Chlorophyllase Biocatalysis in an Aqueous/Miscible Organic Solvent Medium Containing Canola Oil

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ABSTRACT: Chlorophyllase catalyzes the bioconversion of chlorophyll into chlorophyllide by replacing the phytol group with a hydrogen atom. There is an increased interest in the biotechnological application of chlorophyllase for the removal of green pigments from edible oil and its potential as an alternative to the use of the conventional bleaching technique. Partially purified chlorophyllase, obtained from the alga *Phaeodactylum tricornutum*, was assayed for its hydrolytic activity in an aqueous/miscible organic solvent system containing refined-bleached-deodorized (RBD) canola oil, using chlorophyll and pheophytin as substrate models. The results indicated that chlorophyllase biocatalysis could be successfully carried out in an aqueous/miscible organic system containing RBD canola oil. The presence of 20% RBD canola oil decreased the hydrolytic activity of chlorophyllase by 2.2 and 6.7 times, using chlorophyll and pheophytin as substrates, respectively. In addition, acetone acted as an activator of chlorophyllase activity at low concentrations and an inhibitor at higher ones. The optimal reaction conditions for chlorophyllase biocatalysis in the aqueous/miscible organic system were determined to consist of 20% RBD oil and 10% acetone at a 200 rpm agitation speed and at a temperature and substrate concentration of 35°C and 12.6 μM for chlorophyll, and 30°C and 9.3 μM for pheophytin.

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The high content of chlorophyll and its oxidized products in canola oil has a significant commercial impact on the canola industry. The presence of high levels of chlorophyll not only is organoleptically unacceptable but also reduces the oxidative stability of the oil due to the accelerated oxidation of unsaturated FA, especially linolenic acid. Consequently, there is an increase in the oxidative rancidity of the oil, which results in an undesirable color and taste and a reduced shelf life (1). Removal of chlorophyll from oil requires large quantities of bleaching clays, resulting in high processing costs and in notable losses of oil due to its adherence to the clays (2). There is interest in using a biotechnological enzymatic process involving the hydrolytic activity of chlorophyllase for the removal of these pigments, and in its potential as an alternative to the expensive adsorptive bleaching technique.

Chlorophyllase (chlorophyll-chlorophyllidohydrolase, EC 3.1.1.14), thought to be the first enzyme in the chlorophyll degradation pathway, catalyzes the hydrolysis of chlorophylls and related pheophytins into chlorophyllides and pheophorbides, respectively (3). This enzyme also catalyzes the esterification of chlorophyllides and other transesterification reactions depending on the media conditions.

Over the last 10 years, our laboratory has been involved in a systematic study of the optimization of chlorophyllase biocatalysis in nonconventional media, using a wide range of organic solvents, such as ethanol, acetone, propanol, hexane, heptane, and octane (4). In addition, biocatalysis has been performed in various organic systems, including an aqueous/miscible organic solvent system, a biphasic organic system, and a micellar ternary system (5–9). However, the use of a micellar ternary system was found to be the most appropriate reaction medium for the hydrolytic activity of chlorophyllase.

Previous work in our laboratory (8) investigated the biocatalysis of chlorophyllase in the presence of refined-bleached-deodorized (RBD) canola oil; however, increases in canola oil concentrations in the reaction medium were found to decrease the hydrolytic activity of chlorophyllase thereby inhibiting biocatalysis. Kalmokoff and Pickard (10) and Levadoux *et al.* (2) reported that no significant hydrolytic activity of chlorophyllase could be detected in the presence of crude canola oil.

The present work is part of a continuing effort aimed at developing a biotechnological application for chlorophyllase in the removal of green pigments from canola oil. Overall, the present work was aimed at optimizing chlorophyllase-catalyzed hydrolysis of induced chlorophyll and pheophytin in RBD canola oil in an aqueous/miscible organic solvent medium. The specific objectives were to investigate the effects of various parameters including RBD canola oil, acetone, agitation speed, and incubation temperature as well as enzyme and substrate concentrations on chlorophyllase biocatalysis in an aqueous/miscible organic solvent medium containing RBD canola oil.

