

6. Deactivation of immobilized lipase in membrane reactors during continuous hydrolysis of oils containing peroxides

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SUMMARY

Continuous hydrolysis of oils can be accomplished enzymatically in an immobilized lipase membrane reactor. Because the feasibility of such processes is sensitive to enzyme costs, the present study was undertaken to examine the causes of enzyme deactivation in these systems. Thermostable lipase was prepared by fermentation of *Thermomyces lanuginosus* QM 225 and immobilized by simple adsorption to a hydrophilic microporous membrane. The immobilized lipase membrane reactor was operated continuously at 51°C by pumping olive oil or melted tallow through the membrane, and sweeping the partially hydrolyzed oil from the downstream side of the membrane with buffer recycled through a continuous oil/water separator. The stability of reactors as measured by half-life decreased from 40 days to 12 days when the peroxide value of the feed oil increased from 5 mEq/kg to 45 mEq/kg. These results demonstrate the importance of preventing formation of peroxides in oils fed to reactors containing lipase.

INTRODUCTION

World production of tallow, or rendered animal fat, currently ranks 5th among fats and oils behind soybean and palm oils and was surpassed only recently by increases in production of rapeseed and sunflower. U.S. annual production of about 7.4 billion pounds is about half of world production, and with exports of about 2.5 billion pounds per year, the U.S. dominates the international market for tallow. While the other fats and oils in the top ten in world production are used mostly for food, such is not the case with tallow, with only about 1/4 of U.S. production going to domestic human consumption. The highest value-added use of tallow is for the production of oleochemicals, most of which, with the exception of soap, require first the hydrolysis of the fat or oil to its constituent fatty acids and

glycerol (fat-splitting) [6].

In an effort to improve the value and utilization of tallow, we have been developing a continuous process for hydrolysis of tallow with an immobilized lipase [4, 5]. While initial applications of this technology are more likely to occur in the hydrolysis of highly unsaturated oils or in specialty chemicals where the stereospecificity or other specificity of the lipase-catalyzed hydrolysis is essential, we are hopeful that eventually, our process may be able to compete with bulk high-temperature fat-splitting. A critical factor in the commercial success of an immobilized enzyme is the stability of the reactor. Half-lives measured in months are typical and half-lives of years have been reported. In this paper, we show that loss of activity in an immobilized lipase reactor can be significantly correlated with the peroxide value of the fat or oil used to feed the reactor.

MATERIALS AND METHODS

Lipase was assayed by a flow-through pH-stat method [2]. Thermostable, non-specific lipase was obtained by 10-liter batch fermentation of *Thermomyces lanuginosus* QM 225 (NRRL, USDA) as previously described [1, 3]. The culture was harvested by filtration through a multifilament polypropylene cloth, 1 mm thick. The crude culture filtrate was centrifuged and the supernatant fluid was adjusted to pH 3.8 with HCl. The precipitated lipase was collected by centrifugation, neutralized with sodium bicarbonate and freeze-dried. As needed for immobilization, 1 mg/ml of the freeze-dried powder (about 200 IU/mg) was dissolved in 0.02 M trishydroxymethyl aminomethane (Tris) buffer, pH 8.0, with 0.04 M KCl, 0.01 % Triton X-100 (Rohm and Haas, Philadelphia, PA)* and 0.02% sodium azide, and centrifuged. Reagent grade chemicals were used except for sodium azide which was technical grade. The clear, dark brown supernatant fluid was adjusted to pH 5.5 with acetic acid and centrifuged.

