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Purification and properties of urease from the leaf of mulberry, *Morus alba*

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Abstract

Urease was purified from leaves of mulberry (*Morus alba*, L.) by ammonium sulfate fractionation, acetone fractionation and sequential column chromatography including Q-Sepharose HP, Phenyl-Sepharose HP, Superdex 200 HR and Mono Q. The enzyme was purified 5700-fold to apparent homogeneity with a recovery of 3.6%. The molecular mass of the enzyme was determined to be 90.5 kDa by sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis and 175 kDa by gel filtration, indicating that the enzyme was a homodimer. In the western blot analysis, 90.5 kDa subunit of the mulberry leaf urease cross-reacted with antiserum raised against jack bean seed urease. The N-terminal sequence of the first 20 residues of the enzyme revealed that it has a high similarity (80–90%) to ureases from other plant sources, suggesting that the mulberry leaf urease is closely related to other plant ureases. However, the mulberry leaf enzyme showed an optimum pH for activity of 9.0, while the optimum pH of most ureases isolated from plants and bacterial is neutral. In addition, the K_m value for urea was 0.16 mM, which is lower than those of ureases from other sources. It is also proposed that urease activity ingested by browsing silkworm releases ammonia that is subsequently used in silkworm protein synthesis.

Keywords: Mulberry (*Morus alba*); Moraceae; Urease; Ammonia; Urea; Arginine; Sericulture; Silkworm; Enzyme purification

1. Introduction

Urease catalyses hydrolysis of urea to ammonia and carbon dioxide. Although this enzyme is especially abundant in legume seeds, it is possibly widespread in all plants albeit at lower levels of activity (Horgan, Swift & Done, 1983) Seed urease has, however, been purified to homogeneity from several leguminous plants and intensively studied (Reithel, 1971; Polacco & Havir, 1979). Interestingly, leaf urease was also partially purified from soybean, this being serologically and biochemically distinct from that of seed urease (Kerr, Blevins, Rapp & Randall, 1983; Polacco & Winkler, 1984). Genetical and chemical blocking of leaf urease activity also caused necrotic leaf tip associated with urea accumulation (Stebbins, Holland, Cianzio & Polacco, 1991; Krogmeier, McCarty & Bremner, 1989; Eskew, Welch & Cary, 1983), indicating that it has a significant role in urea metabolism. Nevertheless, the information available on properties of plant leaf urease is quite incomplete.

Plant urease has been generally proposed to function in the assimilation of urea derived from ureides or arginine (Reinbothe & Mothes, 1962; Polacco & Holland, 1993). Arginine is one of the predominant free amino acids in the stem of mulberry tree especially during winter, and is considered to function as its major nitrogen storage compound. Indeed the arginine accumulated in stem bark in autumn gradually decreased during the following spring (Suzuki, 1984), suggesting deployment for new grown tissues such as developing leaves. Moreover, when mulberry plants in pots were placed in darkness, arginine rapidly accumulated in its leaves according to a decrease in leaf argi-

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Table 1
Purification of urease from mulberry leaves

Purification step	Total protein (mg)	Total activity (nkat)	Specific activity (nkat/mg protein)	Yield (%)	Purification (fold)
Crude extract	5170	392.4	0.076	100	1
Ammonium sulfate	240	267.9	1.12	68.3	14.7
Acetone	25	122.9	4.92	31.3	64.7
Q-Sepharose HP	3	92.4	30.8	23.5	405
Phenyl-Sepharose HP	0.48	88.4	184	22.5	2420
Superdex 200 HR	0.061	25.7	421	6.5	5540
Mono Q	0.033	14.3	433	3.6	5700

nase activity (Inokuchi & Yamada, 1990). These results indicate that arginine may be actively recycled in the normally developing leaves, where urease may function coordinately with arginase. Urease activity in the leaf is also important if foliar application of urea is considered as a fertilizer. It has been reported that foliar treatment with urea increased the total leaf yield in mulberry plants, improved the leaf nutritional quality and led to an increase of cocoon yield (Fotedar & Chakrabarty, 1985; Sarker & Absar, 1995).