MATERIALS AND METHODS

Production, extraction, and partial purification of chlorophyllase. The marine alga *Phaeodactylum tricornutum* Bohlin (Bacillariphyceae) was cultivated in a seawater medium, using

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the nutrient formula f/2 with a salinity of 2.4%; the biomass was harvested at the exponential phase; the chlorophyllase was extracted and partially purified according to the procedure described previously by Kermasha *et al.* (11).

Preparation of enzyme suspension. The enzymatic suspension was prepared according to the procedure outlined by Kermasha *et al.* (11). The enzyme extract, consisting of 1 mg lyophilized powder, was suspended in 1 mL of Tris-HCl buffer solution (20 mM, pH 8.0) and homogenized at 4°C for 5 min using a tissue grinder (Wheaton, Millville, NJ). The homogenized enzyme extract was analyzed for its protein content, using a modification of the Lowry method (12). The enzyme suspension and subsequent dilutions were freshly prepared prior to the enzymatic assay.

Preparation of substrates. Partially purified chlorophyll was prepared from fresh spinach leaves according to the procedure described previously by Khalyfa *et al.* (13). Chlorophyll was converted to pheophytin according to the method of Fraser and Frankl (14), using a 9.2% HCl (vol/vol) solution. Lyophilized partially purified chlorophyll and pheophytin extracts were dissolved in cold acetone (–20°C) (Fisher Scientific, Pittsburgh, PA) at a concentration of 1 mg/mL (1.12 mM) and subjected to subsequent dilutions to give a concentration range of 0.10–0.64 mM (11). The substrate stock solution and subsequent dilutions were freshly prepared prior to the enzyme assay.

Chlorophyllase assay. The chlorophyllase assays were carried out in an aqueous/miscible organic solvent system containing RBD canola oil with chlorophyll or pheophytin as substrate. The assay was performed according to a modification of the Khamessan *et al.* (6) procedure. The reaction mixture was prepared in a 50-mL Erlenmeyer flask and consisted of 1.03 mL of Tris-HCl buffer solution (20 mM, pH 8.0), 0.30 mL of acetone, 0.60 mL of RBD canola oil, and 0.07 mL of acetone-solubilized substrate; the final concentration of substrate in the reaction mixture was 10.26 μM. The enzymatic reaction was initiated by the addition of 1 mL of the enzymatic suspension, containing 200 μg protein, to the reaction medium. The mixture was incubated at 35 and 30°C for chlorophyll and pheophytin, respectively, with continuous agitation at 200 rpm using an orbital shaker-incubator (New Brunswick Scientific, Edison, NJ) for 2 h. At the defined time, the enzymatic reaction was halted by adding 4 mL of cold acetone (–20°C) and 1.6 mL of NaCl solution (2%, wt/vol). The residual unhydrolyzed substrate was extracted with 2 mL of petroleum ether (b.p. range 35–60°C) and the absorbance of the recovered organic phase was measured at 666 nm (Beckman DU-65 spectrophotometer). Reaction trials were conducted in triplicate; the data presented are means, with SE bars also shown in the figures. The unhydrolyzed substrate concentration was quantified using a calibration curve, ranging from 0 to 11.7 μM, established with standard solutions of either chlorophyll or pheophytin. The enzymatic reaction rate was calculated from the slope of the regression line of concentration of hydrolyzed chlorophyll or pheophytin vs. the reaction time. The specific activity of chlorophyllase was defined as nmol of hydrolyzed chlorophyll or pheophytin per mg protein per min.

Effect of RBD canola oil concentration on chlorophyllase activity. The effect of RBD canola oil content on the specific activity of chlorophyllase was determined by using selected concentrations of RBD canola oil ranging from 0 to 40% (vol/vol) in the enzymatic reaction mixture.

Effect of acetone concentration on chlorophyllase activity. The effect of acetone content on the specific activity of chlorophyllase was determined by using a wide range of acetone concentrations from 0 to 20% (vol/vol) in the enzymatic reaction mixture. Enzyme activity was determined as described above in "Chlorophyllase assay." The total volume of each reaction mixture was adjusted to 3.0 mL by varying the amount of buffer solution added.