Reactors contained double separator screens with silicone rubber gaskets (Millipore Pellicon system). Two filtrate screens were placed on one side and two retentate screens on the other side of a double layer of acrylic microporous membrane having maximum pore diameter 0.2 μm or 0.45 μm (Gelman Versapor, AP-200 or AP-450). Effective area of the double layer membrane was 220 cm^2 . The screens and membranes were pressed tightly together between two 1-inch thick stainless steel plates with 4 studs and with holes drilled to provide an inlet and an outlet to each side of the membrane. Reactors were deaerated with deaerated water, and loaded by recycling the clear, dark brown pH 5.5 supernatant fluid through the reactor with a peristaltic pump at about 2 ml/min for at least 16 h. About 100 IU/ cm^2 of effective membrane area was irreversibly adsorbed at pH 5.5. The unadsorbed enzyme solution was rinsed from the reactor with

0.04 M sodium acetate buffer, pH 5.5, with 0.02% sodium azide and the reactor was placed in a 29-ft³ chamber maintained at 51°C. The chamber also contained reservoirs of the same buffer and of oil, feed pumps for oil and buffer (about 0.04 g/min each), a pressure gauge, a continuous oil/water separator and a buffer recycle pump (about 1 g/min). The pumps and associated Teflon[®] tubing were configured so that a pressure of 3–7 psi forced the oil through the membrane. Buffer recycled from the oil/water separator swept the oil from the downstream side of the membrane [4, 5]. Fresh olive oil or edible tallow was kept under nitrogen to prevent formation of peroxides.

To measure fatty acid concentrations in the feed and product, 8 ml of 67% ethanol (95%)/33% hexane mixture were added to a weighed sample (about 1 g) in a 1-oz. vial. The resulting emulsion was titrated to pH 10.4 endpoint with 0.1 N NaOH. A saturated solution of KCl in methanol was used as the reference electrode filling solution to prevent clogging of the reference junction with insoluble soaps. Activity of each reactor was calculated from the difference between the feed and product fatty acid concentrations multiplied by the flow rate of oil. The initial activity and half-life for each reactor was determined from the zero-time intercept and slope of the semilogarithmic plot of the activity vs. time (simple first order model of deactivation). Reactors were operated at 51°C continuously until activity dropped to about 10 $\mu\text{mol}/\text{min}$. Reactors were washed with hot soapy water followed by 5%

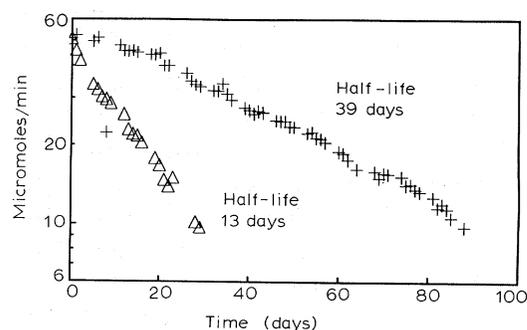


Fig. 1. Free fatty acid produced by reactors fed with fresh olive oil or artificially aged olive oil. +, fresh (peroxide value 5.1 mEq/kg); Δ , artificially aged (45 mEq/kg).

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

sulfuric acid, 5% KOH and water. The washing cycle was repeated until solutions leaving the reactor were clear, then the reactor was reloaded with lipase as before.

To measure the peroxide value, 30 ml of a mixture of 2 parts chloroform to 3 parts acetic acid (v/v) were added to a 5-g sample in a 250-ml flask. One ml of saturated potassium iodide solution was added and 1 min later 30 ml of water were added. The resulting emulsion was titrated immediately with 0.1 N sodium thiosulfate and starch indicator. Edible tallow (peroxide value 1.4 mEq/kg) was purchased as such and used immediately. High-peroxide tallow (36 mEq/kg) was obtained after storage for more than 6 months at room temperature of bleached inedible tallow. Fresh olive oil (Felippo Berio) was purchased at the local grocery store in 1-gallon cans. To artificially age olive oil it was stirred on a hot plate (50–70°C) and sparged with pure oxygen for 28 days. Artificial aging increased the peroxide value of olive oil from 5 mEq/kg to 45 mEq/kg). To remove peroxides from high-peroxide tallow it was sparged with SO₂ gas for about 15 min, or mixed with an equal volume of 1% sodium metabisulfite and centrifuged at 50°C to separate the emulsion. After removal of peroxides, the tallow was shaken at 52°C with buffered enzyme solution and the free fatty acids in samples taken after various times from 1 to 24 h were measured. In the case of SO₂, residual acid was neutralized with sodium hydroxide solution before enzyme was added.

