

Immobilization of hydroperoxide lyase from *Chlorella*

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Hydroperoxide lyase (HPLS) isolated from microalgae is an enzyme that oxidatively cleaves 13-hydroperoxy-*cis*-9-*trans*-11-octadecadienoic acid to a C₁₃ oxocarboxylic acid and a small C₅ fragment. Acetone powder extracts from *Chlorella pyrenoidosa* and *C. fusca* that contained HPLS were partly purified by chromatography on DEAE-Sepharose CL-6B. Five commercially available gels were evaluated for their ability to immobilize the HPLS preparations by determining their capacity for protein binding and the activity and stability of immobilized HPLS. It was found that Reacti-Gel (6X) and Affi-Gels 10, 15, 102 and 501 could bind 60–90% of the available protein. However, HPLS activity was detected only when the enzyme was immobilized on Affi-Gels 10, 15 and 501. The stability of immobilized HPLS during storage at 5 °C for several months was determined. Product yields with repeated use of the immobilized preparations were also determined. These measurements demonstrated that Affi-Gel 10 and 501 are the best gels for the immobilization of HPLS. A pH study of HPLS immobilized on Affi-Gel 501 showed that enzymic activity was retained from pH 6 to pH 9, with maximal activity at pH 6.5.

Introduction

Hydroperoxide lyase (HPLS)² is an enzyme of lipid metabolism that cleaves hydroperoxides of certain polyunsaturated fatty acids to oxocarboxylic acids. There are two broad classes of HPLS with different substrate specificities. A membrane-associated HPLS is found in higher plants and catalyses the cleavage of fatty acid hydroperoxides at the carbon-carbon bond adjacent to the hydroperoxide function and proximal to the carboxy carbon [1]. A water-soluble HPLS isolated from microalgae, mushroom and one species of higher plant cleaves fatty acid hydroperoxides at the carbon-carbon bond distal to the carboxylate and adjacent to the hydroperoxide functionality [1]. Thus HPLS from the microalgae *Chlorella*

and *Oscillatoria* cleaves 13-hydroperoxy-*cis*-9-*trans*-11-octa-decadienoic acid (HPOD) to 13-oxo-*cis*-9-*trans*-11-tri-decadienoic acid and a C₅ fragment [2,3]. We recently reported an improved extraction method for obtaining HPLS from the algae *Chlorella pyrenoidosa* and *C. fusca* [4]. By using this method, HPLS recovery was increased 5-fold compared with methods previously reported [2,3].

The C₁₃ oxocarboxylic acid obtained from the sequential action of lipoyxygenase (LOX; linoleate:oxygen 13-oxidoreductase, EC 1.13.11.12) and HPLS on linoleic acid (LA) can be further oxidized to the corresponding dicarboxylic acid. This diacid product could be used as a starting material for the production of a higher polyamide similar to nylon 13,13, a polymer that has excellent dimensional stability, a low affinity for moisture, a high dielectric constant and desirable melting properties [5]. Moreover, the double bonds in the dicarboxylic acid obtained from enzymically transformed LA would allow further cross-linking or functionalization of the polymer. Currently one C₁₃ dicarboxylic acid, brassylic acid, is obtained by the ozonolysis of erucic acid. However, this process was considered to be too expensive to compete with those of other industrially produced dicarboxylic acids [5].

To facilitate the reuse of HPLS, ways of immobilizing this enzyme are needed. Although there are a variety of published methods for the immobilization of LOX [6–13], there are no reports about the immobilization of HPLS. In this paper a number of commercially available cross-linked agarose gels were evaluated for their potential to produce an enzymically active immobilized HPLS preparation.

