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Review

Insights into the role and structure of plant ureases

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Abstract

The broad distribution of ureases in leguminous seeds, as well as the accumulation pattern of the protein during seed maturation, are suggestive of an important physiological role for this enzyme. Since the isolation and characterization of jack bean urease by Sumner in 1926, many investigations have been dedicated to the structural and biological features of this enzyme; nevertheless, many questions still remain. It has been reported that ureases from plants (jack bean and soybean seeds) display biological properties unrelated to their ureolytic activity, notably a high insecticidal activity against Coleoptera (beetles) and Hemiptera (bugs), suggesting that ureases might be involved in plant defense. Besides the insecticidal activity, canatoxin, a jack bean urease isoform, causes convulsions and death in mice and rats, induces indirect hemagglutination (hemilectin activity) and promotes exocytosis in several cell types. Not only plant ureases but also some microbial ureases (found in *Bacillus pasteurii* and *Helicobacter pylori*) are able to induce activation of platelets in a process mediated by lipoxygenase-derived metabolites. This review summarizes the biological and structural properties of plant ureases, compares them with those displayed by bacterial ureases, and discusses the significance of these findings.

Keywords: *Canavalia ensiformis*; *Glycine max*; Leguminosae; Jack bean; Soybean; Ureases; Canatoxin; Insecticidal; Plant defense

Contents

1. Introduction

Urease (EC 3.5.1.5; urea amidohydrolase) is a metalloenzyme that catalyzes the hydrolysis of urea to yield

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ammonia and carbon dioxide (Dixon et al., 1975); it is found in a wide variety of organisms including plants, fungi and bacteria (Mobley and Hausinger, 1989; Polacco and Havir, 1979; Hirayama et al., 2000; Cox et al., 2000). Although urea is the major substrate of urease, this enzyme is capable of hydrolyzing (albeit poorly) other substrates such as acetamide, formamide, *N*-methylurea, semicarbazide, and hydroxyurea (Dixon et al., 1980a). In urea hydrolysis, plant and microbial ureases exhibit K_m values ranging from 0.1 to >100 mM urea; jack bean urease (JBU) has a K_m value of 2.9 mM (Mobley and Hausinger, 1989). Externally applied as fertilizer, urea becomes accessible to plants only through urease activity (Witte et al., 2002). Since urea is one of the world's most widely used nitrogen fertilizers, its enzymatic hydrolysis is a process of great agricultural importance. In addition, urease is known to be a major cause of pathologies induced by *Helicobacter pylori*, as it allows the bacteria to survive at the low pH of stomach during colonization and therefore plays an important role in the pathogenesis of gastric and peptic ulcers, which in some cases may lead to cancer (Mobley et al., 1995). In agriculture, high urease activity causes significant environmental and economic problems by releasing abnormally large amounts of ammonia into the atmosphere during urea fertilization (Mobley et al., 1995; Mobley and Hausinger, 1989; Sirko and Brodzik, 2000). Besides the importance of urease in making nitrogen available to plants and the impact of microbial ureases in agriculture and medicine, the structure and catalytic mechanism of this enzyme are of interest because of its large enhancement (10^{14}-fold) of the rate of urea hydrolysis and the presence of active-site nickel, which is unique among hydrolytic enzymes (Estiu et al., 2006).

Biochemically, the best-characterized plant urease is that from jack bean (*Canavalia ensiformis*) (Zerner, 1991; Sheridan et al., 2002; Follmer et al., 2004a; Krajewska and Ciurli, 2005). JBU was the first enzyme to be crystallized, and it played an important historical role as proof of the proteinaceous nature of enzymes. Sumner's work, "Isolation and crystallization of the enzyme urease" (Sumner, 1926, Nobel Prize for Chemistry, 1946), represents a fundamental contribution to modern enzymology. JBU is also the first nickel-containing enzyme to be described (Dixon et al., 1975), and it is the only nickel-containing metalloenzyme identified so far in plants (Polacco and Holland, 1993). Unfortunately, neither the three-dimensional structure of JBU nor that of any other plant urease has been determined. The great heterogeneity, insolubility, and high polydispersity of JBU in solution (Fishbein et al., 1969; Fishbein and Nagarajan, 1971; Fishbein, 1969; Follmer et al., 2004a) may be related to the lack of success in determining its structure by X-ray crystallography (Jabri et al., 1992; Sheridan et al., 2002). Moreover, the fact that JBU is not the only urease expressed in *C. ensiformis* (Pires-Alves et al., 2003) raises the possibility that contamination by urease isoforms during JBU purification impedes the production of adequate JBU crystals. Therefore, most of our knowledge about the molecular mechanism of ureolytic catalysis by plant ureases is based on the 3-D structures of bacterial ureases.

This review will summarize the structural properties and physiological significance of plant ureases, notably jack bean and soybean (*Glycine max*) ureases. When appropriate, plant ureases will be compared and contrasted to the bacterial ureases, which are the best-characterized from a molecular point of view.

