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Properties and immobilization of urease from leaves of *Chenopodium album* (C_3)

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Abstract. In *Chenopodium album*, leaf excision and light both increase urease (EC. 3.5.1.5) activity. Dithiothreitol (DTT), reduced glutathione (GSH), cysteine and diazoinedicarboxylic acid bis(N,N-dimethylamide) (diamide) activated the crude enzyme. In contrast, crude urease was inhibited by phenylmethylsulphonyl fluoride (PMSF) and N-α-p -tosyl-L-lysine chloromethyl ketone HCl (TLCK), suggesting the presence of serine and histidine residues in the active site. The enzyme is Ca dependent for its activity and exogenous calmodulin (CaM) did not stimulate it. However the enzyme is strongly inhibited by CaM antagonist fluphenazine, indicating the presence of a Ca-like domain. EGTA, $LaCl_3$ and tetraacetic acid, 3,4,5,-trimethoxybenzoic acid 8-(diethyl-amino)-octyl ester (TMB-8) inhibited urease activity in vivo, and the inhibition was restored by exogenous Ca. Urease was immobilized in gelatin by covalent cross-linking with formaldehyde as organic hardener. The results indicated enhanced resistance to thermal denaturation, increased temperature optima (from 30°C to 40°C), and a rapid rate of substrate saturation were achieved after immobilization. The free urease showed remarkable loss of activity in the presence of sodium dodecyl sulphate, sodium deoxycholate, sodium taurocholate, Triton X-100, and Tween 80. The free enzyme lost 68%, 75% and 81% of its activity in the presence of 5,5´-dithiobis-(2-nitrobenzoic acid) (NBS_2), p-hydroxymercuribenzoate (PHMB) and phenylmercuric acetate (PMA) as thiol reagents. However, the immobilized enzyme was not affected significantly by these compounds. By increasing the incubation time, the activity of immobilized enzyme decreased faster than that of the free one.

Keywords: *Chenopodium album*; Immobilization; Properties; Purification; Urease.

Abbreviations: DTT, dithiothreitol; **GSH,** reduced glutathione; **diamide,** diazoinedicarboxylic acid bis(N,N-dimethylamide); **PMSF,** phenylmethylsulphonyl fluoride; **TLCK,** N-α-p -tosyl-L-lysine chloromethyl ketone HCl; **TMB-8,** tetraacetic acid, 3,4,5,-trimethoxybenzoic acid 8-(diethyl-amino)-octyl ester; **PCM,** phenylmercuric acetate; **NBS_2,** 5,5´-dithiobis-(2-nitrobenzoic acid); **PHMB,** p-hydroxymercuribenzoate; **SDS-PAGE,** sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Introduction

It has been proposed that plant urease functions in the assimilation of urea, normally formed in plants as a result of the hydrolysis of arginine to ornithine catalyzed by the enzyme arginase (Polacco and Holland, 1993; Lea, 1997). The enzyme catalyses the hydrolysis of urea to ammonia and carbon dioxide.

The enzyme has been found in the leaves, roots, and bark of plants with actively growing tissues possessing greater activity than senescing ones (Thompson, 1980; Horgan et al., 1983). The enzyme activity in the leaf is also important if foliar application of urea is considered as fertilizer. It has been proposed that foliar treatment with urea increases total leaf yield in mulberry plants, improves leaf nutritional quality, and leads to an increase of cocoon yield (Fotedar and Chakrabarty, 1985; Sarker and Absar, 1995). Nevertheless, the information available on properties of plant leaf urease is quite incomplete.

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Genetic and chemical blocking of leaf urease activity caused necrotic leaf tip, associated with urea accumulation (Eskew et al., 1983; Krogmeier et al., 1989; Stebbins et al., 1991). This reveals that urease plays a significant role in urea metabolism.

Interestingly, urease has been partially purified from soybean leaf (Kerr et al., 1983; Polacco and Winkler, 1984; Hirayama et al., 2000) and seeds of several leguminous plants (Reithel, 1971; Polacco and Havir, 1979). However, the leaf urease yielded was biochemically distinct from that of seed enzyme (Polacco and Winkler, 1984).