Kinetic studies of chlorophyllase activity. The effects of selected kinetic parameters including enzyme content (32 to 132 μg protein/mL), agitation speed (100 to 200 rpm), incubation temperature (20 to 40°C), and substrate concentration (3.26 to 14.93 μM) on the hydrolytic activity of chlorophyllase were investigated.

RESULTS AND DISCUSSION

Effect of RBD canola oil concentration on chlorophyllase activity. With an aqueous/miscible organic solvent system containing 10% (vol/vol) acetone as the reaction medium, the results (Fig. 1A) show that an increase in RBD canola oil con-

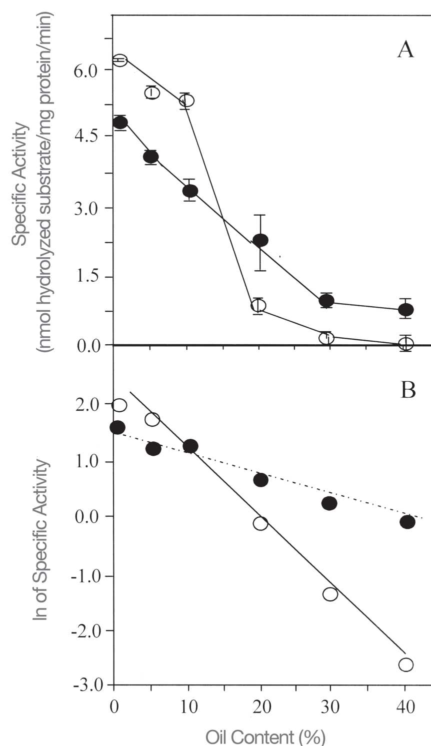

FIG. 1. Effect of refined-bleached-deodorized (RBD) canola oil content on (A) the specific activity of chlorophyllase and (B) the natural logarithm of the specific activity in a system containing chlorophyll (●) or pheophytin (○) as substrate.

TABLE 1
Inhibitory Effect of RBD Canola Oil on the Specific Activity of Chlorophyllase Using Chlorophyll and Pheophytin as Substrates[a,b]

Substrate	k	I_{50}
Chlorophyll	0.0411	18.9
Pheophytin	0.1383	7.8

[a] k is the inactivation constant, defined as 1 divided by the relative percentage (vol/vol) of oil content in the reaction medium; the inactivation of chlorophyllase by canola oil can be described by a first-order kinetic equation: $A_o = A_i\, e^{-kI_o}$, where A_o and A_i were the specific activities of the enzyme at a defined percentage of oil and without oil, respectively, and I_o was the relative percentage of oil content in the reaction medium.
[b] I_{50} was the amount of oil (%, vol/vol) in the reaction medium where the specific activity of the chlorophyllase decreased by 50%. RBD, refined, bleached, deodorized.

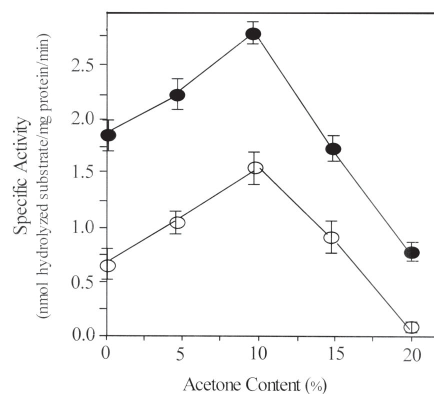

FIG. 2. Effect of acetone content on the specific activity of chlorophyllase in a system containing chlorophyll (●) or pheophytin (○) as substrate.

centration from 0 to 20% decreased the specific activity of chlorophyllase by 2.2 and 6.7 times using chlorophyll and pheophytin as substrates, respectively. These experimental findings suggest that RBD canola oil acted as a chlorophyllase inhibitor. This inhibition may have been due to unfolding of the enzyme conformation, thereby causing a loss in its activity (15). The hydrophobic character of RBD canola oil could also have affected the availability of the substrate to chlorophyllase, hence resulting in an inhibitory effect (8). Kalmokoff and Pickard (10) reported that chlorophyllase hydrolyzed chlorophyll but not pheophytin in a buffer/acetone system containing RBD canola oil. The discrepancy between these experimental results (Fig. 1) and those reported by Kalmokoff and Pickard (10) in terms of the substrate specificity of chlorophyllase could be related to the nature of the enzyme extract and/or the conditions of the enzymatic assay.