2. Structural characteristics of ureases

JBU exists as homotrimers that can associate to form hexamers of identical ~90 kDa subunits containing two nickel ions per subunit (Dixon et al., 1975,1980b; Takishima et al., 1988). Bacterial ureases are similar to JBU in that they are either trimers or hexamers of complex subunits (Mobley et al., 1995). However, while fungal and plant (e.g. jack bean and soybean) ureases are homo-oligomeric proteins of *ca.* 90 kDa subunits, bacterial ureases are multimers of two- or three-subunit complexes (Mobley et al., 1995). The A, B and C chains of *Klebsiella aerogenes* (Fig. 1, bottom),

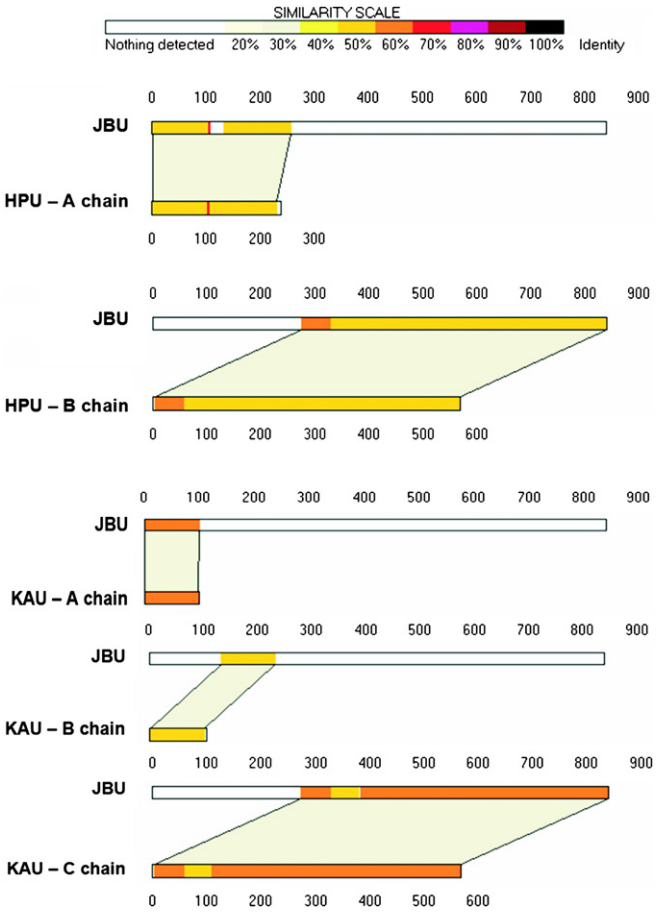


Fig. 1. Alignment of the amino acid sequence of jack bean urease (JBU) and bacterial ureases [*Helicobacter pylori* urease (HPU) and *Klebsiella aerogenes* urease (KAU)]. The similarity of sequence is displayed using the graphical viewer LALNVIEW (Duret et al., 1996).

Bacillus pasteurii and most other bacterial ureases are co-linear with the single chain of fungal and plant ureases. *H. pylori* urease has two subunits: a small polypeptide chain (A chain – 238 residues) homologous to the N-terminal region of JBU and a larger chain (B chain – 569 amino acids) (Fig. 1, upper) containing the catalytic site. Although only bacterial ureases such as *K. aerogenes* (Jabri et al., 1995), *B. pasteurii* (Benini et al., 1999) and *H. pylori* urease (Ha et al., 2001), as well as several mutants and inhibited systems have their tertiary structures successfully solved by X-ray crystallography, there are significant amino acid sequence similarities among all known ureases (Mobley et al., 1995). Comparing the structures of microbial ureases we can establish extensive similarity of both the secondary and tertiary structures (Fig. 2), despite differences in the number of chains.

The sequence of JBU is closely related to the sequence of all bacterial ureases suggesting a common evolutionary origin. Comparison of the amino acid sequence of the major urease subunits from *Proteus mirabilis*, *P. vulgaris* and *H. pylori* shows that they are identical to the sequence of JBU in over 50% of amino acid residues, despite the very distant phylogenetic relation between plants and bacteria (Jones and Mobley, 1989). On the basis of the similarities in sequence and reaction kinetics, it has been assumed that known ureases have a common structure and catalytic mechanism and so we can use much of the information obtained for the structure and catalytic mechanism of bacterial ureases to understand plant ureases.

The best-characterized 3-D structure of an urease is that of the enteric bacterium *K. aerogenes*, which was resolved to 2.2 Å (Jabri et al., 1995). The microbial ureases of *K. aerogenes* and *B. pasteurii* are essentially identical in terms of backbone structure (Benini et al., 1999). The dinuclear active sites of the urease trimers are roughly 70 Å apart and behave independently (Estiu et al., 2006). To catalyze urea hydrolysis at a rate that is $>10^{14}$-fold greater than the spontaneous rate, *K. aerogenes* urease uses a dinuclear nickel active site with the protein providing a bridging carbamylated lysine residue as well as an aspartyl and four histidyl ligands (Jabri et al., 1995; Mobley et al., 1995). Several ureases display a highly conserved amino acid sequence in the region of their catalytic site with respect to both critical residues and adjacent amino acids (Fig. 3, bottom). Fig. 3 shows the catalytic site of *K. aerogenes* and the key residues involved in catalysis: His134, His136, His246, His272, Lys217, and Asp360. The residues His246 and His272 and one water molecule are ligands to nickel 1 (Ni-1), while the residues His134, His136, Asp360 and one water molecule are involved in the binding to nickel 2 (Ni-2). Pearson et al. (2000) suggested that the enzyme mechanism involves O-coordination of urea to Ni-1. The residue His219 stabilizes this interaction and causes further polarization of the carbonyl group. The attack by one water molecule on the carbonyl carbon gives rise to a tetrahedral intermediate, followed by proton donation by His320, resulting in ammonia elimination to yield the products (Pearson et al., 2000).