Urease from jack bean seed, soybean seeds, and bacteria have a lower optimum pH, at pH 7-8 (Kerr et al., 1983; Mobley and Hausinger, 1989), whereas that of most fungal urease ranges between 8-8.5 (Mobley et al., 1995; Lubbers et al., 1996). Interestingly, urease partially purified from soybean leaves had two pH optima (5.3 and 8.8) (Kerr et al., 1983).

There have been few reports on the purification of urease to homogeneity from a plant leaf source despite its significant role in urea metabolism. This is likely owing

to the low abundance of urease in leaf compared with seed (Polacco and Winkler, 1984).

The judicious and efficient utilization of enzymes in industry requires that they be physically or chemically immobilized. This immobilization on a variety of carriers is well documented in the literature: There is adsorption on raw starch (Ray et al., 1994), entrapment in alginate (Kokufuta et al., 1988), immobilization on ion exchanger (Roy and Hedge, 1987; Deleyn and Stouffs, 1990), silica beds (Germain and Crichton, 1980), porous chitosan (Obha et al., 1979) or agarose (Viera et al., 1988; Sheffield et al., 1995).

In the present investigation, we purify and partially characterize urease from *Chenopodium* leaves as a first step towards understanding its properties. We also deal with the immobilization of pure urease in gelatin matrix and its covalent conjugation by organic cross-linkers. The changes in characteristic features of the enzyme brought about by immobilization are studied and their usefulness from an industrial point of view is also evaluated.

Materials and Methods

Material

Seeds of *Chenopodium album* were sterilized by immersion for 10 min in 95% commercial bleach, followed by 40 min in full strength bleach and 20 min in 50% bleach. Discolored or broken seeds were discarded, while the remaining seeds were washed and imbibed in sterile water for 24 h, and then germinated on water agar until the radicle emerged to a length of 1 mm. The seedlings were then placed in a root growth chamber consisting of a 500-ml beaker containing 250 ml of 50% Hoagland's solution (Hoagland and Arnon, 1950) and covered by a sheet of aluminum foil. The seedling roots were suspended through holes in the foil into the water-saturated air. Plants were grown for two weeks at 30°C in an incubator with 12 h/12 h light/dark cycles. Leaves were collected and used for enzyme preparation.

Urease Preparation

Freshly harvested *Chenopodium* leaves were washed with tap water followed by triple distilled water and dried with filter paper. The similar leaves (50 g) were homogenized in a Waring blender with 100 ml of cold, freshly prepared homogenizing medium containing 50 mM K-phosphate (8.0) and 10 mM DTT. The homogenate was filtered through four layers of muslin, and the filtrate was centrifuged at 5,000 *g* for 30 min to get a clear supernatant.

Purification of Urease

The clear supernatant was fractionated with NH_4SO_4 (25-80%). The protein fraction so precipitated, containing urease activity, was dialyzed against 0.02 M Na phosphate buffer, pH 8.0 and loaded onto a DEAE-cellulose column (3.2 × 20 cm), previously equilibrated with the 0.02 M Na phosphate buffer, pH 8.0. After collecting the pass through, the column was washed with two-bed volumes of the same buffer. The adsorbed proteins were eluted using a linear NaCl gradient (0-1 M). Active fractions (0.3 M-0.4 M NaCl) were pooled and concentrated with sucrose. The enzyme preparation was then subjected to gel filtration through a Sephacryl S-200 column (1.6 × 90 cm), which had been equilibrated with 0.02 M Na phosphate buffer, pH 8.0 containing 0.1 m NaCl and 20% glycerol. The urease rich fractions were concentrated and applied on a Sephadex G-200 column (1.6 × 90 cm) pre-equilibrated with the above buffer. Gel filtration resulted in a single peak of activity. Pooled active fractions were concentrated with sucrose and applied to a Con A-Sepharose column (1 × 6.5), which had been washed with 10 bed volume of the binding buffer (0.2 M Na phosphate buffer, pH 8.0 containing 0.5 M NaCl). Proteins were eluted with a 0.05-0.3 M KCl gradient.

