BIOCATALYSIS OF IMMOBILIZED CHLOROPHYLLASE

IN

A TERNARY MICELLAR SYSTEM

by

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SHORT TITLE

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1. INTRODUCTION

Chlorophylls are green pigments distributed in living plant cells, algae and photosynthetic bacteria (Svec, 1991). Chlorophylls can be readily converted in both *in vivo* and *in vitro* to a number of characteristic derivatives. The loss of the central magnesium ion results in the formation of pheophytin. The removal of the carboxymethyl group gives pyropheophytin, whereas the removal of the phytol group yields chlorophyllides and pheophorbides. Chlorophylls, pheophytins and pyropheophytins are lipophilic compounds due to the presence of a long chain alcohol, phytol, whereas, chlorophyllides and pheophorbides are hydrophilic ones (Rüdiger and Schoch, 1991).

The chlorophyll content of edible oils is used as a measure of oil quality. During extraction, there remains in the oil, a portion of the chlorophyll. High levels of chlorophyll result in oxidative rancidity, responsible for the instability as well as the undesirable color and taste in oils (Dahlen, 1973). This is a serious problem in the oil industry and could result to significant commercial losses. Canada, being among the major producers of oil, has a vested interest in solving this problem. Such levels of chlorophyll have been difficult to remove by conventional bleaching methods (Daun, 1982).

Theoretically, vegetable oil could be decolorized more efficiently with the treatment of chlorophyllase. Chlorophyllase is an intrinsic membrane glycoprotein, found in higher plants and algae (Lambers and Terpstra, 1985). This enzyme has been proven to catalyze the bioconversion of chlorophyll and pheophytin into chlorophyllide and

pheophorbide. The enzyme is also capable of catalyzing the synthesis of chlorophyll and pheophytin from chlorophyllide and pheophorbides, respectively in the presence of phytol (Ellsworth, 1971).

In the past, research in our laboratory was directed at the optimization of chlorophyllase biocatalysis, using chlorophyll as a substrate in a wide variety of organic solvents (Khamessan *et al.*, 1993, 1994 and 1995). In addition, preliminary work was conducted for optimizing the biocatalysis of chlorophyllase in the presence of canola oil (Khamessan and Kermasha, 1996). Furthermore, Samaha and Kermasha (1997*a,b*) investigated the use of chlorophyll derivatives as substrates in the biocatalysis of chlorophyllase in a ternary micellar system. These authors have concluded that the poor solubility of chlorophyllase, chlorophyll and its derivatives in aqueous media was improved by the use of the ternary micellar system as it provided a similar model for cell membranes and simple organelles (Khamessan *et al.*, 1993). While the specific activity of chlorophyllase increased in such a system, the use of the free enzyme presented some difficulties such as its dispersion and recovery; these problems could be overcome by the immobilization of chlorophyllase onto an inorganic support (Al-Duri *et al.*, 1995).

The immobilization of the enzyme is of interest as it eliminates intraparticle mass transfer limitations (Poncelet *et al.*, 1993). However, as the author is aware the immobilization of chlorophyllase has not been reported in the past. The immobilization of enzymes by adsorption is considered to be the most economically attractive method of immobilization, for it is simple and inexpensive (Clark, 1994). Adsorption is a physical phenomenon and, in principal, reversible in that both the enzyme and the support can be

recovered unchanged. The binding forces between the enzyme and the support are relatively weak (Sinisterra, 1997). Adsorption of the enzyme is dependent on a number of experimental variables such as pH, nature of the solvent, ionic strength, concentration of enzyme, adsorbent and temperature. A close control of these variables is required for optimal adsorption and retention of activity, owing to the weak binding forces between the enzyme and adsorbent. The major disadvantage of adsorption is desorption of the enzyme from the support, which occurs during use, owing to the weakness of the involved binding forces. However, the use of immobilized enzyme in organic solvent overcomes such problems (Chaplin and Bucke, 1990).

No ideal support for immobilization has emerged to provide a standard for the immobilization of enzymes. Selection of a support and method of immobilization is made by weighing the various characteristics and required features of the enzyme/cell application against the properties, limitations and characteristics of the combined immobilization support (Bickerstaff, 1997).

Throughout this research, the alga *P. tricornutum* was used as a source for the enzyme, chlorophyllase. The enzyme was extracted and partially purified according to the procedure developed in our laboratory by Kermasha *et al.* (1992).

The specific objectives of this research were:

1. To investigate the immobilization of chlorophyllase, using the most appropriate selected inorganic support, and its biocatalysis in a ternary micellar system.

2. To optimize the conditions, including the ratio enzyme to support, incubation time needed for immobilization, immobilization efficiency and specific activity, using the most appropriate selected support.

3. To investigate the effects of different kinetic parameters including pH value, reaction temperature, enzyme stability, selected activators and inhibitors and mass transfer on the hydrolytic activity of immobilized chlorophyllase using chlorophyll and pheophytin as substrates.

4. To compare the effect of recycling on the hydrolytic activity of free and immobilized chlorophyllase in batch and in closed continuous system using chlorophyll and pheophytin as substrates.

2. LITERATURE REVIEW

2.1. Chlorophyllase

2.1.1. Definition

Chlorophyllase (chlorophyll-chlorophyllido-hydrolase, EC 3.1.1.14) is an intrinsic membrane glycoprotein (Lambers and Terpstra, 1985). The enzyme has a high carbohydrate residue connected to the hydrophilic part and an asparagine content associated with the lipophilic part (Terpstra, 1980). Chlorophyllase is found in the photosynthetic membranes of higher plants and algae (Drazkiewicz, 1994). The alga, *Phaeodactylum tricornutum* was reported to be a rich source of the enzyme in comparison with other lower plants (Owens and Falkowski, 1982).

2.1.2. Enzyme Mechanism

Chlorophyllase catalyzes two main reactions; the first is the hydrolysis of chlorophyll and related pheophytin into chlorophyllide and pheophorbide, respectively, as well as the isoprenoid alcohol phytol (Holden, 1976). The second is the biosynthesis of chlorophyll or pheophytin from chlorophyllide and pheophorbide in the presence of phytol (Ellsworth, 1971). Rüdiger *et al.* (1980) proposed the term "chlorophyll synthetase" as a description for the enzyme activity that catalyzes *in vivo* the esterifying reaction between phytol and chlorophyllide. However, chlorophyllase catalyzes *in vitro* the hydrolysis of chlorophyll to chlorophyllide and phytol (Moll and Lutter, 1978).

It is assumed that the chloroplast chlorophyllase is involved in the chlorophyll metabolism in green plants. The enzyme is intimately involved with the stability of chlorophyll molecules in photosynthetic membranes (Ellsworth *et al.*, 1976). Little is known about the site of synthesis of this enzyme. Goodwin (1976) concluded that the chlorophyllase extracted from *Chlorella protothecoides* was synthesized on cytoplasmic ribosomes and was incorporated into the cytoplastomembrane by a protein factor synthesized on chloroplast ribosomes. While, Terpstra (1974) inferred that the chlorophyllase is present in small stromal membranes of the chloroplast and is not very active in large granal membranes.

Terpstra (1977) suggested that the activity of the enzyme was due to the protein part of the chlorophyll-protein complexes. Therefore, one would expect the synthesis and the activity of chlorophyllase to be associated with the synthesis and the presence of chlorophyll-protein complexes (Terpstra, 1977). He also reported that the chlorophyllase-catalyzed chlorophyll hydrolysis was enhanced by the addition of magnesium. The enhancement of chlorophyllase activity upon the addition of magnesium may be due to the interaction of magnesium with the head group of chlorophyllase-associated lipids, which results in a conformational change of the protein. The degree of purified chlorophyll can greatly influence the activity of the enzyme. It was found that, partially purified chlorophylls were more suitable substrates than highly purified chlorophylls (Terpstra and Lambers, 1983). Schoch and Brown (1987) reported that chlorophyllase from *P. tricornutum* hydrolyzed chlorophyll *a* much faster than chlorophyll *b*. This may be due to the higher affinity of chlorophyll *a* to the active site compared to chlorophyll *b*.

Depending on the pH of the environment, chlorophyll *a* or *b* or both undergo hydrolysis by chlorophyllase. In acid media, the enzyme hydrolyzed predominately chlorophyll *b*, whereas chlorophyll *a* is hydrolyzed at a pH above 7.0.

Recently, Heaton *et al.* (1996) reported that the browning of coleslaw tissue is due to chlorophyll degradation, which follows two distinctive pathways (Fig. 1). Pathway A represents the primary loss of the magnesium moiety to form pheophytin, followed by the cleavage of the phytol chain to form pheophorbide, whereas, pathway B involves the primary cleavage of the phytol chain to form chlorophyllide and subsequently pheophorbide. The removal of the central magnesium ion from the porphyrin ring is the result of acidic substitution and/or heat (White *et al.*, 1963). However, Langmeier *et al.* (1993) postulated that there might be a magnesium dechelatase that could perform this function. Cleavages of the phytol chain from chlorophyll can either result from chemical hydrolysis (Schwartz and Lorenzo, 1990) or enzymatic cleavage by chlorophyllase (Amir-Shapira *et al.*, 1987).

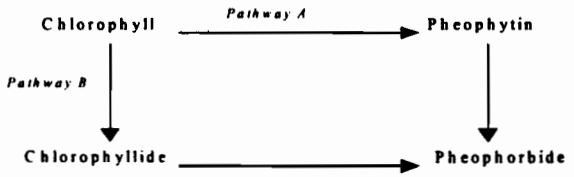

Figure 1. Pathways of chlorophyll degradation in plant tissue (Heaton *et al.*, 1996).

2.2. Chlorophyll and Derivatives

The green color of immature oil seeds is due to the presence of the photosynthetic pigments called chlorophylls. Chlorophylls are a group of tetrapyrrole pigments which

contain an isocyclic ring derived from the C-13 propionic acid side chain of protoprophyrin and are characterized by a central magnesium atom (Scheer, 1991). Investigations on the green pigments which appear in oils have established that there are two chlorophyll components, *a* and *b,* and the products of their decomposition, pheophytins *a* and *b* (Niewiadomski *et al.*, 1965). The ratio of chlorophylls *a* and *b* is usually 3 to 1, respectively. The loss of the central magnesium in chlorophyll yields pheophytin, whereas the removal of the phytol group yields chlorophyllide and pheophorbide. In general, chlorophyll, pheophytin and pyropheophytin are lipophilic compounds due to the presence of a long chain alcohol residue in the molecule usually phytol. On the other hand, chlorophyllide and pheophorbide are hydrophilic compounds (Scheer, 1991).

2.3. Chlorophyll Derivatives in Oils

The exact quantity of chlorophyll depends, to a large extent on the degree of maturity of the oil seeds (Daun, 1982). During extraction, there remains in the oil a portion of the chlorophyll that is very difficult to remove by conventional bleaching methods (Beckman, 1983). Unbleached vegetable oils have variable levels of chlorophyll pigment. Residual chlorophyll contents of up to 5 μg/ml are common. Canola oils, however, may contain up to 15 to 20 μg/ml under normal growing conditions (Mag, 1983), 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 the canola seed to the extent that a significant increase in the chlorophyll content of the oil may result (up to 50 to 60 μg/ml).

The chlorophyll content of edible oil is used as a measure of oil quality. Oils with high levels of chlorophyll and oxidized derivatives can be a major problem in the edible oil industry. Besides giving a product an undesirable color, chlorophyll has been implicated as a pro-oxidant in oxidative rancidity and the subsequent instability of oils (Dahlen, 1973).

2.4. Potential Biotechnological Applications of Chlorophyllase

The Canola Council of Canada considers that the presence of chlorophyll and its oxidized products in canola oil to be of significant commercial impact within the canola industry. Canada being a major producer of canola has a vested interest in solving this problem efficiently (Minguez-Mosquera *et al.*, 1994). The 1992/1993 adverse weather conditions have resulted in unacceptably high levels of chlorophyll in the canola seed, causing considerable concern among the Japanese customers, which are the major importers of Canadian Canola seed (Tautorus and Low, 1993). The present processing costs for oil with high levels of green pigments are very high. Oils of low quality containing high level of green pigment cannot be sold except at very substantial discounts (DeMan, 1990). Crude oils with a chlorophyll content greater than 20 to 30 μg/ml are difficult to refine and oils that exceed this level require special treatment during refining to obtain a product with acceptable levels of color, free fatty acid content and oxidative flavor stability (Suzuki and Nishioka, 1993).

Theoretically, vegetable oil could be decolorized more efficiently by treatment with chlorophyllase as compared to the current conventional procedure. Chlorophyll, a

hydrophobic compound, is converted to chlorophyllide, with a hydrophilic nature, using the hydrolytic activity of chlorophyllase (Mag, 1983). This conversion makes it possible to remove the green pigments from the edible oil into the aqueous media by an extraction technique (DeMan, 1990). Therefore, the development of an enzymatic process, which involves chlorophyllase, may be an alternative technique to the adsorptive bleaching clays used in the canola oil industry (Levadoux *et al.,* 1987).

2.5. Chlorophyllase Biocatalysis in Organic Solvents

The functional properties of chlorophyllase such as pH dependence, substrate specificity and incubation temperature are dramatically altered when the enzyme is suspended in organic solvents. These characteristics are discussed below.

2.5.1. pH Dependance

The optimum pH for chlorophyllase activity from a series of plants has been reported to be from 6 to 8 (McFeeters *et al.*, 1971). The enzyme is classified into three groups: enzymes with an acidic pH optima, such as *Alianthus*, pH 4.5 (McFeeters *et al.*, 1971) and tea leaves, pH 5.8 (Ogura, 1972); enzyme with a neutral pH optima, such as *Chlorella vulgaris*, pH 7.2 to 7.3 (Moll and Stegwee, 1978), sugar-beet, pH 7.1 (Bacon and Holden, 1970) and tobacco, pH 7.0 to 7.5 (Shimizu and Tamaki, 1963); and enzyme with an alkaline pH optima such as *P. tricornutum*, pH 8.5 (Kermasha *et al.*, 1992) and *Chlorella protothecoides*, pH 8.5 (Tamai *et al.*, 1979).

The optimium pH for chlorophyllase activity from *P. tricornutum* was investigated in different systems, including water-miscible (Khamessan *et al.*, 1993),

biphasic (Khamessan *et al.*, 1994) and ternary micellar system (Khamessan *et al.*, 1995; Khamessan and Kermasha, 1996 and Samaha and Kermasha, 1997*a, b*) and reported to be around pH 8.0.

2.5.2. Substrate Specificity

Chlorophyllase has a higher specificity towards chlorophylls *a* and *b* compared to the methyl and ethyl chlorophyllides and pheophorbides (McFeeters, 1975). Ellsworth (1971) reported the use of pheophytin *a*, pheophorbide *a* and methylpheophorbide *a* as substrates for the activity of chlorophyllase. It was also investigated that the carboxymethyl group on position 13 and the hydrogen atoms on position 17 and 18 are essential for the enzyme attack (Holden, 1963).

Mcfeeters (1975) reported small differences in apparent K_m values among magnesium-containing compounds (e.g., chlorophyll *a*, ethyl chlorophyllide *a*) which indicated that the alcohol substituents have only minor effects on substrate binding. However, the apparent K_m values for pheophytin *a*, ethyl pheophorbide *a* and methyl pheophorbide *a* decreased with decreasing size of the alcohol substituent. This showed that, when the magnesium ion was removed from the tetrapyrrole ring, decreasing the size of the alcohol group improved the substrate binding; the data also demonstrated that the major effect of magnesium ion removal was to decrease the apparent K_m.