Jabri and Karplus (1996) reported that all residues in the *K. aerogenes* urease are well ordered except for residues 317–334 on the C chain, which forms a mobile flap that covers the active site (Fig. 4). This flap contains the key residue His320, as well as the Cys319 and changes in the flap conformation, besides controlling the accessibility of the active site, have been suggested as an important structural feature for catalysis (Karplus et al., 1997; Pearson et al.,

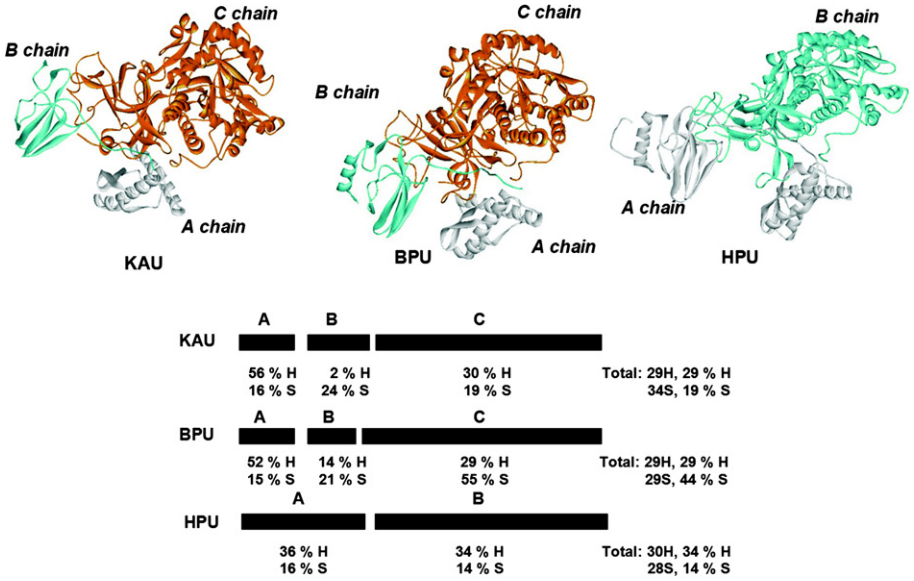
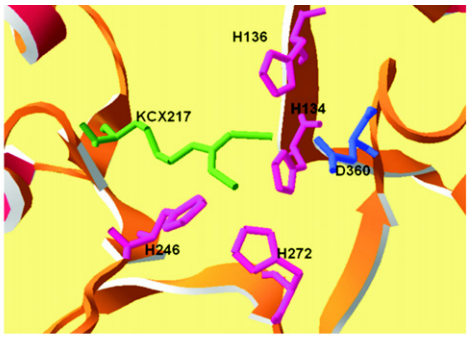
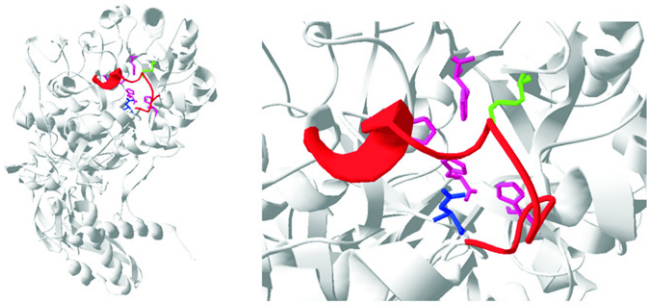


Fig. 2. Similarity of the structures of bacterial ureases [*Klebsiella aerogenes* urease (KAU) (PDB code: 1FWJ); *Bacillus pasteurii* urease (BPU) (PDB code 2UBP); *Helicobacter pylori* urease (HPU) (PDB code 1E9Z)]. The tertiary (top) and secondary (bottom) structures of the monomer subunit of multi-chain ureases are shown. The percentage and total number of helical (H) and beta-strand (S) structures is displayed (bottom). Tertiary structures were built using Swiss-PDB Viewer (Guex and Peitsch, 1997) and secondary structure data were obtained from www.pdb.org.



DTH$_{134}$I HWI	GLK$_{217}$I H	ALH$_{246}$SD	TF H$_{272}$TE	SSD$_{360}$ SQ	*K. aerogenes*
DCH$_{407}$VHYI	GLK$_{490}$LH	NIH$_{519}$TD	TYH$_{545}$SE	SSD$_{633}$SQ	*C. ensiformis (JBU)*
DCH$_{346}$VHYI	GLK$_{429}$LH	NIH$_{458}$TD	TYH$_{484}$SE	SSD$_{572}$AQ	*C. ensiformis (JABURE-II)*
DCH$_{405}$VHYI	GLK$_{488}$LH	NIH$_{517}$TD	TYH$_{543}$SE	SSD$_{631}$SQ	*G. max- embryo*
DCH$_{405}$VHFI	GLK$_{488}$LH	NIH$_{517}$TD	TYH$_{543}$SE	SSD$_{631}$ SQ	*G. max- ubiquitous*
DCH$_{137}$VHYI	GLK$_{220}$L H	AIH$_{249}$S D	SFH$_{275}$VE	TI D$_{363}$ AL	*B. pasteurii*
DTH$_{136}$I H FI	GFK$_{219}$I H	AIH$_{248}$T D	TFH$_{274}$TE	SSD$_{362}$ SQ	*H. pylori*

Fig. 3. Catalytic site of *Klebsiella aerogenes* urease. The key residues involved in the urea hydrolysis reaction in *K. aerogenes* urease, i.e. His134, His136, His246, His272, Lys217, and Asp360, are shown (upper). The residues His246 and His272 and one water molecule (not shown) are ligands to nickel 1 (Ni-1), while the residues His134, His136, Asp360 and one water molecule are involved in the binding to nickel 2 (Ni-2). At the bottom, the amino acid sequence of the region involved in the catalysis reaction (key residues in bold) from several organisms are shown.