Preparation of Immobilized Enzyme

Gelatin powder (5-10% w/v) used for the immobilization of urease enzyme was swelled in 10 ml (50 mM L^{-1}) phosphate buffer (pH 8.0) and heated at 50°C for 5 min for complete solubilization of gelatin. The mixture was cooled and enzyme (0.8 mg protein in 0.2 ml of buffer) was added. After thorough mixing of the enzyme, the required amount (0.6% w/v) of organic cross- linker, gluteraldehyde was added. The mixture was stirred constantly at 28°C and poured on a (7 × 4) cm^2 glass plate to prepare a thin film of the enzyme. The film was stored at 4°C for 18 h for complete cross-linking. The immobilized enzyme film was washed thoroughly with 50 mM L^{-1} phosphate buffer (pH 8.0) and cut into small blocks before subsequent experiments.

Assay of Urease

The reaction mixture contained 0.1 M-Tris-HCl buffer (pH 8.0), 20 mM urea, 2 mM $CaCl_2$, and the enzyme and was incubated for 30 min at 30°C. The reaction was stopped by adding 10 ml of 1 N H_2SO_4 to the mixture. Urease hydrolysis was determined by measuring the amount of ammonia released from urea using the ammonia assay reagent (Kennedy, 1987). The enzyme activity was expressed in units (U) of katals.

For assaying the immobilized enzyme activity, gelatin blocks containing enzyme (approximately 90 µg of protein) were suspended in 1 ml of 50 mM L^{-1} of phosphate buffer (pH 8.0) containing 20 mM urea. The reaction mixture was incubated at 30°C for 30 min with shaking.

Protein Determination

Proteins were determined according to Lowry et al. (1951) using BSA as standard.

Electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). Proteins were stained with Coomassie brilliant blue.

Results and Discussion

The results in Figure 1 show the urease activity of 2-week-old *Chenopodium* after excision. The seedlings were grown under continuous light for two weeks. The leaves were excised and were incubated in either continuous light or dark. The control leaves were obtained from intact seedlings. At the time intervals indicated, leaves were homogenized and the homogenate was used as crude extract after being filtered. The activity in intact leaves was at a steady level. However, the excision of leaves stimulated urease activity with a peak level at 18 h. In comparison with intact leaves, excision stimulated urease activity by 2.1-fold at 18 h in leaves incubated in darkness. The incubation of leaves in light further stimulated urease increase, and it was 4.5-fold higher than the basal level. However, the profile of increase in urease activity was similar in both dark- and light-exposed leaves. On incubation of excised leaves longer than 18 h, urease activity declined. Several reports have shown that injury to plants caused by wounding or excision initiates a set of responses such as the production of hormones like systemin and jasmonic acid (Taiz and Zeiger, 1998), which in turn stimulate the expression of genes regulating different enzymes.

The results in Table 1 show that urease was activated when crude extracts were treated with 10 mM of DTT, GSH, cysteine or diamide. The 30% increase in activity when extracts are treated with DTT is possibly the result of cleavage of a mixed disulfide between urease and some other thiol compound. However, the activation of urease by diamide could be due to the formation of disulfide resulting from oxidation of two cys residues to cystine(Anderson et al., 1998). On the other hand, PMSF and TLCK at 5 mM inhibited urease activity (Table 1), suggesting an essential role of a serine and histidine residue (Piero and Petrone, 1999) respectively, in the catalytic function of this enzyme.

The purification protocol developed for urease of *Chenopodium* is summarized in Table 2. Ammonium sulphate removed about 45% of the contaminating proteins. The enzyme was purified with a yield of 11.5% and 144-fold. In fact the yield value is higher than that reported for urease from leaves of other plants (Hirayama et al., 2000). The purified enzyme displayed a specific activity of 231U mg^{-1} protein. After this final purification step, a single band was detected on SDS-PAGE (Figure 2), indicating that the enzyme had been purified to apparent homogeneity.