The substrate specificity of the enzyme may also change depending on the medium in which the enzyme is present. Khamessan *et al.* (1994) investigated the chlorophyllase-catalyzed hydrolytic activity in different organic solvents and found a

relationship between K_m values and log P which is defined as the partitioning of a given solvent and 1-octonal in a two phase system. The results demonstrated that the affinity of the substrate, chlorophyll, to the enzyme decreased by increasing the log P value i.e., K_m values were higher in the more hydrophobic solvents. A low K_m indicated a higher affinity of the substrate to the enzyme, since maximum reaction velocities will already be attained at low substrate concentration (Dordick, 1989).

2.5.3. Optimum Incubation Temperature

The literature indicated a wide range of optimum temperature for chlorophyllase activity (Shimokawa, 1981). Khamessan *et al.* (1993) reported that an increase in the incubation temperature from 25 to 32.5°C increased the enzyme activity by 30%; however, 70% of this activity was lost when the incubation temperature was raised to 40°C. Holden (1963) demonstrated that the greater part of chlorophyllase was denatured during treatment of 37°C for 30 min.

2.5.4. Inhibition Effects

The conversion of chlorophyll to chlorophyllide by chlorophyllase activity is inhibited by phytol (Terpstra, 1974). Khalyfa *et al.* (1995) showed that phytol acted as a competitive inhibitor of chlorophyllase; whereas Khamessan *et al.* (1994) reported a non-competitive behavior of phytol. Oleic acid inhibited an external chlorophyllase activity in lamellar fragments (Terpstra, 1974); this inhibition may be due to the interference of this long-chain polar carbon molecule with the spatial arrangement of the molecules present in the complexes. However, the Mg^{2+} ion would act as a protecting agent against oleic

acid since, chlorophyllase inhibition by oleic acid was lower in the presence of Mg^{2+} ions than in their absence.

Diisopropyl fluorophosphate (DIFP) has a strong inhibitory effect on chlorophyllase activity (Kermasha *et al.*, 1992; Khamessan *et al.*, 1993; Khamessan *et al.*, 1994 and Khalyfa *et al.*, 1995); however, the K_i value of the purified enzyme was lower (0.78 mM) than that of the partially purified one (5.57 mM). Moreover, Terpstra and Lambers (1983) reported that β-carotene, in the absence of other lipids, inhibited the chlorophyllase-catalyzed hydrolytic activity.

2.5.5. Activating Factors

Several factors were known to activate chlorophyllase: *(a)* divalent cations such as Mg^{2+} (Terpstra and Lambers, 1985); *(b)* lipids such as lecithin and *(c)* a combination of cationic divalent ions and reagents such as dithiothreitol (Terpstra, 1980). Terpstra and Lambers (1983) reported that Mg^{2+} was found to activate purified chlorophyllase in the presence of certain chloroplast lipids. Moreover, mixed spinach chloroplast and several single plant lipids were found to activate chlorophyllase-catalyzed chlorophyll hydrolysis.

Khalyfa *et al.* (1995) showed that the addition of β-carotene, L-α-phosphatidyl-DL-glycerol (PG) and L-α-phosphatidycholine (PC) increased the specific activity of the purified chlorophyllase fraction. Terpstra and Lambers (1983) reported that chlorophyllase activity increased by 50 and 35%, respectively, with the addition of PG and β-carotene. However, in the absence of Mg^{2+}, the negatively charged lipid PG, either

alone or mixed with other lipids, may abolish the chlorophyllase activity (Lambers and Terpstra, 1985). Metal ions, such as Mg^{2+}, are believed to primarily induce conformational changes in the protein and secondly, to interact with the head groups of lipids that bind to the protein in the formation of an active enzyme complex (Lambers and Terpstra, 1985).

2.6. Progress in the Biocatalysis of Chlorophyllase in Organic Media

Biocataylsis of partially purified chlorophyllase from *Phaedactylum tricornutum* was studied using chlorophyll and pheophytin as substrates in a model system composed of a wide range of organic solvents including water-miscible, biphasic (Khassman *et al.*, 1994, 1995 and 1996) and a ternary micellar system containing polysorbates or span (Samaha and Kermasha, 1997*b*).

The hydrolytic activity of chlorophyllase was determined in a water/miscible organic media with different mixtures of acetone and Tris-HCl buffer solutions. The storage stability of the enzyme suspended in different environments containing variable concentrations of acetone, indicated that when chlorophyllase was prepared and stored in higher concentrations of acetone, it retained its hydrolytic activity for a longer time compared to that prepared and stored in aqueous media (Khassman *et al.*, 1994).

In the biphasic organic system, the biocatalysis of chlorophyllase was studied using chlorophyll as a substrate under different mixtures of hexane and Tris-HCl buffer solution. The optimum amount of hexane was reported to be 45%, but the incubation time, enzyme content as well as other kinetic parameters were not encouraging compared

to those of the water/miscible system. As a result, further research was carried out which led to the use of the ternary micellar system (Khassman *et al.*, 1996).

In the past, a major problem in investigating the chlorophyllase activity was the insolubility of chlorophyll in the aqueous phase (Bacon and Holden, 1970) which resulted in a wide variation of kinetic data (McFeeters *et al.*, 1971). However, the poor solubility of chlorophyllase and chlorophyll in aqueous media was improved by using a non-conventional media where surfactants were present (Khamessan *et al.*, 1993 and 1994).

Surfactants are regarded as a special group of lipids containing a polar head-group and a non-polar hydrophobic tail. Enzymes act mostly on or near the water/organic medium interface in living cells (Martinek *et al.*, 1989). In nature, membrane proteins exist at the interface between a phospholipid bilayer and the adjacent aqueous medium (Tanford, 1968); thus the use of enzymes in a micellar system simulated the native membrane environment (De Kruijiff *et al.*, 1980). The use of surfactants in water/organic solvent media, spontaneously formed spherical or ellipsoidal associated micelles, whose dimensions were comparable with the molecular dimensions of protein (Martinek *et al.*, 1989).

Non-ionic surfactants appear to be more applicable agents than ionic-surfactants in the solubilization of intrinsic membrane glycoproteins (Helenius and Simons, 1975). The application of non-ionic surfactants was due mainly to their safety and for enhancing solvent solubility (Ismail *et al.*, 1970). Myers (1988) reported that the non-ionic surfactants possess relatively stronger solubility power which could be related to the

looser packing of the surfactant molecules in the micelles of the non-ionic materials; thus making more avaliable space for the incorporation of added molecules. Another advantage of non-ionic surfactants, is they are highly effective in much lower concentration than the ionic ones (Attwood and Florence, 1983).

Khamessan *et al.* (1995) and Khamessan and Kermasha (1996) investigated the optimization of the hydrolytic activity of chlorophyllase in different ternary micellar systems (Tris-HCl buffer/hexane/surfactant) using chlorophyll as a substrate. The surfactants included a wide range of polysorbates (20, 40, 60, 80 and 85) and Spans (20, 40, 60, 80 and 85). Using polysorbate 80, the enzymatic activity increased compared to that in the biphasic system. The optimum kinetic parameters showed a decrease in enzyme content and incubation time compared to the biphasic solvent. Moreover, the use of Span 85 surfactant instead of polysorbate 80 increased the enzyme activity even more compared to the biphasic system with a greater reduction in incubation time and an increase in the affinity of the chlorophyll substrate towards the enzyme, as indicated by the V_{max} and K_m values. Recently, Samaha and Kermasha (1997 *a, b*) studied the hydrolytic activity of chlorophyllase under the same conditions but using chlorophyll derivatives as substrates.

The overall data illustrated that chlorophyllase has lower affinity for chlorophyll derivatives as substrates than that reported for chlorophyll. In both cases, Span 85 was seen to be the most appropriate surfactant for the hydrolytic activity of chlorophyllase (Khamessan and Kermasha, 1996; Samaha and Kermasha, 1997*b*).

2.7. Enzyme Immobilization

2.7.1. Introduction

The need to improve the biocatalysis and stability of enzymes in organic systems has led to the use of immobilized enzymes. Immobilized enzymes have been a topic of ongoing research since the early 1960s but in recent years the immobilization of enzymes and cells has declined slightly. However, the expansion of biotechnology and the expected developments that occurred from advances in genetic technology, has revitalized the enthusiasm for immobilization of enzymes and cells (Clark, 1994). Past research has provided an enormous array of support materials and methods for immobilization. Much of the expansion may be attributed to developments that provided specific improvements for a given application. Surprisingly, there have been few detailed and comprehensive comparative studies on immobilization and the supports used. Hence, no ideal support for immobilization has emerged to provide a standard for each type of immobilization. Selection of the appropriate support and method of immobilization is made by weighing the various characteristics and required features of the enzyme/cell application against the properties, limitations and characteristics of the combined immobilization support (Bickerstaff, 1997).

2.7.2. Definition

As defined by Trevan (1980), immobilization is the entrapment of a biocatalyst in a distinct phase that allows exchange with, but is separated from the bulk phase in which the substrate, the effector or the inhibitor are dispersed and monitored. The biocatalyst is often insoluble in water and is of high molecular weight. In general, immobilized

enzymes can be classified into three categories; carrier-binding, crosslinking and entrapment methods (Chaplin and Bucke, 1990).

2.7.3. Advantages of Immobilized Enzymes

The immobilization of enzymes results in numerous advantages. First, it has long been recognized as a useful tool for retaining enzymes in bioreactors (Horvath, 1974). Second, the enzyme-support system can be easily removed from the solution without contamination of the reaction mixture by the contents of the enzyme preparation (Tramper, 1985). Third, a single aliquot of enzyme can be repetitively used. Finally, immobilization often improves the operational stability of enzymes (Sang-Woo and Rhea, 1992).

2.7.4. Economic Benefits of Enzyme Immobilization

An important factor determining the use of enzymes in a technological process is their expense. Several hundred enzymes are commercially available at prices that are more expensive than expected (Klein and Ziehr, 1990). Their high initial cost, therefore should only be incidental to their use (Zaborsky, 1973). However, due to denaturation, they do lose activity with time. If possible, they should be stabilized against denaturation and utilized in an efficient manner (Goto *et al.*, 1994). When they are used in a soluble form, they retain some activity after the reaction, which cannot be economically recovered for re-use and is generally wasted (Chaplin and Bucke, 1990). This activity residue remains to contaminate the product and its removal may involve extra purification costs. In order to eliminate this wastage and give an improved productivity, simple and economic methods must be used that enable the enzyme to be separated from the reaction

product (Kurokawa *et al.*, 1993). Immobilization achieves the separation of the enzyme and the product during the reaction using a two-phase system (Malcata *et al.*, 1990); one phase contains the enzyme and the other contains the product. The enzyme is entrapped within its phase, allowing its re-use or continuous use but preventing it from contaminating the product; other molecules including the reactants, are able to move freely between the two phases (Parrado and Bautista, 1994). The most important benefit derived from immobilization is the easy separation of the enzyme from the products of the catalyzed reaction. This prevents the enzyme from contaminating the product, minimizing the downstream processing costs and possible effluent-handling problems (Chaplin and Bucke, 1990).

2.8. Classification and Properties of Immobilization Techniques

There are several means of classifying the various types of immobilized enzymes, such as carrier binding, crosslinking and entrapment. A classification may be based on the nature of the interaction responsible for immobilization which can be achieved either by chemical or physical means (Zaborsky, 1973). The classification presented below combines the nature of interaction responsible for immobilization and the nature of the support.

2.8.1. Support-Binding Method

The support binding method, which consists of the coupling of enzymes to solid supports, is the oldest immobilization technique, and hundreds of papers have been published on this type of immobilization. The support binding method can be further

divided according to the binding mode of the enzyme: physical adsorption, ionic binding and covalent binding (Wingard *et al.*, 1976).

(i) Physical–Adsorption Method

The adsorption of enzymes is considered to be the most economically attractive method of immobilization, for it is simple and inexpensive (Anderson *et al.*, 1990). Adsorption of an enzyme is achieved by bringing an enzyme solution in contact with the absorbent surface under the appropriate conditions and following a period of contact, separating the insoluble enzyme preparation from the material in solution by centrifugation or filtration (Yamanaka and Tanaka, 1979.) Adsorption is a physical phenomenon and, in principal, reversible in that both the enzyme and the support can be recovered unchanged. The binding forces between the enzyme and the support are relatively weak (Basri *et al.*, 1996). The adsorption of the enzyme is dependent on the experimental variables such as pH, nature of the solvent, ionic strength, concentration of enzyme, adsorbent and temperature. A close control of these variables is required for optimal adsorption and retention of activity, owing to the weak binding forces between protein and adsorbent (Sinisterra, 1997).

A major influence on the quantity of enzyme adsorbed to a solid support is the enzyme concentration exposed to the unit surface carrier during the immobilization process. The activity increases with increasing enzyme concentration, approaching a saturation value asymptotically at higher enzyme concentration, with a decrease in specific activity. Both time and temperature are important parameters in adsorption of

enzymes, particularly with porous carriers, since diffusion is an important factor for immobilizing enzymes to such carriers (Tramper, 1985).

The major disadvantage of adsorption is desorption of the enzyme from the carrier, which occurs during use, owing to the weakness of the involved binding forces. This in turn results in a loss of catalytic activity and contamination of the product. High concentration of salts or substrates have been shown to enhance the rate of desorption of the enzyme. Thus, adsorption techniques are of limited reliability when absolute immobilization of an enzyme is desired (Basri *et al.,* 1995).

Inorganic supports used for physical adsorption offer several advantages over their organic counter parts. In principle, inorganic supports have high mechanical strength, resistance to solvents and microbial attack and can be regenerated. Moreover, inorganic supports do not change in structure over a wide range of pH, pressure and temperature (Chiabata *et al.*, 1991). Also, inorganic supports are easy to handle and have excellent shelf life. Examples of inorganic supports that have been used include alumina, bentonites, glass, nickel oxide, silica, titanias, zirconais and magnetic iron oxide powder (Weetall, 1975).

(ii) Ionic-Binding Method

Ionic binding is an old and simple way to immobilize enzymes and is mainly based on the ionic binding of the enzyme to the support containing the ion-exchange residues. The main difference between the physical adsorption and ionic binding is the strength of the linkage of the enzyme to the carrier (Taylor, 1991). The resultant binding

forces result in minimal conformational change of the enzyme leading to immobilized enzyme preparation with high activity retention. Ion exchangers are used as carriers for the ionic binding; most frequently they are organic supports with ion-exchange residues, although there are also inorganic supports with similar ion-exchange residues (Kennedy, 1987).

(iii) Covalent Binding Method

Covalent binding to polymeric supports has been the most thoroughly investigated approach to enzyme immobilization. In essence, a covalent bond is formed between a functional group on the enzyme and a reactive group attached to the surface of the solid phase (Guisan *et al.*, 1991). Two main factors have to be considered when choosing a method for the covalent immobilization of an enzyme: *(a)* the type of functional groups on the enzyme through which the covalent bonds with the support material are formed and hence the chemical reaction to be employed; and *(b)* the physical and chemical characteristics of the support material onto which the enzymes are bound (Birnbaum *et al.*, 1996).

2.8.2. Immobilization by Crosslinking

Immobilization of enzymes by crosslinking is based on the formation of intermolecular crosslinking between the enzyme or cells by means of bi or multi-functional reagents. Crosslinking reagents include glutaraldehyde, diisocyanate derivatives, bis (diazobenzidine) and *N,N'*-ethylenebis (maleimide) (Chibata and Sato, 1991). A major disadvantage of crosslinking is the changes in the conformation of the

active center of the enzyme, giving rise to significant loss of enzymatic activity (Fernadez-Lafuente *et al.*, 1995).

