

IMMOBILIZATION OF HYDROPEROXIDE LYASE FROM CHLORELLA FOR THE PRODUCTION OF C13 OXO-CARBOXYLIC ACID

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1. Introduction

The hydroperoxide, 13-(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (HPOD), obtained from the lipoxygenase (LOX, EC 1.13.11.12) catalyzed reaction of oxygen with linoleic acid (LA), can be cleaved enzymatically to an oxo-carboxylic acid by hydroperoxide lyase (HPLS). Depending upon the source of HPLS, the enzymatic cleavage of (HPOD) produces either a C12 or C13 oxo-carboxylic acid, and a C6 or C5 fragment respectively. Membrane-bound HPLS from higher plants produces the C12 oxo-carboxylic acid (1), whereas the water soluble HPLS from the unicellular algae *Chlorella* and *Oscillatoria* sp. gives the C13 acid (2,3). The C13 oxo-carboxylic acid can be chemically converted to a C13 dicarboxylic acid. This dicarboxylic acid can be used to produce a polyamide similar to nylon 13,13, a polymer that has excellent dimensional stability, low affinity for moisture, high dielectric constant and good melting properties (4).

To facilitate the reuse of HPLS, ways of immobilizing this enzyme are needed. While there are a variety of published methods for the immobilization of LOX (5-7), there are no reports concerning the immobilization of HPLS.

2. Result and Discussion

Because HPLS from algae is water soluble rather than membrane-bound as in higher plants, algae were considered attractive sources for obtaining HPLS. Acetone powder extracts from *Chlorella pyrenoidosa* and *fusca* that contained HPLS were partially purified by chromatography on DEAE Sepharose CL-6B (8). Five commercially available gels were evaluated for their ability to immobilize the purified HPLS preparations (Table 1). Enzyme assays were performed by following the decrease in absorption at 234 nm (9). This method is not specific to HPLS, because it does not differentiate between HPLS and hydroperoxide dehydrase (HD), which will also cause a decrease in absorbance at 234 nm. A specific spectrophotometric assay for HPLS based upon the ability of yeast alcohol dehydrogenase (ADH) to reduce aldehydes to alcohols in the presence of NADH has been reported by Vick (9). Initial rates, measured at 234 nm, were comparable with those determined with ADH and NADH at 340 nm, indicating that HD was not contaminating our HPLS preparations.

The enzyme activity is represented by the percent yield of product in phosphate buffer at pH 7.0 according to Equation (1), where A_t is the absorption after 5 min, and A_0 is the absorption of a sample identically treated with gel that contained no immobilized HPLS.

$$\text{Yield \%} = \frac{(A_0 - A_t)100}{A_0} \quad (1)$$

It was found that Reacti-Gel (6X), Affi-Gel 10, 15, 102 and 501 could bind 60-90% of the available protein. However, HPLS activity was only detected when the enzyme was immobilized on Affi-Gels 10, 15 and 501 (Table 1). Moreover, the activity was lost after the Affi-Gel 15 was used one time. Better retention of HPLS activity was obtained by immobilization on Affi-Gel 10 and 501. HPLS activity was retained at a higher level with both of these gels, even after two reaction cycles, with HPLS from *C. fusca*.

The stability of immobilized HPLS (on Affi-Gel 10 and 501) during storage at 4 °C for several months was determined. Fig. 1 shows a plot of the percent yield of product after HPLS was stored up to 4 months. The data show that there was no detected loss of activity over this period.

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

Gel	<i>Chlorella</i> Sp	% Protein immobilized	Yield %	
			1st Cycle	2nd Cycle
Reacti-Gel	<i>Fusca</i>	56.7	0	---
Affi-Gel 10	<i>Pyrenoidosa</i>	83.2	55.3	5.7
	<i>Fusca</i>	79.4	56.7	51.1
Affi-Gel 15	<i>Pyrenoidosa</i>	82.2	21.7	4.9
	<i>Fusca</i>	59.6	32.9	10.6
Affi-Gel 102	<i>Pyrenoidosa</i>	82.7	0	---
	<i>Fusca</i>	61.2	0	---
Affi-Gel 501	<i>Pyrenoidosa</i>	87.2	67.1	21.5
	<i>Fusca</i>	82.1	59.0	65.0
	<i>Fusca</i> (immob. x 2)	86.2	65.6	66.0

Also, product yields with repeated use of the immobilized preparations were determined (Figure 2). HPLS immobilized on Affi-Gel 501 could be used with little loss of activity up to five cycles in phosphate buffer at pH 7.0. After five recycles, this preparation began to lose activity. After the seventh recycle 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 recycles.