Table 3 represents the effect of Ca ion on urease activity in vitro. Supplementation of the reaction mixture with 1 mM $CaCl_2$ results in a 2.1-fold increase in the urease activity. However, EGTA, which is a specific Ca chelator (Beers and Duke, 1990), inhibited 76% of the activity, indicating that urease is Ca dependent and that addition of Ca to the reaction mixture reversed the EGTA effect. Exogenous calmodulin (CaM) did not stimulate the enzyme. CaM is known to be activated by binding to the ion, and is able to associate with various CaM-dependent proteins (Kurosaki, 1997). Table 3 shows that at 1×10^{-4} mM CaM has no effect on urease activity, but the enzyme is strongly inhibited by 1×10^{-2} mM CaM due to binding to CaM. Treating the pure urease with 5×10^{-3} the CaM antagonist fluphenazine (Joseph and Srivastava, 1995) resulted in inhibition of the enzyme activity, indicating the presence of a CaM-like domain.

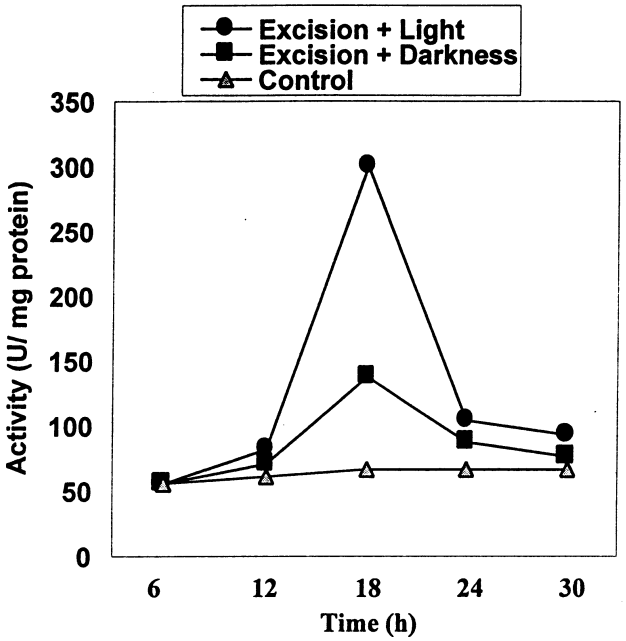

Figure 1. Effect of excision on urease activity of *Chenopodium* leaves.

Table 1. Effect of thiol protecting agents, diamide and some reagents on the crude urease extract. Specific activities are calculated from the mean value of triplicate assay ± s.d.

Additive	Concentration (mM)	Activity (U mg^{-1}protein)	% Activity
None	–	230±0.5	100
DTT	5	300±0.8	130.4
GSH	5	248±1.1	123.5
Cysteine	2	267±1.0	116.1
Diamide	2	280±0.9	121.7
PMSF	5	82±0.5	35.7
TLCK	5	54±0.3	23.5

$LaCl_3$ is known as a Ca channel blocker, and TMB-8 is known as an intracellular Ca antagonist (MacRobbie, 1988). Therefore the effect of EGTA, La3, and TMB-8 in vivo on urease activity was examined. This was achieved by incubating the leaves with these compounds individually for 12 h followed by measuring urease activity. The results in Table 4 show that the three compounds inhibited urease in vivo. Addition of Ca to the medium containing any of these compounds caused the enzyme to be reactivated. The effect of EGTA on urease in vivo appears to indicate that the presence of a minimal Ca level in the apoplast space is necessary for urease activity. However, it is difficult to determine if the increase of cytosolic Ca occurs by an influx from the apoplast solution into the cytoplasm or by an efflux from intracellular organelles.

The activities of free and immobilized urease were assayed at various temperatures (10-60°C) under standard assay conditions. The results (Figure 3) showed that the temperature optima of immobilized enzyme shifted slightly towards high temperature (30-40°C). Increases in temperature optima had been found in immobilized enzymes (Yoshida et al., 1989; Sadhukhan et al., 1993), but the reverse effect was shown by immobilized urease (Sungur et

Figure 2. Purity of urease from leaves of *Chenopodium album* by SDS-PAGE.