MVC$_{592}$HHLDREIPEDLAFAH	*C. ensiformis (JBU)*
MVC$_{491}$HKLNREIPEDLAFAS	*C. ensiformis (JABURE-II)*
MVC$_{590}$HHLNREIPEDLAFAC	*G. max- embryo*
MVC$_{590}$HHLNKNIPEDVAFAE	*G. max- ubiquitous*
MVC$_{322}$HHLKQNIPEDVAFAD	*B. pasteurii*
MVC$_{321}$HHKDKSIKEDVQFAD	*H. pylori*
MVC$_{319}$HHLDPDIAEDVAFAE	*K. aerogenes*

Fig. 4. Structural features of the ureolytic property of *Klebsiella aerogenes* urease. A mobile flap (red) (residues 317–334 on the C-chain) covering the active site of *K. aerogenes* urease have been proposed to be an important factor for the catalysis (upper). Residues of the catalytic site are colored as in Fig. 3. At the bottom, the amino acid sequence of this mobile flap region in plants and bacterial ureases and the position of the cysteines residues (e.g. Cys319 in *K. aerogenes* urease) inside these sequences are shown. These cysteines are involved in many reactions that result in enzyme inhibition (by thiol-blockage) due to proximity of this residue of the catalytic site. The protein structures were built up using Swiss-PDB Viewer (Guex and Peitsch, 1997) and using the PDB code 2KAU.

1997; Estiu et al., 2006). Contrasting with *K. aerogenes* urease, where the flap is closed, in the structure of native *B. pasteurii* urease this mobile flap is found in an open conformation indicating a relative flexibility of this structural motif (Musiani et al., 2001). In addition, this region contains a cysteine residue (Cys319 and Cys322 in *K. aerogenes* and *B. pasteurii* urease, respectively) that is involved in many reactions that lead to inhibition of the enzyme, e.g. metal binding (especially Hg ions). Pearson et al. (1997) have investigated the structure of *K. aerogenes* urease variants altered at position Cys319 by site-directed mutagenesis. Although the Cys319 is not essential for catalysis, structural comparisons among the Cys319 variant and wild-type structures have revealed crucial elements of urease catalysis. The most apparent structural changes in the Cys319 variants involve motilities, e.g., it was observed in urease variants changes in the hydration of the active site nickel ions and in the position and flexibility of the active site flap. Fig. 4 (bottom, last sequence) shows the position of Cys319 in the amino acid sequence of the mobile flap (residues 317–334) of *K. aerogenes* urease. In addition, there is a highly conserved amino acid sequence in this region in plant and bacterial ureases, all displaying the cysteine residue in a similar position (Fig. 4, bottom).

2.1. Urease: nickel active site

Although JBU contains 2 equiv. of nickel per 90 kDa monomer, the metal content in canatoxin, an urease isoform from jack bean, remains unclear. Using particle induced X-ray emission technique, it was demonstrated that canatoxin carries one atom of zinc and only one nickel per monomer, that could be related to its lower urease activity (Follmer et al., 2002,2004b). Zerner (1991) reported very interesting experiments showing that JBU partially depleted of nickel by exhaustive dialysis could be loaded with zinc to make a substituted urease containing one atom of each metal. This new urease displays 0.83 Zn + 0.89 Ni per subunit and about one-tenth of the ureolytic activity of the native enzyme. In addition, some microbial ureases were reported to display mononickel active sites (Mobley et al., 1995). Thus, if we assume that canatoxin indeed has one equivalent of nickel per monomer, although it may be loosely bound, then it is plausible to say that canatoxin could be a variant form of urease containing a hybrid "zinc–nickel" active site. Of course, we cannot exclude other possibilities such as one subunit having two nickel and the other two zinc. Although the idea of the presence of a hybrid nickel/zinc site in canatoxin sounds interesting, additional studies about the structure of the active site of canatoxin are necessary to verify this hypothesis.

Park and Hausinger (1993) reported that the mutant of *K. aerogenes* urease (H134A), with only one (Ni-1) of the two nickel ions found in the catalytic site of the native enzyme (Ni-1 and Ni-2), is inactive and binds approximately half the normal complement of nickel (Park and Hausinger, 1993). In addition, the geometry of the active site of H134A was characterized, showing that Ni^{2+} holds a position in an octahedron coordinated by two histidylimidazoles, in contrast to the pseudotetrahedral geometry observed for Ni-1 in the native urease (Park and

Hausinger, 1996). So it was suggested that Ni-2 is required for the formation of active urease and also that Ni-2 has an important role in the establishing the proper Ni-1 coordination geometry. Unfortunately, the geometry of the active site of mononickel ureases remains unknown, and in this respect, canatoxin is a promising candidate for investigation.

Dixon et al. (1975) showed that when jack beans are grown in a nutrient solution containing a low nickel concentration, the ureolytic activity of the expressed urease decreases in proportion to the reduction of the nickel concentration. As observed for JBU, soybean urease is inactive when soybean cell cultures are grown in the absence of nickel (Polacco, 1977). Although active soybean seed urease is not formed in the absence of nickel ions, synthesis of the apoprotein is independent of the presence of the metal (Winkler et al., 1983). From these findings one important question arises: can the apo-urease be activated by incubation in the presence of nickel? It was reported that *K. aerogenes* apo-urease can be activated *in vitro* by incubating the enzyme with nickel ions plus CO_2 (provided as bicarbonate), but only ~15% forms active enzyme (Park and Hausinger, 1996; Yamaguchi et al., 1999). Accordingly, in Park and Hausinger's model for this activation (Park and Hausinger, 1996), urease apoprotein binds nickel in the absence of bicarbonate to yield inactive and non-carbamylated Ni-urease containing about 2 equiv. of nickel per subunit that can be removed very slowly by treatment with EDTA. Apoprotein alternatively reacts reversibly with CO_2 to generate the Lys217-carbamylated protein (CO_2–urease). This protein binds Ni to form two distinct Ni–CO_2–urease species: ~15% of the molecules are active, likely identical to native enzyme (denoted as Ni–CO_2–ureaseA) and ~85% of them are inactive species (Ni–CO_2–ureaseB) containing labile Ni that is removed by extended treatment with EDTA (Yamaguchi et al., 1999). As apo-ureases can bind a variety of metal ions and plant ureases are continually exposed to large amounts of metals other than nickel, the correct metal incorporation could play an important role in the inactivation/activation of this enzyme.

