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Optimization of Chlorophyllase-catalyzed Hydrolysis of Chlorophyll in Monophasic Organic Solvent Media

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Abstract The effects of selected reaction parameters, including solvent hydrophobicity, initial water activity, agitation speed, temperature and enzyme concentration, on the biocatalytic efficiency of a chlorophyllase enzymatic extract from *Phaeodactylum tricornutum* in neat organic solvent media were investigated. The highest chlorophyllase specific activity of 322 nmol hydrolyzed chlorophyll per gram of protein per minute and bioconversion yield of 91% were obtained in the reaction mixture of hexane/2-octanone (98.3:1.7, *v*/*v*), at a controlled initial water activity of 0.90. $R_{O/A}$ value, which is the ratio of the specific activity in the organic solvent to that in the aqueous/miscible organic solvent medium, was 1.5×10^{-3}. To reduce the substrate diffusional limitations, the appropriate agitation speed and enzyme concentration were determined. The optimum temperature for maximal enzymatic activity and activation energy were 35°C and 105.0 kJ/mol, respectively. Although the catalytic efficiency of chlorphyllase in the neat organic solvent mixture was lower than that in the aqueous medium, its half-life time in the first environment at temperature ranging from 35 to 50°C was increased by 5.0 to 15.0 times.

Keywords Chlorophyllase · Biocatalysis · Neat organic solvent media · Kinetics

Introduction

Chlorophyllase (chlorophyll–chlorophyllido–hydrolase, EC 3.1.1.14), which is the first enzyme in the chlorophyll degradation pathway in vivo, catalyzes the hydrolysis of chlorophylls and pheophytins into their hydrophilic chromophore moieties chlorophyllides and pheophorbides, respectively [1]. Chlorophyllase has been shown to be a glycosylated

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protein associated to plastid membranes and belong to the α/β-hydrolase fold family of enzymes [2]. The use of chlorophyllase in the removal of green pigments from edible oils could be of great interest [3]. The development of a biotechnological process involving the use of chlorophyllase for the bioconversion of the hydrophobic chlorophylls and their derivatives into their hydrophilic chromophore moieties was reported previously by our group [4, 5].

A major research thrust in our laboratory was aimed at the immobilization [6, 10] and the optimization of the hydrolytic activity of chlorophyllase in various nonconventional media, including aqueous/miscible organic solvent [11], biphasic [12], and ternary micellar systems [13]. The use of a micellar ternary system containing Span 85 was found to be the most appropriate medium for the hydrolytic activity of chlorophyllase [13]. However, the poor solubility of chloropylls and the low stability of chloropyllase limited the enzyme biocatalytic efficiency for the removal of green pigments. The use of neat organic solvents as reaction medium may provide a better solvation of the hydrophobic substrates, an increase in the enzyme thermostability and an easier separation of the enzyme from the reaction products [14, 15, 16]. As far as the authors are aware, no work has been reported to date on the chlorophyllase biocatalysis in neat organic solvents.

The present work is aimed at the optimization of chlorophyllase biocatalysis in neat organic solvent media, by investigating the effects of various parameters, including nature of solvent, initial water activity (a_w), diffusional limitations, reaction temperature, and substrate concentration.

Materials and Methods

Biomass Production, Extraction, and Partial Purification of Chlorophyllase

The production of biomass of the alga *Phaeodactylum tricornutum* Bohlin (*Bacillariphyceae*) was carried out according to a modification of the method described by Kermasha et al. [17]. *P. tricornutum* was maintained in a sea water medium, with a salinity of 2.4% (*w*/*v*), containing the nutrient formula *f*/2 defined by Guillard and Ryther (1962) at 18°C with constant light. The alga was grown in a 50 L aquarium containing 30 L of sea water *f*/2 medium at constant pH 8.0 with light periods of 18 h at 18°C and dark periods of 6 h at 10°C. After 8 days of growth, the alga cells were harvested by centrifugation (5,000×*g*, 10 min, 4°C). The extraction and partial purification of chlorophyllase extract were carried out according to the procedure described by Kermasha et al. [17]. The enzymatic preparation was lyophilized in 20 mM Tris–HCl buffer at pH 8.0. The enzymatic 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, USA). The homogenized enzyme extract was analyzed for its protein content, using a modification of the Lowry method [18]. The enzyme suspension and subsequent dilutions were freshly prepared before the enzymatic assay.

