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Co-oxidation of Carotene and Crocin by Soyabean Lipoxygenase Isoenzymes

Franziska Weber, Gudrun Laskawy and Werner Grosch*

Deutsche Forschungsanstalt für Lebensmittelchemie, München (BRD)

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Co-oxidation von Carotin und Crocin durch Lipoxygenase-Isoenzyme aus der Sojabohne

Zusammenfassung. Drei in Sojabohnen vorkommende Lipoxygenasen wurden chromatographisch getrennt: L-1 (pH-Optimum 9,0), L-2 (pH 6,5), L-3 (pH 6,5). Gemessen wurden die Geschwindigkeiten mit denen diese Enzyme β-Carotin oder Crocin in Gegenwart von Linolsäure oder Linoleylsulfat co-oxydieren. Die Carotinoid-Umsätze wurden auf die jeweilige Lipoxygenase-Aktivität bezogen.

β-Carotin/Linolsäure: 55% (L-2), 43% (L-3), 6% (L-1),
Crocin/Linolsäure: 17,8% (L-2), 14,3% (L-3), 3,3% (L-1),
Crocin/Linoleylsulfat: 24% (L-3), 4,2% (L-1).

Für die Crocin-Bleichung wurde die Abhängigkeit der Reaktionsgeschwindigkeit von der Enzym-, Crocin- und Linolsäure-Konzentration bestimmt.

Zur Erklärung der Unterschiede zwischen den pH 6,5- (L-2, L-3) und der alkalischen Lipoxygenase (L-1) wird angenommen: L-2 und L-3 bilden besonders aktiv Radikale, welche die Polyene co-oxydieren können. Beide Enzyme besitzen in der Nähe des aktiven Zentrums eine hydrophobe Bindungsstelle für das β-Carotin.

Summary. Three lipoxygenases that occur in soya beans were separated chromatographically: L-1 (optimum pH = 9.0), L-2 (pH 6.5), L-3 (pH 6.5). The velocities with which these enzymes co-oxidise β-carotene or crocin in the presence of linoleic acid or linoleyl sulphate were measured. The carotenoid turnover was related to each lipoxygenase activity.

β-carotene/linoleic acid = 55% (L-2), 43% (L-3), 6% (L-1),
crocin/linoleic acid = 17,8% (L-2), 14,3% (L-3), 3.3% (L-1),
crocin/linoleyl sulphate = 24% (L-3), 4,2% (L-1).

The relationship between the reaction rate of the crocin bleaching and the concentrations of the enzyme, crocin and linoleic acid was determined. To explain the differences between the pH-6.5 (L-2, L-3) and the alkaline (L-1) lipoxygenases it is supposed that L-2 and L-3 form specially active radicals that are able to co-oxidise polyenes. Both enzymes possess a hydrophobic bonding position, in the neighbourhood of the active site, for β-carotene.

Introduction

The enzyme lipoxygenase (linoleic acid: oxygen oxidoreductase EC 1.13.1.13.) which is found in leguminoses as well as in many other plants bleaches carotenoids in the presence of the substrates [1]. Christopher *et al.* [2, 3] and Verhue and Francke [4] have separated four lipoxygenase isoenzymes from soya beans and have isolated and characterised three of them (Table 1).

Table 1. Properties of lipoxygenase isoenzymes from soya after Christopher *et al.* [2, 3] and Verhue and Francke [4]

Designation of the enzymes according to Christopher *et al.* [2, 3] and Verhue and Francke [4]	L-1 A_1/A_2	L-2 E_1	L-3 E_2
Optimum pH	8.5—9.5	6.6	6.5—7.0
Molecular weight	10^5	10^5	10^5
Turnover number mole linoleic acid (min^{-1} mol^{-1})	15800	6500	
Stability; $t_1/_2$ at 69° C	25 min	0,7 min	
Activity towards methyl linoleate	no	yes	yes

The three lipoxygenases can be separated very easily by chromatography using a DEAE cellulose column [3]. In this way it is evident that the pH 6.5 lipoxygenases-2 and -3 co-oxidise β-carotene much more easily than the alkaline lipoxygenase-1 [5].

This work shows how the bleaching velocities differ for β-carotene and for water soluble crocin using lipoxygenases-1, -2 and -3.