In the silkworm, the mulberry leaf urease could be directly involved in silk production. Urease activity was found in the digestive tract of insects fed with mulberry leaves and urea secreted from the midgut epithelium was decomposed into ammonia by this enzyme activity. Ammonia produced from urea was reabsorbed and used for silk-protein synthesis in the insect (Hirayama, Sugimura & Shinbo, 1999). The urease activity in the digestive tract is likely to be of mulberry leaf origin since urease activity was not detected in the insect intestine when the insect was fed with artificial foods containing no urease (Hirayama et al., 1999).

There has been no report on 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 the leaf compared with seed urease. For example, in soybean, the leaf enzyme activity is found at level 1/1000 that of the seed enzyme (Polacco & Winkler, 1984). Interestingly, urease activity was not detected in mulberry seeds, while the specific activity of mulberry leaf extract was more than ten times that of soybean leaf extract.

In the present study, we have purified and partially characterized urease from mulberry leaves as a first step towards understanding its role in the plant tissue and also the possible involvement in the metabolism of the silkworm.

2. Results and discussion

A typical purification procedure for urease from the mulberry leaves is summarized in Table 1. In the final step of the purification, the enzyme was eluted from Mono Q column at 0.35 M KCl (see Fig. 1), resulting in a near homogeneous preparation as judged by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis (see Fig. 2(A)). The mulberry leaf urease activity was always eluted as a single symmetrical peak in column chromatography (data not shown), suggesting that it was the only enzyme responsible for this activity in the plant leaf.

SDS–PAGE of the purified enzyme indicated that the mulberry urease was composed of a single polypeptide subunit with apparent molecular mass of 90.5 kDa when compared with migration of standards (Fig. 2(A)). This value was very near to the subunit size of legume seed ureases (90–93.5 kDa) (Reithel, 1971; Polacco & Havir, 1979; Takishima, Suga & Mamiya, 1988). A single type of urease subunit appears to be the conserved feature among all eukaryotes (Mobley & Hausinger, 1989; Lubbers, Rodriguez, Honey, & Thornton, 1996). On the other hand, many bacteria seem to have one large and two small subunits, whereas the urease of *Helicobacter* species is composed of two subunits (Mobley & Hausinger, 1989; Mobley, Island & Hausinger, 1995). The native molecular mass of the mulberry leaf urease was 175 kDa based on gel filtration (Fig. 2(B)), suggesting that the enzyme exists

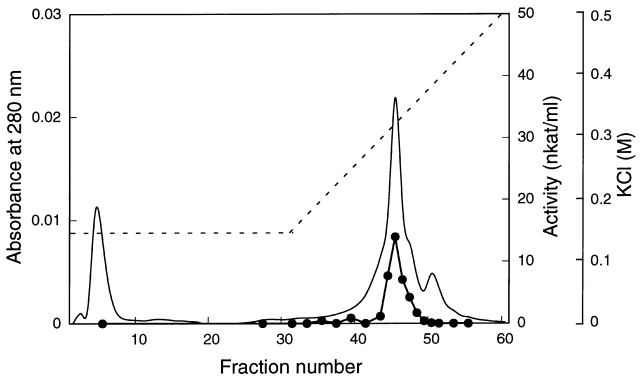

Fig. 1. Elution profile of mulberry leaf urease on a Mono Q 5/5 column. The protein obtained from Superdex 200 HR column chromatography was loaded onto a Mono Q column and eluted in 0.15–0.5 M KCl gradient (– – –). Fractions were monitored at 280 nm for protein (———) and assayed for enzyme activity (●).