Chlorophyllase Assay

Partially purified chlorophyll, used as substrate, was prepared from fresh spinach leaves according to the procedure described previously by Khalyfa et al. [19]. Before each enzymatic assay, a stock solution of chlorophyll 24 μM was freshly prepared in a selected organic medium equilibrated at a specific water activity (a_w) value. In 2 mL vials

containing varying amounts of the lyophilized chlorophyllase preparation (0.6 to 2.0 mg protein), equilibrated at a selected a_w value, the hydrolysis reaction was initiated by adding 1.5 mL of the substrate stock solution. Reaction mixtures were incubated for 2 to 6 h at 35°C under a constant agitation of 200 rpm in an orbital incubator shaker (New Brunswick Scientific Co., Inc., Edison, NJ, USA). Control trials, without enzyme, were carried out in tandem with the enzymatic reactions. At selected time intervals, 200 μL aliquots were withdrawn from the reaction mixture and diluted with hexane. The residual unhydrolyzed chlorophyll was measured spectrophotometrically at 663 nm, by using an apparent molar extinction coefficient of 25.25×10^9 cm^2/mol. Both enzymatic reactions and controls were run in triplicate trials. Chlorophyllase activity was defined as nanomole of hydrolyzed chlorophyll per gram of protein per minute.

Effect of Organic Solvent Mixture

Chlorophyllase-catalyzed chlorophyll hydrolysis was carried out in 2-octanone as well as in selected organic solvent mixtures (98.3:1.7, *v*/*v*), including iso-octane/2-octanone, heptane/2-octanone, hexane/2-heptanone, hexane/2-octanone, hexane/2-butanone, pentane/2-octanone, 3-nonanone/2-octanone, and hexane/acetone. Enzymatic reactions were monitored at defined time intervals during a 2- to 4-h reaction period as described previously for the chlorophyllase assay.

Effect of Initial Water Activity

The effect of initial water activity (a_w) on chlorophyllase activity was investigated in the range of 0.44 to 0.97. Before the enzyme activity measurement, the initial a_w of the selected organic solvent media and the solid enzyme was equilibrated by placing each one of them separately in sealed vessels containing saturated aqueous salt solutions [20], including: K_2SO_4 (a_w=0.97), $ZnSO_4$ (a_w=0.90), NaCl (a_w=0.75), and K_2CO_3 (a_w=0.44). Water activity equilibration was performed over a 60-h period at room temperature. Enzymatic reactions were carried out as described previously in the hexane/2-octanone mixture.

Mass Diffusional Limitations

External diffusional limitations were investigated by carrying out the enzymatic assay at agitation speeds varying from 0 to 200 rpm in an orbital incubator shaker. Internal diffusional limitations were investigated by carrying out the enzymatic assay using selected enzymatic powders with a wide range of enzyme concentrations varying from 250.4 to 1,000.0 mg solid enzyme per gram of powder. The enzymatic powders were prepared by mixing an adequate volume of the chlorophyllase extract with BSA solution. The chlorophyllase assay was carried out, as described previously, in the hexane/2-octanone mixture (98.3:1.7, *v*/*v*) at an initial a_w of 0.9.

Effect of Temperature on Chlorophyllase Activity and Stability

The effect of reaction temperature on chlorophyllase activity was investigated at temperatures varying from 25 to 45°C. The chlorophyllase thermal stability in neat organic solvent medium was studied by incubating the solid enzyme in the hexane/2-octanone mixture at selected temperatures of 35, 40, 45, and 50°C. After different periods of incubation time (15 to 60 min), the solid enzyme was recovered by centrifugation

(5,000×*g*, 4 min) and assayed for its residual enzyme activity as described previously, using standard reaction conditions (35°C, a_w=0.9, 200 rpm). A comparative study with the aqueous medium was also carried out.

Kinetic Parameters

Chlorophyllase activity was investigated at a wide range of chlorophyll concentrations, ranging from 6 to 30 μM, in the hexane/2-octanone mixture. The enzymatic reaction was carried out using the optimized conditions (35°C, a_w=0.9, 400 mg solid enzyme per gram of powder, 200 rpm) and was monitored over the course of a 2.5-h reaction period.

Characterization of Reaction Components

The characterization of reaction components of chlorophyllase-catalyzed hydrolysis of chlorophyll was performed by spectrophotometrical analysis. The residual substrate and enzymatic end product, recovered by extraction with an aqueous/acetone solution (70:30, *v*/*v*), were scanned spectrophotometrically from 300 to 800 nm, using the Beckman spectrophotometer.

