Pigment Removal from Canola Oil Using Chlorophyllase

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Frost-damaged or prematurely harvested canola seed (rapeseed) may yield oil with a high chlorophyll content (50-60 μg/ml). Enzymatic hydrolysis of chlorophyll, added to buffer/surfactant, buffer/acetone or buffer/acetone/canola oil, to produce water-soluble chlorophyllide (green pigment) was studied using a crude chlorophyllase preparation (acetone-dried chloroplasts) from 15 to 20-day-old sugar beet seedlings. In buffer/surfactant, the optimum pH for enzyme activity was temperature dependent. At 30 C and 0.24% Triton X-100 (or 30% acetone), chlorophyllase showed maximum activity toward a crude chlorophyll preparation over the range of pH 8-10. At 60 C, the activity was more than twofold higher, with a sharp maximum at ~pH 8. Mg^{2+} enhanced the activity with an optimal concentration of 50 mM. At pH 7.5, 50 C and in the presence of only 6% acetone, the enzyme showed high affinity for chlorophyll (Km = 15μM or 13.5 μg/ml), suggesting that the natural chlorophyll concentrations found in green canola oils might facilitate high enzymatic efficiencies. The crude enzyme was stable in buffer/acetone at pH 7.5 and 50 C for at least two hr.

With acetone concentrations as low as 6%, maximum enzyme activities in buffer and buffer/canola oil required intensive mixing (homogenization) of the various substrate, enzyme and liquid phases. In general, the rate and extent of chlorophyll hydrolysis were greater in buffer than in buffer/oil. In both reaction systems, chlorophyll hydrolysis slowed down with time due to accumulation of phytol, which proved to be a competitive inhibitor (K_i = 11 μM or 3.3 μg/ml). The other hydrolysis product, chlorophyllide, did not affect enzymatic activity.

Crude canola oil used in the reconstitution of green oil did not support enzymatic chlorophyll hydrolysis without prior degumming and desoaping. The optimum buffer/oil ratio of the reaction mixtures was above 2/1 (v/v).

Residual chlorophyll levels of up to 5 μg/ml are not uncommon in unbleached vegetable oils. Canola (rapeseed) oils, however, may contain up to 15-20 μg/ml under normal growing conditions (1), as the crop must be harvested prematurely to minimize pod shatter and prevent extensive seed loss. Adverse growing conditions (e.g. early frost) can damage canola seed to the extent that a significant increase in chlorophyll content of the oil may result (up to 50-60 μg/ml). The presence of high chlorophyll pigment levels is not only organoleptically unacceptable in a food product, but also reduces the oxidative stability of oils (2,3), as shown by the accelerated oxidation of methyl linoleate (4). Removal required large quantities of bleaching clays (5), resulting in high processing costs and in significant losses of oil through adherence to the clays. As an alternative to the adsorptive bleaching method, we propose the development of a biotechnological process involving chlorophyll hydrolysis by the enzyme chlorophyllase. The chromophore (green) moiety is rendered water soluble and extracted from the oil.

Chlorophyllase (chlorophyll-chlorophyllido-hydrolase, EC 3.1.1.14) is a ubiquitous plant enzyme which hydrolyzes chlorophyll and the related pheophytin into their component moieties chlorophyllide and pheophorbide, respectively, plus the isoprenoid alcohol phytol (6). The enzyme is a glycoprotein (7) of around 39,000 molecular weight (rye, tea, 8; sugar beet, 9) located in the thylakoid membrane of chloroplasts. The poor solubility of the enzyme and its substrate chlorophyll in aqueous solutions, precluding hydrolytic activity, is partially overcome by including up to 30% acetone (8) or surfactant (10) in reaction systems. Mg^{2+} (11) and some chloroplast lipids (12) have been identified as activators of enzyme activity.

In this paper, we present a study on the reaction conditions for enzymatic hydrolysis of chlorophyll added to buffer and oil. The concept that chlorophyllase can be used in an oil environment was established, and may constitute the first step toward a biocatalytic process of pigment removal.

MATERIALS AND METHODS

Plants. Sugar beet seedlings (*Beta vulgaris* var. Salohill) were grown under greenhouse conditions in sufficient quantities to yield 500-700 g of leaf material at the four-leaf stage (approximately 15-20 days of growth). Seeds were obtained from the Alberta Sugar Company, Taber, Canada.