2.8.3. Immobilization by Entrapment

The immobilization of enzyme by entrapment can be sub classified into *(i)* fibre entrapment *(ii)* gel matrix and *(iii)* microencapsulation.

(i) Fibre Entrapment

This method of immobilization involves the entrapment of enzymes within the microcavities of synthetic fibres (Hartmeier, 1986). The enzyme molecule is usually entrapped in fibres, continuously produced by the conventional wet spinning techniques for the manufacture of man-made fibres. This method has several advantages over gel entrapment. High surface area for enzyme binding can be obtained by using very fine fibres (Kennedy, 1987). The fibres are resistant to weak acids and alkali, high ionic strength and some organic solvents and can show good resistance to microbial attack depending on the polymer used. The most common polymer used is cellulose acetate, owing to its low cost, good biological and chemical resistance (Kurokawa *et al.*, 1993).

(ii) Gel Matrix

Enzymes can also be entrapped in the interstitial spaces of cross-linked polymers; so as to prevent the escape of the enzyme from the matrix (Long *et al.*, 1996).

Hydrogels are often used to immobilize enzymes due to their minimal conformational changes and loss in activity upon immobilization. However, they still introduce important mass transfer resistance for the substrate to be converted into the

product that can be sent out of the gel (Park and Hoffman, 1990). Certain types of hydrogels exhibit stimuli-responsive properties so that the gel matrix will swell and deswell due changes in temperature. This in turn leads to the "on/off" enzyme activity in the gel matrix. This reversible, thermal feedback control of the immobilized enzyme activity is due to changes in pore sizes and their interconnections and thus, to the relative amount of free water in the hydrogel pores (Dong and Hoffman, 1986).

(iii) Microencapsulation

In 1964, Chang developed microencapsulation and since then, it proved to be a promising immobilization technique widely used in various fields of biotechnology, medicine, pharmacology and agriculture (King *et al.*, 1987).

The encapsulation of an enzyme in thin, semipermeable membranes is more advantageous than bead entrapment (Chang *et al.*, 1996). Here, the encapsulating membrane controls the chemical species exchanged with the medium and prevents thereby enzyme leakage and microbial contamination (Poncelet *et al.*, 1994). The small microcapsule size and thin membrane also minimize mass transfer limitations (Poncelet *et al.*, 1993).

2.9. Reactors for Immobilized Enzymes

The main task of a bioreactor is, by ensuring adequate relative movement between the biocatalysts and their surrounding medium, to increase external mass transfer to such a degree that the external diffusional limitations play no role (Hulst *et al.*, 1985). In order to create optimal hydrodynamic conditions, the reactor has to provide for retention of the

immobilized biocatalyst (Neufeld *et al.*, 1991). The design of enzyme reactors and the choice of the reactor type are perhaps the key elements in any process using immobilized biocatalysts. In practice, it is necessary to analyze the advantages and disadvantages of any particular reactor and to design it according to the intended application (Leung *et al.*, 1997). Some of the different types of bioreactors will be mentioned below.

2.9.1. Stirred Reactors

The stirred reactor is the type most widely employed in fermentation techniques; as the high stirring efficiency ensures rapid mixing and highly efficient oxygen transfer in the case of aerobic fermentation (Hulst *et al.*, 1985). Major advantages are its cheap and simple use as well as its use for batch-wise or continuous reactions (Greiner and Konietzny, 1996). In the batch procedure, the biocatalysts are separated or filtered off at the end of the reaction for use in the next batch; while, in the continuous procedure, the immobilized biocatalysts must either be retained by a sieve across the outlet or continuously re-entered together with fresh substrate (Bouwer *et al.*, 1997).

2.9.2. Loop Reactors

In loop reactors, the contents are forced to circulate loop-wise; this is achieved by a cylinder, which directs the flow, produced either by air, propeller stirrer or liquid jet, into a closed loop (Balcao *et al.*, 1996). An advantage of the loop reactor is that mass transfer can be achieved with only little or moderate expenditure of energy and the biocatalysts are exposed to little shearing force (Balcao *et al.*, 1996).

2.9.3. Bed Reactors

Bed reactors are very popular for carrying out reactions involving particulate biocatalysts and are mostly used for continuous processing. The reactors are termed as packed bed, fluidized bed reactors and well-mixed layer reactors depending on the nature of the particles constituting the bed and the way in which the substrates flow through the reactor (Arica *et al.*, 1998).

(a) Packed Bed

The packed bed reactor is the most popular of all bioreactors for immobilized biocatalyst since it permits the use of the catalysts at the highest possible density; as a consequence, relative to the avaliable reactor volume, the highest possible substrate conversion per unit time is attainable (Huang *et al.*, 1996). The volumetric productivity is therefore usually higher in the bed reactor than in any other type of reactor. However, by no means are all immobilized biocatalysts suitable for use in a packed bed (Miyakawa *et al.*, 1997).

(b) Fluidized Bed

In contrast to the packed bed, the fluidized bed reactor is only loosely filled with biocatalyst particles. The substrate enters from below and is forced upward through the fluidized bed, whereby the biocatalysts are kept in a state of loose suspension by the substrate flow, given the condition that the density of the particles is greater than that of the substrate. Furthermore, retention of the biocatalysts in fluidized bed is only possible if the medium is not too viscous and the flow rate is not too high (Huang *et al.*, 1996).

2.9.4. Membrane Reactors

The membrane reactors are used for separating low molecular weight from high molecular substances by means of a membrane with extremely fine pores. This type of reactor is useful for enzyme reactions where the size of the enzyme molecules differs considerably from that of the product molecules (Bouwer *et al.*, 1997).

An advantage of the membrane reactor is that the biocatalysts are used in their native state, hence avoiding exposure to any inactivation (Miyakawa *et al.*, 1997). In this way, it becomes possible for reactions to proceed continuously or to be repeated (Greiner and Konietzny, 1996).

2.10. Factors Affecting the Kinetics and Properties of Immobilized Enzymes

2.10.1. Conformational and Steric Effects

A change in enzyme activity on immobilization is attributed to conformational changes in the enzyme structure or to steric hindrances in the immediate vicinity of the enzyme molecules (Kennedy, 1987). It is known that the properties of the active and allosteric sites of an enzyme molecule depend strongly on the three dimensional structure of the protein molecule. Thus, when an enzyme is adsorbed or covalently bound to a solid support, the interaction with the support likely results in a modification of the enzyme conformation (Wiseman, 1985).

Steric hindrance, on the other hand, is caused by the shielding effect of the matrix, which renders certain parts of the enzyme molecule less accessible to the substrate. Although, it is often difficult to distinguish between the shielding of the active site and

the reduced diffusivity of the substrate in the porous medium, the shielding or steric effects have been proposed to explain the decrease in the activity of immobilized enzymes (Chaplin and Bucke, 1990). In order to reduce the shielding of the active or allosteric sites, which may accompany the binding of an enzyme to a carrier, a spacer can be used to keep the enzyme at a certain distance from the matrix (Wiseman, 1985).

2.10.2. Nature of Support

The selection of the right support for the biocatalysis of the immobilized enzyme is of the upmost importance, as enzyme efficiency depends largely on the support and its linkage to it (Bickerstaff, 1980). The selected support should have a well-developed internal structure, a large surface area, high porosity and a reasonable pore size distribution. The support should have high affinity or capacity for enzymes and suitable chemical structure (% hydrophobicity) to provide maximum enzyme activity and enzyme substrate contact. The support also should be thermally stable, chemically durable, resistant to contamination and available at a reasonable cost (Al-Duri *et al.*, 1995).

Porosity, particle size distribution and chemical composition are the other factors to be considered because they determine the type and number of surface functional groups that exist on the support surface. The functional groups are responsible for the support participating in the immobilization process and chemical transformation. They also determine the functional properties, such as hydrophilicity and surface heterogenity (Al-Duri *et al.*, 1995).

2.10.3. Michaelis Constant

The Michaelis constant, K_m reflects the affinity between the enzyme and substrate and immobilization of an enzyme can result in the increase or decrease of this parameter. A decrease in the K_m value of an immobilized enzyme leads to a faster rate of reaction than that obtained for its free counterpart. Also, the K_m of an immobilized enzyme could decrease if the charge on the support and substrate are opposite. On the other hand, an increase in K_m upon immobilization implies the use of a higher substrate concentration to achieve the same rate of the reaction obtained with the free enzyme (Sinisterra, 1997).

2.10.4. Maximum Reaction Velocity

The maximum reaction velocity (V_{max}) is influenced by the concentration of its substrate. At high substrate concentrations, the reaction practically obeys zero-order kinetics and proceeds with a constant velocity (V_{max}). At very low substrate concentrations, the speed of the reaction is approximately proportional to the substrate concentration (Hartmeier, 1986).

2.10.5. pH-Activity Profile

When the enzymes are immobilized, the optimum pH of an enzyme reaction may be altered. The changes in optimum pH and pH-activity curve depends on the state of ionization and dissociation of an enzyme and its environment (Clark, 1994). In the support-binding method, when the matrix is charged, the kinetic behavior of the immobilized enzyme may differ from that of the free enzyme (Bailey and Chow, 1974). This is due to the partition effects that cause different concentrations of charged species in the microenvironment of the immobilized enzyme and in the domain of the bulk

solution, owing to electrostatic interactions with fixed charges on the support; resulting in a shift in the optimum pH, with a displacement of the pH-activity profile of the immobilized enzyme towards more alkaline or acidic pH values for negatively or positively charged matrixes, respectively (Kennedy, 1987).

2.10.6. Temperature-Activity Profile

In some cases, changes in the optimum temperature may occur upon immobilization. The optimum temperature of the enzyme immobilized by entrapment, ionic and covalent binding becomes higher than that of the native enzyme (Kennedy, 1987). This may be due to diffusional effects, which protects the enzyme against heat denaturation making the actual temperature in the microenvironment lower than that of the bulk solution (Clark, 1994). The heat tolerance may also be obtained when the enzyme molecule is fixed on a support by ionic or covalent bonds. In some cases, the conformational changes and the steric hindrances play an important role on the increased enzyme's tolerance to heat denaturation (Wingard *et al.*, 1976).

2.10.7. Heat and Storage Stability

Although it is not established that there is a correlation between heat stability and the immobilization method, it has been observed that covalent bonding, entrapment methods usually lead to immobilized enzyme preparations with improved stabilities (Chaplin and Bucke, 1990). The heat stability also depends on the medium used to test the enzymes. In some cases, the immobilized enzyme show higher heat stability than the free enzyme at temperatures higher than 50°C. This stability was improved by both

substrates and preferentially by the products. These substances protect the enzyme against heat denaturation (Wingard *et al.*, 1976).

2.10.8. Partition Effects between the Micro and Macroenvironment

The partition effect is created when hydrophobic, hydrophilic and electrostatic interactions between the support and the substrate and/or effector often produce an unequal distribution of these species between the immediate vicinity of the bound enzyme (microenvironment) and the bulk solution (macroenvironment) (Hartmeier, 1986). The micro- and macroenvironment concentrations of the substrate and effectors can also differ when both the support and the species are electrically charged (Wingard *et al.*, 1976).

2.10.9. Mass Transfer

Mass transfer arises from diffusional resistance to the transport of substrate from the bulk solution to the catalytic sites and from the diffusion of products of the reaction back to the bulk solution (Hartmeier, 1986). Such diffusional resistances are classified as either: internal or intraparticle mass transfer effect when the enzyme is located in a porous medium. External or interparticle mass transfer effects occur between the bulk solution and the outer surface of the enzyme-matrix (Mogensen and Vieth, 1973).

In the case of diffusional resistances, the concentration differences in the system are caused by the respective depletion or accumulation of the substrate and product as a result of the chemical reaction in the enzymatic microenvironment (Poncelet *et al.*, 1992). The extent of substrate depletion and product accumulation in the matrix usually depends

on the size of the species involved. Large molecules have a relatively small diffusivity in the porous medium so that they usually encounter significant diffusional resistances (Nakamura *et al.*, 1996).

2.11. Choice of Immobilization Method

Although, many methods of immobilization techniques have been developed and applied to many enzymes, it is well recognized that no one method can be regarded as the universal method for enzymes (Hartmeier, 1986). This is because of the widely different chemical characteristics and composition of enzymes. Therefore, for each application of an immobilized enzyme it is necessary to find a procedure, which is simple and inexpensive to perform, and which gives a product with good retention of activity and high operational stability (Klein and Ziehr, 1990).

When immobilization is accompanied by a chemical reaction, as in the crosslinking and covalent binding methods, conformational changes in an enzyme must be kept to a minimum to avoid partial deactivation due to involvement of the active site in the immobilization reaction (Basri *et al.*, 1996). This requires the use of the mildest conditions possible to effect immobilization. Once an enzyme is successfully immobilized by chemical means, the operational stability of the immobilized enzyme is high, owing to the strength of the bonds between enzyme molecules (in the case of crosslinking) or between the enzyme and carrier (in the case of covalent binding), and the reluctance of these bonds to disruption by substrate or salt solutions (Birnbaum *et al.*, 1981). In general, crosslinking and covalent binding are not suitable methods for large

scale industrial applications because of the lack mechanical stability of the immobilized enzyme (Chibata *et al.*, 1991).

Adsorption is an attractive method for enzyme immobilization owing to the mild conditions involved in the binding reaction. However, since the binding forces are generally weaker than the chemical binding methods, the operational stabilities are lower through loss of enzyme from the matrix as a result of changes in the ionic concentration, pH, substrate concentration or temperature of the reaction medium (Cho and Rhee, 1993). The use of organic solvent media should minimize such lost, since the enzyme clutches to the support (Anderson *et al.*, 1990).

Immobilization by entrapment results in high retention of activity due to lack of binding between the enzyme and carrier. However, limitation of enzyme activity can occur owing to diffusion effects of large molecular weight substrates and products. Thus, the use of entrapment method must be limited to reactions involving small molecular weight substrate and product molecules (Cao *et al.*, 1995).

3. MATERIAL AND METHODS

3.1. Biomass Production, Extraction and Partial Purification of Chlorophyllase

The biomass of the alga *P. tricornutum* Bohlin (Bacillariphyceae) was cultivated using the incubation medium described by Khalyfa *et al.* (1992) and harvested at the stationary phase. The chlorophyllase fraction was extracted and partially purified according to the procedure described by Kermasha *et al.* (1992).

3.2. Protein Measurement

The partially purified chlorophyllase extract was assayed for protein content, using a modification of the Lowry method (Hartee, 1972). Bovine serum albumin (Sigma Chemical, St-Louis, MO) was used as a standard for calibration.

3.3. Preparation of Enzyme Suspension

The enzymatic suspension was prepared according to the procedure of Khamessan *et al.* (1994). The partially purified extract was suspended in Tris-HCl buffer (20 mM, pH 8.0) containing 4 mM $MgCl_2$ and 5 mM dithiothreitol and solubilized by homogenization using a tissue grinder (Wheaton, Millville, NJ). The enzyme suspension was then diluted with the same buffer to give a final concentration of 5 μg protein per ml. The enzyme suspension and subsequent dilutions were freshly prepared on a daily basis for the enzyme assays.

3.4. Preparation of Substrates

A chlorophyll extract was obtained from spinach leaves and partially purified (Khalyfa *et al.*, 1992). The chlorophyll substrate solution (1 mg/ml) was then prepared

using the partially purified chlorophyll extract according to the method described by Khamessan *et al.* (1994). The pheophytin substrate solution (2 mg/ml) was prepared according to the procedure of Samaha and Kermasha (1997). Stock substrate solutions and subsequent dilutions were freshly prepared prior to the enzymatic assays.