Results and Discussion

Effect of Organic Solvent Mixture

The chlorophyllase-catalyzed hydrolysis of chlorophyll was carried out using a wide range of selected binary organic solvent mixtures (98.3:1.7, *v*/*v*), which are varying in their degree of hydrophobicity, as denoted by their log *P* values. The use of 1.7% of cosolvent was the minimum amount necessary to ensure an adequate solubilization of the partially purified chlorophyll extract, used as substrate. The specific activities of chlorophyllase and bioconversion yield are shown in Table 1. No significant chlorophyllase activity could be obtained in both 3-nonanone/2-octanone mixture and 2-octanone, with log *P* of 2.89 and 2.40, respectively. The highest enzyme-specific activity of 322 nmol hydrolyzed chlorophyll per gram of protein per minute and bioconversion yield of 91% were obtained in the hexane/2-octanone mixture (log *P* of 3.47), it corresponds to a $R_{O/A}$ value of 1.5×10^{-3}, where $R_{O/A}$ is the ratio of the specific activity in the organic solvent to that in the aqueous/miscible organic solvent medium [11]. Although the log *P* of the reaction mixture remained higher than 3.0, using 2-heptanone or 2-butanone as cosolvents with hexane, the results (Table 1) show important decreases in enzyme activity and bioconversion yield as compared to those obtained with 2-octanone as a cosolvent. The results also show that lower specific activity and bioconversion yield were obtained with the most hydrophobic mixtures of isoctane/2-octanone and heptane/2-octanone, denoted by their higher log *P* values, as compared to those obtained in the hexane/2-octanone mixture.

The overall findings (Table 1) indicate clearly that there was no correlation between the specific activity of chlorophyllase and the Log *P* value of the organic solvent mixture. Similar results have been reported for the porcine pancreatic lipase-catalyzed esterification of tributyrin with hexanol, using mixtures of hexane and a wide range of cosolvents [21]. With a concomitant increase in solvent hydrophobicity, a higher enzymatic activity is generally expected; this phenomenon was attributed to the fact that the hydrophilic solvents have higher tendency to strip off the water from the surface of the enzyme, which is

Table 1 Effect of selected organic solvent mixtures on the specific activity and the bioconversion yield of chlorophyllase-catalyzed hydrolysis of chlorophyll.

Solvent mixture[a]	Log P value[b]	Specific activity[c]	Maximum bioconversion yield (%)[d]
Iso-octane/2-octanone	4.45	292.50 (33.5)[e]	76.70
Heptane/2-octanone	3.96	155.90 (8.5)[e]	51.70
Hexane/2-octanone	3.47	322.40 (24.8)[e]	90.70
Hexane/2-heptanone	3.45	49.80 (10.1)[e]	6.90
Hexane/2-butanone	3.05	16.70 (4.3)[e]	19.80
Pentane/2-octanone	2.98	109.50 (16.1)[e]	36.30
3-Nonanone/2-octanone	2.89	<0.01	<0.01
Hexane/acetone	2.86	37.50 (31.7)[e]	34.50
2-Octanone	2.40	<0.01	<0.01

[a] Solvent mixture at a ratio solvent to cosolvent of 98.3:1.7 (*v*/*v*).

[b] Log *P* value defined as the partition coefficient of the solvent mixture between water and 1-octanol. Log *P* of the solvent mixture was calculated according to the empirical formula log *P* mixture = *X*1 log *P*1 + *X*2 log *P*2, in which *X*1 and *X*2 are the mole fractions of solvents 1 and 2.

[c] Specific activity was defined as nmol of hydrolyzed chlorophyll per gram of protein per minute.

[d] Maximum bioconversion yield was calculated as the difference between the initial chlorophyll concentration and the residual one, obtained at the reaction equilibrium, divided by the initial concentration of chlorophyll multiplied by 100.

[e] Standard deviation was calculated for triplicate trials.

essential for its catalytic activity [15, 16, 22]. Because the a_w of the organic system was controlled, the rational of solvent dependence, which is based on the differences in the retention of the essential enzyme-bound water, could not be applied to the experimental findings (Table 1). Such results may indicate a direct effect of the organic solvent on the enzyme itself by binding in or near its active site, resulting in conformational changes [23]. The effect of substrate–solvent interactions on the availability of substrate to the enzyme may also account for the dependence of the specific activity of chlorophyllase on the solvent nature [15, 24]. In addition, the chlorophyllase activity in organic solvent media may have also been affected by the partition of the hydrophobic by-product phytol, which acted as an enzyme inhibitor, between the micro- and macroenvironment of the solid enzyme [11].