Table 2. Purification of urease from *Chenopodium album* leaves.

Fraction	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1}protein)	Fold
Crude extract	280	170	1.6	1
$(NH_4)_2SO_4$ (25-80%)	164	94	1.7	1.1
DEAE-cellulose	114	61	1.9	1.2
Sephacryl S-200	89.0	12	7.4	4.6
Sephadex G-200	66.5	0.8	83.1	51.9
ConA-Sepharose	23.1	0.1	231	144.4

Table 3. Effect of Ca^{+2} ions, CaM and fluphenazine (CaM antagonist) in vitro on pure urease from *Chenopodium* leaves. Specific activities are calculated from the mean value of triplicate assay ± s.d.

Additive	Concentration (mM)	Activity (U mg^{-1}protein)	Relative activity (%)
None	–	225±1.0	100
$CaCl_2$	1	472±0.9	210
EGTA	0.5	54±0.6	24
EGTA + $CaCl_2$	0.5 + 1	296±0.8	131
EGTA + $CaCl_2$ + CaM	$0.5 + 1 + 1 \times 10^{-4}$	220±1.1	98
EGTA + $CaCl_2$ + CaM	$0.5 + 1 + 1 \times 10^{-2}$	153±0.9	68
Fluphenazine	5×10^{-3}	178±1.0	79

Table 4. Effect of EGTA, $LaCl_3$, and TMB-8 in vivo on urease activity in leaves of *Chenopodium*. The cotyledons were incubated with each compound for 12 h followed by measuring urease activity. Specific activities are calculated from the mean value of triplicate assay ± s.d.

Additive	Concentration (mM)	Urease activity (U mg^{-1}protein)	Relative activity (%)
None	-	228±1.0	100
EGTA	20	93±0.8	40.8
EGTA + Ca^{2+}	20 + 1	221±1.2	97.0
$LaCl_3$	10	15±0.7	6.8
$LaCl_3$ + Ca^{2+}	10 + 1	224±0.5	98.2
TMB-8	2	25±1.1	11.0
TMB-8 + Ca^{2+}	2 + 1	216±0.6	94.8

al., 1992). The increase in temperature optima for the activity of immobilized enzyme could be due to the fact that actual temperature in the micro-environment of the gel matrix was lower than in the bulk solution (Kennedy, 1987).

The thermoinactivation kinetics at 70°C of native and immobilized enzyme showed remarkable achievement of thermostability by the immobilized form (Figure 4), with enhanced half-life. The high thermostability of immobilized urease is consistent with the results obtained for other enzymes (Sheffield et al., 1995). This increased tolerance to thermal denaturation, therefore, had been imparted by the gel entrapment covalent cross-linking of the enzyme protein.

Sodium dodecyl sulphate, sodium deoxycholate, and sodium taurocholate are known surfactants (Naeshima and Beevers, 1985). The effect of these compounds on urease was examined, and the results are shown in Table 5. These results demonstrate that the activity of immobilized urease was not reduced significantly in the presence of the various tested compounds, while the free enzyme showed remarkable loss of activity in the presence of dodecyl sulphate. In addition, the free enzyme had lost 68%, 75% and 81% of its activity in the presence of thiol reagents NBS_2, PHMB, and PMA, respectively (Mukhopadhyay, 1997) (Table 4), due to the destruction of thiol groups present at the catalytic site of the enzyme. In contrast, the immobilized enzyme in the presence of thiol reagents restored most of its activity. This phenomenon could be the effect of diffusion hindering additives from reaching the enzyme or by the masking effect of gelatin matrix on the active site of the enzyme.