Isolation of Chloroplasts. Crude chlorophyllase was prepared by isolating chloroplasts from the sugar beet leaves according to the method of Tanaka et al. (8), with some modifications. Batches of leaf material (500 g wet weight) were homogenized in 580 ml of 0.35 M NaCl for 2 min at 4 C using a Waring blender (model 33BL 12, Dynamic Corp. of America, New Hartford, Connecticut) at top speed. The homogenate was filtered through two layers of cheesecloth to remove the coarse debris. Chloroplasts were sedimented by centrifugation of the filtrate at 20,000 × g for 20 min, and washed three times by suspending in 200 ml of 80% (v/v) acetone (−15 C), followed by a final washing in 200 ml of hexane (−15 C). The washings removed pigments that would interfere with the chlorophyllase assay. Each time the chloroplasts were recovered by centrifugation (5000 × g, 4 C), and the final pellet was freeze dried and ground to a fine powder. The chloroplast preparation was stored at −15 C, and showed no loss in chlorophyllase activity for up to four months.

Preparation of chlorophyll. A crude chlorophyll sample was prepared from the above pooled 80% acetone extracts by dioxane precipitation according to the method of Iriyama et al. (13) and stored in the dark at −15 C. A qualitative evaluation of the chlorophyll

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precipitate by thin layer chromatography (TLC) indicated that it consisted predominantly of chlorophyll *a* and a minor amount of chlorophyll *b*. Stock solutions containing known concentrations of chlorophyll were prepared in acetone for use in enzyme reactions.

Quantitation of chlorophyll. Absorption spectra (400-700 nm) of the above described crude chlorophyll preparation and a purified chlorophyll *a* (U.S. Biochemical Corp., Cleveland, Ohio) dissolved in acetone/petroleum ether (b.p. 30-60 C) (1/4, v/v) were very similar, with maxima at 660 nm. Chlorophyll content of enzyme reaction samples was determined spectrophotometrically in petroleum ether extracts, using the absorbance coefficient (A = 0.092 μg/ml/cm) determined with the purified chlorophyll *a* at 660 nm. All concentrations are expressed as chlorophyll *a* equivalents (μg/ml).

Preparation of chlorophyllide. Chlorophyllide was produced by enzyme-catalyzed hydrolysis of crude chlorophyll in a 20-ml aqueous acetone system (2.5 mg enzyme/ml, 6% acetone). Reactions were stopped by the further addition of 20 ml acetone, and the residual chlorophyll was extracted with 30 ml petroleum ether. The precipitated enzyme was quantitatively removed by filtration of the aqueous-acetone phase (containing chlorophyllide) through a sintered glass filter. A total of 200 ml of the aqueous acetone phase was collected, concentrated to 40 ml by rotary evaporation and the residual water removed by freeze drying. The residue containing the chlorophyllide was redissolved in 10 ml of cold 80% acetone and stored in the dark at −15 C. Exposure of the solution to light during chlorophyllide recovery was kept to a minimum. The absorbance coefficient (A = 0.060 μg/ml/cm) of chlorophyllide in distilled water at 665 nm was determined with a known quantity of chlorophyll subjected to complete enzyme hydrolysis.

Oils. Crude unrefined and refined-bleached-deodorized (RBD) canola oil were obtained from CSP Foods Ltd., Saskatoon, Saskatchewan. Crude oil was subjected to a distilled water wash to remove phosphatides and proteinaceous materials, followed by treatment with 15% (w/v) NaOH to remove fatty acids. The water and NaOH to oil ratio was 1/9 (v/v). Both treatments were carried out at 60 C for 30 min with agitation. The resultant solids were removed by centrifugation. The semi-refined oil was stored at −15 C. Green oils were prepared by the addition of crude chlorophyll to oil, in most cases at 50-60 μg/ml, followed by heating to 60 C to evaporate the acetone added with the chlorophyll.

Phytol. A stock solution of 2.5 mg/ml phytol (Sigma Chemical Co., St. Louis, Missouri) in acetone was prepared and stored at −15 C.