Materials and methods

Materials

Soybean LOX type 1-B, yeast alcohol dehydrogenase (ADH), LA, NADH and DEAE-Sepharose CL-6B were pur-

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² Abbreviations used: ADH, alcohol dehydrogenase; HPLS, hydroperoxide lyase; HPOD, 13-hydroperoxy-*cis*-9-*trans*-11-octadecadienoic acid; LA, linoleic acid; LOX, lipoyxygenase.

chased from Sigma (St. Louis, MO, U.S.A.). Affi-Gels 10, 15, 102 and 501 were obtained from Bio-Rad Laboratories (Hercules, CA, U.S.A.). Reacti-gel (6X) was from Pierce (Rockford, IL, U.S.A.). Water was purified to a resistance of 18 M Ω ·cm with a Barnstead (Dubuque, IA, U.S.A.) NANOpure system. All other reagents were of the highest purity available.

Preparation of HPOD

HPOD was obtained by adding four 100 μ l aliquots of LOX (10 mg/ml) at 30 min intervals over a 2 h period to a solution of 40 mg of LA in 50 ml of borate buffer (pH 9.0) at 0 °C, with controlled constant oxygen bubbling at 5 ml/min. The mixture was acidified to pH 3.0 with 1.0 M HCl and extracted three times with 5 ml of diethyl ether. The combined ether layers were dried over anhydrous sodium sulphate and filtered, and then the ether was evaporated under a stream of nitrogen. The residue was redissolved in ethanol and analysed by TLC [7] and HPLC with a Waters MCL1 (Waters Co., Milford, MA, U.S.A.) instrument equipped with a UV detector and a Varex Mark III evaporative light-scattering detector, and a CHROMSPHER SI (200 mm \times 30 mm) column (Chrom-pack, Raritan, NJ, U.S.A.) run isocratically with hexane/isopropanol (98:2, v/v). Results indicated that the conversion into the hydroperoxide exceeded 90%.

Growth of algae and isolation of HPLS

Chlorella pyrenoidosa (strain 211/8b) was obtained from the American Type Culture Collection. *C. fusca* was obtained from the Culture Collection at UTEX (Austin, TX, U.S.A.). Cells were grown in a 4-litre flask containing 2 litres of growing medium, as reported by Sorokin and Krauss [14]. Air containing 5% CO₂ was bubbled into the flasks (3–5 l/min), and the flasks were shaken at 125 cycles/min. The algae received 16 h of light at 25 °C and 8 h of darkness at 20 °C. Cultures were harvested after 4 days of growth in the late-exponential phase. The culture broth was centrifuged at 2000 g to yield 9–12 g of wet cells, which were washed with distilled water and lysed by the acetone-powder method [4]. Typically, the cells were pelleted at 12000 g and transferred to a blender containing 300 ml of acetone at –70 °C. After 5 min of blending the acetone was filtered; the filter cake was washed with cold acetone under nitrogen and dried *in vacuo*.

HPLS was extracted from the acetone powder by stirring for 5 min with 10–15 ml of potassium phosphate buffer, pH 7.0. Particulate matter was removed by centrifugation at 12000 g. The supernatant was loaded on a 15 cm \times 5 cm DEAE-Sepharose CL-6B column, and HPLS was eluted by isocratic elution with 50 mM potassium phosphate, pH 7.0, containing 15 mM NaCl. A total of 30–40 fractions (10 ml) was collected. Active fractions

were determined by conducting assays with ADH and NADH at 340 nm [15]; these were stored at –20 °C.

Immobilization of HPLS

Typically, 10–12 ml of gel, suspended in isopropanol or acetone, was placed in a 30 ml disposable polypropylene column fitted with a porous polyethylene disc (Bio-Rad, Richmond, CA, U.S.A.). The solvent was removed by vacuum without allowing the gel to dry completely, and the weight of the gel was determined. The gel was washed five times with 10 ml of cold water and stabilized at the desired pH by washing five times with 10 ml of buffer. The gel was transferred to a 250 ml polypropylene flask and then mixed with the semi-purified HPLS from DEAE chromatography [5 ml of HPLS (40–60 μ g of protein/ml) per g of gel].

Affi-Gels 10, 15 and 501 For these gels HPLS immobilization was performed at pH 7.0 in 50 mM phosphate buffer. The coupling mixture was shaken at 100 rev./min for 1.5 h at room temperature and afterwards transferred to a polypropylene column. The gel was washed three times with 5 ml of cold water. The remaining active sites of Affi-Gels 10 and 15 were blocked by reacting them with 10 ml of 1 M ethanolamine/HCl (pH 8.0) for 1 h. This last procedure was not necessary for Affi-Gel 501.