A quantitative determination (Table 1) of chlorophyllase inactivation was obtained by calculating the I_{50} value, calculated as the concentration of RBD canola oil required to decrease the chlorophyllase activity by 50%. The I_{50} value was obtained by plotting the logarithm of specific activity vs. the RBD canola oil content (Fig. 1B). Table 1 indicates that the I_{50} value obtained with chlorophyll was higher than that found with pheophytin as substrate, suggesting that the inhibitory effect of the oil was dependent on the nature of the substrate. These findings may be attributed to the effect of oil on enzyme conformation, perhaps resulting in a change in the affinity and catalytic activity of chlorophyllase for the substrate (8). The effect of RBD canola oil on the partition coefficients of the substrates could also be an explanation for these findings, as the presence of RBD canola oil may have rendered pheophytin less available than chlorophyll to the enzyme due to differences in the polarities of the substrates (7,8).

Effect of acetone concentration on chlorophyllase activity. The effect of acetone, ranging from 0 to 20% (vol/vol), on chlorophyllase activity in a water/miscible organic solvent system containing 20% (vol/vol) RBD canola oil was investigated. The results (Fig. 2) show that the presence of 5% acetone in the reaction medium increased the specific activity of chlorophyllase by 1.2 and 1.6 times for chlorophyll and pheophytin as substrates, respectively, whereas the addition of 10% acetone increased the specific activity of chlorophyllase by 1.5 and 2.4 times, respectively. However, at acetone concentrations higher than 10%, the specific activity of chlorophyllase decreased.

The increase in the specific activity of chlorophyllase at acetone concentrations lower than 10% may be explained by the role of acetone as a co-solvent in the reaction medium. Acetone may have increased the solvation of chlorophyll and pheophytin in the aqueous phase, thereby increasing their availability to the enzyme (16). The increase in the specific activity of chlorophyllase may also have been due to the dissociation of chlorophyll dimers and oligomers into monomers in the presence of acetone (11). Khamessan *et al.* (6) reported that the addition of acetone at a concentration below 30% to the biphasic reaction medium increased chlorophyllase activity. The variations in the optimal acetone concentrations reported by Khamessan *et al.* (6) and our present findings may be due to the presence of the nonpolar solvent hexane in the former biphasic reaction medium containing Tris-HCl and RBD canola oil.

Decreases in the specific activity of chlorophyllase in the presence of acetone concentrations higher than 10% could have been due to protein precipitation by acetone, rendering the enzyme less accessible to the substrate (17). In addition, conformational changes in the enzyme may have occurred owing to disruption of hydrogen bonds and hydrophobic interactions (18–20). The phenomenon of chlorophyllase inhibition by a high concentration of acetone was also reported by Khamessan *et al.* (6). Similarly, Rosell *et al.* (21) demonstrated a continuous loss up to approximately 75% in the activity of penicillin acylase as the solvent concentration of *N*,*N*-dimethylformamide increased to 50%, but at higher concentrations there was a dramatic decrease in enzyme activity.

Effect of enzyme concentration on chlorophyllase activity. The effect of enzyme concentration on the hydrolytic activity of chlorophyllase and bioconversion is shown in Table 2. With chlorophyll as substrate, the hydrolytic activity increased linearly with increasing chlorophyllase concentration, whereas with pheophytin, the increase in activity was to a lesser extent. The correlation coefficients (R^2) for these linear relationships

TABLE 2
Effect of Chlorophyllase Concentration on Hydrolytic Activity and Bioconversion Using Chlorophyll and Pheophytin as Substrates

Enzyme concentration	Hydrolytic activity[a]		Bioconversion (%)[b]	
(μg proteins/mL)	Chloro[c]	Pheo[d]	Chloro[c]	Pheo[d]
33	0.065	0.025	23.9	22.2
66	0.135	0.036	33.1	23.9
99	0.188	0.050	34.4	24.5
133	0.248	0.061	35.4	26.2