3.5. Enzyme Assay

The enzymatic assay was carried out according to the method described by Khamessan *et al.* (1994) using the ternary micellar system: a mixture (70:30, v/v) of Tris-HCl buffer (20 mM, pH 8.0) and hexane containing 75 μM Span 85. The specific activity of chlorophyllase was defined as μmol hydrolyzed chlorophyll or pheophytin per mg protein per min.

3.6. Immobilization of Chlorophyllase

Five inorganic supports, including alumina oxide (8-200 mesh), celite-545 (70-200 mesh), Dowex-1-chloride (100-200 mesh), glass beads (8-120 mesh) and silica gel (60-230 mesh) (Sigma Chemical, Co.), were investigated throughout this study. The immobilization of chlorophyllase was carried out by suspending 1 mg of chlorophyllase (0.35 mg protein) in 1 ml of different media, including water, Tris-HCl buffer solution (20 mM, pH 8.0) and the ternary micellar system, followed by the addition of different supports. After gentle stirring at 4°C for 60 min, the suspension was filtered (Whatman #541; Sigma Chemical Co.) and lyophilized (Triantafyllou *et al.*, 1997).

In order to evaluate the immobilization efficiency, the immobilized enzyme was washed three times with 0.5 ml of the selected medium. The protein contents of the

washed solution and of the supernatant before and after the immobilization were considered in determining the amount of bound enzyme.

3.6.1. Effect of the Ratio of Enzyme to Support

The effect of different ratios of enzyme to support was investigated by suspending 1 mg of chlorophyllase (0.35 mg protein) in 1 ml Tris-HCl buffer solution (20 mM, pH 8.0) followed by the addition of silica gel ranging in concentration from 1 to 5 mg. After gentle stirring at 4°C for 60 min, the suspension was filtered (Whatman #541; Sigma Chemical Co.) and lyophilized (Triantafyllou *et al.*, 1997). The immobilization efficiency and specific activity were determined as previously mentioned.

3.6.2. Effect of Incubation Time on the Immobilization of Chlorophyllase

The effect of incubation time on the immobilization of chlorophyllase was performed by suspending 1 mg of chlorophyllase (0.35 mg protein) in 1 ml Tris-HCl buffer solution (20 mM, pH 8.0) and then adding 4 mg of silica gel. After gentle stirring at 4°C for an incubation period ranging from 15-60 min, the suspension was filtered (Whatman #541; Sigma Chemical Co.) and lyophilized (Triantafyllou *et al.*, 1997). The immobilization efficiency and specific activity were determined as described.

3.7. Characterization of Chlorophyllase Activity

3.7.1. Effect of pH on Chlorophyllase Activity

The effect of pH on the activity of chlorophyllase in its free and immobilized state was investigated by performing the enzymatic assays according to the method described by Khamessan *et al.* (1994); the reaction medium was composed of the ternary micellar

system consisting of a mixture of Tris-HCl buffer solution (20 mM) at pH values ranging from 7.0 to 9.0 and hexane containing 75 μM Span 85, free or immobilized chlorophyllase extract and chlorophyll. The specific activity of the free and immobilized chlorophyllase was measured after 60 min.

3.7.2. Effect of Incubation Temperature on Chlorophyllase Activity

The effect of temperature was determined on the immobilization of chlorophyllase by incubating enzymatic assays at different temperatures ranging from 20 to 50°C.

3.7.3. Effect of Shaker Speed on Chlorophyllase Activity

The effect of shaker speed ranging from 25 to 200 rpm on the specific activity of the free or immobilized chlorophyllases extracts using chlorophyll or pheophytin as substrate was determined.

3.7.4. Effect of Membrane Lipids on Chlorophyllase Activity

Membrane lipids, ranging from concentrations of 0 to 25 μg, were studied for their effects on the specific activity of the free and immobilized chlorophyllases extracts; the membrane lipids included β-carotene, $_{L}$-α-phosphatidylcholine (PC) and $_{L}$-α-phosphatidyl-$_{DL}$-glycerol (PG) (Sigma Chemical Co.) as well as a mixture of the three compounds at a ratio of (1:1:1, v/v/v).

3.7.5. Effect of Magnesium Chloride on Chlorophyllase Activity

The effect of magnesium chloride ($MgCl_2$) on the chlorophyllase activity of the free and immobilized enzymatic extracts was investigated at concentrations of 0 to 12 mM per assay.

3.7.6. Effect of Diisopropyl Fluorophosphate on Chlorophyllase Activity

The effect of diisopropyl fluorophosphate at concentrations ranging from 0 to 12 mM on the chlorophyllase activity of the free and immobilized enzymatic extracts was determined using the assay procedure described above.

3.7.7. Effects of Substrate Concentration on Chlorophyllase Activity

The effect of chlorophyll (0.10 to 0.34 μM) and pheophytin concentrations (0.02 to 0.20 μM) on the specific activity of the free and immobilized chlorophyllase extracts (5 μg protein) was investigated.

3.7.8. Bioconversion of Chlorophyll and Pheophytin by Chlorophyllase Activity

The bioconversion of chlorophyll (0.12 μM) and pheophytin (0.1 μM) into chlorophyllide and pheophorbide, respectively, by the activity of the free and immobilized chlorophyllase (5 μg protein) was investigated. Enzymatic assays were performed using the method previously mentioned to determine the specific activity of chlorophyllase for each substrate.

3.8. Effect of Temperature on the Storage Stability of Chlorophyllase Activity

The free and immobilized chlorophyllase extracts were suspended in a ternary micellar system and stored at 4.0, 25.0 and 35.0°C with continuous shaking at 150 rpm for different periods of time (0 to 20 h). The chlorophyll substrate (0.12 μM) was then added to the enzyme suspensions after different periods of storage and the enzymatic assays were conducted at 35°C for 60 min at 150 rpm. The same study was performed using the substrate pheophytin (0.10 μM); however, the enzymatic suspension was stored

at 4.0, 27.5 and 35.0°C with continuous shaking at 175 rpm and the enzymatic assays were performed at 27.5°C for 60 min at 175 rpm.

3.9. Effect of Temperature and Reaction Time on Chlorophyllase Activity

The effect of temperature on the activity of the free and immobilized chlorophyllase extracts at different reaction times was investigated. Enzymatic assays were performed in the ternary micellar system containing either chlorophyll (0.12 μM) or pheophytin (0.10 μM) as substrate. Each assay was incubated for different time periods (1 to 10 h) at a broad range of temperatures (20 to 50°C) and the enzymatic activity was measured at various intervals.

3.10. Effect of Recycling on the Specific Activity of Chlorophyllase

The partially purified chlorophyllase extracts in their free or immobilized states were suspended in Tris-HCl buffer solution (20 mM, pH 8.0) and added to the ternary micellar reaction medium containing either chlorophyll or pheophytin as substrate. After 60 min, the enzymatic assay was stopped and the reaction mixture was filtered to obtain the enzyme extract, which was then reused again in a freshly prepared reaction medium. The procedure was repeated until both the free and immobilized chlorophyllase extracts showed no activity. The relative activity was expressed as a percentage of the specific activity of the enzymes after each cycle over the initial specific activity of the enzyme.

3.11. Biocatalysis of Immobilized Chlorophyllase in Continuous System

The chlorophyllase activity of the immobilized enzymatic extract was investigated using a continuous system. The continuous system consisted of a double jacketed column with outlets connected to a water-bath maintained at 27.5 and 35°C. The top end of the

column was attached to a peristaltic pump while the opposite bottom end of the column contained the immobilized chlorophyllase extract bound to filter paper placed on the interior of the column. On the column exterior, solvent resistant tubing connected the bottom outlet of the column to a three-way valve, which permitted collection of the end product and connected back to the peristaltic pump. The double-jacketed column rested on a stirring plate and a magnetic stir bar placed inside the column allowed homogenous mixing of the ternary micellar system with chlorophyll or pheophytin as substrates. The reaction mixture was assayed for enzyme activity during a period of 5-60 min. In addition, the enzymatic activity of the immobilized chlorophyllase extract was determined in batch mode, using 50 µl of the immobilized enzymatic extract (5 µg protein/ml), 70 µl of chlorophyll or pheophytin substrate solution and 880 µl of the ternary micellar system in a 50 ml Erlenmeyer flask fitted with a ground glass stopper; continuous shaking was performed at 150 and 175 rpm for chlorophyll and pheophytin, respectively, at corresponding temperatures of 35.0 and 27.5°C for a period of 5-60 min.

4. RESULTS AND DISCUSSION

4.1. Immobilization of Chlorophyllase on Different Inorganic Supports

Table 1 shows the specific activity of the immobilized chlorophyllase and the immobilization efficiency of the enzyme on five different inorganic supports, using three different environments. These supports were selected on the basis of their inertness, non-toxicity and other numerous advantages including, high mechanical strength, resistance to solvents and regenerability (Yokoseki *et al.*, 1982). The results show that the use of the silica gel support produced the highest immobilization efficiency (74 to 84 %) as well as the highest specific activity (0.13 to 0.34 μmol hydrolyzed chlorophyll per mg protein per min); in addition, the results indicated that in general 80% of the protein extract remained bound to the silica gel support after the enzyme assays. Although the ternary micellar system was suggested as an environment for chlorophyllase biocatalysis (Khamessan and Kermasha, 1996; Samaha and Kermasha, 1997*b*), the results (Table 1) indicated that the buffer medium was the most appropriate one for the immobilization of the enzyme since its specific activity increased by three-fold compared to that of the free biocatalyst.

On the other hand, the results (Table 1) show that a lower immobilization efficiency and specific activity were observed when chlorophyllase was immobilized onto the other supports. These findings suggest that the interaction between those carriers and the enzyme was relatively weak which may have resulted in a desorption phenomena as enzyme efficiency depends largely on the support and its bonding with the enzyme (Sinisterra, 1997). The decrease in enzyme activity after immobilization

Table 1. The immobilization of chlorophyllase by adsorption on different supports, using different environments.

Support	Unbound protein (mg)	Immobilized protein (mg)	Immobilization efficiency (%)[a]	Specific activity[b]
Free enzyme	0.31 (± 0.05)[c]	-	-	0.11 (± 0.01)[c]
Enzyme suspended and washed in deionized water				
Alumina	0.09 (± 0.07)[c]	0.22 (± 0.04)[c]	68.37 (± 0.16)[d]	0.08 (± 0.02)[c]
Celite	0.13 (± 0.09)[c]	0.18 (± 0.12)[c]	57.56 (± 0.39)[d]	0.05 (± 0.03)[c]
Dowex[e]	0.08 (± 0.04)[c]	0.23 (± 0.04)[c]	72.66 (± 0.17)[d]	0.06 (± 0.03)[c]
Glass beads	0.08 (± 0.07)[c]	0.23 (± 0.06)[c]	73.76 (± 0.25)[d]	0.10 (± 0.05)[c]
Silica gel	0.06 (± 0.07)[c]	0.25 (± 0.05)[c]	79.73 (± 0.20)[d]	0.13 (± 0.07)[c]
Enzyme suspended and washed in Tris-HCl buffer solution (20 mM, pH 8.0)				
Alumina	0.11 (± 0.08)[c]	0.21 (± 0.09)[c]	69.53 (± 0.31)[d]	0.10 (± 0.05)[c]
Celite	0.10 (± 0.10)[c]	0.21 (± 0.09)[c]	68.24 (± 0.32)[d]	0.02 (± 0.03)[c]
Dowex[e]	0.08 (± 0.04)[c]	0.23 (± 0.07)[c]	70.88 (± 0.24)[d]	0.05 (± 0.07)[c]
Glass beads	0.09 (± 0.04)[c]	0.22 (± 0.04)[c]	71.08 (± 0.17)[d]	0.18 (± 0.03)[c]
Silica gel	0.07 (± 0.09)[c]	0.24 (± 0.05)[c]	84.56 (± 0.22)[d]	0.34 (± 0.09)[c]
Enzyme suspended and washed in ternary micellar system				
Alumina	0.11 (± 0.04)[c]	0.20 (± 0.04)[c]	64.61 (± 0.16)[d]	0.07 (± 0.09)[c]
Celite	0.12 (± 0.04)[c]	0.19 (± 0.04)[c]	61.89 (± 0.16)[d]	0.05 (± 0.05)[c]
Dowex[e]	0.14 (± 0.10)[c]	0.17 (± 0.08)[c]	52.71 (± 0.26)[d]	0.06 (± 0.03)[c]
Glass beads	0.09 (± 0.12)[c]	0.21 (± 0.07)[c]	69.25 (± 0.25)[d]	0.11 (± 0.08)[c]
Silica gel	0.08 (± 0.05)[c]	0.23 (± 0.07)[c]	73.79 (± 0.25)[d]	0.25 (± 0.12)[c]

[a]The immobilization efficiency was determined as the relative percentage of adsorbed enzymatic protein to that present in the free enzyme extract.

[b]Specific activity was expressed as μmol of hydrolyzed chlorophyll per mg protein per min.

[c]Standard deviation (SD) of samples was performed in triplicate.

[d]Standard deviation (SD) was calculated from the percent relative standard deviation values of unbound protein (RSD1) and immobilized protein (RSD2) according to the equation: $[(RSD1)^2 + (RSD2)^2]^{1/2}$ x immobilization efficiency (Harris, 1987).

[e]Dowex-1-chloride ion-exchange resin is a strongly basic anion.

can be caused by other factors such as changes in enzyme-substrate interactions upon binding to a support, steric hindrance caused by the interaction of the substrate with the immobilized enzyme, partitioning and diffusional effects (Taylor, 1991).

The overall results suggest that the adsorption of chlorophyllase onto silica gel was dependent on the chemical nature of the support, type of enzyme and the nature of the solvent (Cho and Rhee, 1993). Kondo *et al.* (1993) reported that the adsorption of porcine trypsin, horseradish peroxidase and bovine catalase to the surface of silica depends on the presence of polar groups in the enzyme molecules which are attracted to the silanol surface of silica; silica gel is an amorphous inorganic polymer, possessing negatively charged siloxane groups (Si-O-Si) in the bulk and silonal groups (Si-OH) on its surface (Cestari and Airoldi, 1997). In an aqueous solution, water competes for the silonal surface, so that the amount of protein molecules adsorbed depends on the attraction between the protein molecules and the silica interface (Nagata *et al.*, 1998). Our findings suggest that the adsorption of chlorophyllase at the silica could have occurred by the attraction of the hydrophilic part of the enzyme to the silonal surface of silica. Other characteristics of silica gel, which enhance its adsorption capacity, include a well-defined porosity, high surface area and high mechanical, chemical and thermal stability (Nagata *et al.*, 1998).

4.1.1. Effect of the Ratio of Enzyme to Support

Table 2 shows that the effects of different ratios of silica gel support to chlorophyllase. The results indicate that the highest immobilization efficiency and specific activity were obtained when the ratio of enzyme to support was 1 to 4.

Table 2. Immobilization of chlorophyllase by adsorption using different ratios of silica particles.

Enzyme/Support ratio	Unbound protein (mg)	Immobilized protein (mg)	Immobilization efficiency (%)[a]	Specific activity[b]
Free enzyme	0.31 (± 0.05)[c]	-	-	0.18 (± 0.11)[c]
(1:1)	0.07 (± 0.06)[c]	0.24 (± 0.08)[c]	77.67 (± 0.29)[d]	0.19 (± 0.12)[c]
(1:2)	0.07 (± 0.08)[c]	0.24 (± 0.09)[c]	78.29 (± 0.31)[d]	0.21 (± 0.07)[c]
(1:3)	0.07 (± 0.06)[c]	0.24 (± 0.12)[c]	78.54 (± 0.41)[d]	0.28 (± 0.10)[c]
(1:4)	0.06 (± 0.09)[c]	0.25 (± 0.06)[c]	81.79 (± 0.23)[d]	0.38 (± 0.06)[c]
(1:5)	0.07 (± 0.10)[c]	0.24 (± 0.06)[c]	78.06 (± 0.23)[d]	0.29 (± 0.08)[c]

[a]The immobilization efficiency was determined as the relative percentage of adsorbed enzymatic protein to that present in the free enzyme extract.