Thin Layer Chromatography. TLC was carried out according to the method of Sievers and Hynninen (14). Samples were prepared by extracting chlorophyll from 2-ml enzyme reaction mixtures into 2 ml petroleum ether, leaving chlorophyllide behind. The two fractions were concentrated by evaporation and spotted at the base of 5 × 20 cm K2 cellulose plates (Whatman Chemical Separation Inc., Clifton, New Jersey) which had been sectioned previously into 9-mm wide strips. The mobile phase consisted of pyridine and petroleum ether (9/1, v/v). Chromatograms were developed until the solvent front had reached 17 cm (10-15 min), after

TABLE 1

Cellulose Thin Layer Chromatography of a Chlorophyllase Reaction Mixture, Fractionated by Petroleum Ether and Aqueous Acetone Liquid/Liquid Extraction

Sample	Rf	Color
Petroleum ether fraction	0.93	green
Aqueous acetone fraction	0.39[a]; 0.21[b]	green; lime-green
Chlorophyll *a* standard	0.93	blue-green
Crude chlorophyll	0.93; 0.86[c]	green; lime-green

[a]Identified as chlorophyllide *a*, based on spectrophotometric, chromatographic and chromatic information.

[b]Identified as chlorophyllide *b*, based on spectrophotometric, chromatographic and chromatic information.

[c]Identified as chlorophyll *b*, based on spectrophotometric, chromatographic and chromatic information.

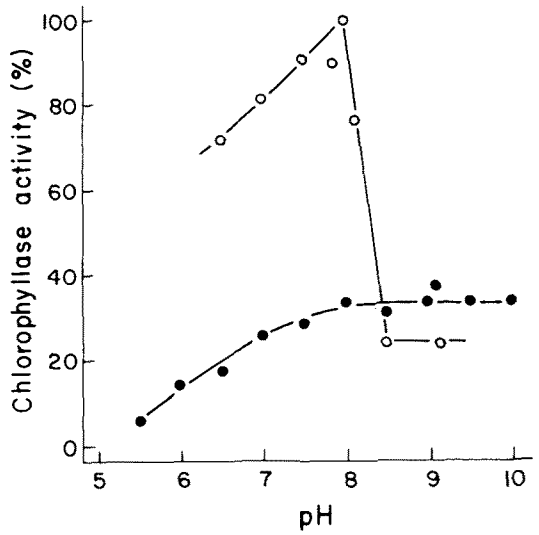

FIG. 1. Effect of pH on chlorophyllase activity at 30 and 60 C. Assays were carried out with 0.012 M sodium-acetate-phosphate-borate buffers containing 0.24% Triton X-100, 30 C (●) and 60 C (○).

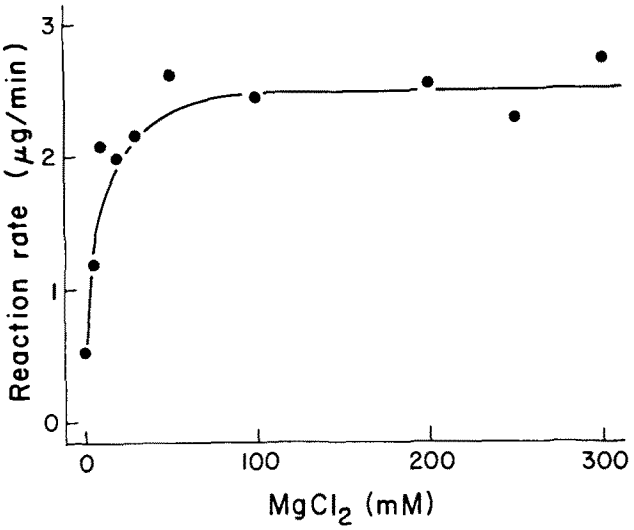

FIG. 2. Effect of Mg^{2+} on chlorophyllase activity. Assays were carried out with TRIS buffer containing 0.12% Triton X-100 at 60 C.

which they were examined under long wave U.V. light.

