probably because, under stronger alkaline conditions, reduction may increase, as was also found by *Simpson*[16]. A slight decrease in total activity could be seen in going from pH 8 to 11 (Figure 2) but at the same time the product specificity was clearly improving substantially in favour of the production of 13-*ZE*-HPOD. Because of this better product specificity and the somewhat higher activity of LOX-1 at pH 10.0 compared to pH 11, pH 10 was considered optimal. In literature, most measurements are reported to be done at pH 9.0 but with this crude extract clearly better results were found at pH 10.0. *Martini* and co-workers[10] also found better results in the range pH 10–11 but these authors did not continually adjust the pH, and found a decrease of approximately 1 pH unit after addition of the substrate. The original pH value of 11 would therefore correspond with a pH 10, which we found to be optimal.

Incubation with different dissolved oxygen concentrations

Adequate oxygen supply plays an essential role in hydroperoxide formation, catalysed by LOX-1[17], and a low oxygen concentration may eventually lead to an anaerobic reaction

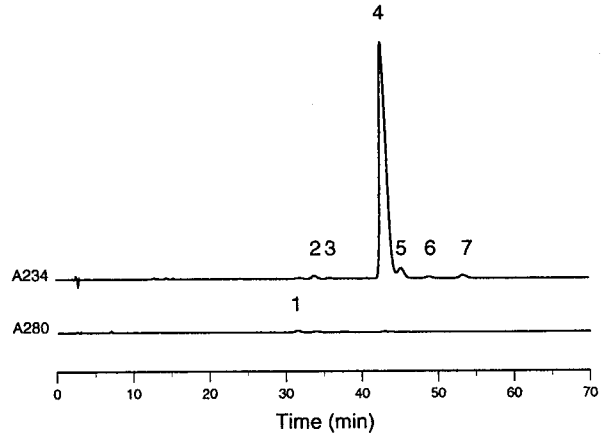


Figure 4. RP-HPLC analysis of products formed by the incubation of hydrolysed safflower oil with soybean flour at pH 10.0. Analysis was done with a CP-Spher C18 column (5 μm, 4.6 × 250 mm, Chrompack) with THF / MeOH / H_2O / HOAc (25 / 30 / 45 / 0.1, v / v) at a flow rate of 1.0 ml / min with UV detection at 234 nm and at 280 nm. Peak identification (see text) 1: 13-OXOD; 2: 13-HOD; 3: 9-HOD; 4: 13-ZE-HPOD; 5: 9-EZ-HPOD; 6: 13-EE-HPOD; 7: 9-EE-HPOD.

Table I Effect of different pH values on product composition.

pH	13-OXOD	13-HOD	9-HOD	13-*ZE*-HPOD	13-*EE*-HPOD	9-*EZ*-HPOD	9-*EE*-HPOD
8	0.7	2.2	2.3	77.7	1.3	13.5	2.3
9	0.6	2.4	2.2	84.7	1.3	7.0	1.9
10	0.5	3.1	2.4	87.7	1.0	3.9	1.5
11	0.5	4.4	2.5	86.9	0.9	3.7	1.2

resulting in unwanted side-products. At air saturation, the biocontroller was arbitrarily set to a dissolved oxygen concentration of 100%. The effect of varying the dissolved-oxygen concentration was investigated at 0, 10, 25, 50, 75, and 100% air saturation with hydrolysed safflower oil and crude LOX-1 at pH 10.0 and 10°C. The parameters were kept constant by the controller. The maximal variation in the dissolved oxygen concentration was 10% of the set value. To achieve anaerobiosis nitrogen gas was extensively bubbled through the reaction media. The total conversion was almost the same (Figure 5) for 25, 50, 75 and 100% air saturation. At 10% air saturation, there was clearly less production of 13-HPOD compared to the other concentrations and at 0% air saturation the yield of 13-HPOD was, of course, extremely low. The anaerobic cycle starts somewhere between 0 and 10% air saturation.

In the product compositions (Table II), small increases in 13-HPOD could be seen in going from 25 to 100% air saturation. No linoleic acid could be detected after the reactions. At 10% air saturation, only 53% of the linoleic acid was converted and the amount of 13-HPOD was clearly lower than at higher saturation levels. At 0% air saturation, the product composition contained almost the same quantities of 13- and 9-HPOD and a relatively large amount of 13-OXOD; the conversion of linoleic acid was only 17%. This conversion could probably occur because some oxygen was still present in one of the solutions which were pumped into the fermentor. From these results, it was concluded that 100% air saturation was optimal, although slightly lower oxygen concentrations do not dramatically affect the reaction. No significant differences were found when reactions were carried out with pure oxygen instead of at 100% air saturation.