2.2. Construction of the urease metallocenter

The activation of the nickel metalloenzyme urease is a complex process. For bacterial and plant apo-ureases, the participation of several accessory proteins is required in order to assemble an active urease by a process of selective incorporation of the nickel. These activating proteins appear to act as urease-specific chaperones (Mobley et al., 1995). The best-characterized activation process is that from *K. aerogenes,* where the cellular activation process is mediated by the auxiliary proteins UreD, UreE, UreF, and UreG (Mobley et al., 1995; Soriano and Hausinger, 1999; Kuchar and Hausinger, 2004). In conjunction with the three genes that encode the structural subunits (UreA, UreB and UreC), a total of seven genes is necessary to express active *K. aerogenes* urease. Besides the genes for the activatory enzymes, most plants possess only a single urease gene for the structural subunit, e.g. potato (*Solanum tuberosum*), tomato (*Lycopersicon esculentum*), several other solanaceous species, and also Arabidopsis (*Arabidopsis thaliana*) (Witte et al., 2005a). Jack bean is the first plant identified to contain a family with at least three urease-related genes encoding the isoforms: JBU, canatoxin (Carlini and Guimarães, 1981) and JBURE-II (Pires-Alves et al., 2003). In addition, soybean has two distinct urease genes (*Eu1* and *Eu4*), that encode an embryo-specific and an ubiquitous urease, respectively.

The accessory proteins form many complexes with urease apoprotein, resulting in an increase of the ureolytic properties of the enzyme by an activation process (Mobley et al., 1995). Compared with bacterial ureases, little is known about the pathways of activation of ureases in plants. It has been proposed that the function of the bacterial metallochaperone UreE may have been taken over in plants by the UreG protein (Freyermuth et al., 2000; Witte et al., 2005b). A role for the putative plant urease accessory proteins was demonstrated for an UreG ortholog from soybean (identified as *Eu*3 gene) (Freyermuth et al., 2000). The sequence of the *ureG* ortholog from *A. thaliana* was identified and the amino acid sequences deduced from the *A. thaliana* and soybean cDNAs show great similarity to bacterial UreG proteins (Witte et al., 2005b). In addition, Bacanamwo et al. (2002) isolated plant orthologs of the bacterial urease accessory genes *Ure*D and *Ure*F from soybean, tomato and *A. thaliana*.

Functional complementation of activation complexes has been demonstrated in defective microbial urease, suggesting a possible role of other accessory protein orthologs in activation of plant ureases. Mutation in the *UreG* gene of the *K. aerogenes* urease operon can be partially complemented by a potato *UreG* ortholog (Witte et al., 2001). Similar results were obtained for a mutant of *Ure*F from *Schizosaccharomyces pombe* that was partially complemented by a soybean protein that is similar to microbial UreF (Bacanamwo et al., 2002). Three accessory genes that are necessary for urease activity have been identified in *A. thaliana*, encoding the urease accessory proteins *At*UreD, *At*UreF, and *At*UreG (Witte et al., 2001; Bacanamwo et al., 2002). Simultaneous coexpression of *At*urease, *At*UreD, *At*UreF, and *At*UreG in *Escherichia coli* led to activation of plant urease in bacterial cells and in cell-free extracts, showing that these proteins are the core components for plant urease activation (Witte et al., 2005b).

2.3. Metals modulating urease activities

The high affinity of ureases for metal ions (e.g. Hg^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+}) as well as their inhibitory effects (Hughes et al., 1969) upon the ureolytic and insecticidal activities of the enzyme raises the possibility of metals being modulators of urease activities. Follmer and Carlini

(2005) suggested that JBU aggregation could be induced by binding of metal. The Cu^{2+}-induced polymeric forms of JBU have no urease or insecticidal activities, similar to aggregated forms of JBU induced by freezing and thawing cycles (Follmer et al., 2004a). Chemical modification of histidine residues of JBU by treatment with diethylpyrocarbonate decreases its affinity for Cu^{2+} and inhibits oligomerization induced by this metal, resulting in a protection of insecticidal properties of JBU from the inactivation by Cu^{2+} (Follmer and Carlini, 2005). In addition, it was shown that certain ions, notably Hg^{2+} and Cu^{2+}, are inhibitors of urease activity, and it was suggested that this inhibition might involve blockage of thiol groups in the protein (Hughes et al., 1969). Cu^{2+} can bind to many sites on the *K. aerogenes* urease in addition to the usual metallocenter, but most of the adventitious metal is removed by treatment with EDTA (Yamaguchi et al., 1999). Several studies hypothesized that nonproductive metal binding arises by coordination to Cys319 localized in the mobile flap (317–334) of *K. aerogenes* urease (Pearson et al., 1997; Martin and Hausinger, 1992). On the other hand, Yamaguchi et al. (1999) reported that the mutation C319A does not prevent the irreversible inactivation of the enzyme by Cu^{2+} (Yamaguchi et al., 1999), suggesting that an affect other than blockage of thiol is involved. Cys319 in *K. aerogenes* urease is located at the active site (in *K. aerogenes* urease, the sulfur atom is positioned 6.7 and 6.1 Å from Ni-1 and Ni-2, respectively) (Pearson et al., 1997; Martin and Hausinger, 1992) and is surprisingly conserved in the amino acid sequences of both microbial and plant ureases (Fig. 4). In addition, due to its reactivity, this cysteine is one of the most important targets for urease inhibition (Pearson et al., 1997; Todd and Hausinger, 1991; Zhang et al., 2006; Krajewska et al., 2004).