Affi-Gel 102 The procedure for this gel was similar to that described for the Affi-Gels above, except that the coupling was performed at pH 6.0 in 50 mM phosphate buffer. The DEAE extract was premixed with 10 mg of coupling reagent, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide/HCl (Bio-Rad, Richmond, CA, U.S.A.), and the pH was readjusted to 6.0 before mixing with the gel. The coupling time for this gel was 3 h at room temperature. After that, the gel was filtered and washed three times with 5 ml of cold water.

Reacti-Gel The coupling of HPLS with Reacti-gel (6X) was at pH 8.00 in 100 mM borate buffer. The immobilization procedure was conducted as described previously for LOX [7].

After immobilization and the washing cycle, all gels were washed three times with 10 ml of 50 mM phosphate buffer, pH 7.0, and stored at 4 °C in 50 mM phosphate buffer, pH 7.0, containing 0.02% NaN₃.

Affi-Gel 501 regeneration

The Affi-Gel 501 containing inactive immobilized HPLS was regenerated as follows. The gel was transferred to a polypropylene column and washed five times with 10 ml of 50 mM sodium acetate, pH 5.0, followed by washing five times with 10 ml of 4 mM mercuric acetate in sodium

acetate buffer to remove the bound protein. The gel was then washed five times with 10 ml of 50 mM sodium acetate buffer and equilibrated at pH 7.0. This was followed by five washes with 10 ml of phosphate buffer, pH 7.0, before the gel was re-used for immobilization.

Percentage of protein loaded on the gel

Protein concentration was determined with the Bio-Rad protein reagent. Albumin, globulin protein standard (Sigma, St. Louis, MO, U.S.A.) was used for calibration. Protein concentration was determined after chromatography, before incubation and after the coupling to the gel was completed. The gel was separated from the storage buffer and washed as described in the immobilization procedure. The volume of the combined storage buffer and wash was measured and the amount of recovered protein was determined. The percentage of protein loaded on the gel is calculated as the difference between the initial and final protein concentration, divided by the initial amount and multiplied by 100.

Assay of immobilized HPLS

The immobilized enzyme preparation (400 mg) was placed in a Pasteur pipette with a cotton filter at the end of the tip and washed four or five times with 2 ml of phosphate buffer until no UV absorption was observed for NaN_3 (above 240 nm) or free protein (280 nm). After removal of the washing buffer, the gel was mixed with 1.2 ml of 35 μM HPOD in 50 mM potassium phosphate buffer, pH 7.0. The ends of the pipette were closed and the mixture was shaken gently in a vertical rotatory shaker at 3 rev./min for 5 min. The HPOD solution was eluted through the cotton filter into a cuvette and the absorption at 234 nm was determined.

The percent yield was determined from:

$$\text{Yield (\%)} = 100(A_0 - A_t)/A_0$$

where A_t is the absorption after 5 min and A_0 is the absorption of a sample identically treated but in which the gel contained no immobilized HPLS.

Specific HPLS assays at 340 nm with yeast ADH and NADH were performed as described by Vick [15]. These assays demonstrated that the loss of absorption at 234 nm is due almost entirely to the action of HPLS on HPOD.

Results and discussion

The ability to obtain a stable enzyme preparation is crucial for potential biosynthetic applications. One method of increasing enzyme stability is to immobilize the enzyme on an appropriate support. There are a variety of commer-

cially available gels with different chemical properties: Figure 1 shows the structure of the reactive functional group that characterizes each gel chosen for this study. In addition, the far right column of Figure 1 shows the functional group in the protein that reacts with the gel functionality to form a stable cross-link.