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Experimental

1. *Materials and Reagents*

β-carotene (Hoffmann La Roche); linoleic acid and methyl linoleate (Nu Chek Prep) Tween 80 (Schuchardt); EDTA (Merck Titriplex III) $LiAlH_4$ (Merck); Amidosulphonic acid (Merck); Piperazine-N-N'-bis (2-ethane sulphonic acid) (PIPES; Sigma); Sephadex G-50 (Pharmacia). DEAE cellulose (Serva). Soya beans (Harburger Ölwerke, Brinckmann and Mergell); Crocin: 0.5 g of saffran (bought locally) was defatted with ethyl ether in a Soxhlet apparatus. The residue was extracted with 3 $\times$ 20 ml of methanol, and the methanol was evaporated from 0.5 ml of this solution. The resulting residue was dissolved in 25 ml of water or in 25 ml of buffer solution. The concentration was determined by measurement of the absorbance at 440 nm (10^{-3} μmole/ml crocin $\triangle A_{440} = 0.1335$ after [6]. Linoleyl sulphate was synthesised according to Allen [7].

2. *Separation of Lipoxygenases-1, -2 and -3 from a Soy Extract**

(All procedures at 2—4° C). 10 g of ground soya (defatted with light petroleum) was stirred for 30 min with 100 ml of 0.01 M Na phosphate buffer, pH 7.0, then filtered through one layer of cheesecloth and centrifuged (30 min; 15,000 $\times$ g). 50 ml of the extract was pipetted on to a Sephadex G-50 column (23 cm $\times$ 2.5 cm), which was washed through with 0.05 M Na phosphate buffer, pH 6.5; 30 ml of eluent in the range of V_0 (V_0 being determined by chromatography of dextran blue) was collected and centrifuged until clear. 25 ml of this solution was used on a DEAE cellulose column and eluted with 0.01 M Na phosphate buffer, pH 7.0, which had a linear NaCl gradient. Further experimental details can be found under "Figures". The lipoxygenase activity in the eluate was measured at pH 6.5 and 9.0 as described in 5.1.

3. *Ammonium Sulphate Preparation (ASP)*

Proteins were precipitated from the clear centrifuged extract produced under 2. using ammonium sulphate in the saturation range 30—60%. This solution was centrifuged, the residue was dissolved in 0.01 M Na phosphate buffer, pH 7.0 and centrifuged again until clear.

4. *Protein Determination*

Protein was measured routinely by the absorbance at 280 nm or 230 nm. For more accurate measurements the biuret method, as described by Beisenherz *et al.* [8], was used.

5. *Determination of the Lipoxygenase Activity (Modification of the Surrey [14] Procedure)*

Test 5.1: Linoleic acid emulsion: 0.25 ml of linoleic acid dissolved in 20 ml of water with 0.1 ml of Tween 80 using 1 N NaOH, pH adjusted to 7.0 with HCl and diluted to 100 ml with water.

Substrates: Each 25 ml of linoleic acid emulsion was diluted with 75 ml of 0.1 M Na phosphate buffer, pH 6.5 or with 0.1 M Na borate buffer, pH 9.0.

Determination: 1 ml of the substrate and 1 ml of 0.1 M Na phosphate buffer, pH 6.5 or 0.1 M Na borate buffer, pH 9.0 (each buffer containing 0.36 μl of Tween 80 and 0.25 mg of EDTA) were pipetted into a 1-cm silica cell. The reaction was started by the addition of 10 μl of the enzyme solution and the increase in Extinction A_{234} between 15 and 30 sec at T = 23° C was measured.

Test 5.2: Substrate: 25 ml linoleic acid emulsion from Test 5.1 diluted with 75 ml of 0.1 M PIPES buffer, pH 6.5.

Determination: The following solutions were pipetted into a 1-cm silica cell: 1 ml of substrate with 1 ml of 0.1 M PIPES-buffer, pH 6.5 or with 1 ml of 0.1 M PIPES buffer, pH 6.5 which contained 0.8 mM $CaCl_2$. The procedure was then as described in Test 5.1.

Test 5.3: Substrates: 10 mg of linoleyl sulphate dissolved in 20 ml of 0.1 M Na phosphate buffer at pH 8.15 and corrected to pH 6.5 with conc. HCl. 10 mg of linoleyl sulphate dissolved in 20 ml of 0.1 M Na borate buffer, pH 9.0. The substrates were diluted with corresponding buffers to the optimum linoleyl sulphate concentrations.