[a]Hydrolytic activity, defined as μmol of hydrolyzed substrate/L of reaction medium/min, determined during the first 15 min of the reaction.
[b]Relative percentage of bioconversion was defined as the difference between the initial and residual substrate concentrations divided by the initial concentration of substrate, multiplied by 100.
[c]Substrate chlorophyll.
[d]Substrate pheophytin.

were 0.99 and 0.85 for chlorophyll and pheophytin, respectively; these relationships were used to interpret the degree of mass transfer limitations (22). These results suggest that the hydrolytic reaction with chlorophyll as substrate was kinetically dependent on the chlorophyllase concentration, whereas with pheophytin, it was limited by the mass transfer rate of the substrate from the hydrophobic oil phase into the buffer phase where catalysis occurred. The differences in mass transfer rates between the two substrates could in turn have affected their diffusion into the reaction medium and subsequent interaction with the enzyme (11, 23).

Table 2 shows that an increase in chlorophyllase concentration from 33 to 133 μg protein/mL increased the bioconversion yield of chlorophyll into chlorophyllide from 23.9 to 35.4%, whereas the bioconversion yield of pheophytin into pheophorbide increased from 22.2 to 26.2% (22). The low overall bioconversion yields obtained for both substrates may have been due to inactivation of chlorophyllase with time (24) and/or the presence of the by-product phytol that acted as an enzyme inhibitor (5,11).

Effect of agitation speed on chlorophyllase activity. In using an aqueous/miscible organic solvent system containing 10% acetone and 20% RBD canola oil, an increase in agitation speed, from 100 to 200, increased the specific activity of chlorophyllase by 1.7 and 2.8 times with chlorophyll and pheophytin as substrates, respectively (Fig. 3). These results could be attributed to a reduction in size of the oil particles at high agitation speeds and a concomitant increase in interfacial area, thereby enhancing enzyme/substrate interactions (25,26). In an oil/aqueous system, the interfacial area was found to be dependent on the intensity of agitation, oil concentration and physical/interfacial properties of both phases (26). Levadoux *et al.* (2) and Khamessan and Kermasha (8) also reported that intensive mechanical mixing of the reaction medium was required to obtain maximal chlorophyllase activity in a buffer/canola oil environment.

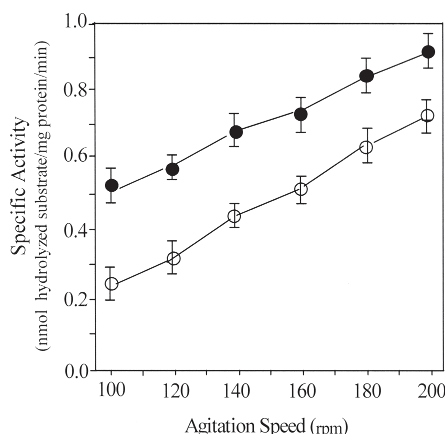

FIG. 3. Effect of agitation speed on the specific activity of chlorophyllase, using chlorophyll (●) or pheophytin (○) as substrate.

Effect of incubation temperature on chlorophyllase activity. The effect of temperature on the specific activity of chlorophyllase, using chlorophyll and pheophytin as substrates, is shown in Figure 4A. The maximal specific activity of chlorophyllase was obtained at 35 and 30°C for chlorophyll and pheophytin, respectively. However, at 40°C, chlorophyllase retained 40 and 16% of its maximal activity using as substrates chlorophyll and pheophytin, respectively. These experimental findings suggested that the stability of chlorophyllase was higher in the presence of chlorophyll than in the presence of pheophytin. The differences in the optimal temperatures for enzyme activity with chlorophyll and pheophytin as substrates may be due to the degree of polarity of each substrate, which could affect its diffusion in the reaction medium. With chlorophyll as substrate, a similar optimal temperature of 35°C was reported for chlorophyllase activity in a micellar ternary system containing 20% RBD canola oil (7,8).