Table I shows the results of the HPLS immobilization experiments with the gels listed in Figure 1. Although Reacti-Gel bound protein at pH 8.0, the resulting preparation had no HPLS activity. In experiments conducted at pH 7.0, Reacti-Gel bound less protein, and the immobilized preparation also had no HPLS activity. All of the Affi-Gels shown in Table I were able to bind protein from HPLS preparations. However, no HPLS activity was detected on Affi-Gel 102, and HPLS activity on Affi-Gel 15 was low. Moreover, the activity was lost after the Affi-Gel 15 had been used once. Better retention of HPLS activity was obtained by immobilization on Affi-Gel 10 and 501. With both of these gels, HPLS activity was retained at a higher level, even after two reaction cycles, with the HPLS isolated from *C. fusca*.

There are two advantages to the use of Affi-Gel 501 for the immobilization of HPLS: first, Affi-Gel 501 requires

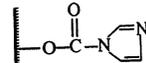
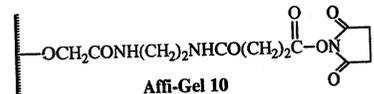
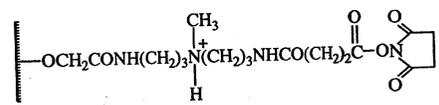
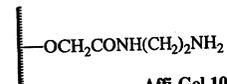
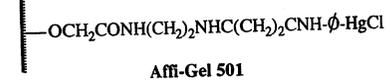
Gel Structure	Specificity
 <p>Reacti-Gel (6X)</p>	-NH ₂
 <p>Affi-Gel 10</p>	-NH ₂
 <p>Affi-Gel 15</p>	-NH ₂
 <p>Affi-Gel 102</p>	-COOH
 <p>Affi-Gel 501</p>	-SH

Figure 1

Schematic representation of the tested gels showing the ligand covalently bonded to the cross-linked agarose matrix and its protein functional group specificity. The coupling reagent was 1-ethyl-3-(3-dimethylamino-propyl)carbodi-imide/HCl (EDAC).

Table 1 Comparison of the capacities of commercial cross-linked agarose gels to couple covalently with HPLS from *Chlorella*

The entries correspond to experiments with different batches of immobilized HPLS.

Gel	Species	Protein immobilized (%) ^a	Yield (%) ^{ab}	
			1st cycle	2nd cycle
Reacti-Gel	<i>C. fusca</i>	56.7	0	—
Affi-Gel 10	<i>C. pyrenoidosa</i>	83.2 ^c	55.3	—
		79.5	29.7	5.7
	<i>C. fusca</i>	79.4 ^b	56.7	6.4
		63.4	53.4	51.1
Affi-Gel 15	<i>C. pyrenoidosa</i>	61.0	69.2	48.5
	<i>C. fusca</i>	82.2 ^c	21.7	64.4
		59.6 ^d	32.9	4.9
Affi-Gel 102	<i>C. pyrenoidosa</i>	82.7 ^c	0	10.6
	<i>C. fusca</i>	61.2 ^d	0	—
Affi-Gel 501	<i>C. pyrenoidosa</i>	87.2 ^c	0	—
		82.1 ^d	67.1	21.5
	<i>C. fusca</i>	89.7	59.0	65.0
		86.2 ^e	60.9	58.9
			65.6	66.0

^aThe protein load on the gel was determined using the method described in the Materials and methods section.

^bThe percentage yield was determined with 400 mg of gel at pH 7.0 in phosphate buffer as described in the Materials and methods section.

^cThe protein concentration for these preparations was 46 µg/ml.

^dThe protein concentration for these preparations was 25 µg/ml.

^eImmobilization was conducted with regenerated Affi-Gel 501.

no blocking of the unreacted active sites after HPLS immobilization; secondly, the reaction of thiol groups with the mercaptide-forming ligand of Affi-Gel 501 is reversible, and the gel can be regenerated by washing the protein off the gel with mercuric acetate. The regenerated gel gave an immobilized preparation that had an activity equivalent to that obtained with fresh Affi-Gel 501 as shown in the last entry of Table 1.

Table 2 gives the results of protein leakage studies during the storage of *C. fusca* HPLS immobilized on Affi-Gels 10 and 501 at 5 °C. For both immobilized preparations there was minimal protein loss (less than 2%)

Table 2 Results of a typical experiment to determine protein loss during gel storage at 5 °C

Abbreviation: n.d., not detected.