Determination: The following solutions were pipetted into a 1-cm silica cell: 1 ml of the substrate and 1 ml of 0.1 M Na phosphate buffer, pH 9.0. The procedure was then as described in Test 5.1.

Test 5.4: Linoleic acid emulsion: 9.8 mg of linoleic acid and 9.8 mg of Tween 80 were dissolved in a few ml of water by the addition of N NaOH solution and the pH was adjusted to pH 7.5 or pH 8.5. This was then diluted to 100 ml with 0.1 M of Tris/HCl buffer, pH 7.5 or 8.5.

Incubation: 4 ml of linoleic acid emulsion were stirred magnetically at 18.5° C. After 5 min, the reaction was started by the addition of 0.1 ml of enzyme solution. 1 and 3 min after the reaction had begun 1-ml samples were taken and pipetted into 3 ml of methanol. Determination of the hydroperoxide concentration using a method similar to that of Ames and King [9]: The

* The DEAE-cellulose chromatography of the soya bean proteins described in [5] was carried out by this procedure.

methanol solution was diluted to 5 ml and acidified with 6 drops of methanol/HCl. After 1 min, 0.01 ml of 3.6% iron-(II)-sulphate (dissolved in 3.6% HCl) was added and 30 sec later 0.5 ml of 20% KSCN solution was pipetted into the well-mixed sample. The absorbance of the thiocyanate complex at 490 nm was measured against 60% methanol after 150 sec. The measured absorbance was corrected by reference to the blank.

Test 5.5: Linoleic acid emulsion as 5.4 but with 19.6 mg of linoleic acid and without Tween 80. $CaCl_2$ solution: 110 mg of $CaCl_2 \cdot 2H_2O$ dissolved in 100 ml of 0.1 M Tris/HCl buffer, pH 7.5.

Incubation: 2 ml of linoleic acid emulsion were stirred with 2 ml of $CaCl_2$ solution at 18.5° C. The reaction was started after 5 min by the addition of 0.1 ml of enzyme solution. Sampling and determination of the hydroperoxide concentration were as reported under Test 5.4.

The linoleic turnover was determined by using a molar absorbance coefficient at 234 nm of $25000\ l \cdot mole^{-1} \cdot cm^{-1}$.

6. *Determination of Carotene Co-oxidation*

Linoleic acid substrate as Test 5.1. β-carotene emulsion after Ben-Aziz *et al.* [10]: 10 mg of β-carotene and 0.36 ml of Tween 80 were dissolved in 10 ml of chloroform. 1 ml of this solution was evaporated and the residue was dissolved in 10 ml of water containing 2.5 mg/ml of EDTA. 1 ml of the resulting solution was diluted with 9 ml of 0.1 M Na phosphate buffer, pH 6.5 or with 0.1 M Na borate buffer, pH 9.0.

Determination: The following solutions were pipetted into a 1-cm glass cell: 1 ml of substrate and 1 ml of β-carotene emulsion. The reaction was started by the addition of 10 μl of enzyme solution and the decrease in E_{460} was measured between 15 and 30 sec at T: 23° C. The measured absorbance differences were used to calculate the μM concentration of carotene with the use of a standard graph (Fig. 1).

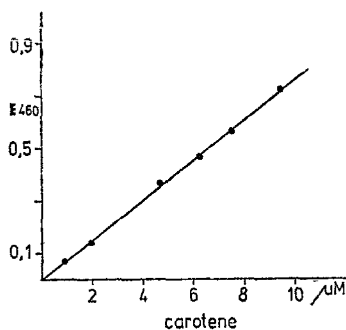


Fig. 1

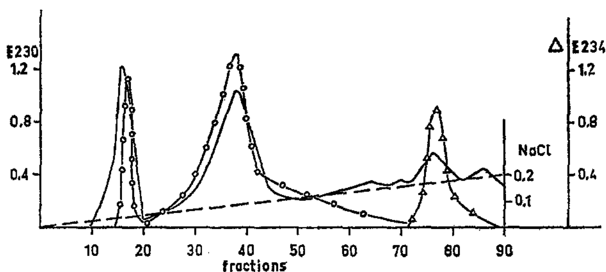


Fig. 2

Fig. 1. Calibration curve: absorbance at 460 nm against carotene concentration in Test 6 (see "Experimental"). 1 mM linoleic acid, 0.3 μl/ml Tween 80, 0.1 M Na phosphate buffer, pH 6.5