Because the experimental findings (Table 1) indicated that the highest specific activity and bioconversion yield were obtained using the neat organic solvent mixture of hexane/2-octanone (98.3:1.7, *v*/*v*) as the reaction medium, further studies were carried out with such environment.

Effect of Initial Water Activity

In organic solvent media, water plays an important role in maintaining the active enzyme conformation and in modulating the equilibrium reaction [16, 24]. The effect of initial water activity (a_w) on both specific activity of chlorophyllase and bioconversion yield was investigated. Figure 1 shows that an increase in the initial a_w of the reaction system, from 0.44 to 0.90, increased the specific activity and bioconversion yield to 260 nmol hydrolyzed chlorophyll per gram of protein per minute and 89%, respectively. However, further increase in the initial a_w to 0.97 resulted in a slight increase in the specific activity to 285 nmol hydrolyzed chlorophyll per gram of protein per minute and a decrease in the bioconversion yield to 69%. This decrease may be due to the aggregation of enzyme solid

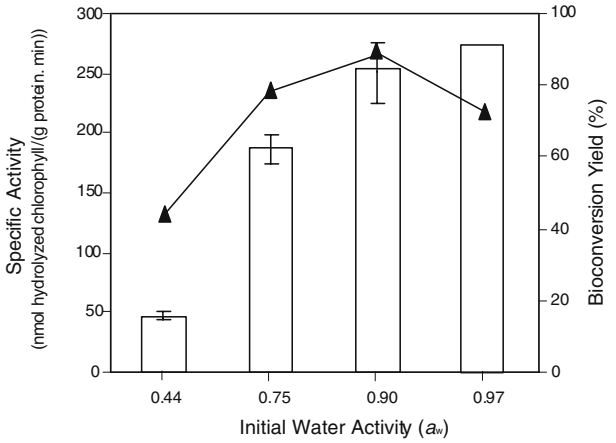

Fig. 1 Effect of initial water activity (a_w) on chlorophyllase specific activity (*empty square*), and bioconversion yield (*filled triangle*) in the neat organic solvent mixture composed of hexane/2-octanone (98.3:1.7, *v*/*v*)

particles during the hydrolysis reaction, which could have limited the accessibility of the substrate to the enzyme active site. On the other hand, the lower enzyme-specific activity and bioconversion yield, obtained at lower a_w values ($\leq$0.75), may be attributed to a decrease in chlorophyllase flexibility, which seemed to require a higher amount of water to maintain its active conformation [14, 16]. The a_w value needed for the optimal catalytic activity has been reported to be dependent on the nature of enzymes as well as on their environments [16, 20]. The mass action effect of water, as a reactant, in the hydrolysis reaction of chlorophyll could also be an explanation for the low specific activity and bioconversion yield at lower initial a_w values [16]. The overall experimental results (Fig. 1) indicate that the initial a_w value of 0.9 was the most appropriate one for the chlorophyllase-catalyzed hydrolysis of chlorophyll and consequently used for further investigations.

Mass Diffusional Limitations

In a heterogeneous system, the enzyme catalysis may be affected by the diffusion rate of substrate through the boundary layer at the surface of the enzyme particles, external diffusion, as well as in their inside, internal diffusion [25]. It is therefore important to ensure that the diffusional limitations do not affect the kinetics of chlorophyllase-catalyzed hydrolysis of chlorophyll in the neat organic solvent mixture.

The degree of external diffusional limitations was examined by varying the agitation speed of the reaction mixture from 0 to 200 rpm. The results (Fig. 2) show that an increase in the agitation speed from 0 to 200 rpm resulted in a 75% increase in the specific activity of chlorophyllase. This increase may be attributed to a decrease in the size of enzyme particles and/or in the thickness of the boundary layer surrounding the solid enzyme, which could have led to an increase in the rate of the external substrate diffusion [25]. The overall results reveal that the external diffusional limitations occurred within the agitation speed range of 0 to 200 rpm. A high agitation speed of 200 rpm was therefore selected for all subsequent investigations.