Chlorophyllase assay. Initial reactions were carried out in stoppered 16 × 125 mm test tubes containing 6 ml total volume of TRIS-HCl buffer (0.1 M, pH = 7.5), $MgCl_2$ (50 mM) plus 10 mg of chlorophyllase, 50-60 μg/ml chlorophyll and acetone (up to 30% v/v) or Triton X-100. Acetone proved to be the preferred solubilizing agent for chlorophyll because Triton X-100 caused excessive foaming and activity depression. The tubes were incubated in a heated water bath, normally at 50 C and agitated by a wrist action shaker. At regular time intervals, 1-ml aliquots were removed over a period of maximally 6 min, placed in ice and the reaction stopped by the addition of 4 ml acetone. Residual chlorophyll was extracted into 6 ml petroleum ether followed by centrifugation (5,000 × g, 5 min) to obtain a clean separation of the two solvent phases. The petroleum ether phase was recovered and its volume recorded for calculation of the dilution factor. Chlorophyllase activity (initial reaction rate) was determined by following the decline in chlorophyll concentration in the recovered petroleum ether fractions. Enzyme activity is expressed as μg chlorophyll hydrolyzed per min per mg chlorophyllase.

In later reactions, improved mixing of the substrate and enzyme phases was accomplished by homogenization, as described below for reactions that include an oil phase.

Chlorophyll hydrolysis in oil. Preliminary work on the hydrolysis of chlorophyll in canola oil was carried out using the above chlorophyllase assay, with the inclusion of an oil fraction. Unless otherwise indicated, RBD oil was used. The buffer to oil ratio varied among experiments. Reaction mixtures (20 ml) were incubated at 50 C with constant homogenization in a closed 100-ml chamber of a Sorvall Omni mixer (Sorvall Inc., Norwalk, Connecticut) at a rheostat setting of 50. For smaller reaction volumes (5 ml) of both oil and buffer systems, an Ultra-turrax homogenizer (Terochem, Edmonton, Canada) at a rheostat setting of 70 was used. The reaction chamber consisted of a 25 × 52 mm glass vial with the homogenizer probe inserted through a plastic cap and sealed with a rubber washer. The acetone concentration of the buffer phase was 6% (v/v) and the enzyme concentration varied from 1-2.5 mg/ml. Chlorophyll levels indicated in the text are based on the total reaction volume. Reactions were stopped by the addition of acetone (2 times the volume of the reaction mixture), and the chlorophyll extracted with petroleum ether (3 times the volume of the reaction mixture) for spectrophotometric quantitation as previously described.

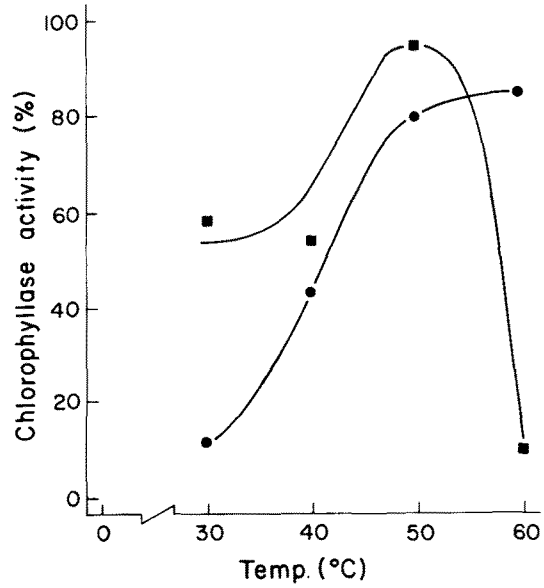

FIG. 3. Effect of temperature on chlorophyllase activity. Fixed-time 10-min assays were carried out with TRIS buffer containing 6% (●) and 30% (■) acetone.

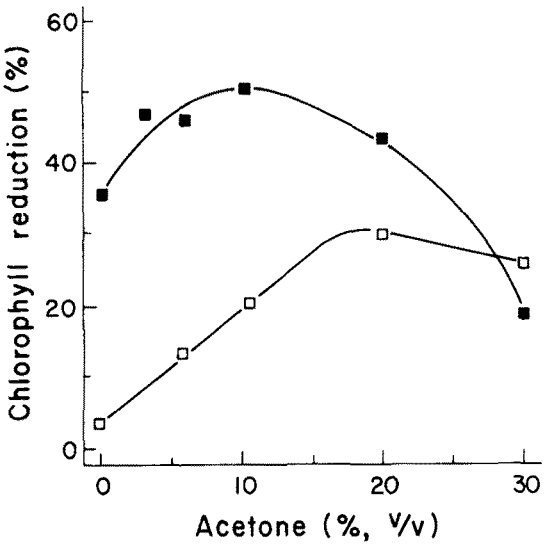

FIG. 4. Effect of acetone concentration on chlorophyll removal from oil. Reaction mixtures of buffer/RBD oil (1/1, v/v) containing 50 μg/ml chlorophyll were incubated with chlorophyllase at 50 C for 60 min in agitated test tubes (□) or in a homogenizer (■).