[b]Specific activity was expressed as μmol of hydrolyzed chlorophyll per mg protein per min.

[c]Standard deviation (SD) of samples was performed in triplicate.

[d]Standard deviation (SD) was calculated from the percent relative standard deviation values of unbound protein (RSD1) and immobilized protein (RSD2) according to the equation: $[(RSD1)^2 + (RSD2)^2]^{1/2}$ x immobilization efficiency (Harris, 1987).

Kermasha and Bisakowski (1998) and Cao *et al.* (1992) have reported similar findings when lipases were immobilized using a wide range of supports. These findings (Table 2) suggest that by increasing the support to enzyme ratio to a certain limit, a larger surface area for more enzyme molecules to bind is provided, which in turn would enhance the rate of enzymatic activity (Klein and Ziehr, 1990).

4.1.2. Effect of Incubation Time

The effect of incubation time on the immobilization efficiency and specific activity of chlorophyllase was investigated (Table 3). The results indicate that the optimum incubation time for the immobilization of chlorophyllase on the silica gel support was 60 min; the results also indicated that, in general, 80% of the protein extract remained bound to the silica gel after the enzymatic assays. However, Table 3 also indicates that a decrease in immobilization efficiency and specific activity was observed beyond the 60 min. Cao *et al.* (1992) and Triantafyllou *et al.* (1997) have reported that the optimum incubation time for the immobilization of lipases using a wide range of supports was 30 min.

4.2. Characterization of Chlorophyllase Activity

4.2.1. Effect of pH on Chlorophyllase Activity

Figure 2 shows that the optimum pH for the hydrolytic activity of both the free and immobilized chlorophyllase extracts was 8.0; however, the specific activity of the immobilized chlorophyllase was higher than that obtained for the free one. Khamessan *et al.* (1995) and Khamessan and Kermasha (1996) reported the same optimum pH for the free chlorophyllase activity. These results suggest that the immobilization of the

Table 3. Effect of incubation time for immobilization of chlorophyllase by adsorption onto silica.

Time (min)	Unbound protein (mg)	Immobilized protein (mg)	Immobilization efficiency[a]	Specific activity[b]
Free enzyme	-	0.31 (± 0.05)[c]	-	0.18 (± 0.03)[c]
15	0.12 (± 0.06)[c]	0.19 (± 0.07)[c]	60.87 (± 0.23)[d]	0.27 (± 0.02)[c]
30	0.12 (± 0.08)[c]	0.19 (± 0.02)[c]	61.29 (± 0.10)[d]	0.29 (± 0.02)[c]
45	0.08 (± 0.10)[c]	0.23 (± 0.10)[c]	72.35 (± 0.33)[d]	0.32 (± 0.07)[c]
60	0.07 (± 0.07)[c]	0.24 (± 0.06)[c]	75.85 (± 0.21)[d]	0.37 (± 0.08)[c]
75	0.08 (± 0.02)[c]	0.23 (± 0.10)[c]	74.54 (± 0.34)[d]	0.27 (± 0.09)[c]
90	0.09 (± 0.09)[c]	0.21 (± 0.07)[c]	70.70 (± 0.25)[d]	0.27 (± 0.04)[c]

[a]The immobilization efficiency was determined as the relative percentage of adsorbed enzymatic protein to that present in the free enzyme extract.

[b]Specific activity was measured as μmol hydrolyzed chlorophyll per mg protein per min.

[c]Standard deviation (SD) of samples was preformed in triplicate.

[d]Standard deviation (SD) was calculated from the percent relative standard deviation values of unbound protein (RSD1) and immobilized protein (RSD2) according to the equation: $[(RSD1)^2 + (RSD2)^2]^{1/2}$ x immobilization efficiency (Harris, 1987).

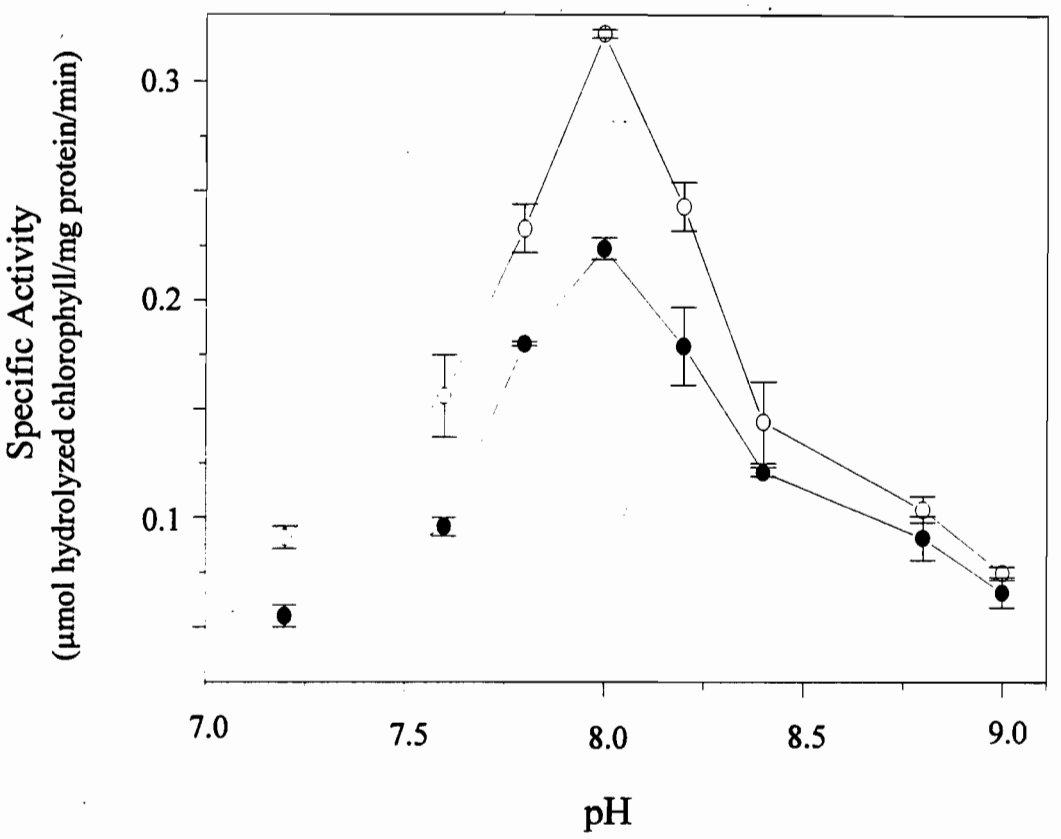

Figure 2. Effect of pH on the specific activity of free (●) and immobilized (○) chlorophyllase in a ternary micellar system.

enzyme have no effect on the optimum pH for chlorophyllase activity. The literature (Basida *et al.*, 1998) indicated that some immobilized enzymes showed no shift in their pH optimum but exhibited narrow or broader changes in their pH-activity profile. A broader profile can be explained by the enzyme's increased resistance to changes in pH upon immobilization, while a narrower profile maybe explained by the opposite trend.

4.2.2. Effect of Incubation Temperature on Chlorophyllase Activity

The results (Figure 3) show that while the specific activity of the immobilized chlorophyllase was higher than that of the free enzyme; the optimum temperature for maximal activity of the immobilized chlorophyllase was found to be 35°C, the same as that exhibited by the free enzyme. The results also showed that the hydrolytic activity of the free and immobilized chlorophyllase extracts decreased gradually from 35 to 45°C and a point was reached at which both the free and the immobilized chlorophyllase extracts showed the same hydrolytic activity. These findings suggest that an increase in temperature beyond a certain point could cause desorption of the immobilized chlorophyllase from its support, resulting into a similar activity as that obtained for the free one. Al-Duri *et al.* (1995) and Basri *et al.* (1995) have reported that the immobilization of lipases on a wide range of hydrophobic supports did not alter the optimum temperature (40°C) of the enzymatic reaction; however, beyond this temperature, the activity of the immobilized enzyme decreased to the level of that of the free one. A similar trend was observed with the immobilization of trypsin by diazo-binding with an amino acid copolymer (Kennedy, 1987). However, Taylor (1991) indicated that in some cases the optimum temperature for maximal activity for an

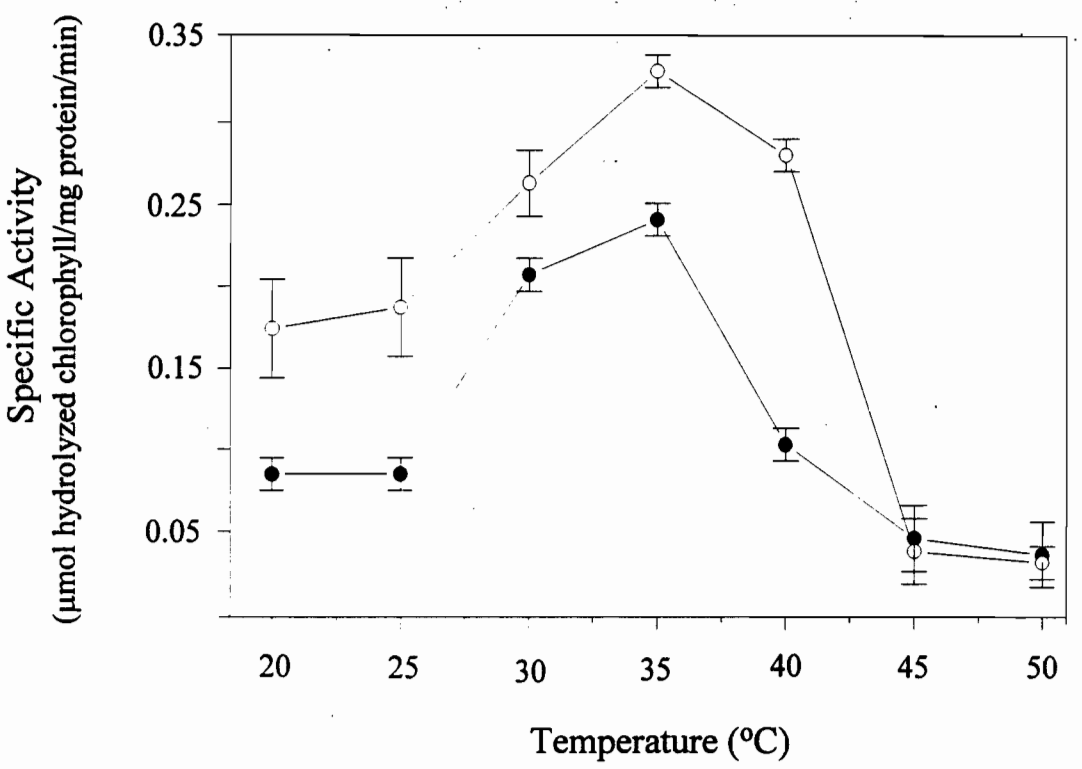

Figure 3. Effect of temperature on the specific activity of free (●) and immobilized (○) chlorophyllase in a ternary micellar system.

immobilized enzyme can be higher than that required for a free one; this may be due to the diffusional effects associated with the support, since the actual temperature in the microenvironment could be lower than that of the bulk solution.

4.2.3. Effect of Shaker Speed on Chlorophyllase Activity

The results (Fig. 4A) indicate that the activity of free and immobilized chlorophyllase extracts, using chlorophyll as a substrate, was enhanced by increasing the shaker speed up to 150 rpm; however, the activity of the free and immobilized chlorophyllase, using pheophytin as a substrate (Fig. 4B), reached its maximum at a speed of 175 rpm. The overall results suggest that the speed of the shaker showed the same effect on the activity of both the free and immobilized chlorophyllase.

Previous research in our laboratory (Khamessan and Kermasha, 1994; Samaha and Kermasha, 1997*b*) indicated similar findings for the free chlorophyllase activity. Khamessan *et al.* (1995) suggested that the increase in chlorophyllase activity may be due to either an increase in mass transfer in the reaction medium or to an increase in surface area as the micelles were broken up by agitation due to increasing speed.

4.2.4. Effect of Membrane Lipids on Chlorophyllase Activity

Figure 5 shows the effects of individual membrane lipids on the activity of the free and immobilized chlorophyllase extracts. The results indicate that the use of the appropriate amounts of individual membrane lipids, including β-carotene, $_{L}$-α-phosphatidylcholine (PC) and $_{L}$-α-phosphatidyl-$_{DL}$-glycerol (PG) as well as a mixture of all three components at a ratio of 1:1:1 (v/v/v) increased the activity of the free

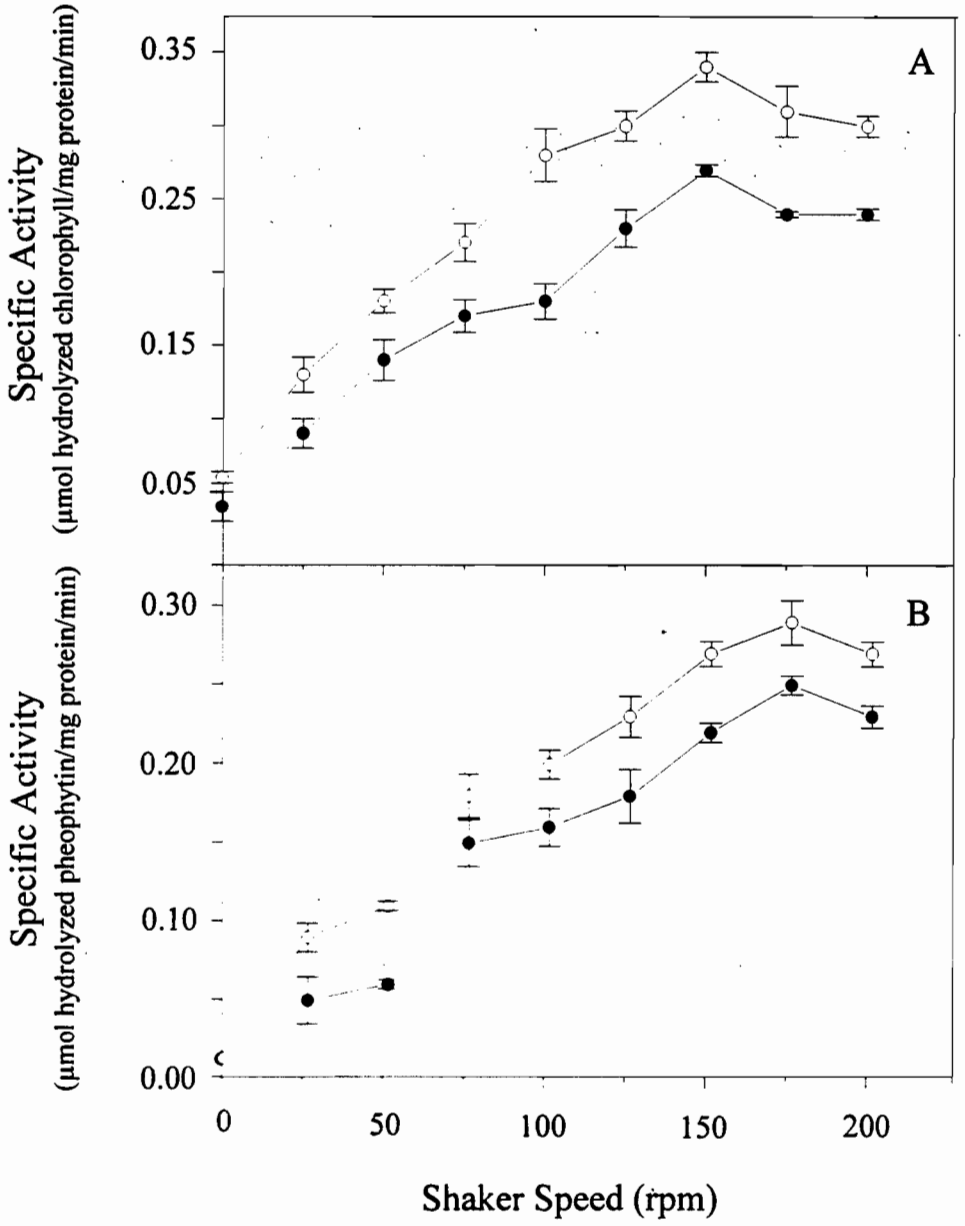

Figure 4. Effect of shaker speed on the specific activity of free (●) and immobilized (○) chlorophyllase in a ternary micellar system, using chlorophyll (A) and pheophytin (B) as susbtrates.