Internal diffusional limitations were studied by investigating the effect of chlorophyllase concentration in the enzymatic powder (250 to 1,000 mg solid chlorophyllase extract per gram of powder containing BSA) on its activity. Table 2 shows that the chlorophyllase activity increased linearly (R^2=0.998) with the increase of enzyme concentration from 250 to 516 mg solid enzyme per gram of powder, followed by a slower increase at a higher

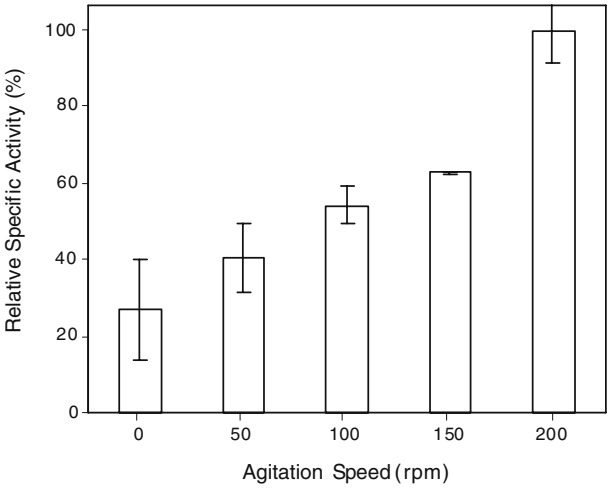

Fig. 2 Effect of agitation speed on chlorophyllase specific activity in the neat organic solvent mixture composed of hexane/2-octanone (98.3:1.7, *v*/*v*)

chlorophyllase concentration of 1,000 mg solid enzyme per gram of powder. As a result, the specific activity of the enzyme remained almost unchanged in the chlorophyllase concentration range of 250 to 516 mg solid enzyme per gram of powder, with an average of 285 nmol hydrolyzed chlorophyll per gram of protein per minute, and then decreased at a higher concentration. These results indicate that the internal diffusional limitations occurred at chlorophyllase concentrations higher than 516 mg solid enzyme per gram of powder. A solid enzyme concentration in the range of 250 to 516 mg solid enzyme per gram of powder was used for further investigations.

Effect of Reaction Temperature

The effect of reaction temperature (25 to 45°C) on the specific activity of chlorophyllase-catalyzed hydrolysis of chlorophyll in the neat organic solvent mixture of hexane/2-octanone was investigated. The results (Fig. 3a) show that the maximal activity of chlorophyllase from *P. tricornutum* was obtained at reaction temperature of 35°C. Similar optimal temperature was also reported for the same enzyme in the aqueous/miscible organic solvent [5] as well as in the ternary micellar system [6, 13]. This optimal temperature of 35°C falls within the range of 30 to 40°C reported in literature for chlorophyllase from other plant

Table 2 Effect of enzyme concentration on chlorophyllase activity in hexane/2-octanone mixture.

Enzyme concentration (milligram of solid enzyme per gram of powder)	Chlorophyllase activity[a]	Specific activity[b]
250.4	17.5 (1.2)[c]	280.0 (10.4)[c]
399.8	27.8 (2.6)[c]	278.2 (26.9)[c]
464.1	34.2 (3.0)[c]	294.4 (30.7)[c]
515.9	37.3 (3.2)[c]	284.6 (24.8)[c]
1,000.0	59.2 (8.1)[c]	236.8 (31.9)[c]

[a] Chlorophyllase activity was defined as nanomole hydrolyzed chlorophyll per gram of enzymatic preparation per minute of hydrolysis.

[b] Specific activity was defined as nanomole of hydrolyzed chlorophyll per gram of protein per minute.

[c] Standard deviation was calculated for triplicate trials.