RESULTS AND DISCUSSION

Detection of enzymatic chlorophyll hydrolysis. Spectrophotometric monitoring of chlorophyll hydrolysis relies on the virtually complete separation of the hydrolysis product chlorophyllide from the substrate by liquid/liquid extraction with aqueous acetone and petroleum ether (10,15). Both liquid phases may be used (8,15), but we found the decrease of chlorophyll in the petroleum ether fraction a more reliable measure of enzyme activity. In addition, chlorophyll appears to be less sensitive to oxidation than chlorophyllide, obviating the need for antioxidants in the reaction mixture (8).

Chlorophyllide formation was qualitatively confirmed by the generation of an absorption maximum at 665 nm (compared to 660 nm for chlorophyll), as well as by the presence of a green pigment in the aqueous acetone phase. Examination of this liquid extraction phase by TLC showed that the pigment had much lower relative mobility than chlorophyll (Table 1).

Control experiments with heat-inactivated chlorophyllase showed very slow chlorophyll hydrolysis (Fig. 5), thus confirming the catalytic function of the biological preparation.

Source of chlorophyllase. In a limited screening, sugar beet leaves proved to contain higher levels of

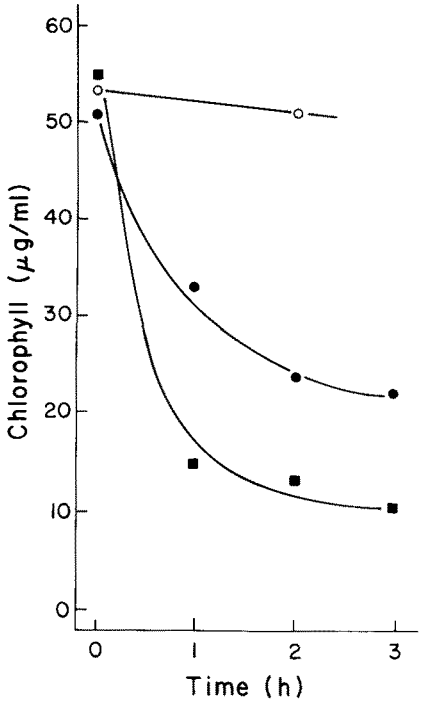

FIG. 5. Time course of chlorophyll hydrolysis in buffer and oil systems. Reactions were carried out with homogenization at 50 C in buffer using active (■) and heat inactivated (5 min, 95 C) chlorophyllase (○). And in buffer/oil (2/1, v/v) using active chlorophyllase (●). The buffers contained 6% acetone.

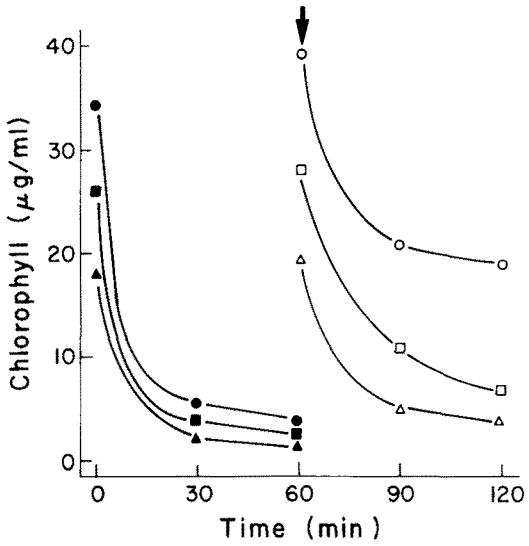

FIG. 6. Time course of two-stage chlorophyll hydrolysis in buffer at various initial concentrations. Reaction mixtures contained 18 (▲), 26 (■) and 34 (●) μg/ml chlorophyll, and were replenished with the same concentrations after 60 min of incubation (arrow). Conditions were as described in Fig. 5.