Fig. 2. DEAE-cellulose chromatography of an extract from soya beans. DEAE cellulose column (32 cm × 2.5 cm) which was equilibrated against 0.01 M Na phosphate buffer, pH 7.0. Sample: 184 mg protein. Elution (32 ml/h) was performed with a linear NaCl gradient (0—0.2 m) in the buffer. Each fraction contains 8.75 ml. —— protein; ○—○ lipoxygenase activity at pH 6.5; △—△ lipoxygenase activity at pH 9.0; --- NaCl gradient

7. *Determination of Crocin Co-oxidation*

Test 7.1: Linoleic acid substrate as Test 5.1. Crocin solution: 200 μg of crocin were dissolved in 10 ml of 0.1 M Na phosphate buffer, pH 6.5 or 0.1 M Na borate buffer, pH 9.0 (both buffers contained 3.6 μl of Tween 80 and 2.5 mg EDTA).

Determination: The following solutions were pipetted into a 1-cm glass cell: 1 ml of linoleic acid substrate and 1 ml of crocin solution. The reaction was started by the addition of 10 μl of enzyme solution and the decrease in E_{440} was measured between 15 and 30 sec at T = 23° C. The crocin turnover was calculated using the correlation: 1 μmole crocin in water causes an absorbance $E_{440}^{1\,cm} = 0.1335$ [6].

Test 7.2: Linoleyl sulphate solution produced as in Test 5.3. Crocin solution: 200 μg of crocin dissolved in 10 ml of 0.1 M Na phosphate buffer, pH 6.5 or in 0.1 M Na borate buffer, pH 9.0.

Determination: The following solutions were pipetted into a 1-cm glass cell: 1 ml of linoleyl sulphate solution and 1 ml of crocin solution. Further procedure as in Test 7.1.

Results and Discussion

The separation of a soya extract into three protein fractions with lipoxygenase activity (L-1, L-2, and L-3, Fig. 2), as already described by Christopher *et al.* [3], was improved. The elution buffer and the buffer in which the sample was dissolved had different compositions (see the pH values and the ionic strengths of the buffers under the heading "Experimental"). This difference caused a complete separation of the L-3 from the L-2 on the DEAE cellulose column (Fig. 2). The separation of a soya extract, published in [5], was also carried out.

When linoleic acid is emulsified with Tween 20, with L-3 and L-2 at pH 6.5 and with L-1 at pH 9.0 the acid is oxidised with maximum velocity [2, 3]. The catalysts were also affected by the presence of Ca^{2+} ions [11]. The three lipoxygenase activities were until now determined only in the presence of both Tween and Ca^{2+} ions [3].

To characterise the separated lipoxygenases the linoleic acid oxidation was measured in the presence of Tween 80 only, Ca^{2+} ions only and also with both substances present (Table 2).

L-1: The rate of reaction is only 15% less if Tween 80 is substituted for Ca^{2+} ions.

L-2 and L-3: The optimum pH values for the linoleic acid/Tween was 6.5 and that for the Ca linoleate substrate 7.5. Under this optimal pH condition the activity of L-2 is about 80% higher and that of L-3 about 20% higher in the presence of Tween 80 than when using Ca^{2+} ions. There is also a difference between L-2 and L-3 when both effectors are dissolved in the substrate (pH 6.5). L-2 was not influenced by the Ca^{2+} ions, although L-3 was inhibited by Ca^{2+} ions (Nr. 4 and 5 in Table 2). This observation confirms the results of Christopher *et al.* [3].

These results therefore show that the activities of the three enzymes, even in the best case (L-3), are not higher with Ca^{2+} ions than with Tween 80. The further investigations were therefore carried out using a linoleic acid/Tween substrate.

It is therefore important to note that the activity of the pH-6.5-lipoxygenases is a function of the detergent concentration, in contrast with the pH-9.0 enzyme, which is only slightly dependent on concentration (Fig. 3). Because of these results a concentration of 0.3 µl/ml of Tween 80 was used in the following activity determinations.