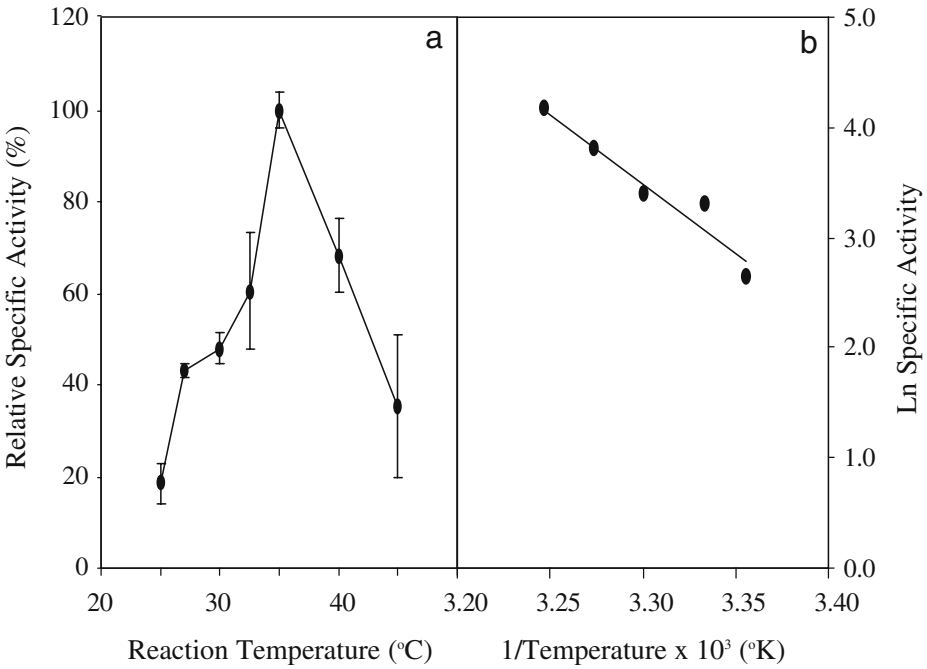

Fig. 3 Effect of reaction temperature on chlorophyllase specific activity in the neat organic solvent mixture composed of hexane/2-octanone (98.3:1.7, *v/v*): **(a)** relative specific activity versus reaction temperature and **(b)** Arrhenius plot

sources [7]. However, the decrease in chlorophyllase activity, obtained at temperature above 35°C (Fig. 3a), was much lower than that reported for the same enzyme in the aqueous/miscible organic solvent mixture [5] and the ternary micellar system [6]. This limited decrease in chlorophyllase activity in the neat organic solvent mixture may be due to a better enzyme stability as compared to that in other reaction media.

The first part of the curve (Fig. 3a) follows the Arrhenius law, as indicated by the linearity of the plot of the logarithm of specific activity versus the reciprocal of the reaction temperature (Fig. 3b). From this plot (Fig. 3b), an activation energy (E_a) of 105.0 kJ/mol was obtained for the chlorophyllase-catalyzed hydrolysis of chlorophyll in the neat organic solvent mixture. This E_a is higher than that obtained for the same reaction (66.9 kJ/mol), but in the aqueous/miscible organic solvent containing 10% acetone [5]. The increase in E_a in the neat organic solvent mixture may be due to the use of a suspended solid enzyme.

Thermal Stability

The thermal stability of chlorophyllase-catalyzed hydrolysis of chlorophyll was investigated at 35, 40, 45, and 50°C, using two reaction media, the neat organic solvent mixture of hexane/2-octanone (98.3:1.7, *v/v*) and the aqueous/miscible organic solvent containing 10% acetone (Fig. 4). At 35 and 40°C, the chlorophyllase showed (Fig. 4a) more than 87 and 70% of its initial specific activity, respectively, after 60 min of incubation; however, in the aqueous medium, the enzyme retained only 15 and 2% of its initial specific activity, respectively. The overall experimental findings indicate an increase in the thermal stability of chlorophyllase in the neat organic solvent mixture as compared to that in the aqueous medium.

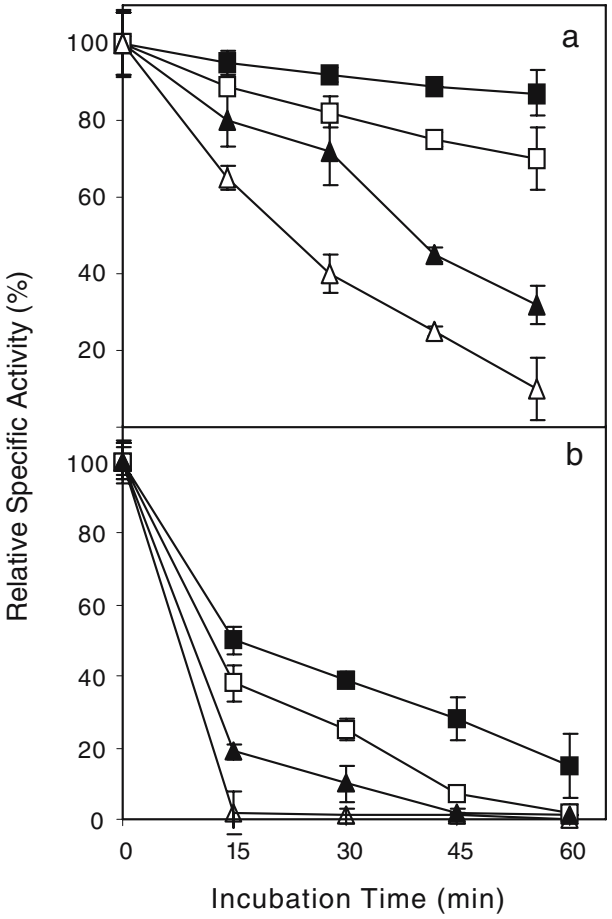

Fig. 4 Thermal stability of chlorophyllase in organic and aqueous media: relative specific activity versus incubation time at different incubation temperatures, 35 (*filled square*), 40 (*empty square*), 45 (*filled triangle*), and 50°C (*empty triangle*) in **(a)** neat organic solvent mixture composed of hexane/2-octanone (98.3:1.7, *v*/*v*) and **(b)** aqueous/miscible organic solvent containing 10% acetone