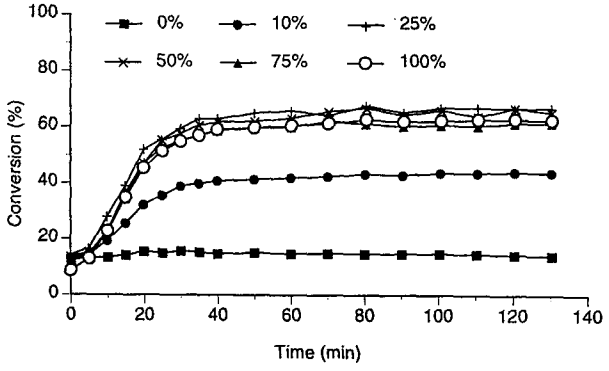


Figure 5. Substrate conversion into hydroperoxides at different oxygen concentrations.

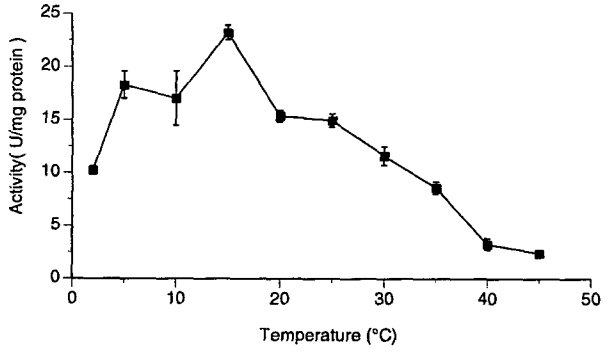


Figure 6. Crude lipoxygenase activity as a function of the reaction temperature.

Incubations at different temperatures

The optimum temperature for the crude enzyme was determined with linoleic acid as substrate (Figure 6). An important factor that could contribute to the effect of temperature on enzyme activity, is that the oxygen content of air-saturated water is temperature-dependent. The oxygen content decreases from 386 μM at 5°C via 206 μM at 40°C to 69 μM at 70°C[18]. It was found that the optimum temperature for the crude lipoxygenase lies around 15°C.

In a temperature range of 5 to 40°C, we studied the conversions on a 2-l scale. Measurements below 5°C were not feasible because of the insolubility of fatty acids at these temperatures. Hydrolysed safflower oil (to yield a final linoleic acid concentration of 1 g/l) was incubated with a crude LOX-1 extract (100 U/l) at pH 10.0 at 100% air saturation at

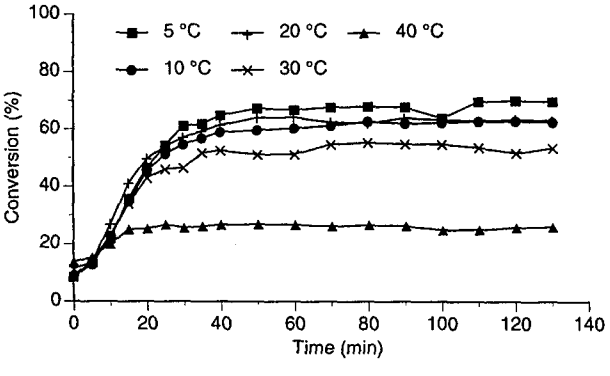


Figure 7. Substrate conversion into hydroperoxides at different temperatures.

Table II Effect of dissolved oxygen concentration on product composition.

Oxygen conc. (%)	13-OXOD	13-HOD	9-HOD	13-*ZE*-HPOD	13-*EE*-HPOD	9-*EZ*-HPOD	9-*EE*-HPOD
0	6.7	11.4	12.8	31.7	6.2	24.7	6.5
10	3.2	4.6	6.7	66.5	3.0	12.8	3.2
25	0.7	3.1	2.3	85.8	1.3	5.2	1.7
50	0.5	3.1	2.3	86.6	1.2	4.8	1.6
75	0.5	3.0	2.3	86.8	1.2	4.8	1.6
100	0.5	3.1	2.4	87.7	1.0	3.9	1.5

Table III Effect of temperature on product composition.