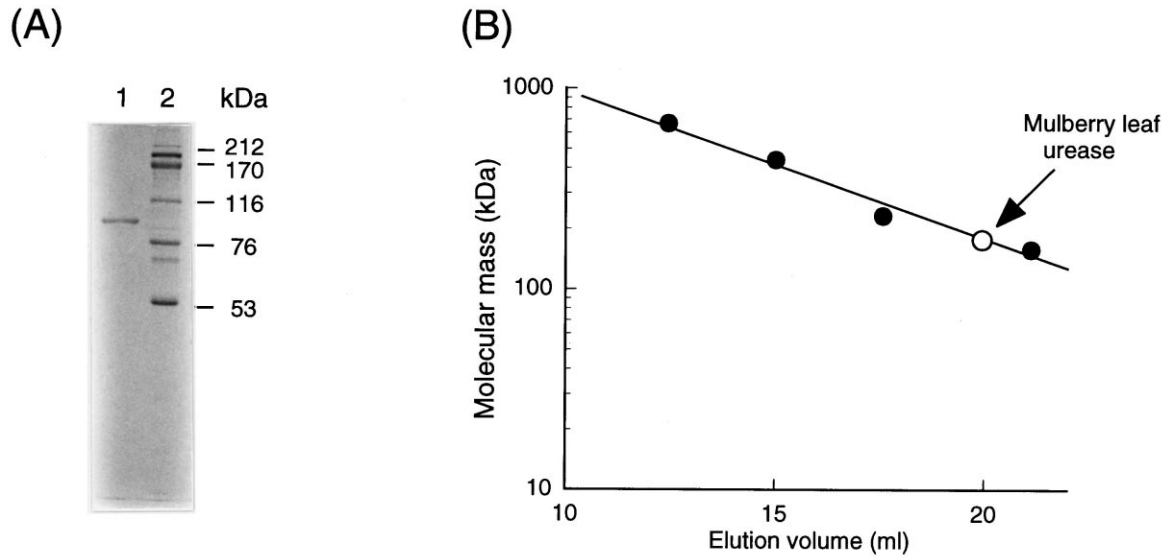

Fig. 2. (A) SDS–PAGE analysis of the purified mulberry leaf urease. Protein sample from the final purification step was denatured in sample buffer and subjected to electrophoresis is on 10% SDS–PAGE (Laemmli, 1970). Lane 1, Mono Q fraction; Lane 2, molecular mass markers (myosin, 212 kDa; α_2-macroglobulin, 170 kDa; β-galactosidase, 116 kDa; transferrin, 76 kDa; glutamate dehydrogenase, 53 kDa). (B) Estimation of molecular mass of mulberry leaf urease. A native molecular mass 175 kDa was determined by gel filtration on a Superdex 200HR 10/30 column. The standard proteins used were thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; aldolase, 158 kDa.

as a homodimer composed of two 90.5 kDa subunits, while legume seed ureases are 480–590 kDa indicating that the native enzymes are hexamers (Polacco & Havir, 1979; Andrews, Blakely & Zerner, 1984). Urease from the yeast, *Shizosaccaromices pombe*, was also estimated to be a homodimer of a single 102 kDa subunit (Lubbers et al., 1996).

Western blotting was carried out with antiserum raised against jack bean seed urease, which recognized the 93 kDa subunit of the jack bean enzyme (see Fig. 3). The antiserum also specifically recognized 90.5 kDa subunit of mulberry leaf urease obtained after acetone fractionation, but none of the numerous contaminating proteins present in the partially purified preparation. Leaf urease activity decreased upon incubation with the jack bean seed urease antibody, incubation with pre-immune serum had no effect on leaf urease activity (data not shown). These data indicate that mulberry and jack bean ureases contain common antigenic determinant(s), suggesting that these enzymes are closely related.