3. Roles of ureases in nitrogen metabolism

Many species of leguminosae have high levels of urease activity in their seeds. For example, the extractable protein from soybean is at least 0.2% urease (dry-weight), whereas in jack bean, urease comprises 0.15% (Polacco and Sparks, 1982). Whereas in mammals urea is a nontoxic waste form for excess ammonia, in plants significant quantities of nitrogen flow through urea (urea is 47% nitrogen), which can be recycled only by urease action (Polacco and Holland, 1993; Sirko and Brodzik, 2000). In plants, urea derives primarily from arginine, which is the major nitrogenous transport and storage compound in plants (Polacco and Holland, 1993). Urea can also be generated from ureide (allantoate, allantoin) catabolism; e.g. it was demonstrated that ureidoglycolate, a product of allantoate degradation, is a urea precursor (Todd and Polacco, 2004; Munõz et al., 2006). In soybean, *in situ* leaf degradation of ^{14}C-labelled allantoin confirmed that both urea and CO_2/NH_3 are direct products of ureide degradation and the growth of plants in the presence of the specific urease inhibitor phenylphosphorodiamidate caused urea accumulation in soybean (Todd and Polacco, 2004). In addition, it was suggested that in soybeans, generation of urea from ureides result of the action of commensal bacteria associated with the plant (Polacco and Holland, 1993).

The primary role of the ureases is to allow the organisms to use external or internally generated urea as a nitrogen source (Mobley and Hausinger, 1989; Mobley et al., 1995; Andrews et al., 1984). In combination with arginase, urease has been proposed to function in the utilization of the seed protein reserves during germination (Thompson, 1980). Nevertheless, the physiological relevance of ureases, at least in certain plants, is still unclear. For example, Brodzik et al. (2000) reported no significant alteration in the growth pattern of tobacco plants expressing *H. pylori* urease, which caused a twofold increase in the ureolytic activity and an eightfold increase in ammonia levels of the transgenic plants as compared to controls.

In soybean, the dispensability of ureases is still in question (Goldraij et al., 2003). Soybean contains an ubiquitous urease (encoded by *Eu4*) that is synthesized in all tissues, as well as an embryo-specific urease (encoded by the gene *Eu1*) that is confined to the developing embryo and is retained in mature seeds where its activity is roughly 1000-fold greater than that of ubiquitous urease (Polacco and Holland, 1993; Goldraij et al., 2003). Urease-negative mutant soybean plants accumulated urea in all tissues, as well as in necrotic leaf tips (Stebbins et al., 1991). Similar results were obtained for soybean using foliar application of urea plus an inhibitor of urease (Krogmeier et al., 1989) as well as for urease-negative plants induced by absence of nickel (Eskew et al., 1984). On the other hand, soybean mutants lacking the embryo-specific urease do not exhibit any of the abnormalities associated with urea accumulation such as those reported when the ubiquitous urease is lost (e.g. necrotic leaf tips due to urea burn). Therefore, it has been suggested that embryo-specific urease probably has no essential physiological function related to urea hydrolysis (Stebbins et al., 1991). *In vitro* studies with developing cotyledons of pea (Lea et al., 1979) and soybean seedlings (Thompson et al., 1977; Stebbins and Polacco, 1995) indicated that urease(s) play little or no role in embryo nutrition. Since the developing soybean embryo does not generate urea, the obvious question is why the developing soybean embryo would invest in a very active ureolytic protein when it never ''sees'' urea. The abundant seed urease appears to be nonassimilatory and it has been hypothesized that it may be involved in a plant defense function. Polacco and Holland (1993) suggested that wounding or infection of the immature embryo will lead to arginase release from ruptured mitochondria, generating a large quantity of urea from arginase degradation. Urea would be then converted to ammonia, which would have a deleterious effect upon herbivores.

4. Canatoxin

In 1981, Carlini and Guimarães isolated canatoxin, a highly toxic protein from jack beans that induces convulsions and death in mice and rats when injected intraperitoneally. Canatoxin was subsequently identified as an isoform of urease (Follmer et al., 2001) which can be isolated separately from JBU using their different affinities for metal ions (Follmer et al., 2004b). Unlike JBU, canatoxin is a homodimer of 95 kDa subunits (Carlini and Guimarães, 1981). Corroborating these findings, Pires-Alves et al. (2003) reported the presence of a family of urease-related genes in *C. ensiformis* with at least three members: canatoxin, JBU and JBURE-II. Fig. 5 shows the similarity of amino acid sequences of jack bean ureases. Although the amino acid sequence of canatoxin remains incomplete (only internal peptides of canatoxin were sequenced), we can observe a high identity with all jack bean ureases, with JBURE-II sequence sharing 82% of identity with JBU. In addition JBURE-II displays a high similarity with other plant ureases such as embryo-specific and ubiquitous ureases of soybean (82% and 79% of amino acid identity, respectively) and the putative urease of *A. thaliana* (72% of identity). The expression of JBU and JBURE-II genes is induced in seedlings and in leaves treated with abscisic acid, a phytohormone involved in seed maturation and wound reponse. The obvious question from these findings is what is the role of these different ureases in the plant? Do these enzymes have physiological properties other than ureolytic activity?