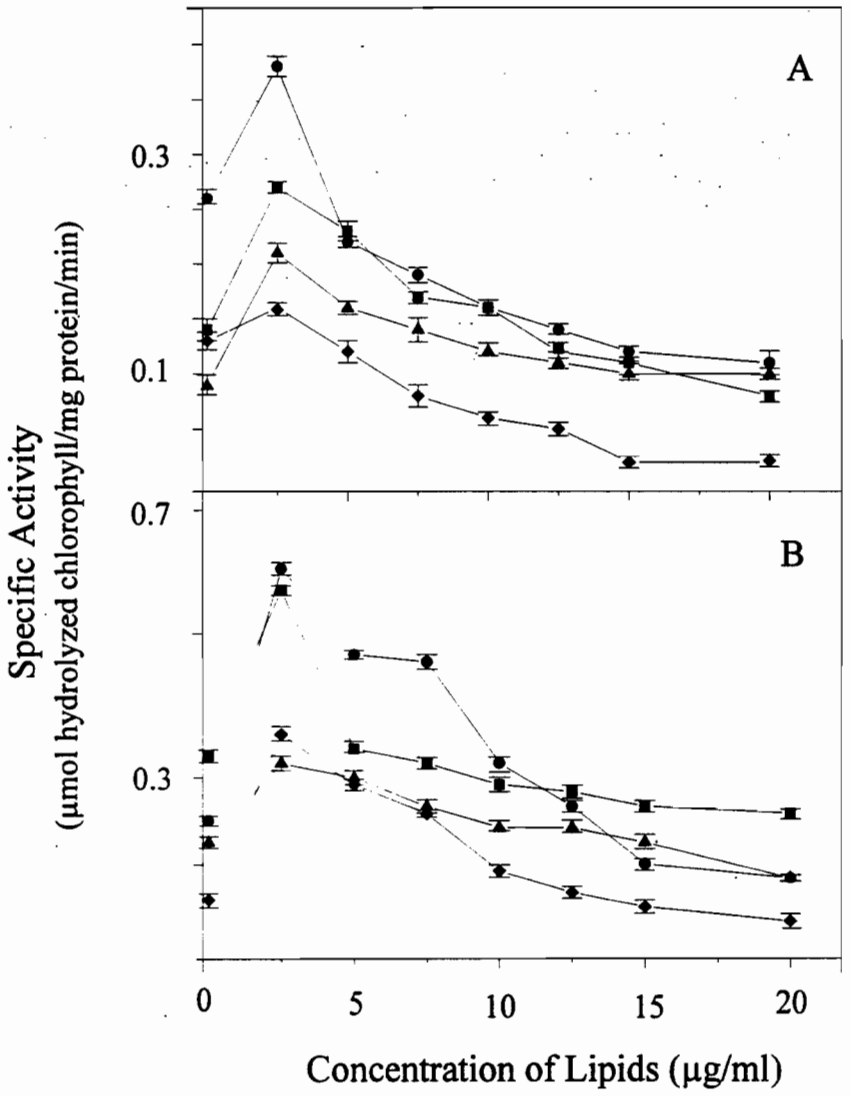

Figure 5. Effect of L-α-phosphatidylcholine (●), a mixture of the three lipids including L-α-phosphatidylcholine, β-carotene and phosphatidyl-DL-glycerol (PG) at a ratio of (1:1:1) (◆), β-carotene (▲) and L-α-phosphatidyl-DL-glycerol (PG) (■) on the specific activity of free (A) and immobilized (B) chlorophyllase in a ternary micellar system.

chlorophyllase extract by 45, 50, 34 and 43%, respectively and that of the immobilized one by 55, 59, 48 and 56%, respectively.

Khalyfa *et al.* (1995) investigated the effects of the membrane lipids on the chlorophyllase activity in an aqueous medium and reported that 40 μg of β-carotene, 20 μg of PG and 60 μg of PC increased the enzyme activity by 95, 61 and 50%, respectively; in addition, a mixture of these lipids decreased and subsequently inhibited the chlorophyllase activity. The difference between our findings and those reported by Khalyfa *et al.* (1995) may be due to the nature of the reaction media or to the degree of purification of chlorophyllase.

Terpstra and Lambers (1983) suggested that membrane lipids could affect the conformation of membrane enzymes such as chlorophyllase; these authors indicated that chlorophyllase activity increased by 50 and 35% with the addition of PG and β-carotene, respectively. The same authors also reported that lipids such as (PG) and digalactosyl diacylglycerol were shown to enhance the aggregation of chlorophyll molecules in an aqueous environment thereby stimulating the formation of crystalline chlorophylls. However, research preformed in our laboratory (Samaha and Kermasha, 1997*b*) suggested that the use of a ternary micellar system might prevent such aggregation.

4.2.5. Effect of Magnesium Chloride on Chlorophyllase Activity

The results (Fig. 6) show that the optimum amount of $MgCl_2$ required for maximal activity of free and immobilized chlorophyllase extract was 5 mM. Figure 6 also

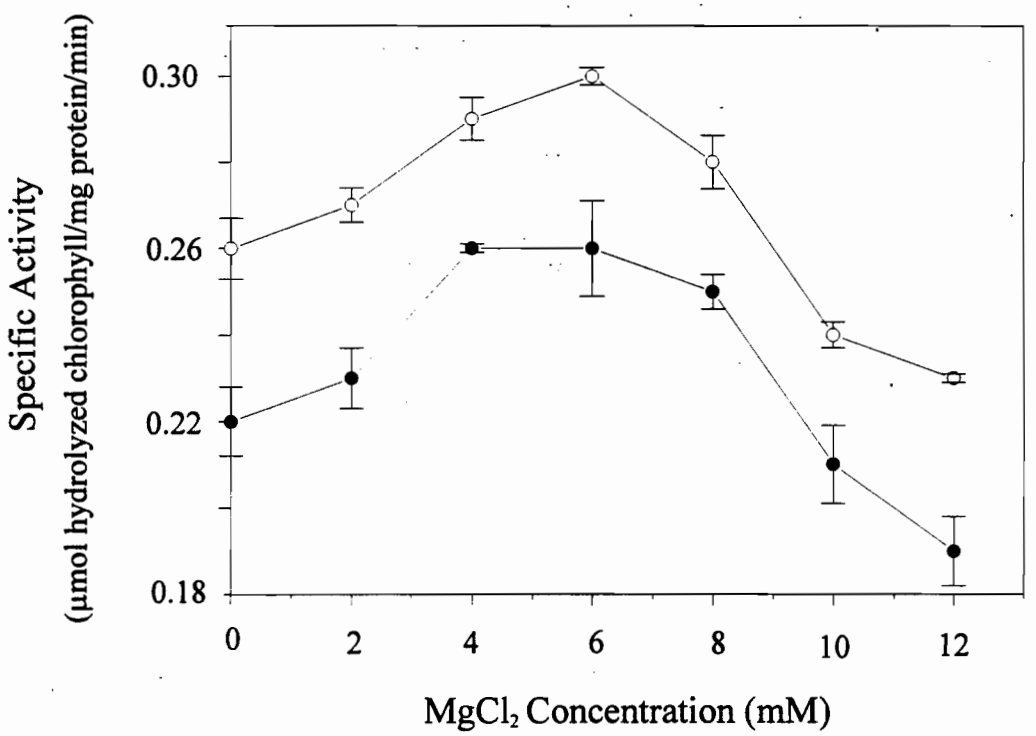

Figure 6. Effect of $MgCl_2$ concentrations on the on the specific activity of free (●) and immobilized (○) chlorophyllase in a ternary micellar system.

shows that the use of higher concentrations of $MgCl_2$ did not further enhance enzymatic activity.

Terpstra (1977) reported that the chlorophyllase-catalyzed chlorophyll hydrolysis, in the presence of spinach chloroplast lipids, was greatly enhanced by the presence of Mg^{2+} combined with dithiothreitol. However, the enhancing effect of Mg^{2+} did not occur when purified chlorophyll was added to the reaction medium in the absence of lipid. Lambers and Terpstra (1985) reported that Mg^{2+}induced conformational changes in the protein resulting in the formation of an active enzyme complex.

4.2.6. Effect of Diisopropyl Fluorophosopahte (DIFP) on Chlorophyllase Activity

Table 4 shows that the K_{mapp} values for the free and immobilized chlorophyllase extracts in the presence of DIFP were 0.10 and 0.16 μM, respectively, while in its absence, the K_m values were 0.06 and 0.01 μM, respectively. These findings suggest that DIFP had a higher inhibitory effect towards free chlorophyllase than the immobilized one. The results also show the V_{maxapp} values obtained for the free and immobilized chlorophyllase extracts in the presence of DIFP were 0.10 and 0.28 μmol of hydrolyzed chlorophyll per mg protein per min, respectively whereas without DIFP the V_{max} values were 0.15 and 0.38 μmol of hydrolyzed chlorophyll per mg protein per min, respectively.

Fig. 7 demonstrates that DIFP showed an irreversible type inhibition for the chlorophyllase biocatalysis. Khalyfa *et al.* (1992) reported similar findings for DIFP on chlorophyllase activity in its free state. Lambers and Terpstra (1985) stated that organophosphosphous compounds such as DIFP are potent irreversible inhibitors that

Table 4: Kinetic parameters for chlorophyllase activity in the presence of diisopropyl fluorophosphate (DIPF).

	Absence of DIFP			Presence of DIFP		
	K_m [a]	V_{max} [b]	(V_{max}/K_m) [c]	K_{mapp} [a]	V_{maxapp} [b]	(V_{maxapp}/K_{mapp}) [c]
Free enzyme	0.06	0.15	2.50	0.10	0.10	1.00
Immobilized enzyme	0.01	0.38	38.00	0.16	0.28	1.75

[a] Michaelis constant of chlorophyllase was measured and defined as μM of chlorophyll.

[b] Maximum velocity of chlorophyllase was determined and defined as μmol hydrolyzed chlorophyll per mg protein per min.

[c] Enzyme efficiency is defined as the ratio of V_{max}/K_m or V_{maxapp}/K_{mapp}.

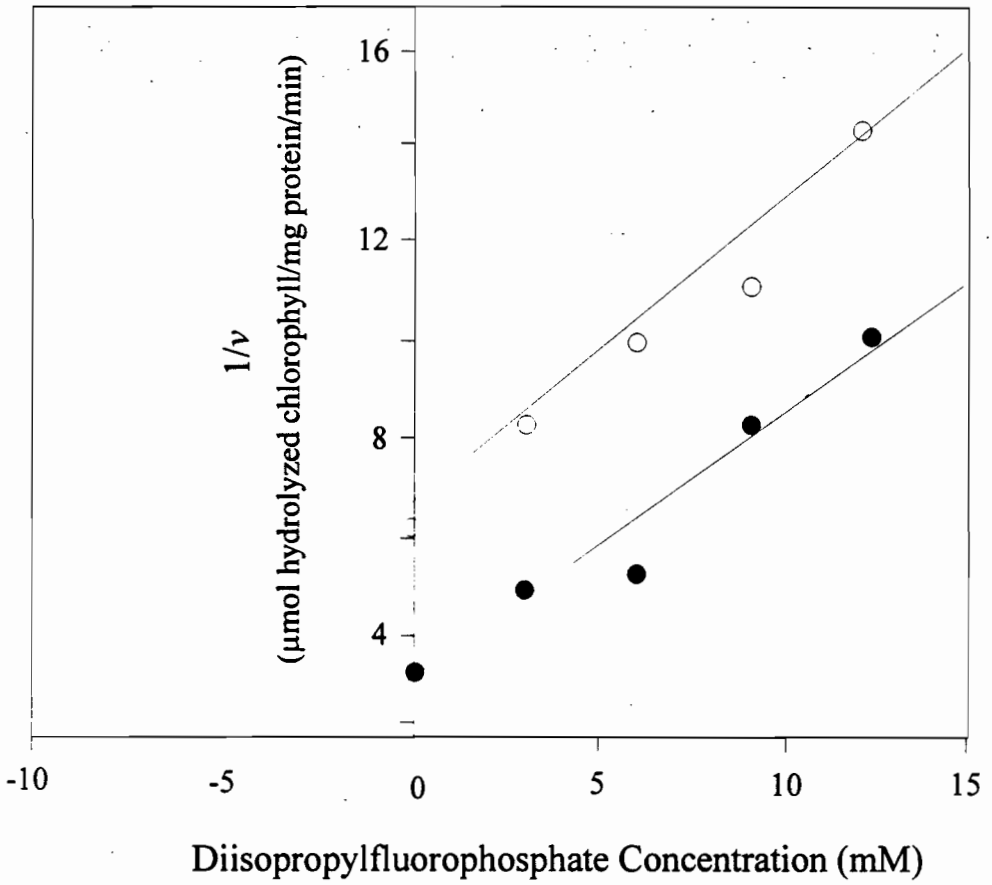

Figure 7. Effect of diisopropyl fluorophosphate concentration on chlorophyllase activity ($1/v$) of free (●) and immobilized (○) enzymatic extracts.

usually inactivate enzymes by covalently binding to their active sites. Moreover, the authors also reported that many irreversible inhibitors including DIFP attach to –SH groups (in cysteine side chains) often found at the active sites of enzymes. Alkylating agents, such as iodacetate and iodoacetamide, form covalent linkages with essential –SH groups (Terpstra *et al.*, 1986).

4.2.7. Enzyme-Substrate Interaction on Chlorophyllase Activity

The kinetic values of K_m and V_{max} (Table 5) for the activity of the free and immobilized chlorophyllase extracts, using chlorophyll and pheophytin as substrates, were obtained from the Lineweaver-Burk plots of $1/v$ versus 1/[S]. The results indicate a decrease in the K_{m1} value from 0.06 μM for the free enzyme to 0.01 μM with the immobilized one, using chlorophyll as a substrate. A decrease in the K_{m2} value of an immobilized enzyme suggests that a faster rate of reaction can be achieved in comparison to that obtained with the free one (Sinisterra, 1997). However, when pheophytin was used as a substrate, an increase in the K_m value from 0.05 μM for the free chlorophyllase to 0.09 μM with the immobilized one was observed; these findings suggest the need of a higher enzyme concentration to achieve the same rate of reaction obtained as that obtained with the free enzyme (Taylor, 1991). Kennedy (1987) reported a decrease in the K_m value when ficin was immobilized by peptide binding on carboxy methyl cellulose azide in comparison to that obtained with the free enzyme. In addition, the hydrolysis of maltose by immobilized glucoamylase showed a decrease in the K_m value of the enzyme upon immobilization (Bickerstaff, 1997).