Days after coupling	Protein lost			
	Affi-Gel 10 ^a		Affi-gel 501 ^b	
	(µg) ^c	(%) ^d	(µg) ^c	(%) ^d
10	4.4	0.15	28.1	0.95
40	10.1	0.27	32.1	0.98
70	n.d.		n.d.	

^aAffi-Gel 10 (10.5 g) containing 3.7 mg of protein from *C. fusca*.

^bAffi-Gel 501 (9.3 g) of containing 3.3 mg of protein from *C. fusca*.

^cProtein in water rinses of the immobilized gel after three washes with 5 ml of phosphate buffer, pH 7.0.

^dBased on total protein in the gel.

during the first 40 days. After this time no further leakage of protein into the storage buffer was detected.

Table 3 gives the relative activity of *C. fusca* HPLS immobilized on Affi-Gels 10 and 501 after recycling the preparations several times. HPLS immobilized on Affi-Gel 501 could be used with little loss of activity for up to five cycles in phosphate buffer at pH 7.0. After five cycles this preparation began to lose activity. After the seventh cycle the gel retained only 11% of its original activity. The Affi-Gel 10 preparation had a lower capacity for recycling, losing 85% of its original activity after only five cycles.

The stability of HPLS immobilized on Affi-Gels 10 and 501 was determined when the preparations were stored at 5 °C. Figure 2 shows a plot of the percentage yield of

Table 3 Change in relative activity of immobilized HPLS from *C. fusca* with re-use

Assays were performed with 600 mg of Affi-Gel containing 354 µg of protein per g of gel and 1.2 ml of 35 µM HPOD, as described in the Materials and methods section. Data are the means ± S.E.M. for three repetitions.

Cycle number	Relative activity (%)	
	Affi-Gel 10	Affi-Gel 501
1	100	100
2	97 ± 3	99 ± 1
3	76 ± 6	99 ± 1
4	31 ± 7	98 ± 2
5	12 ± 4	97 ± 1
6	—	56 ± 9
7	—	13 ± 2

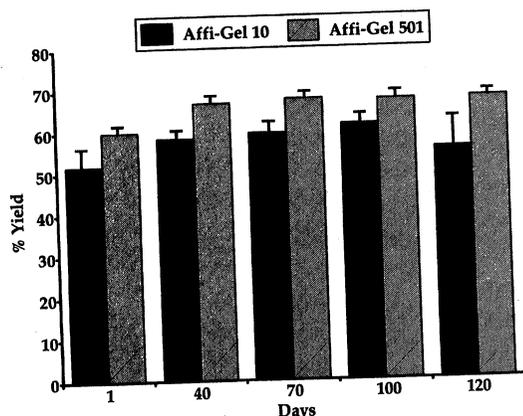


Figure 2

Influence of storage time at 4°C on the percentage yield of oxo-product with HPLS from *C. fusca* immobilized on Affi-Gels 10 and 501. The immobilized HPLS preparations were stored in 50 mM phosphate buffer, pH 7.0, containing 0.02% Na₂S₂O₃. The HPLS concentrations were 0.32 mg of protein per g of Affi-Gel 10, and 0.35 mg of protein per g of Affi-Gel 501. Each data point was determined with 0.4 g of the corresponding gel and 1.2 ml of 35 μM HPOD. The data are the means ± S.E.M. for two determinations.

product after HPLS was stored for up to 4 months. The results show that there was no detected loss of activity over this period.