Among the gel tested Affi-Gel 501 appears to be the most promising. In addition to its better capacity for recycling, the reaction of sulfhydryl groups with the

mercaptide-forming ligand of Affi-Gel 501 is reversible, and this gel can be regenerated by washing the protein off 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.

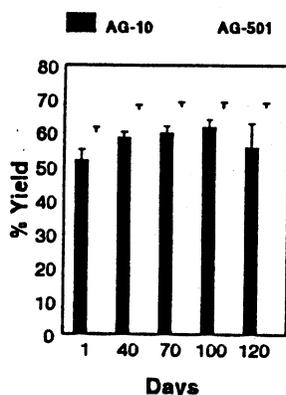


Fig. 1: Stability of HPLS from *C. fusca* storage at 4°C.

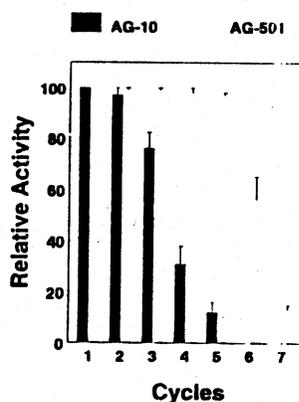


Fig. 2: Relative activity of immobilized HPLS from *C. fusca* with reuse.

Enzymatic activity for the immobilized preparation on Affi-Gel 501 was determined from pH 5 up to pH 9, with maximal activity at pH 6.5. These results are consistent with an earlier study of HPLS from the alga *Oscillatoria*, which showed maximal activity at pH 6.4 (3).

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casein. The weak β -casein band could have been due to the 3% FBS in the medium. Plasmins, which are present in serum, hydrolyze α_s -casein and β -casein but not k-casein (27). k-Casein holds the casein micelles containing α_s -casein and β -casein in colloidal suspension and protects them from precipitation by calcium ions (23,33). The appearance of k-casein in the medium suggests that the mechanism of micelle formation was functioning in both explant and primary cells.

Whey protein content of explant cells increased ($P < 0.006$) through 96 h while secretion of α -lactalbumin into the medium decreased ($P < 0.09$) after 48 h (Fig. 6). This suggests a breakdown in the secretion mechanism of explant cells. The primary cells maintained ($P > 0.10$) their cellular whey protein concentration but continued to increase ($P < 0.0001$) secretion through 72 h. The β -casein content (Fig. 7) of primary cells remained constant ($P > 0.10$) over the 96 h period while that of explants increased ($P < 0.0001$). The increase in whey and β -casein concentration within explant cells occurred simultaneously with the increases in DNA content discussed above (Fig. 1). Others (1,29,30) have observed an association of cellular DNA concentration with changes in protein synthesis. They suggested (1) that this association was due to amplification of specific genes.

Confluent primary cells contained 8.7% less ($P < 0.0001$) whey protein and secreted the same ($P > 0.40$) amount of α -lactalbumin compared to explant cells (Fig. 6). However, confluent primary cells contained 46.3% less ($P < 0.0001$) β -casein and secreted 42.8% less ($P < 0.004$) β -casein than explant cells (Fig. 7). Explant cells secreted less ($P < 0.03$) k-casein than confluent or growing primary cells particularly so during the first 24 h of incubation (Fig. 7).

Whey protein content of explants was depressed ($P < 0.04$) by bST, IGF-I, and bST + IGF-I compared to control (234, 238, 234, and 247 mean channel, respectively). A similar effect with bST and IGF-I on total milk protein synthesis was observed in an earlier experiment using explants from mid- to late-lactation cattle (21). In this experiment, hormones had no effect ($P > 0.10$) on protein content or release by primary cells, but others (14) reported an enhancement of protein synthesis by primary bovine mammary cells with IGF-I.

These results show that primary bovine mammary epithelial cells, grown to confluence on collagen, are similar to mammary tissue explants with respect to changes in DNA content with time, hormonal treatment, and the number of differentiated cells in the total cell population. Differences in whey protein content and secretion were small. Explant cells contained and secreted more β -casein than primary cells but secretion trends for β -casein and k-casein were similar after 48 h for both cell types. These results suggest that primary cell cultures are comparable to explant cultures when used to study mechanisms of DNA and milk protein synthesis and secretion.

ACKNOWLEDGMENTS

Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may be suitable. We thank Marsha Atkins for her excellent technical assistance.

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