Table 5. Optimum kinetic parameters for free and immobilized chlorophyllase.

Substrate	Free enzyme			Immobilized enzyme		
	V_{max} [a]	K_m [b]	(V_{max}/K_m) [c]	V_{maxapp} [a]	K_{mapp} [b]	(V_{maxapp}/K_{mapp}) [c]
Chlorophyll	0.15	0.06	2.5	0.38	0.01	38.0
Pheophytin	0.09	0.05	0.6	0.04	0.09	0.5

[a] Maximum velocity of chlorophyllase was defined as μmol hydrolyzed chlorophyll or pheophytin per mg protein per min.

[b] Michaelis constant was defined as μM of chlorophyll or pheophytin.

[c] Enzyme efficiency was defined as the ratio of V_{max}/K_m or V_{maxapp}/K_{mapp}.

Table 5 demonstrates an increase in the V_{max} value from 0.15 to 0.38 μmol of hydrolyzed chlorophyll per mg protein per min after the immobilization of chlorophyllase, using chlorophyll as substrate; this increase may be due to an increase in the stability of both the substrate and enzyme, thereby enhancing their interaction at the interface (Kermasha and Khamessan, 1996). However, the results show that a decrease in the V_{max2} value from 0.09 to 0.04 μmol of hydrolyzed pheophytin per mg protein per min was observed for the immobilized chlorophyllase using pheophytin as a substrate. Kennedy (1987) reported that the V_{max} value for glucoamylase, immobilized by adsorption, was about 10 times higher than that obtained for the free enzyme. However, the immobilization of the enzyme β-D-fructofuranosidase by entrapment resulted in a 10-fold decrease in its V_{max} value.

The overall results suggest that the immobilized chlorophyllase has a higher affinity towards chlorophyll compared to that exhibited for pheophytin. Similar findings (Khamessan *et al.*, 1995; Khamessan and Kermasha, 1996 and Samaha and Kermasha, 1997*b*) were reported using the free chlorophyllase.

4.2.8. Bioconversion of chlorophyll and pheophytin by chlorophyllase activity

Table 6 shows the percentage conversion of the substrates chlorophyll and pheophytin into their respective end-products, chlorophyllide and pheophorbide by the chlorophyllase activity of the free and immobilized extracts. The percentage bioconversion was defined as the percentage of hydrolyzed chlorophyll or pheophytin after the enzymatic reaction compared to that before the enzymatic reaction. The results

Table 6. The bioconversion of chlorophyll and pheophytin, respectively into chlorophyllide and pheophorbide by chlorophyllase activity.

Substrate	Initial substrate (μM)[a]	Hydrolyzed substrate (μM)[b]	Bioconversion (%)[c]	Specific activity[d]
Chlorophyll				
Free enzyme	24.86 (± 0.06)[e]	6.35 (± 0.03)[e]	25 (± 0.13)[f]	0.19 (± 0.04)[e]
Immobilized enzyme	24.86 (± 0.06)[e]	9.43 (± 0.07)[e]	38 (± 0.29)[f]	0.39 (± 0.12)[e]
Pheophytin				
Free enzyme	34.80 (± 0.10)[e]	7.75 (± 0.05)[e]	22 (± 0.16)[f]	0.16 (± 0.07)[e]
Immobilized enzyme	34.80 (± 0.10)[e]	9.07 (± 0.06)[e]	26 (± 0.20)[f]	0.19 (± 0.05)[e]

[a]The amount in μM of initial chlorophyll or pheophytin before enzymatic reaction.

[b]The amount in μM of hydrolyzed chlorophyll or pheophytin after enzymatic reaction.

[c]The percentage bioconversion of chlorophyll or pheophytin into chlorophyllide or pheophorbide, respectively.

[d]The specific activity was measured as the μmol hydrolyzed chlorophyll or pheophytin mg protein^{-1} min^{-1}.

[e]Standard deviation (SD) for samples was performed in triplicate.

[f]Standard deviation (SD) was calculated from the percent relative standard deviation values of hydrolyzed substrate (RSD1) and initial substrate (RSD2) according to the equation: $[(RSD1)^2 + (RSD2)^2]^{1/2}$ x bioconversion rate (Harris, 1987).

show that a higher percentage bioconversion was obtained with the immobilized enzyme (37.93 and 26.06% chlorophyll and pheophytin, respectively) compared to that with the free one (25.54 and 22.27% chlorophyll and pheophytin, respectively). These findings suggest that a higher percentage bioconversion may occur for the immobilized chlorophyllase extract due to the occurrence of a lower diffusional resistance involved in the transportation of the substrate from the bulk solution to the enzyme as well as the diffusion of end-product from the enzyme back to the bulk solution (Kennedy, 1987).

4.3. Effect of Temperature on the Storage Stability of Chlorophyllase Activity

The results show that the free (Fig. 8a) and immobilized (Fig. 8b) chlorophyllase extracts exhibited almost no activity after 10 hours of storage at 35°C using chlorophyll as substrate. The results also show that a considerable loss of activity was observed for the free and immobilized chlorophyllase extracts after 15 hours of storage at 25°C. In addition, the experimental findings indicate that the free and immobilized chlorophyllase fractions stored at 4°C for up to three hours demonstrated an initial increase in activity followed by a subsequent decrease of 25 to 33% which stabilized after 10 hours of incubation. A similar trend was observed for the chlorophyllase activity of the free (Fig. 9a) and immobilized (Fig. 9b) enzymatic extracts using pheophytin as substrate. Moreover, the overall results indicate that the specific activity of chlorophyllase in its free and immobilized states was lower after storage at all temperatures using pheophytin as substrate in comparison to that obtained using chlorophyll. The overall findings also demonstrate that the stability of the enzymatic activity of the free and immobilized chlorophyllase extracts was temperature dependent as indicated by the loss of enzymatic

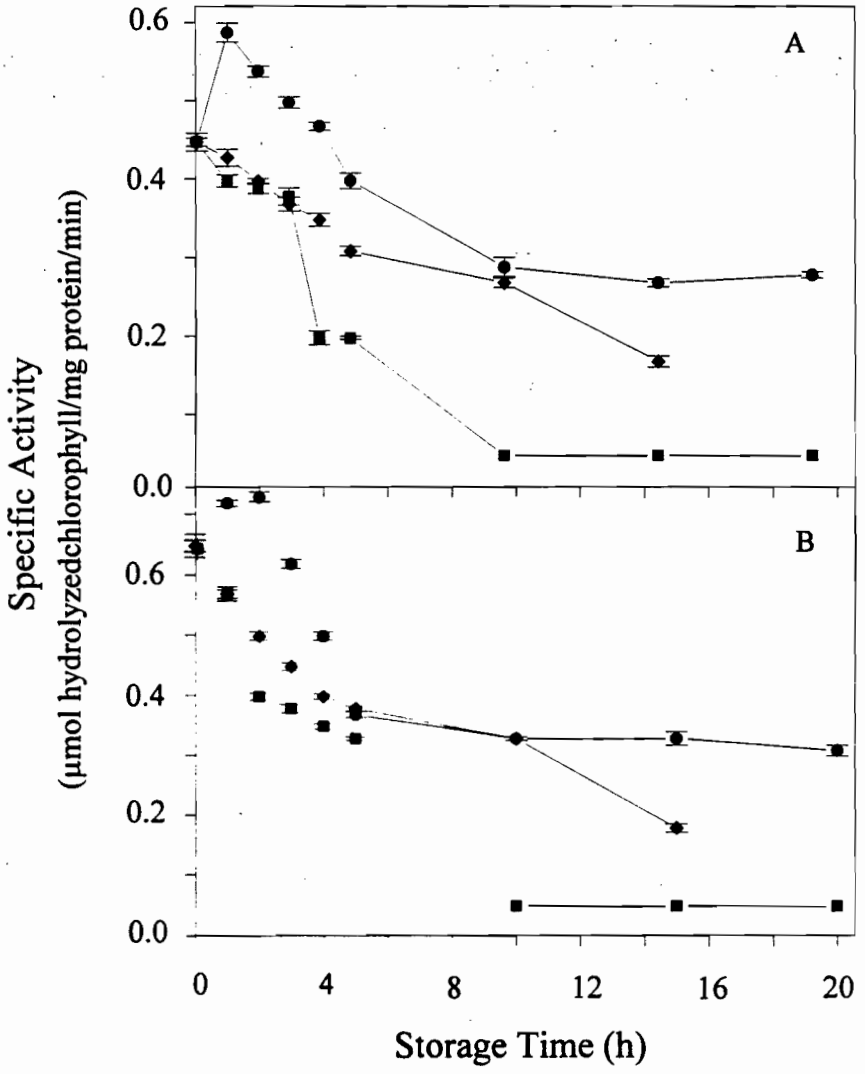

Figure 8. Effect of temperature at 4°C (●), 25°C (♦) and 35°C (■) on the storage stability of the specific activity of the (A) free and (B) immobilized chlorophyllase extracts in a ternary micellar system, containing chlorophyll.

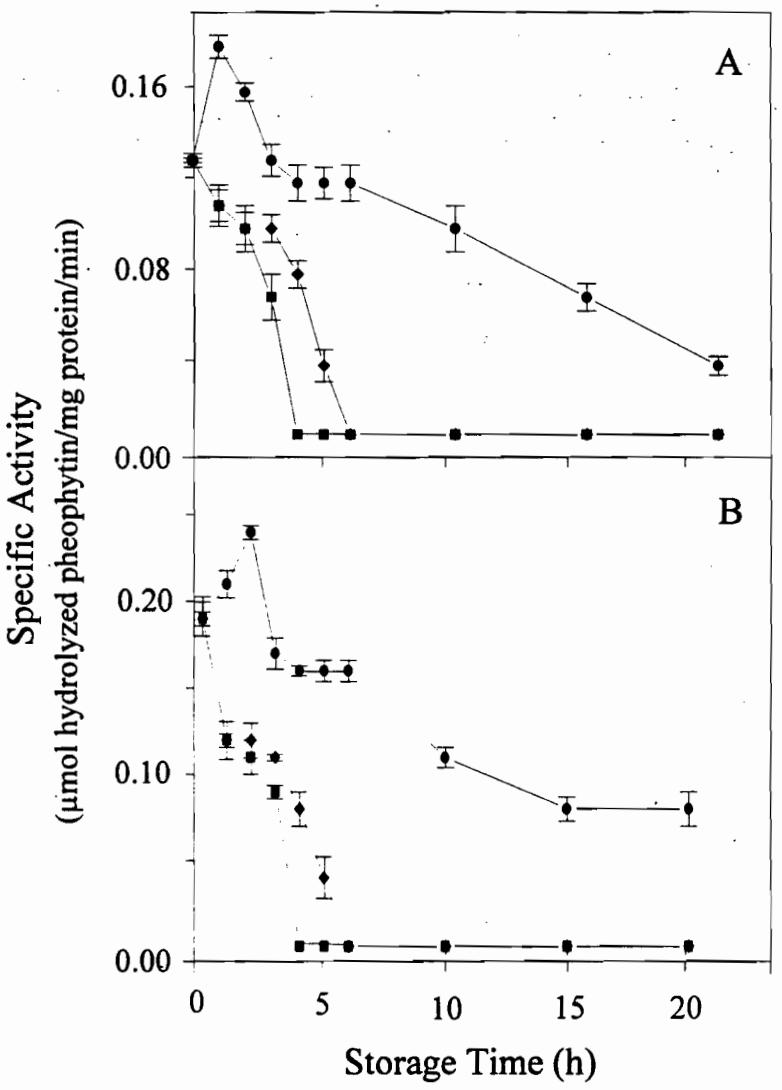

Figure 9. Effect of temperature at 4°C (●), 25°C (◆) and 35°C (■) on the storage stability of the specific activity of the (A) free and (B) immobilized chlorophyllase extract in a ternary micellar system, containing pheophytin.

activity with a concomitant increase in storage temperature. Previous research in our laboratory by Khamessan and Kermasha (1996) reported similar findings for the chlorophyllase activity of free enzymatic extract.

4.4. Effect of Temperature and Reaction Time on Chlorophyllase Activity

Figures 10 and 11 show that the highest initial specific activity of the free and immobilized chlorophyllase fractions with chlorophyll and pheophytin as substrates, respectively, was obtained using an incubation temperature of 30°C, followed by that exhibited at 20 and 40°C. In addition, the results also show that 60 min was considered to be the optimal reaction time at all temperatures for chlorophyllase activity in its free and immobilized states with both the substrates. Khamessan and Kermasha (1996) reported that the optimum temperature and reaction time for chlorophyllase activity in its free state was 35°C for 60 min, respectively, using chlorophyll as a substrate while Samaha and Kermasha (1997*b*) indicated that the optimal enzymatic activity was at 27.5°C for 60 minutes, using chlorophyll derivatives including pheophytin as substrates. The overall findings demonstrate that a continuous decrease in chlorophyllase activity was observed with a concomitant increase in reaction time from 1 to 6 h. The results (Fig. 10 and Fig. 11) also show that although changes in temperature from 20 to 40°C showed little effect on chlorophyllase activity, the specific chlorophyllase activity decreased at temperatures above 40°C. Khamessan and Kermasha (1996) indicated that an increase in chlorophyllase activity at temperatures above 20°C could be due to either the conversion of the enzyme structure into the appropriate conformation or the dehydration of a hydrophilic group of the surfactant thereby favoring the formation of micelles; however,

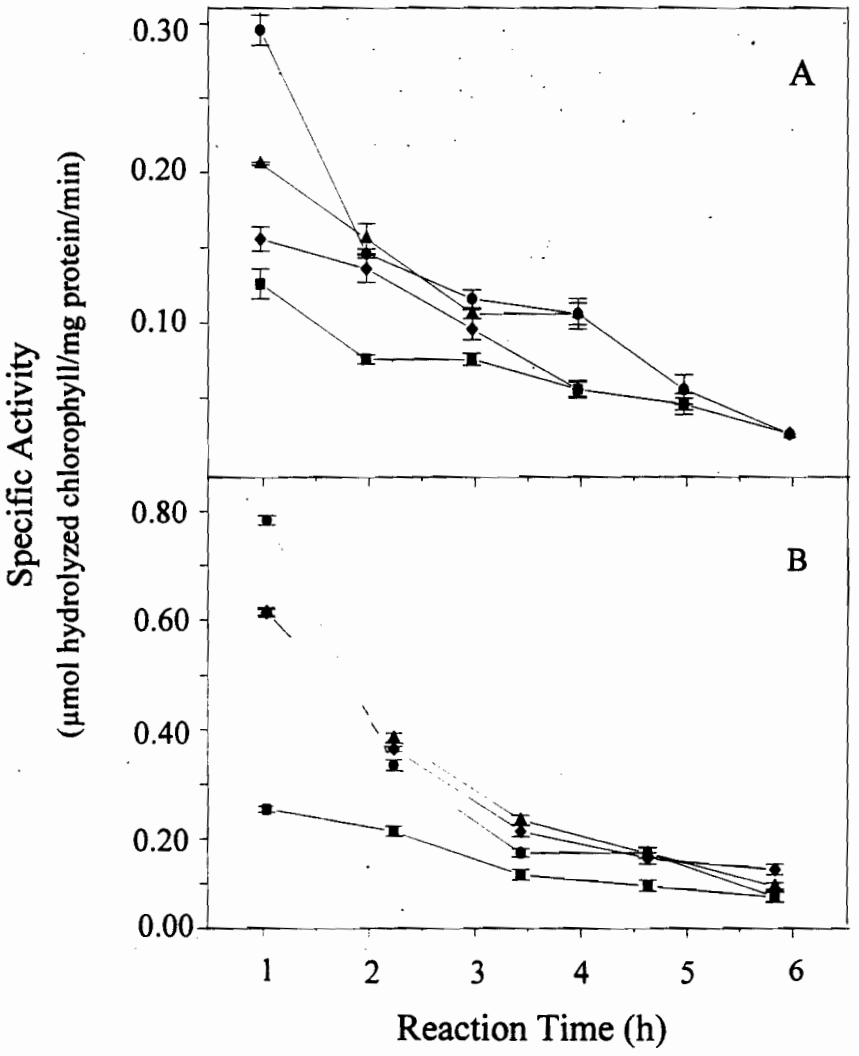

Figure 10. Effect of temperatures at 20°C (▲), 30°C (●), 40°C (♦) and 50°C (■) and reaction time on the specific activity of the (A) free and (B) immobilized chlorophyllase extracts in a ternary micellar system using chlorophyll.