The first 20 amino acids of 90.5 kDa subunit of the mulberry leaf urease were determined and the results were compared with the relevant amino acid sequences of ureases from jack bean seed, soybean seed and soybean seedling axis (see Fig. 4), revealing a high structural similarity, the first 20 amino acids of the

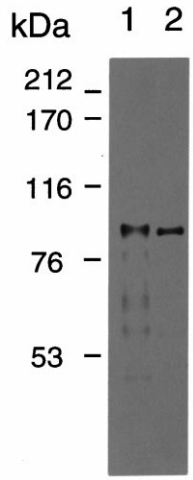

Fig. 3. Immunoblot analysis of mulberry leaf urease. Jack bean seed urease (Sigma, type c-3) (Lane 1) and partially purified mulberry leaf urease by acetone fractionation (Lane 2) were run on a SDS–PAGE (7.5%), transferred to PVDF membrane and immunostained with antiserum produced against the jack bean urease. Molecular mass markers are the same as those shown in Fig. 2(A).

	1	5	10	15	20
Mulberry (leaf)	M	K L T P R E I E K L D L H N A G F L A
Soybean (seedling axis)	*	* * S * * * * * * * * * * * * * Y * *
Soybean (seed)	*	* * S * * * V * * * G * * * * * Y * *
Jack bean (seed)	*	* * S * * * V * * * G * * * * * Y * *

Fig. 4. Sequence alignment of N-terminal amino acid sequence of mulberry leaf urease compared with those of other plant ureases. Shown are the N-terminal 20 amino acid residues of mulberry leaf urease (the present study); soybean ureases from seedling axes and seeds (Torisky, Griffin, Yenofsky & Polacco, 1994); jack bean seed urease (Takishima et al., 1988). Asterisks represent amino acid residues identical to those of mulberry leaf urease.

mulberry leaf urease showed a high degree of conservation with those of jack bean seed urease (80% identity), soybean ureases from seeds and seedling axes (80% and 90% identity), respectively. However, compared with the subunit of microbial ureases, N-terminal sequence of the mulberry leaf urease showed a relative lower structural similarity (40–70% identity) (data not shown). The high similarity of the amino acid sequences between plant ureases suggests that these enzymes are closely related.

The mulberry urease was stable over the range of pH 6–10 when incubated for 2 h at 30°C but is extremely unstable at low pH; enzymatic activity was completely destroyed by incubation with sodium acetate buffer (pH 4) for 30 min (data not shown). The optimum pH for urease activity was around 9.0 (see Fig. 5). This is the highest value among the ureases purified so far. Ureases from jack bean seed, soybean seed and most bacteria have lower pH optimum, at pH 7–8 (Fishbein, 1969; Kerr et al., 1983; Mobley & Hausinger, 1989), whereas the optimum pH of most fungal ureases is within the range of 8–8.5 (Lubbers et al., 1996). Interestingly, urease partially purified from soybean leaves had two pH optimum (5.3 and 8.8) (Kerr et al., 1983).

Under the optimum buffer condition (pH 9.0), reaction velocity plots against urea concentration displayed a normal Michaelis–Menten curve. Lineweaver–Burk plots revealed a K_m of 0.16 mM for urea. A considerable variation of K_m values has been observed among legume seed ureases, ranging from 3 to 250 mM (Fishbein, 1969; Reithel, 1971; Kerr et al., 1983; Andrews et al., 1984). The mulberry leaf urease seems therefore to be suitable for metabolizing urea, which is normally present at a low level in the tissue.

In summary, urease was purified to apparent homogeneity from mulberry leaves and partially characterized. The immunoblot analysis and the N-terminal amino acid sequence indicated considerable structural similarity to leguminous plant ureases. However, the kinetic properties were appreciably different, suggesting a distinct physiological role for the leaf enzyme. We consider that the mulberry leaf enzyme functions even in the digestive tract of the silkworm fed with the mulberry leaves. It has been reported that the pH in the insect midgut is 8–11 (Moriyama, Enomoto, Kato & Iwanami, 1984) and that urea concentration is 2–3 mM (Hirayama et al., 1999). The optimum pH shift to higher values and the apparent K_m for urea (0.16 mM) would allow the leaf enzyme to work efficiently in this environment. Similarly, urease purified from the gastrointestinal microorganism *Helicobacter pylori* was found to have a K_m for urea of 0.17 mM, enabling the organism to inhabit the gastric mucosal lining, where a low concentration of urea (1.7–3.4 mM) was supplied from the serum (Hu & Mobley, 1990). However, further studies are needed for a deeper understanding of the functions of the leaf enzyme.