Figure 3 shows the pH profile of HPLS from *C. fusca* after immobilization on Affi-Gel 501. There was a significant loss of activity below pH 5. HPLS activity is nearly constant in the pH range 6.0–9.0, although the best activity was observed at pH 6.5. This result is consistent with an earlier study of HPLS from the alga *Oscillatoria*,

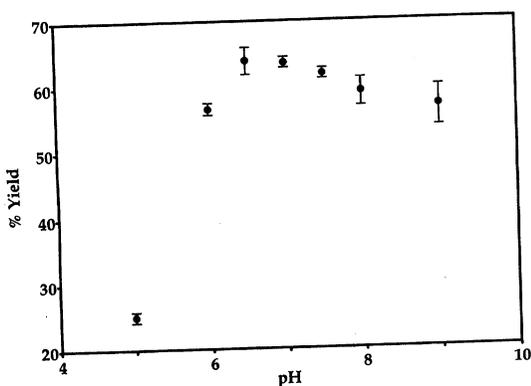


Figure 3

Influence of pH on the activity of HPLS from *C. fusca* immobilized on Affi-Gel 501 (0.35 mg of protein per g of gel) with an equimolar mixture of 100 mM citrate, phosphate and borate/HCl buffers. Each assay was conducted with 0.4 g of gel containing 0.13 mg of protein. The data are the means ± S.E.M. for at least three determinations.

which showed maximal activity at pH 6.4 [3]. The solubility of the HPOD is very low at below pH 6; the detergent Tween-20 (approx. 0.07%, v/v) was used to solubilize the substrate at pH 5.0. However, even in the presence of Tween-20 little HPLS activity was observed at pH 5.0. A control experiment using Tween-20 at pH 7 demonstrated that Tween-20 does not have an inhibitory effect on HPLS activity. The pH study was not extended above pH 9.0, because Affi-Gel released material at higher pH that has UV absorption in the region where HPOD absorbed, interfering with the determination of the product yield. The fact that the immobilized HPLS has good activity at pH 8 and pH 9 is important, because LOX has its best activity in this pH range. Accordingly this observation is relevant in the design of a bioreactor involving both enzymes.

The results of this study indicate that Affi-Gels 10 and 501 are excellent supports for immobilizing HPLS from *C. fusca* compared with the other gels tested. Affi-Gel 501 shows the best capacity for recycling and requires only a single-step procedure for immobilization of HPLS. In addition the spent gel can be regenerated. Our focus now is to study the capacity of this gel to immobilize other important lipid-modifying enzymes.

Acknowledgements

Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of similar nature not mentioned.

References

- Gardner, H.W. (1991) *Biochim. Biophys. Acta* **1084**, 221–239
- Vick, B.A. and Zimmermand, D.C. (1989) *Plant Physiol.* **90**, 125–132
- Andrianarison, R.H., Beneytout, J.L. and Tixier, M. (1989) *Plant Physiol.* **91**, 1280–1287
- Núñez, A., Foglia, T.A. and Piazza, G.J. (1995) *Biotechnol. Techn.* **9**, 613–616
- Van Dyme, D.L. and Blase, M.G. (1990) *Biotechnol. Prog.* **6**, 273–276
- Piazza, G., Brower, D.P. and Parra-Diaz, D. (1994) *Biotechnol. Appl. Biochem.* **19**, 243–252
- Parra-Diaz, D., Brower, D.P., Medina, M.B. and Piazza, G.J. (1993) *Biotechnol. Appl. Biochem.* **18**, 359–362
- Battu, S., Cook-Moreau, J. and Beneytout, J.L. (1994) *Biochim. Biophys. Acta* **1211**, 270–276
- Cuperus, F.P., Kramer, G.F.H., Derksen, T.P. and Bouwer, S.T. (1995) *Catalysis Today* **25**, 441–445

- 10 Battu, S., Rabinovitch-Chable, H. and Beneytout, J.L. (1994) *J. Agric. Food Chem.* **42**, 2115–2112
- 11 Maguire, N.M., Mahon, M.F., Molloy, K.C., Read, G., Roberts, S.M. and Sik, V. (1991) *J. Chem. Soc. Perkin Trans. I*, 2054–2056
- 12 Laakso, S. (1982) *Lipids* **17**, 667–671
- 13 Hearn, M.T.W., Harris, E.L., Bethell, G.S., Hancock, W.S. and Ayers, J.A. (1981) *J. Chromatogr.* **218**, 509–518
- 14 Sorokin, C. and Krauss, R.W. (1958) *Plant Physiol.* **33**, 109–113
- 15 Vick, B.A. (1991) *Lipids* **76**, 315–320

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