Co-oxidation of β-carotene or Crocin in the Presence of Linoleic Acid

At the optimum Tween concentration, L-2 and L-3 bleach the carotene very quickly (Table 3). The carotene turnover using L-2 was 55% and L-3 was 43%,

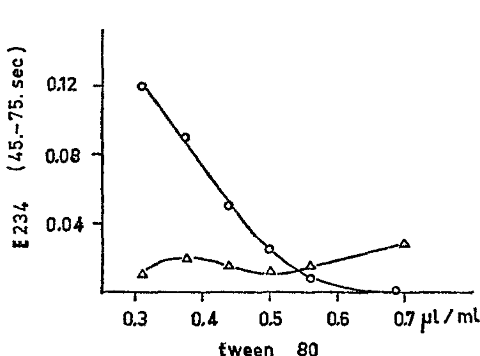


Fig. 3

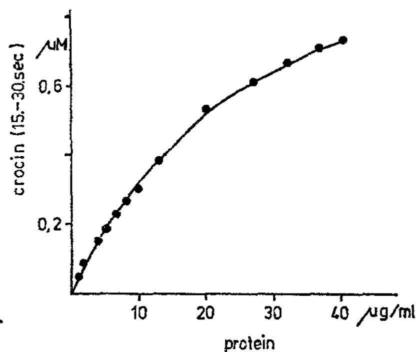


Fig. 4

Fig. 3. Influence of Tween 80 on the lipoxygenase catalysis. Test 5.1 (see "Experimental") with 1.7 µg/ml protein (ASP). ○—○ pH 6.5; △—△ pH 9.0

Fig. 4. Crocin co-oxidation as a function of enzyme concentration. Test 7.1 (see "Experimental") at pH 6.5 with ASP and 4,73 µM crocin

Table 2. Influence of Ca^{2+} ions and Tween 80

No.	Reaction systems						Test No.	L-1	L-2	L-3
	Linoleic acid	Tween 80 (µl/ml)	$CaCl_2$	Protein (µg/ml)			in „Experimental“	$\triangle A^{1.-3.\ min}_{490\ nm}$		
				L-1	L-2	L-3				
1	340 µM	98	—	0.32	2.1	0.72	5.4	0.206 (pH 8.5)	0.062 (pH 7.5)	0.014 (pH 7.5)
2	340 µM	—	0.36 mM	0.32	2.1	0.72	5.5	0.176 (pH 8.5)	0.272 (pH 7.5)	0.197 (pH 7.5)
3	340 µM	98	—		2.1	0.72	5.4		0.486 (pH 6.5)	0.238 (pH 6.5)
								$\triangle A^{15.-30.\ sec}_{234\ nm}$		
4	1 µM	0.125	—	—	0.44	0.46	5.2	—	0.038 (pH 6.5)	0.148 (pH 6.5)
5	1 µM	0.125	0.4 mM	—	0.44	0.46	5.2	—	0.035 (pH 6.5)	0.052 (pH 6.5)

Table 3. Co-oxidation of β-carotene or crocin

No.	Reaction system							Test-No. in "Experimental"	Turnover (15.—30. sec)		
	Linoleic acid	Tween 80 (µl/ml)	β-carotene	Crocin	Protein (µg/ml)				L-1 pH 9.0	L-2 pH 6.5	L-3 pH 6.5
					L-1	L-2	L-3				
									Linoleic acid		
6	1 mM	0.30	—	—	0.27	0.40	0.46	5.1	2.2 µM	3.6 µM	4.4 µM
									β-carotene		
7	1 mM	0.30	9.34 µM	—	0.27	0.40	0.46	6	0.14 µM	2.0 µM	1.9 µM
								β-carotene per linoleic acid turnover	6.4%	55%	43%
									Crocin		
8	1 mM	0.30	—	9.34	0.27	0.40	0.46	7.1	0.075 µM	0.646 µM	0.634 µM
								Crocin per linoleic acid turnover	3.3%	17.8%	14.3%

related to the linoleic acid oxidation in the lipoxygenase catalysis (Nr. 6, Table 3). The alkaline lipoxygenase L-1 destroys β-carotene 9 times more slowly than the L-2. It reaches about 6% of the linoleic acid oxidation rate. By comparison with the hydrophobic β-carotene, the water soluble polyene glycoside crocin was bleached more slowly by pH-6.5-lipoxygenase L-2 and by L-3 (No. 8 in Table 3). The crocin bleaching rates by these enzymes lie below 20% (relative to the lipoxygenase catalysis in Experiment No. 6, Table 3).