Temp. (°C)	13-OXOD	13-HOD	9-HOD	13-*ZE*-HPOD	13-*EE*-HPOD	9-*EZ*-HPOD	9-*EE*-HPOD
5	0.4	2.6	2.2	88.9	0.9	3.9	1.1
10	0.5	3.1	2.4	87.7	1.0	3.9	1.5
20	0.7	3.8	2.3	85.4	1.4	4.6	1.9
30	1.0	6.1	3.1	78.5	2.1	6.2	3.0
40	2.1	12.6	9.4	52.5	4.9	12.5	6.0

temperatures of 5, 10, 20, 30 and 40°C (Figure 7). The extent of the conversion of linoleate into HPOD was highest at the three lowest temperatures. At 5°C, a conversion of 70% could be detected, while, at 10 and 20°C, the conversion reached approximately 64%. In the GC analyses, no residual linoleic acid was found, except in samples from incubations at 30 and 40°C where the conversion was found to be 55 and 27%, respectively. This could mean that, because of the higher temperatures, the reaction is less regio- and stereospecific. The decreased oxygen content at lower temperatures may be a contributing factor.

From literature data[19], it is known that temperature also plays a role in the ratio of the isomers that are formed in a lipoxygenase reaction. At a temperature of 0°C and pH 9.0 13-monohydroperoxides are formed almost exclusively, whereas at higher temperatures more of the 9-isomer will be formed. At these higher temperatures, slightly more autoxidation products could be detected at the start of this experiment. Table III gives the product compositions at the different temperatures. With increasing temperatures, the relative amount of 13-HPOD decreased, while the production of side-products, especially 9-HPOD, increased. It seems that at these higher temperatures, the enzyme does not work as effectively as at lower temperatures. The lower activity and the lower oxygen concentration may be responsible for this decreasing specificity. The stereochemistry of derivatized 9-HPOD was analyzed by CP-HPLC. The *R* and *S* enantiomers were found to be present in almost the same ratio at all temperatures. We can therefore conclude that the presence of 9-HPOD at higher temperatures is most probably due to instability of the enzyme substrate complex or to autoxidation, and not to some other LOX activity. Stereochemical analysis of 13-HPOD showed that more than 95% of 13*S*-HPOD was formed. A temperature of 5°C was optimal in terms of the highest conversion rate and the desired product composition and yield.

Large-scale reaction of the optimized reaction conditions

A 2-l large-scale reaction was performed under optimized reaction conditions. The reaction parameters used were pH 10.0, 5°C and air saturation. In a 7-l bioreactor a LOX-1 extract was added with a total activity of 2000 U/l. Hydrolysed safflower oil was pumped into the bioreactor with a speed of 4 ml/min to a final linoleic acid concentration of 40 g/l. Using these reaction parameters, a conversion of up to 80% was produced after 2-h incubation with a product compostion of > 95% 13-*ZE*-HPOD and a configuration that was > 95% *S*.

Conclusion

This work describes the optimization of different parameters in the synthesis of hydroperoxides under industrially interesting conditions, *i.e.*, defatted soybean-flour as a source of LOX-1, hydrolysed safflower oil as substrate, and air instead of pure oxygen gas. The following conclusions can be made.

1. The activity of the crude LOX-1 preparation decreased strongly at 4°C during the first few days but thereafter its activity decreased less steeply. By adjusting the amount of enzyme the total activity could be kept constant during the incubation period.
2. Activity measurements confirmed that the optimum pH lies in the basic range. The best result in terms of product specificity for 13-HPOD occured at pH 10.0.
3. Acidification to pH 3.5 was necessary to stop the reaction and to allow extraction of the products. Extraction should either be done immediately after this acidification, or the sample should be kept in a freezer as at higher temperature there was significant formation of oxo compounds.
4. There were only small differences in product specificity at oxygen concentrations of 25 to 100% air saturation. At 10% air saturation the conversion was lower and the product specificity deteriorated. Under the conditions, the anaerobic reaction starts at somewhere between 0 and 10% air saturation. It is concluded that the oxygen concentration of air-saturated buffer should be kept as high as possible to give the best results, although minor fluctuations do not cause dramatic changes in product profiles. No clear differences could be detected when pure oxygen was used instead of air saturation.
5. By increasing the reaction temperature from 5 to 40°C, a decrease of 13-HPOD and an increase of 9-HPOD was found. From chiral analysis, it is concluded that the latter increase is not due to an increase in LOX-2 activity. Finally, at 5°C, the best results were found with respect to product specificity.
6. By combining the results obtained here, we conclude that the reaction has a consolidated optimum at 5°C, pH 10.0 and 100% air saturation of the buffer. Applying the reaction parameters to a 40 g/l scale experiment showed 80% conversion into > 95% 13-*ZE*-HPOD with a configuration of > 95% *S*.

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