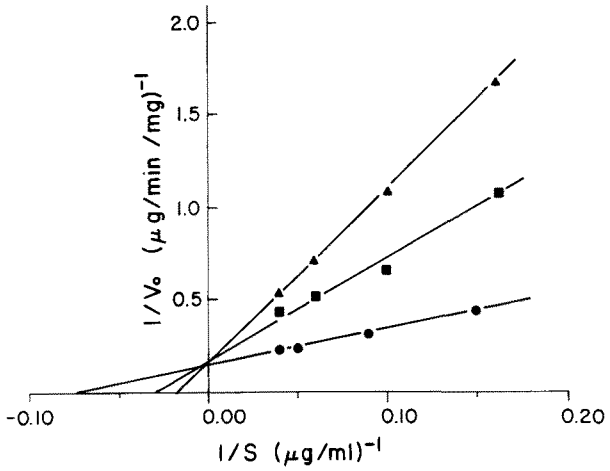

FIG. 7. Lineweaver-Burk plots showing competitive inhibition of chlorophyllase by phytol. Reaction conditions using buffer were as described in Fig. 5, with sampling at 30-sec intervals over a 3-min period. Control, no phytol (●), 22 μM phytol (■) and 34 μM phytol (▲). Km = 15 μM (13.5 μg/ml) chlorophyll and V_{max} = 6.2 μg/min/mg. Ki = 11 μM (3.3 μg/ml) phytol.

chlorophyllase than some other suggested souces (16) such as alfalfa and ryegrass. Around 5 g of crude chlorophyllase was routinely recovered per kg of leaf material. The specific activity varied considerably among preparations.

Activity of the chlorophyllase toward purified chlorophyll *a* was much lower than toward crude chlorophyll, inferring that the acetone and hexane washings of the chloroplasts removed lipid fractions deemed essential for catalytic activity (12).

Effect of various reaction parameters on chlorophyllase activity. Maximum enzyme activity at 30 C occurred at pH 8-10 (Fig. 1), as previously observed by Holden (17). Raising the temperature to 60 C resulted in a two-threefold increase in activity with a sharp maximum at ~ pH 8, above which the enzyme was denatured. To ensure chlorophyllase stability, a pH of 7.5 was used in further work.

Of the cations required for activity, $Mg^{2+}$was found to activate crude chlorophyllase several fold (Fig. 2). The optimum concentration (~50 mM) was significantly higher than that (12 mM) reported for purified chlorophyllase (12). The metal ion is believed to induce primarily conformational changes in the protein and, secondly, to interact with headgroups of lipids that bind tightly to the protein for the formation of an active enzyme complex (18).

The temperature optimum of chlorophyllase activity depended on the concentration of the substrate-solubilizing agent. At the frequently used (8, 19) high acetone concentration of 30% the enzyme was inactivated above 50 C, whereas in 6% acetone the activity was maintained up to at least 60 C (Fig. 3). In comparison, Tanaka et al. (8) found that their purified chlorophyllase was unstable at 30 C in concentrations of acetone above 10%, suggesting that the high thermostability of our enzyme preparation was related to the presence of impurities. The above temperature/acetone interaction-study with crude chlorophyllase revealed further (data not shown) that the optimum acetone concentrations for activity at 30 C and 50-60 C were ~20% and ~6%, respectively. The latter conditions were adopted for routine use.

Chlorophyll hydrolysis in oil. Preliminary work with oils focused on crude canola oil, because it was perceived that the presence of surface active compounds (gums) might facilitate pigment hydrolysis by

chlorophyllase, analogous to the use of Triton X-100 in buffer systems. Repeated attempts with crude oil (2 parts buffer/1 part oil) failed, and only after removal of phospholipids and proteinaceous materials (degumming) did chlorophyllase show some effect: ~10% chlorophyll reduction in 30 min at 50 C. Desoaping with NaOH improved the efficacy of the system. Therefore, it appeared that further optimization studies were best performed with the purest oil available, i.e. RBD oil.

Effect of acetone on chlorophyll hydrolysis in oil. In order to shift the partition coefficient of chlorophyll more in favor of the buffer solution or to increase the affinity of chlorophyll for the oil/buffer interface and consequently improve the proximity of the enzyme and the substrate, the role of acetone in the buffer phase was examined. Furthermore, it was tested whether increasing the interfacial suface area by mechanical homogenization, as compared to shaking, would enhance enzyme activity by physically improving the contact between enzyme and substrate. Figure 4 shows that an interactive effect exists between the acetone concentration of the buffer phase and the intensity of mixing. Homogenization of the reaction mixture gave good results even in the absence of acetone, and in general resulted in greater chlorophyll reductions (~50% over the first hour) than mixing by agitation (~30%). Repeated experiments established that inclusion of 5-10% acetone supported maximum hydrolysis in buffer/oil (1/1-2/1, v/v) systems subjected to homogenization.