The different activities of the enzymes towards β-carotene and crocin correspond to the difference in the substrate specificity towards linoleic acid and to methyl linoleate (see Table 1); as reported by Christopher *et al.* [2, 3] and Verhue and Francke [4]. L-2 and L-3, which can oxidise not only the free acid but also the hydrophobic ester, also very quickly destroy the hydrophobic β-carotene.

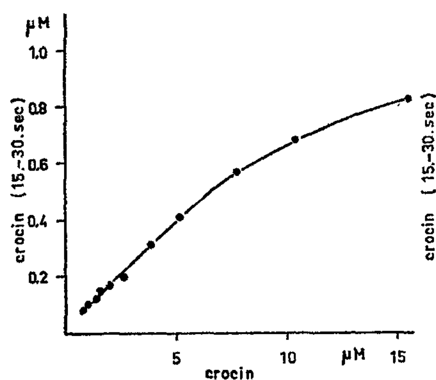
Teng and Smith [12] have shown that lipoxygenases form peroxyl radicals from linoleic acid and oxygen. These radicals can co-oxidise other substances occuring in the reaction system. The high co-oxidation potential of the pH-6.5-lipoxygenase can be explained by a special ability to develop peroxyl radicals. Additionally it is possible that L-2 and L-3 can orientate the β-carotene near the active site in such a way that the peroxyl radicals formed at the active site (or other reactive intermediates) could directly attack the polyene.
Crocin with its large carbohydrate residues cannot come so closely into contact with this hydrophobic area as can β-carotene. Therefore the bleaching rate decreases if β-carotene is substituted by water soluble crocin.

Kinetic Patterns of the Crocin Destruction

The rate of the carotene bleaching can only be measured when the polyene is emulsified by Tween 80; however, high concentrations of Tween 80 inhibit the pH-6.5-lipoxygenases (Fig. 3). Therefore kinetic measurements of the carotene destruction are only possible for a very small concentration range of the polyene. With water-soluble crocin, which needs no detergent, we can study the co-oxidation kinetic in the presence of the Tween 80 at the low concentration necessary for emulsifying the linoleic acid. The dependence of the crocin bleaching on the lipoxygenase concentration was first examined (Fig. 4). As Christopher *et al.* [3] have reported for L-3, no rectilinear relationship was observed between the quantity of lipoxygenase taken for assay and the rate of crocin destruction. Analogous results for the influence of crocin on the co-oxidation rate are plotted in Fig. 5. Increased crocin concentrations increased the bleaching velocity. Under the chosen conditions the rate of reaction is first order with respect to crocin in the range 1 to 6 μM crocin. Above 6 μM crocin the influence of the polyene concentration on the reaction rate decreases. Probably the first step of the crocin bleaching, the lipoxygenase catalysis, becomes more and more the rate limiting reaction.

Of special interest, however, is the influence of the linoleic acid concentration on the crocin bleaching. Unfortunately in the direct spectrophotometric assay we cannot determine the dependence of the reaction rate upon the linoleic acid concentration in the presence of excess crocin. In order to get an insight, the crocin co-oxidation was measured at two concentrations of crocin. The results were plotted (Fig. 6) together with a lipoxygenase kinetic taken under the same conditions. Fig. 6 shows that the linoleic acid oxidation and the crocin bleaching increase in the same way with increasing linoleic acid concentrations. The range in which the lipoxygenase linoleic acid oxidation kinetic follows the same course as the crocin bleaching kinetic is greater the higher the crocin concentration. In this range it is evident that certain reaction steps of the lipoxygenase catalysis initiate the crocin destruction and thus determine its reaction rate. At higher linoleic acid concentrations this relationship is not so clearly seen, since the crocin bleaching rate is braked by the low crocin concentration.

At concentrations lower than 0.4 mM linoleic acid, the lipoxygenase catalysis and the crocin destruction are both inhibited (Fig. 6). Experiments on the relationship of the kinetic of pH-6.5-lipoxygenases to the ratio linoleic acid/Tween 80 show that the inhibition comes from the Tween 80 (Fig. 7). When the Tween 80 proportion is smaller then the sigmoidal behaviour changes to a Michaelis-Menten type kinetic. The sigmoidal behaviour in the presence of Tween characterises the 6.5-lipoxygenases, for Ben-Aziz *et al.* [13] reported only the Michaelis-Menten type kinetic for the pH-9-enzyme at different Tween concentrations.