In both reaction media, the thermal inactivation of chlorophyllase was found to follow the first order kinetic behavior (data not shown). From the logarithmic plots of the thermal inactivation kinetics, the half-life times of chlorophyllase were estimated at different temperatures and are given in Table 3. These experimental findings indicate that the use of the neat organic solvent mixture, within the investigated temperature range (35 to 50°C), resulted in an increase in the half-life time of the chlorophyllase by 5.0 to 15.0-fold as compared to that in the aqueous one. The use of a suspended solid enzyme may explain the increase in the thermal stability of chlorophyllase in the neat organic solvent mixture as compared to the aqueous/miscible organic solvent mixture. In addition, this increase in thermal stability may be due to the presence of a restricted amount of water in the neat organic solvent mixture, which may have resulted in an increase in the enzyme structure rigidity and a decrease in the rate of covalent processes involved in irreversible inactivation [15, 16]. Using molecular dynamics simulations, Hartsough and Merz [27] also demonstrated that the high thermal stability of an enzyme in organic solvent media may be due to an increase in its intramolecular stabilizing interactions as well as to a decrease in its surface area to its molecular size ratio.

Table 3 Half-life times for chlorophyllase-catalyzed hydrolysis of chlorophyll in neat organic solvent and in aqueous medium.

Temperature (°C)	Half-life time (min)[a]	
	Neat organic solvent[b]	Aqueous medium[c]
35	346.6 (±20.1)[d]	23.7 (±2.4)[d]
40	117.5 (±8.4)[d]	10.9 (±1.5)[d]
45	41.5 (±4.3)[d]	8.4 (±1.1)[d]
50	18.7 (±2.0)[d]	2.6 (±0.5)[d]

[a] Half-life time is defined as the incubation time required to obtain a 50% decrease in the initial chlorophyllase activity.

[b] Chlorophyllase thermal stability in neat organic solvent medium was carried out by incubating the solid enzyme in the hexane/2-octanone mixture (98.3:1.7, *v*/*v*) at selected temperatures.

[c] Chlorophyllase thermal stability in aqueous medium was carried out by incubating the solid enzyme in the aqueous/miscible organic solvent containing 10% acetone at selected temperatures

[d] Standard error was defined as the standard deviation divided by the square root of the number of samples and was calculated, using SigmaPlot statistical software, based on the logarithm of remaining specific activity of chlorophyllase as a function of incubation time for triplicate trials.

Kinetic parameters

The kinetic parameters for the chlorophyllase-catalyzed hydrolysis of chlorophyll in the neat organic solvent mixture of hexane/2-octanone (98.3:1.7, *v*/*v*) were determined, using the optimized conditions. The results (Fig. 5) indicate that the kinetics of chlorophyllase in the neat organic solvent mixture followed the Michaelis–Menten model, as indicated by the linearity of the corresponding Lineweaver–Burk plot. The apparent kinetics parameters, K_{mapp} and V_{maxapp}, estimated by nonlinear regression, were 14.2 μM and 362.7 nmol hydrolyzed chlorophyll per gram of protein per minute, respectively. As a result, the catalytic efficiency of chlorophyllase, defined as V_{max}/K_{mapp}, in the neat organic solvent mixture was calculated as 0.025 L per gram of protein per minute, which is lower than that (30.1 L per gram of protein per minute) obtained in the aqueous/miscible organic solvent

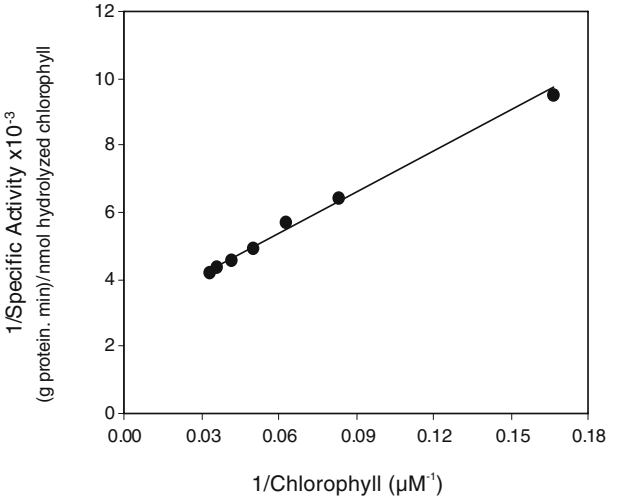

Fig. 5 Effect of chlorophyll concentration on chlorophyllase specific activity in the neat organic solvent mixture composed of hexane/2-octanone (98.3:1.7, *v*/*v*): the corresponding Lineweaver–Burk plot