Using the optimum reaction conditions for chlorophyllase, time courses of chlorophyll hydrolysis in straight buffer and in buffer/oil were compared (Fig. 5). The presence of oil with its affinity for chlorophyll impaired the hydrolysis considerably. Although the reactions were fast during the first hour, the subsequent drop in activity still left ~20% and ~45% chlorophyll unhydrolyzed in buffer and buffer/oil, respectively, after 3 hr of incubation. An additional treatment of the oil phase with a fresh buffer/enzyme solution resulted in a further reduction, but not below ~20% residual chlorophyll after 3 hr. These observations suggested that the chlorophyllase was either undergoing a form of inactivation over time or was suffering from product inhibition. Both possibilities were addressed in a time course study with staggered chlorophyll additions of various concentrations (Fig. 6). Up to 35 μg/ml (initial concentration), was fully hydrolyzed after 60 min (residual material absorbing at 660 nm could not be confirmed as chlorophyll *a* or *b* by visible range spectroscopy and TLC). However, replenishment with fresh chlorophyll at 26 μg/ml and above showed slower hydrolysis during the second hour, resulting in incomplete conversion. It was concluded that chlorophyllase remains active for at least 2 hr at 50 C, but that accumulation of a product causes inhibition, manifesting itself strongly above 50 μg/ml chlorophyll.

Product inhibition of chlorophyllase. Of the two products of chlorophyll hydrolysis, phytol proved to be a strong competitive inhibitor of chlorophyllase (Ki = 11 μM or 3.3 μg/ml; Fig. 7), whereas chlorophyllide had no effect on its activity. Phytol, therefore, was likely responsible for the limited hydrolysis of high levels of chlorophyll observed in the previous experiments (Figs. 5 and 6). Although several other inhibitors of chlorophyllase have been identified (20), the importance of product inhibition, especially for processing applications, has not been recognized previously.

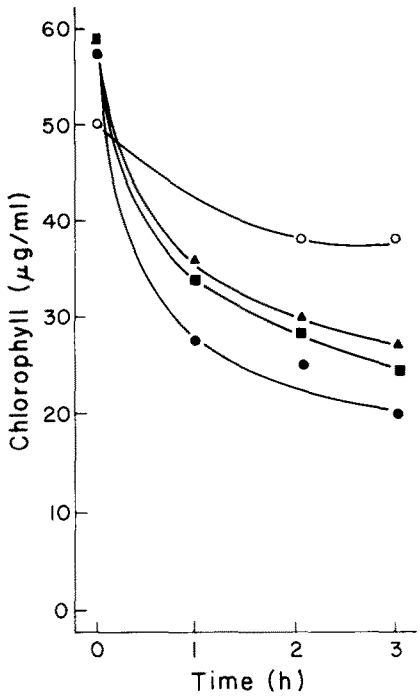

FIG. 8. Effect of buffer/oil ratio on the time course of chlorophyll hydrolysis. Reaction conditions were as described in Fig. 5. Buffer/oil ratios 2/1 (●), 1/1 (■), 1/2 (▲) and 1/9 (○).

The apparent Km value of 15 μM chlorophyll was slightly greater than that reported for rye chlorophyllase (Km = 12 μM) determined in 30% acetone (8) and tree-of-heaven chlorophyllase (Km = 10.3 μM) determined in 0.2% Triton X-100 (10). This might reflect a greater mass transfer resistance, due to poor chlorophyll solubility in 6% acetone.

Effect of water content. The minimum water requirement for effective enzymatic treatment of oil will have a major bearing on the industrial feasibility of the process. Figure 8 shows that a reduction in the water (buffer) content of the reaction system from 66% to 10% (v/v) caused a marked drop in the rate of chlorophyll removal. The positive relationship between the rate of hydrolysis and the water content reflects a dependence on interfacial surface area (mass transfer), as was also demonstrated by changing the intensity of homogenization. Consequently, process design and energy input factors eventually will dictate the optimum aqueous/organic phase ratio.

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