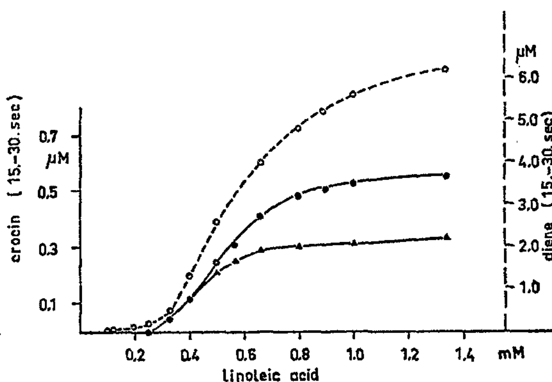


Fig. 5

Fig. 6

Fig. 5. Crocin co-oxidation as a function of crocin concentration. Test 7.1 at pH 6.5 was carried out with 8.1 µg/ml protein (ASP); 1 mM linoleic acid

Fig. 6. Crocin co-oxidation as a function of linoleic acid concentration. Test 7.1 at pH 6.5 was carried out with 8.5 µg/ml protein (ASP) and two different crocin concentrations: ▲—▲ 4.73 µM crocin; ●—● 9.45 µM crocin; ○---○ turnover of linoleic acid as function of linoleic acid concentration. Test 5.1 at pH 6.5 was carried out with 8.5 µg/ml protein (ASP)

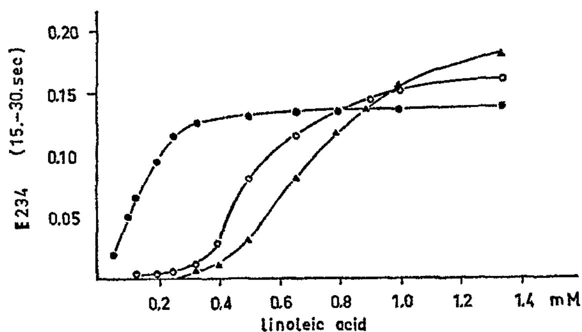


Fig. 7. Linoleic acid oxidation at pH 6.5 as a function of linoleic acid and Tween 80 concentration. Test 5.1 (see "Experimental") was carried out with 10 µg/ml protein (ASP). Ratio: linoleic acid per Tween 80: ●—● 2.5 (µl/µl); ○—○ 1.0 (µl/µl); ▲—▲ 0.85 (µl/µl)

Crocin Bleaching in the Presence of Linoleyl Sulphate

To eliminate the influence of the detergent on the lipoxygenase Allen [7] substituted the linoleic acid with water soluble linoleyl sulphate. With this substrate the crocin destruction could be followed without Tween 80. To test this possibility the lipoxygenase activities of L-1 and L-3 towards linoleyl sulphate were measured. It was surprising that the reaction rates of the two lipoxygenases reached a maximum

with increasing enzyme concentrations (Fig. 8). Probably the lipoxygenases associate totally unspecifically with the linoleyl sulphate molecules. In this way the substrate concentration decreases with increasing protein concentration. This results in a slow-