The effect of urea concentration on the activity of both free and immobilized enzyme was examined (Figure 5). Immobilized urease utilized about five times less substrate concentration than free urease, as is evident from Figure 5. The mass transfer resistance and steric hindrance created by enzyme immobilization restricted the transport of substrate from the bulk solution to the catalytic sites and diffusion of products back to the solution. Hence, only a little amount of substrate concentration was required by

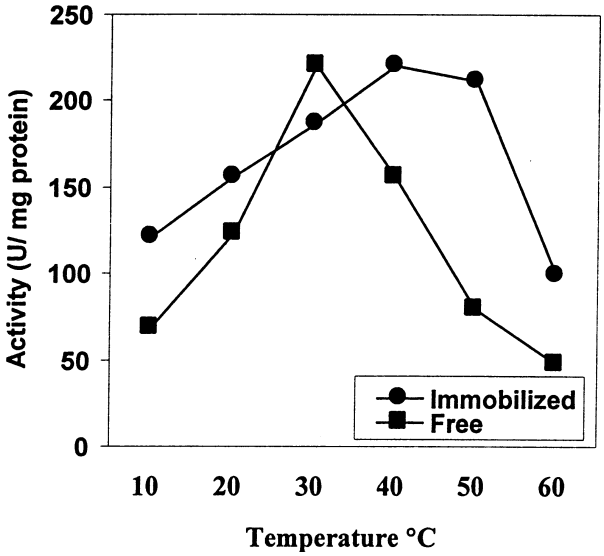

Figure 3. Effect of temperature on the activity of both free and immobilized urease.

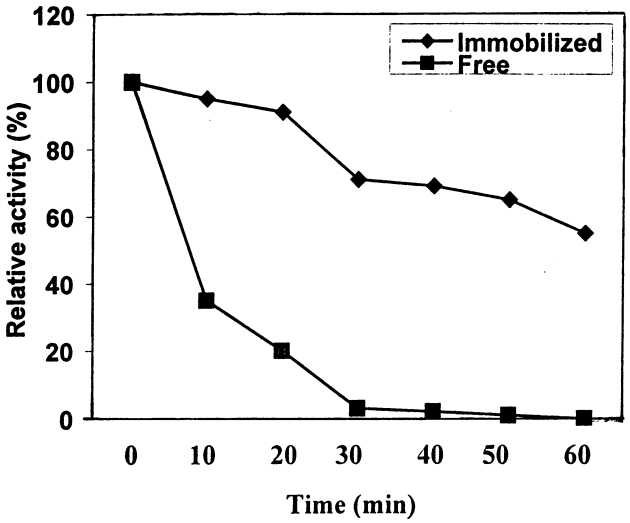

Figure 4. Thermoinactivation kinetics of both free and immobilized urease exposed to 70°C.

Table 5. Effect of thiol reagents and surfactants on the activity of immobilized and free urease of *Chenopodium* leaves. The enzyme was incubated for 30 min at 30°C. Specific activities (U mg^{-1} protein) are calculated from the mean value of triplicate assay ± s.d.

Additive	Concentration (mM)	Immobilized		Free	
		Activity	% Control	Activity	% Control
None	-	230±1.1	100	221±0.3	100
Thiol reagents					
PHMB	2	223±0.9	97	55±1.0	25
NbS_2	2	221±0.4	96	71±0.9	32
PMA	2	219±5.0	95	42±0.6	19
Surfactants					
Na deoxychlorate	0.05	216±0.2	94	130±0.8	59
Na dodecyl sulphate	0.05	212±0.4	92	141±1.2	64
Na taurocholate	0.05	223±0.7	97	77± 95	35
Triton X-100	0.05	214±0.3	93	157±0.7	71
Tween 80	0.05	207±0.2	90	86±1.0	39

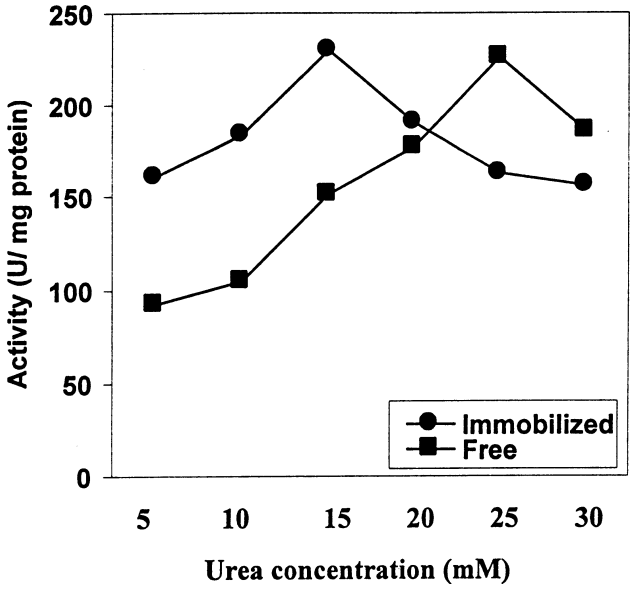

Figure 5. Effect of urea concentration on the activity of free and immobilized urease.