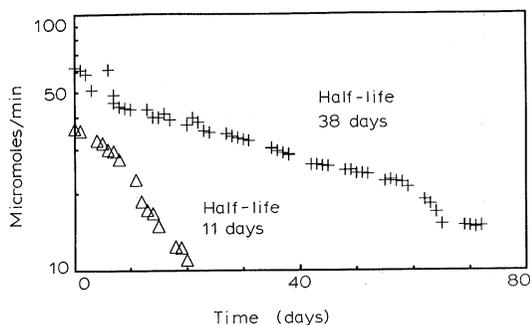


Fig. 2. Free fatty acid produced by reactors fed with edible tallow or high-peroxide tallow. +, edible (peroxide value 1.4 mEq/kg); Δ, high-peroxide (36 mEq/kg).

RESULTS AND DISCUSSION

To investigate the effect of peroxide value of oils hydrolyzed in immobilized enzyme reactors, data from continuous operation reactors fed with two different oils at two peroxide levels were obtained. In comparing new or acid/base-washed membranes, no significant differences in initial activity or half-life of reactors were observed. Initial activities of reactors fed with fresh or artificially aged olive oil were about 50 μmol/min (Fig. 1). Initial activities of reactors fed with edible tallow or high-peroxide tallow were also about 50 μmol/min (Fig. 2). Half-lives of reactors fed with fresh olive oil or edible tallow were about 40 days (Figs. 1, 2 and 3). Half-lives of reactors fed with artificially aged olive oil or high-peroxide tallow were about 12 days (Figs. 1, 2 and 3). The fact that results with tallow and olive oil were essentially the same shows that the effect is due to peroxides and not some other difference between edible and inedible tallow.

There was no detectable activity in emulsions of SO₂-treated tallow with lipase solution. Activity in emulsions of bisulfite-treated tallow with lipase solution was as good or better than in untreated controls. Emulsions made with bisulfite-treated tallow were more difficult to separate than those made with untreated tallow. An attempt to operate the membrane reactor with bisulfite-treated tallow was unsuccessful due to high pressure and poor separation in the continuous oil/water separator.

Peroxides form in oils as a result of the reaction of molecular oxygen with unsaturated fatty acids.

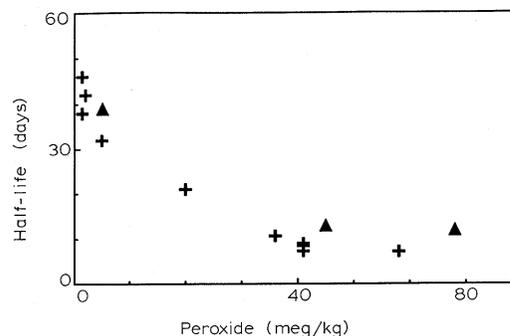


Fig. 3. Effect of peroxides on half-lives of immobilized lipase reactors. Δ, olive oil; +, tallow.

Peroxides are readily reduced and react with free sulfhydryl groups or disulfide bridges commonly found in most enzymes. A rough calculation indicates that with artificially aged olive oil, in every 8-h period, the moles of peroxides pumped through the reactor were in excess of the moles of sulfur in the total protein in the reactor by about 100-fold. Therefore, it was somewhat unexpected, first that the initial activities were the same with low or high peroxide oils, and second that the half-life was as long as 12 days with high peroxide oils. We believe that diffusion of reactants and products into and out of the membrane plays a role in limiting the rate of hydrolysis [4, 5], so perhaps the rate of deactivation by peroxides could be limited by diffusion. On the other hand, some other mechanism for reduction of peroxides may be operating in the system. Regardless of the origin or mechanism of action of peroxides, it is clear that to achieve economical continuous enzymatic fat-splitting with immobilized lipase, it will be essential to prevent their formation during the storage, handling and processing of the raw fat or oil.

ACKNOWLEDGEMENT

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