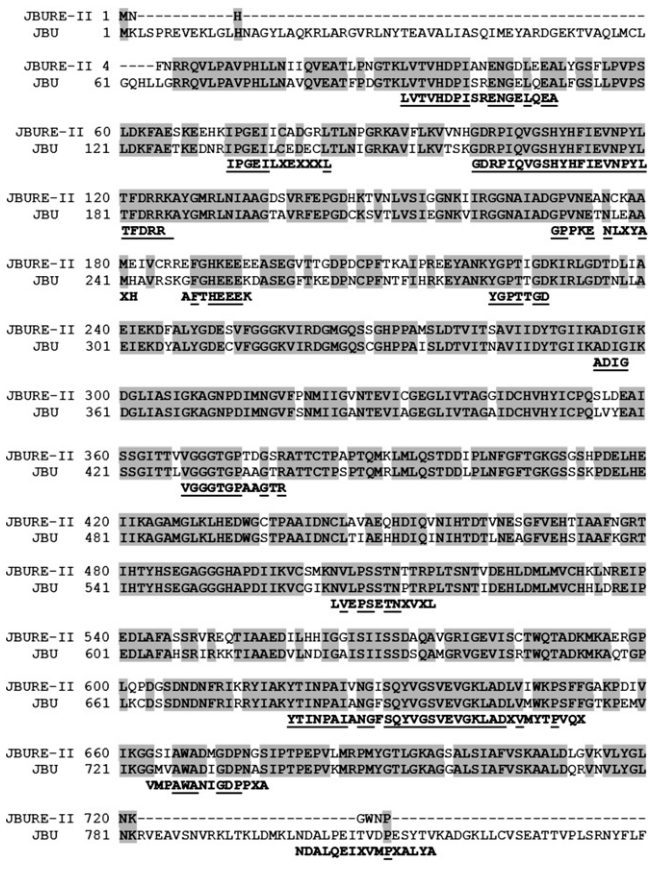


Fig. 5. Alignment of amino acid sequence of ureases from jack bean (*Canavalia ensiformis*). JBURE-II and JBU share 82% of identity (identical residues between JBURE-II and JBU are highlighted). The sequence of internal peptides of canatoxin are shown in *bold* and the residues conserved in all three ureases are underlined.

Canatoxin displays insecticidal activity against Coleoptera (beetles) and Hemiptera (bugs) (Carlini and Grossi-de-Sa, 2002). Studies by Carlini's group indicate that canatoxin is at least as toxic to insects as α-amylase inhibitors, proteinase inhibitors and some lectins, and it is 40-fold more potent than arcelin for the bruchid *Zabrotes subfasciatus* or the variant forms of vicilins for *Callosobruchus maculatus* (Carlini and Grossi-de-Sa, 2002; Carlini et al., 1997). One important feature of canatoxin is its potent insecticidal effect on two economically important hemipteran pests, *Nezara viridula* and *Dysdercus peruvianus* (Carlini and Grossi-de-Sa, 2002; Follmer et al., 2004c; Stanisçuaski et al., 2005), which are not susceptible to the insecticidal activity of Cry toxins from *B. thuringiensis* (Carlini and Grossi-de-Sa, 2002; Schnepf et al., 1998). *N. viridula* (Southern green soybean stinkbug) is also known in some countries as the green vegetable bug. It occurs throughout the Americas, in various Pacific Islands, Japan, Australia, New Zealand, and Africa (Clausen, 1978). *N. viridula* is a cosmopolitan pest with important economic effects on several crops including soybean, peas, cucumber, lettuce, tobacco, and several others (Todd and Herzog, 1980; Jackai et al., 1990). Although this species has been chemically controlled mainly with organophosphate insecticides such as methyl-parathion and monocrotophos for more than 40 years, *N. viridula* is one of the few examples of pentatomids that have become resistant to insecticides (Georghiou and Lagunes-Tejeda, 1991). Another economically important insect susceptible to canatoxin is the hemipteran *D. peruvianus* (cotton stainer bug), which feeds on cotton seeds, resulting in damage to the seed and staining of the cotton fibers. In addition, this insect is a vector for phytopathogenic bacteria and fungi that may cause significant losses on cotton plantations (Gallo, 1988). It has been demonstrated that canatoxin is about 25-fold more toxic to *D. peruvianus* than to *C. maculates,* making this protein one of the most potent of all plant insecticidal proteins (Stanisçuaski et al., 2005).

5. Insecticidal properties of ureases and biotechnological perspectives

In order to understand the relation between the different activities displayed by ureases, Follmer et al. (2004b,c) analyzed the insecticidal activity of the jack bean ureases (JBU and canatoxin), the soybean embryo-specific urease (SBU), and the urease from *B. pasteurii*, using *D. peruvianus* as the insect model. Only the plant ureases were toxic in the feeding trials. JBU was slightly less toxic than canatoxin but

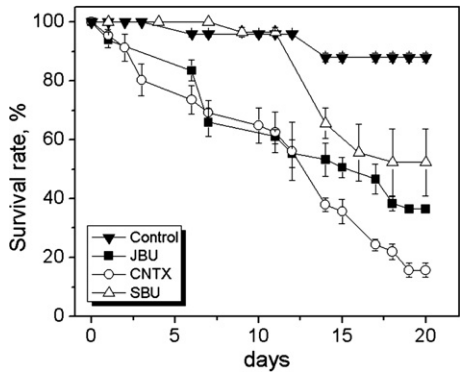


Fig. 6. Insecticidal activity of plant ureases on *Dysdercus peruvianus*: jack bean urease (JBU), canatoxin (CNTX) and soybean embryo-specific urease (SBU). The insects (second instar) were fed on artificial seeds composed of ureases in 20 mM sodium phosphate pH 7.0, 1 mM EDTA, and cottonseed meal to give a final concentration of urease of 0.05% (w/w). Data adapted from Follmer et al. (2004b,c).

threefold more potent than SBU (Fig. 6) (Follmer et al., 2004b, c). Besides being lethal, both ureases induced severely detrimental effects in surviving insects, reducing gain in body weight and delaying the development of nymphs into adults. Interestingly, the insecticidal effects of the plant ureases were not modified when the proteins were pre-treated with the urease inhibitor *p*-hydroxymercuribenzoate, clearly indicating that insecticidal and ureolyitc activities are unrelated. Therefore, these findings suggest that insecticidal activity is a characteristic of plant ureases, and they provide important evidence for a possible defense role of these proteins.