medium [11] and that (2,560.2 L per gram of protein per minute) reported for the ternary micellar system [6]. The decrease in the catalytic efficiency of enzymes in organic solvent media may be attributed to an increase in the rigidity of the enzyme structure in this environment containing a restricted amount of water as well as to a substrate partitioning effect [4, 16]. In addition, because the by-product phytol is hydrophobic, its inhibitory effect on chlorophyllase activity may have increased in the neat organic solvent mixture, affecting hence the catalytic efficiency of the enzyme [3, 11, 12].

Characterization of Reaction End Products

The characterization of reaction components of chlorophyllase-catalyzed hydrolysis of chlorophyll in the neat organic solvent mixture was performed by spectrophotometric scanning. The spectrophotometric analysis of the partially purified chlorophyll, used as substrate, shows (Fig. 6a) absorbance maxima (λ_{max}) at 429 and 662 nm, whereas that of the end product (Fig. 6b) exhibited a λ_{max} at 434 and 665 nm. The λ_{max} at 429 and 434 nm could be attributed to the presence of carotenoids in both substrate and end product [28]. However, the λ_{max} of 662 nm of chlorophyll (Fig. 6a) is within the characteristic absorption region of the isomer chlorophyll *a*, which has a λ_{max} between 662 and 666 nm [29]. The experimental findings (Fig. 6) may indicate the presence in the partially purified chlorophyll preparation of a higher portion of chlorophyll *a* than that of chlorophyll *b*. However, the λ_{max} of chlorophyllide end product at 665 nm (Fig. 6b) is slightly different

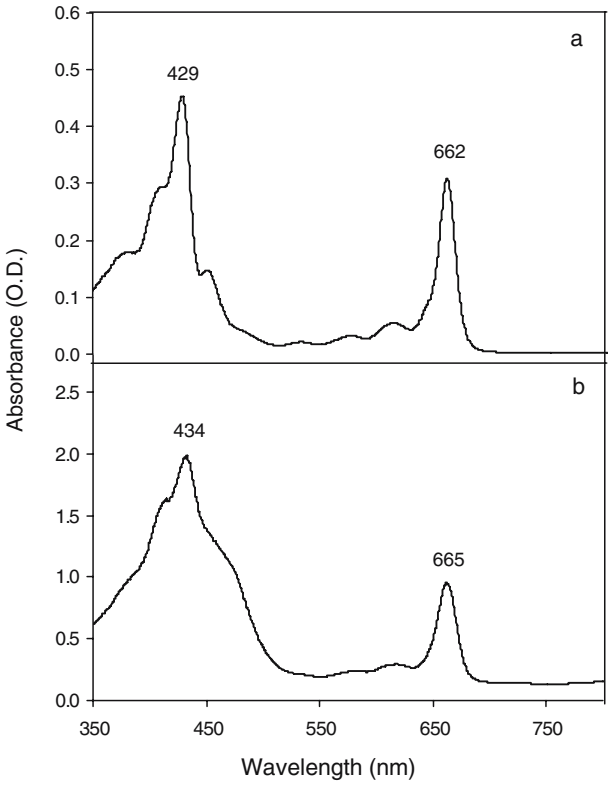

Fig. 6 Spectrophotometric scanning of **(a)** partially purified chlorophyll preparation used as substrate, and **(b)** its corresponding hydrolyzed end product, chlorophyllide

from that reported in literature [12, 30], where its λ_{max} was found to be at 666 to 667 nm. The slight difference between the experimental findings (Fig. 6) and those reported in literature may be due to the difference in the degree of purity of chlorophyll preparation and/or to the nature of organic solvents used for the extraction of chlorophyllide end product fraction.

Conclusion

The overall results, gathered in the present study, demonstrated that the chlorophyllase-catalyzed hydrolysis of chlorophyll could be successfully carried out in a neat organic solvent mixture. Although the catalytic efficiency of the enzyme decreased in this reaction environment, a high increase in its thermal stability was obtained as compared to that in the aqueous medium.

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