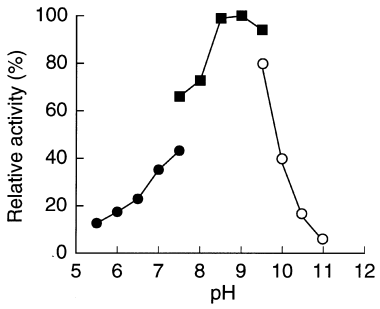

Fig. 5. Effect of pH on mulberry leaf urease activity. Buffers used were 0.1 M phosphate (●), 0.1 M Tris–HCl (■), 0.1 M glycine–NaOH (○).

3. Experimental

3.1. Plant material and chemicals

Mulberry leaves (*Morus alba* L. cv. Shin-ichinose) harvested from field grown trees were rapidly frozen in liquid nitrogen and stored at −80°C. Q-Sepharose HP, Phenyl-Sepharose HP, Superdex 200HR 10/30, and Mono Q 5/5 were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Ammonia assay reagent (171-C) and Jack bean urease (type C-3) were obtained from Sigma (St. Louis, USA). All other chemicals used were of the highest purity commercially available.

3.2. Assay of urease activity

The standard reaction mixture containing 0.1 M-Tris–HCl buffer (pH 9.0), 30 mM urea and the enzyme in a total volume of 100 μl was incubated at 30°C. The reaction was stopped by adding 10 μl 1 N H_2SO_4 to the mixture. Urea hydrolysis was determined by measuring the amount of ammonia released from urea using the ammonia assay reagent (171-C). The efficacy of the assay under conditions used here was confirmed using an ammonium sulfate solution of known concentration as a control. Urease activity is expressed in units of Katals. The pH optimum was determined using the same assay method with 0.1 M phosphate buffer (for the pH range 5.5–7.5), 0.1 M Tris–HCl buffer (for the pH range 7.5–9.5), and 0.1 M glycine–NaOH buffer (for the pH range 9.5–11). The pH stability was investigated by incubating the enzyme for 30 min to 2 h at pH between 3 and 11 in 0.1 M sodium acetate buffer (pH 3, 4, 5), 0.1 M phosphate buffer (pH 6, 7), 0.1 M Tris–HCl buffer (pH 8, 9), and 0.1 M

glycine–NaOH buffer (pH 10, 11) and measuring the remaining activity under the standard condition. In order to determine the apparent K_m for urea, urease was assayed in a reaction mixture containing variable concentrations of urea.