Although the detailed mechanism of action concerning the insecticidal activity of these ureases is still unknown, it has been demonstrated, at least for canatoxin, that a proteolytic activation by insect cathepsin-like digestive enzymes is required to produce entomotoxic peptide(s) (Ferreira-DaSilva et al., 2000). Only insects in which cathepsins B (cysteine protease) and D (aspartyl protease) are the main digestive enzymes (e.g. *C. maculatus* and *Rhodnius prolixus*) were susceptible to canatoxin. In contrast, insects in which trypsin (serine protease) is the digestive enzyme were not affected by canatoxin. The lack of insecticidal activity of *B. pasteurii* urease may be explained by the fact that the region comprising the sequence of the entomotoxic peptide released from canatoxin by insect cathepsins is absent in microbial ureases due to the three-chain structure of the microbial urease subunit. In plant ureases, this region corresponds to a fragment located between UreB and UreC chains of *B. pasteurii* urease (Follmer et al., 2004c).

A similar process occurs in another class of insecticidal proteins, the Cry toxins produced by *B. thuringiensis*. As the Cry toxins are ineffective in Hemiptera, the urease and Cry toxins might be complementary in their effects for different groups of insects. The mode of action of Cry toxins has been well reviewed (Schnepf et al., 1998; Aronson and Shai, 2001; Whalon and Wingerd, 2003). When insect larvae ingest these proteins, the prototoxins are solubilized and processed to toxins by midgut proteases. Knowles (1994) proposed on the basis of *in vitro* studies, that the toxin binds to receptors on cells lining the larval midgut, inserts into the cell membrane and forms ion channels. These channels are somewhat cation-selective, lead to loss of the transmembrane potential, followed by cell lysis and insect death (Knowles, 1994).

6. Other activities of plant ureases

Canatoxin is highly toxic in rats and mice when administered intraperitoneally ($LD_{50} = 1–2$ mg/kg) but inactive when given orally (Carlini and Guimarães, 1981; Follmer et al., 2001). As observed for the insecticidal activity, canatoxin pre-treated with *p*-hydroxymercuribenzoate retains its toxic activity in mammals despite complete loss of its ureolytic activity. On the other hand, neither JBU nor SBU is lethal to mice or rats when given intraperitoneally (maximal dose tested 20 mg/kg) (Follmer et al., 2004c). Therefore, there is no correlation between the insecticidal activity of ureases and the intraperitoneal toxicity in mice, until now a property displayed only by canatoxin. Canatoxin is also able to induce an increase in the levels of gonadotropin and insulin when administered at subconvulsant doses (Ribeiro-DaSilva et al., 1989; Ribeiro-DaSilva and Prado, 1993). *In vitro* studies revealed that canatoxin displays pro-inflammatory activity and induces exocytosis in several cell types, including platelet, synaptosomes, pancreatic islets, macrophages, neutrophils and mast cells (Carlini et al., 1985; Olivera-Severo et al., 2006a). Lipoxygenase metabolites are involved in most pharmacological effects of canatoxin, since they are blocked by lipoxygenase inhibitors such as nordihydroguaiaretic acid and esculetin, but not by cyclooxygenase inhibitors (Carlini et al., 1985; Ghazaleh et al., 1997). In addition, canatoxin is able to dispupt Ca^{+2} transport by Ca^{+2}, Mg^{+2}-ATPase of the sarcoplasmic reticulum membrane vesicles (Ghazaleh et al., 1997).

In light of these findings, a number of studies have addressed the question of whether biological activities are properties common to all ureases or only to canatoxin, how these data might be relevant to the role of ureases in plants and bacteria. The plant ureases (JBU, canatoxin and SBU) as well as the microbial ureases (BPU) are able to induce activation of rabbit blood platelets (Follmer et al., 2004c). JBU and SBU show similar potency as inducers of platelet aggregation, with $EC_{50} = 22.2$ μg/mL and 15.8 μg/mL, respectively (Follmer et al., 2004c). BPU, on the other hand, is a 20-fold less potent, with $EC_{50} = 400$ μg/mL (Follmer et al., 2004c). *H. pylori* urease also induces platelet activation (Olivera-Severo et al., 2006a,b). The time course of the platelet response to the two ureases is very similar, indicating a collagen-type shape-change reaction. Olivera-Severo et al. (2006b) reported that platelet aggregation induced by BPU is mediated by lipoxygenase-derived eicosanoids and secretion of ADP from the platelets through a calcium-dependent mechanism. As observed for the insecticidal activity

and for toxicity in mammals, the ability to activate platelets was not modified in *p*-hydroxymercuribenzoate-treated proteins, confirming the idea that this property is independent of the ureolytic activity. The relevance of these data, especially for the pathogenesis of bacterial infections, remains unclear.

7. Concluding remarks

Ureases from plant and microbial sources belong to a group of multifunctional proteins displaying not only the capacity for ureolysis, but also toxic effects in insects and the ability to activate blood platelets. The newly described properties of plant ureases may shed new light on the physiological roles of these proteins in the source organisms, reinforcing the possibility of plant ureases having a protective role against phytopathogens through an entirely different mechanism, unrelated to the release of ammonia.

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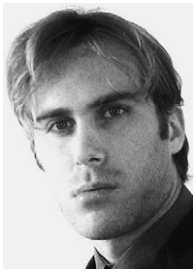

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