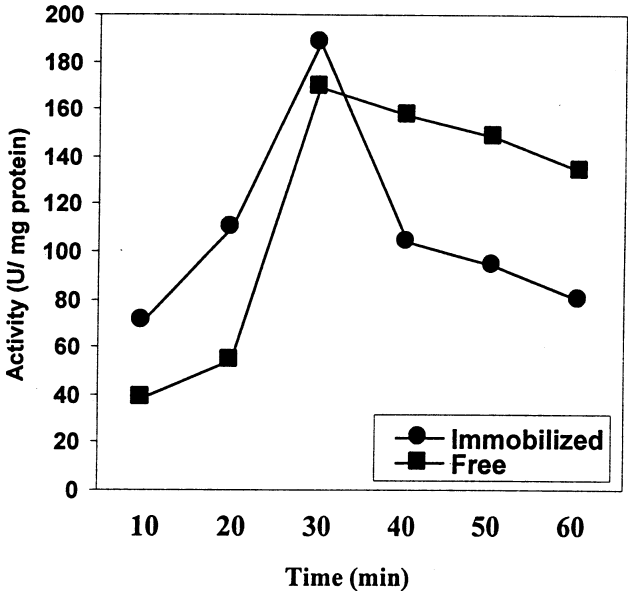

Figure 6. Effect of incubation time on the activity of free and immobilized urease.

the immobilized enzyme to achieve the same rate of reaction obtained with free enzyme.

To analyze the effect of diffusion limitations on reaction yield, incubation of both free and immobilized enzyme was varied from 10 to 60 min, keeping other operational conditions (pH and temperature) constant. The obtained results are illustrated in Figure 6 and indicate that the activity of thiol reagents immobilized urease decreased faster than the free enzyme one. Since other parameters were kept constant, it could be concluded that the diffusional barrier of the gelatin matrix affecting the extent of enzyme-substrate reaction was responsible for the faster activity decrease of immobilized urease. Similar results were obtained for immobilized urease (Sungur et al., 1992).

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藜葉片尿素酶之特性及固定化作用

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當藜葉片被切除或光照時其尿素酶的活性會增加， Dithiothreitol（一種氫硫基化合物）、還原態的麩胱肽、半胱氨酸、以及醯二氨可活化此粗抽的酵素。相反的，此粗抽的酵素活性受 PMSF 及 TLCK 之抑制，此結果建議於其活性部位含有絲氨酸及組織氨酸。此酵素活性需鈣離子，外加鈣調素 (CaM) 並不激化其酵素活性，然而鈣調素拮抗物 fluphenazine 強烈的抑制其活性，此結果建議存在一個 Ca-like domain。EGTA、$LaCl_3$ 及 TMB-8 於活體內抑制尿素酶的活性且外加的鈣可以恢復此受抑制的活性。利用甲荃作為有機硬化劑可將尿素酶經共價結合至 gelatin 上，固定化作用結果導致該酵素增加抗熱解離作用、增加溫度的最適值（30°C to 40°C）、及快速的基質飽合作用。於 sodium deoxycholate、sodium taurocholate、Triton X-100 及 Tween 80 下此自由態的酵素活性顯著減少，於含 thiol 藥劑如 NBS_2、PHMB 及 PMA，其活性亦分別減少 68%、75% 及 81%，然而固定化的酵素活性並沒有顯著受這些化合物的影響。增加反應時間，固定化的酵素活性減少速率比自由態的酵素活性減少速率為快。

關鍵詞：藜；固定化作用；特性；純化；尿素酶。