3.3. Purification of urease from mulberry leaves

All purification procedures were carried out at 0–4°C, unless stated otherwise. Frozen mulberry leaves (500 g) were homogenized using a Physcotron homogenizer (NITI-ON, Japan) in 1.5 l buffer A [0.1 M Tris–HCl, 5 mM EDTA, 0.1% 2-mercaptoethanol, pH 7.5] containing 20 g polyvinylpyrrolidone. The slurry was centrifuged at 28,000 g for 20 min and the resultant supernatant was considered to be a crude urease preparation. The supernatant was heated in a 60°C water bath for 17 min, then rapidly cooled on ice. After centrifugation at 28,000 g for 20 min, the resultant precipitate was discarded and solid ammonium sulfate was added to 20% saturation. The pellet was discarded after centrifugation at 28,000 g for 20 min with the supernatant brougt to 40% ammonium sulfate saturation. After centrifuging the supernatant at 28,000 g for 20 min, the pellet was resuspended in minimum volume of buffer A, and dialyzed against the same buffer for 12 h. Redissolved proteins were precipitated with acetone using the following cut: 0–45 and 45–60% (v/v). The precipitated urease in acetone 45–60% (v/v) was resuspended in minimum volume of buffer A, and dialyzed against the same buffer. The dialyzed solution was loaded on a Q-Sepharose HP column (1.6 × 10 cm) which had been equilibrated with buffer A. After extensively washing with this buffer, the column was eluted with a linear gradient of KCl (0–1 M) in buffer A at a flow rate 2 ml/min. Urease was eluted at 0.35 M KCl and the pooled active fraction was dialyzed against buffer B [Tris–HCl 20 mM, 1 mM EDTA, 1 mM 2-mercaptoethanol, pH 7.5] containing 1 M KCl. The dialyzed solution was then applied to a Phenyl-Sepharose HP column (1.6 × 10 cm) that was previously equilibrated with buffer B containing 1 M KCl at a flow rate of 1.0 ml/min. Urease, bound to the resin under these conditions, and was released when the KCl concentration was reduced to 0 M. The eluate was concentrated with an Ultrafree 15 (Millipore, USA). The solution obtained in the above step was further purified by HPLC (Model Bio-LC System, TOSOH, Tokyo) using a Superdex 200HR column (1.0 × 30 cm) previously equilibrated with buffer B containing 0.15 M KCl at room temperature. Fractions of 0.5 ml were collected at a flow rate of 0.5 ml/min, immediately placed on ice, and then assayed for urease activity. The urease active fractions were combined and directly applied to a Mono Q 5/5 column that had been equilibrated with buffer B containing 150 mM KCl at room temperature. The protein was eluted with a linear gradient of KCl (0.15 to 0.5 M) in buffer B at a flow rate of 0.5 ml/min. The urease active fractions were pooled and stored at 4°C.

3.4. Protein assay

Protein concentration was determined by using a commercial protein assay kit (Coomassie® Plus Protein Assay Reagent, Pierce) with bovine serum albumin as the standard.

3.5. Electrophoresis

SDS–PAGE was performed according to Laemmli (1970). Proteins on the gel were stained with Coomassie Brilliant Blue R-250. Subunit molecular mass was determined by comparison to a standard curve using a high molecular weight calibration kit (myosin, 212 kDa; 2-macroglobulin, 170 kDa; β-galactosidase, 116 kDa; transferrin, 76 kDa; glutamate dehydrogenase, 53 kDa) (Pharmacia, Sweden).

3.6. Molecular mass determination of native enzyme

The molecular mass of the native enzyme was determined by gel filtration through a Superdex 200 HR column as described in the purification procedure. Prior to application, the column was calibrated using standards of known molecular mass (thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; aldolase, 158 kDa) (Pharmacia, Sweden).

3.7. N-terminal amino acid sequence

Purified enzyme was subjected to SDS–PAGE (7.5%), electroblotted onto PVDF membrane according to the procedure of Towbin, Staehelin & Gordon (1979), and visualized by Coomassie Brilliant Blue staining. The 90.5 kDa protein band was cut out and subjected to N-terminal sequence analysis by automated Edman degradation on a gas phase sequencer (Model LF-3400, Beckman, USA). Phenylthiohydantoin (PTH) derivatives of individual amino acids were identified by reverse phase HPLC.

3.8. Preparation of antibody

Approximately 0.5 mg of commercial jack bean urease (type C-3) was emulsified in complete adjuvant and injected subcutaneously into a female white New Zealand rabbit. Four weeks after the initial dose, another 0.5 mg of antigen was injected. The rabbit was bled 2 weeks after the last injection. The blood was allowed to clot over night at 4°C and serum was stored at −30°C.

3.9. Immunoblotting

Proteins separated by SDS–PAGE (7.5%) were transferred to PVDF membrane as described above. The membrane was stained using the Bio-Rad Immuno blot assay kit (Bio-Rad). Protein on blot was reacted with antiserum against jack bean urease and visualized with goat anti-rabbit IgG conjugated with alkaline phosphatase.

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