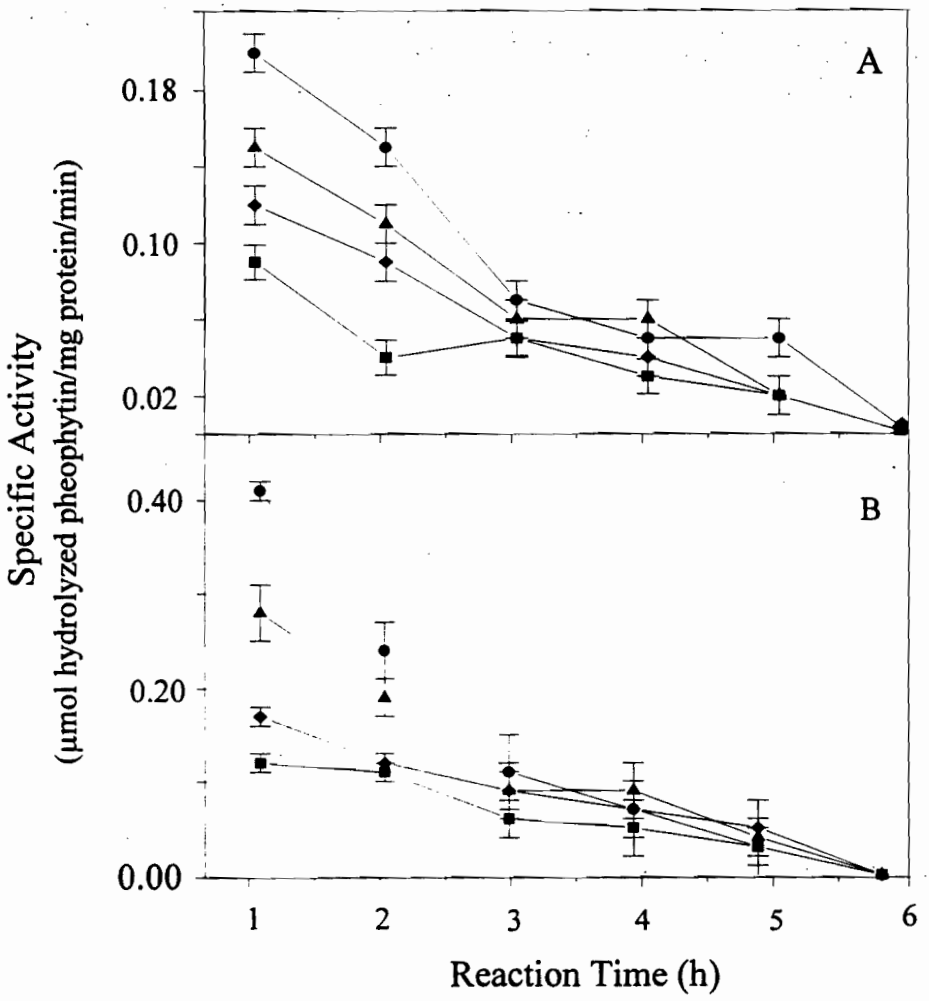

Figure 11. Effect of temperatures at 20°C (▲), 30°C (●), 40°C (♦) and 50°C (■) and reaction time on the specific activity of the (A) free and (B) immobilized chlorophyllase extracts in a ternary micellar system using pheophytin.

an increase in temperature above 40°C could result in the denaturation of the enzyme and/or the formation of a rigid micellar structure.

Overbeek *et al.* (1984) indicated that the temperature plays an essential role in micellar behavior, since the solubility of non-ionic surfactants is highly temperature dependent. Ottewill (1984) also reported that the solubility behavior of surfactants was anomalous; as temperature was allowed to reach a certain value, a sudden increase in the solubility of the surfactants would occur. In addition, Attwood and Florence (1983) indicated that the influence of temperature on enzyme activity could be related to changes in the properties of micelles. Myers (1988) stated that the effect of temperature on the properties of non-ionic surfactants was complex; an increase in temperature might not only favor the formation of micelles by producing a decrease in the hydration of the hydrophilic group, but also disable micelle formation by disrupting the structured water surrounding the hydrophobic group. This finding may explain the lower temperature required for optimal chlorophyllase activity using pheophytin in the micellar system compared to that using chlorophyll since the former substrate may be better solubilized in non-ionic surfactants at lower temperatures than the latter one (Samaha and Kermasha, 1997*a, b*).

4.5. Effect of Recycling on the Relative Activity of Chlorophyllase

The effect of recycling the free and immobilized enzymatic extracts (Fig. 12) on the chlorophyllase activity was investigated using chlorophyll and pheophytin as substrates. The results show that the initial residual activity of the free and immobilized chlorophyllase extracts was 80 and 100%, respectively, using both substrates. The results

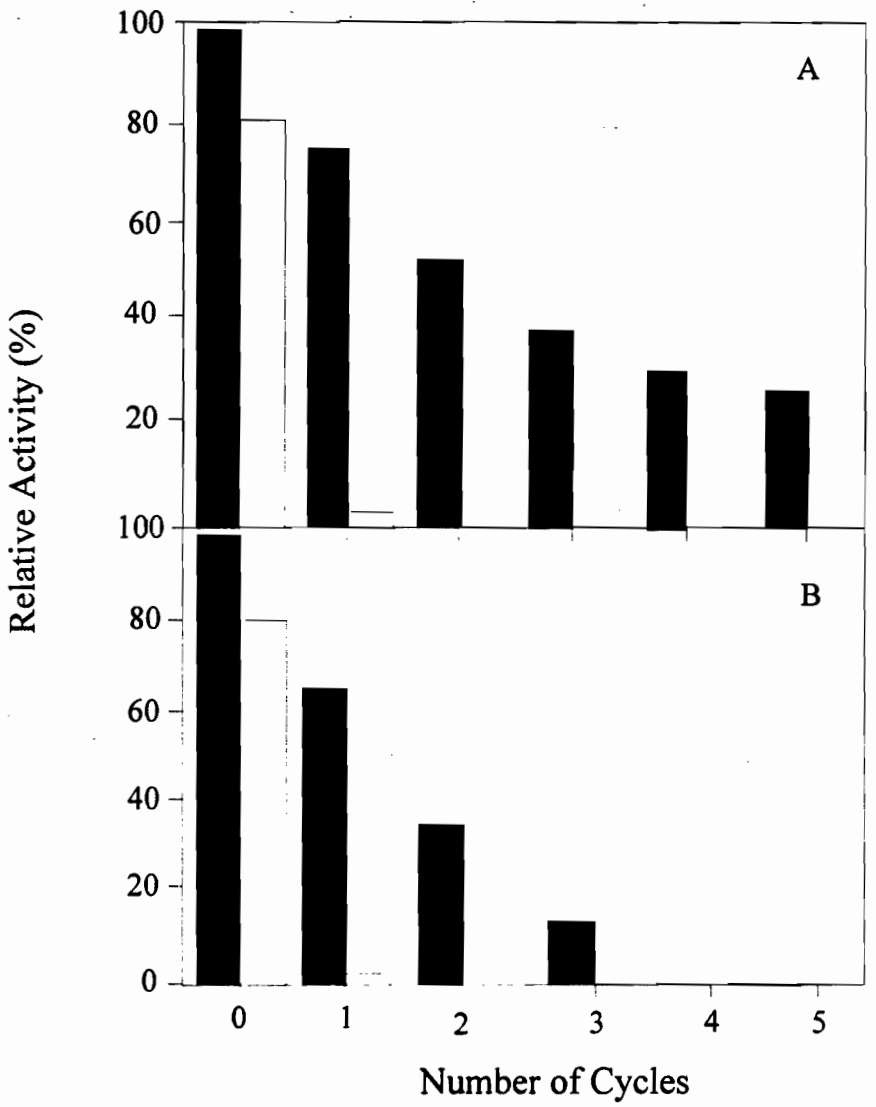

Figure 12. Effect of recycling on the relative activity of free (□) and immobilized (■) chlorophyllase extracts using (A) chlorophyll and (B) pheophytin as substrates.

also show that after the first cycle, the residual activity of the free chlorophyllase extract decreased dramatically by 97.5% and 98% for chlorophyll and pheophytin, respectively. However, the experimental findings also indicate that the activity of the immobilized chlorophyllase extract decreased by only 24 and 36% with chlorophyll and pheophytin, respectively, after the first cycle. In addition, Figure 12 shows that the relative activity of the immobilized chlorophyllase extract continued to decrease steadily reaching a minimum of 26% after five cycles using chlorophyll and 12% after three cycles using pheophytin, as substrate. The overall results show that higher activity was observed when recycling the immobilized chlorophyllase extract with chlorophyll in comparison to that obtained with pheophytin, as substrate.

Bickerstaff (1980) reported that immobilization restricts the movement of the backbone and side chains of the enzyme molecule thereby preventing intermolecular interaction and unfolding of the polypeptide chain. These advantages increase the stability of the enzyme and retain its activity after repeated use.

4.6. Biocatalysis of Immobilized Chlorophyllase in Continuous and Batch Systems

Figures 13a and 13b show that the specific activity of the immobilized chlorophyllase extract using chlorophyll and pheophytin, respectively, was maximal during the first 5 min of reaction time in the continuous system and continued to decrease gradually with time obtaining a minimum at 35 min. These findings suggest that the high stirring efficiency of the magnetic stir bar as well as the pressure of the pump resulted in a homogenous mixture containing the ternary micellar system and substrate thereby resulting in maximum formation of the enzyme-substrate complex during the first

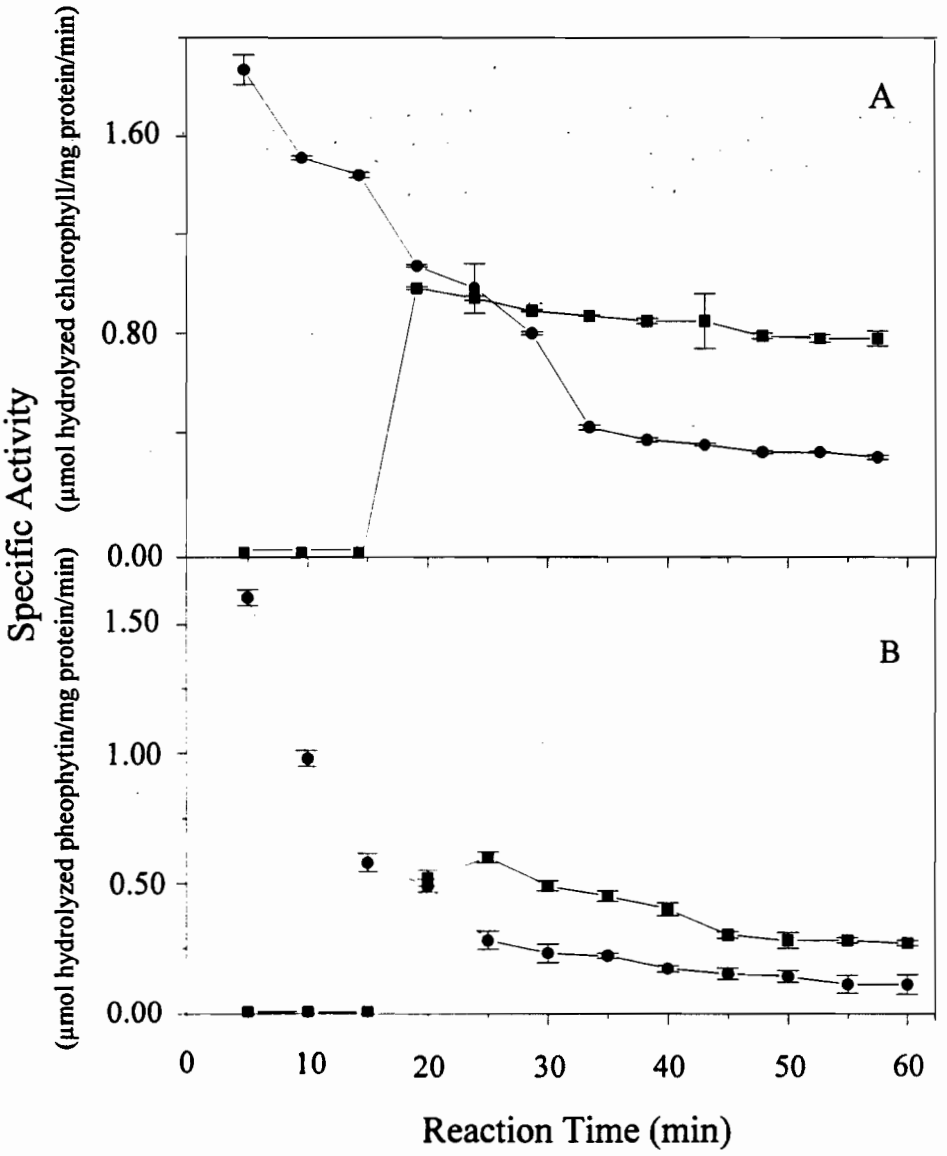

Figure 13. Effect of time on the specific activity of immobilized chlorophyllase extract with (A) chlorophyll and (B) pheophytin using the continuous (●) and batch (■) systems.

5 min. In addition, these findings suggest that the accumulation of the end product could have resulted in a product feed back inhibition thereby decreasing the specific activity with time.

Figures 13a and 13b also show that the enzymatic assays performed by the batch procedure resulted in a gradual increase with time in chlorophyllase activity giving rise to a higher specific activity than that obtained by the continuous system. These findings suggest that the increase in specific activity over time may be due to a longer period of incubation needed for the formation of the enzyme substrate complex in the batch system due to a lesser degree of homogeneity than that found in the continuous closed system.

5. CONCLUSION

The results gathered in this study for the immobilization of chlorophyllase using selected supports showed that the adsorption of chlorophyllase onto silica gel has the highest immobilization efficiency of 85% as well as specific activity of 0.34 μmol hydrolyzed chlorophyll per mg protein per min, respectively. In addition, the results indicated that the immobilized chlorophyllase has a higher affinity for chlorophyll than that exhibited for pheophytin. The addition of magnesium chloride and membrane lipids showed an activating effect on both free and immobilized chlorophyllase extracts. However, low amounts of diisopropyl fluorophosopahte was needed to inhibit the chlorophyllase activity.

Preliminary research performed on the biocatalysis of immobilized chlorophyllase, using batch and continuous closed systems suggested that there was a reduction in substrate concentration or possible feed back inhibition resulting in a decrease in the specific activity of the enzyme in the continuous closed system. However, in the batch system, an increase in specific activity over time suggested the need of longer periods of time required for the formation of the enzyme substrate complex due to a lesser degree of homogeneity than that found in the continuous closed system.

The immobilization of chlorophyllase by physical adsorption could be considered as an appropriate technique for further investigation of the biocatalysis of the immobilized enzyme and its potential applications in the removal of green pigments from edible oils.

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