From the Arrhenius plots (Fig. 4B), which were based on the first part of the two curves represented in Figure 4A, the activation energy (E_a) values were determined to be 62.9 and 66.9 kJ/mol for the hydrolysis of chlorophyll and pheophytin, respectively; the slight difference in the E_a values for the two substrates indicates that the hydrolytic reaction rate could have had a higher degree of sensitivity to temperature with pheophytin compared with that observed with chlorophyll. These experimental findings (Fig. 4A) are in the same range as those reported in the literature (40 to 200 kJ/mol) for other biocatalytic reactions, such as peroxidase (28) and tyrosinase (29).

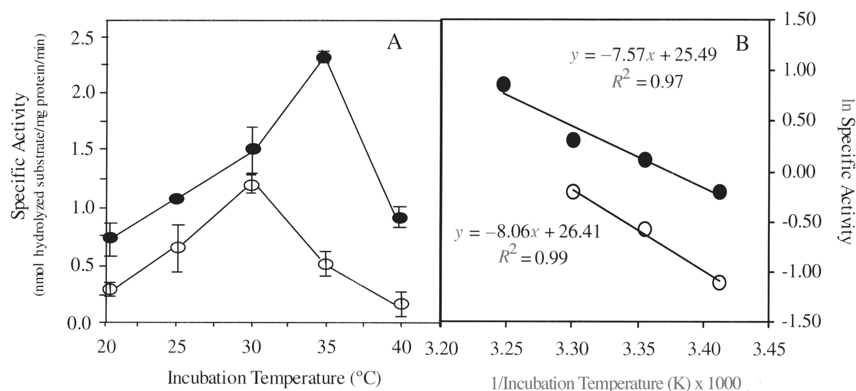

FIG. 4. Effect of incubation temperature on the specific activity of chlorophyllase, using chlorophyll (●) or pheophytin (○) as substrate: (A) incubation temperature vs. specific activity and (B) Arrhenius plot.

Effect of substrate concentration on chlorophyllase activity. The chlorophyllase reaction was studied in relation to substrate concentration over different periods of time. The results showed that the concentration of hydrolyzed chlorophyll (Fig. 5A) and pheophytin (Fig. 5B) increased with reaction time, with maximal hydrolysis of the substrate after approximately 3.3 h of incubation time. In the presence of 3.3 μM of either chlorophyll or pheophytin, the maximal bioconversion yield was 38%. The results also indicate that the initial reaction rate increased with increasing substrate concentration but then decreased once it reached its maximum at concentrations of 12.6 and 9.3 μM for chlorophyll and pheophytin, respectively. This decrease in chlorophyllase activity could be attributed to enzyme inhibition by the by-product phytol (11). Phytol was reported to exhibit a competitive inhibitory effect on the chlorophyllase reaction due to its influence on the equilibrium state of the hydrolysis reaction of chlorophylls and pheophytins into chlorophyllides and pheophorbides, respectively (5). The phytol molecule also could have decreased the hydrolytic activity of the enzyme by binding to its active site (2).

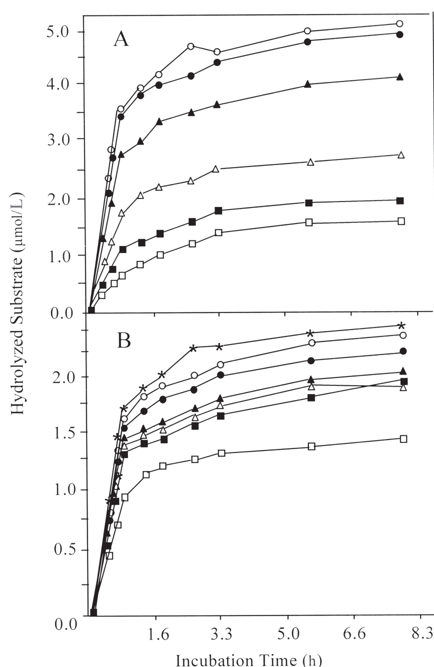

FIG. 5. Effect of initial substrate concentration on the hydrolysis of chlorophyll (A) and pheophytin (B), using seven substrate concentrations: 3.3 μM (□), 5.6 μM (■), 7.9 μM (△), 9.3 μM (★), 10.2 μM (▲), 12.6 μM (○), and 14.9 μM (●).

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