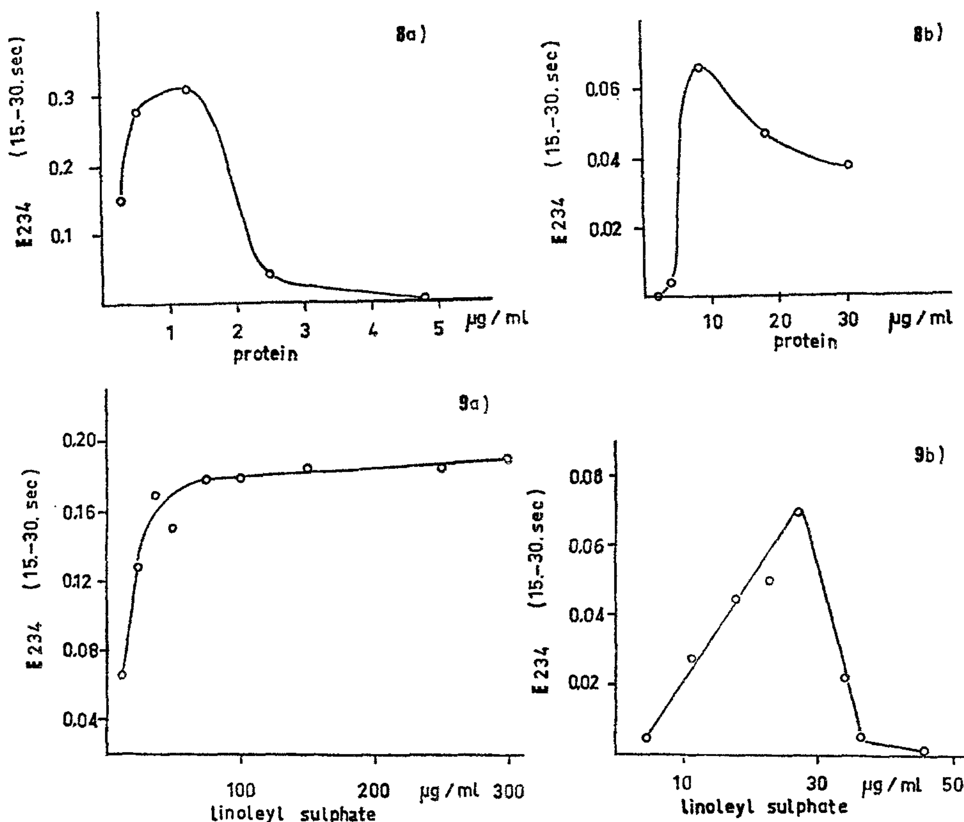


Fig. 8. Lipoxygenase catalysis with linoleyl sulphate as substrate: Influence of the protein concentration. a Test 5.3 at pH 9.0 with L-1 (50 µg/ml linoleyl sulphate), b Test 5.3 at pH 6.5 with L-3 (25 µg/ml linoleyl sulphate)

Fig. 9. Lipoxygenase catalysis: Variation of the linoleyl sulphate concentration. a Test 5.3 at pH 9.0 with 0.27 µg/ml L-1, b Test 5.3 at pH 6.5 with 8.3 µg/ml L-3

Table 4. Co-oxidation of crocin in the presence of linoleyl sulphate

Reaction system				Test-No. in „Experimental"	Turnover (15.—30. sec)	
Linoleyl sulphate	Crocin	Protein (µg/ml)				
		L-1	L-3		L-1 pH 9.0	L-3 pH 6.5
					Linoleyl sulphate	
174 µM	—	—	8.4	5.3	—	2.8 µM
216 µM	—	0,27	—	5.3	6.8 µM	—
					Crocin	
174 µM	9.34 µM	—	8.4	7.2	—	0,671 µM
216 µM	9.34 µM	0.27	—	7.2	0.283 µM	—
		Crocin per linoleyl sulphate turnover			4.2%	24%

ing down of the reaction. Although only semi purified enzymes were used in these tests, it was nevertheless clear that high concentrations of L-3 are, in comparison to L-1, needed for maximum activity towards linoleyl sulphate (Fig. 8). A varying concentration of linoleyl sulphate results in differences between L-1 and L-3 (Fig. 9). Only the activity of the L-3 is inhibited by the substrate.

Together, these kinetic patterns show that it is extremely problematic to substitute linoleic acid/Tween with linoleyl sulphate. Thus we have measured the crocin co-oxidation in the water-soluble system linoleyl sulphate/crocin under the previously determined best conditions and plotted the linoleyl sulphate turnover against this (Table 4). This experiment also showed that the pH-6.5-lipoxygenase L-3 is 7 times as active as the pH-9-lipoxygenase L-1 for polyene destruction. These experiments show that the differences between L-1 and L-3 activities in the polyene destruction stay the same when the hydrophobic system carotene/linoleic acid is substituted by the water soluble system crocin/linoleyl sulphate. The particular efficiency of the pH-6.5-lipoxygenases in forming reactive intermediates that can co-oxidise other substances (polyenes, chlorophyll etc.) is clearly shown.

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PD Dr. W. Grosch
Deutsche Forschungsanstalt
für Lebensmittelchemie
D-8000 München 40, Leopoldstr. 175
Bundesrepublik Deutschland