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Effect of chemical modification of histidines on the copper-induced oligomerization of jack bean urease (EC 3.5.1.5)

Cristian Follmer[a,*], Célia R. Carlini[a,b]

[a] *Graduate Program in Molecular and Cellular Biology, Center of Biotechnology, Universidade Federal Rio Grande do Sul, Porto Alegre, CEP 91.501-970, RS, Brazil*
[b] *Department of Biophysics, IB, Universidade Federal Rio Grande do Sul, Porto Alegre, CEP 91.501-970, RS, Brazil*

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Abstract

Aggregation of jack bean urease (JBU) is associated with alterations of its biological properties, notably the ureolytic and entomotoxic activities. We investigated the influence of metals on protein oligomerization and biological properties. Besides protein aggregation, Cu^{2+} induces inhibition of both ureolytic and insecticidal activities of JBU. Chemical modification of histidine residues in JBU with diethylpyrocarbonate (DEPC) decreases its affinity for Cu^{2+} and inhibits oligomerization induced by this metal. Furthermore, this modification protects the insecticidal properties of JBU from being inactivated by Cu^{2+}. Although DEPC-treated JBU displayed lower ureolytic activity, the modified protein is less susceptible to inhibition by Cu^{2+} when compared to native enzyme. Our findings show that Cu^{2+} promotes JBU aggregation and differently of other heavy metals studied here, it apparently inhibits the ureolytic activity by inducing protein polymerization along with blockage of sulfhydryl groups.

Keywords: Urease; *Canavalia ensiformis*; Oligomerization; Diethylpyrocarbonate; Copper; Insecticide; Histidine; *Dysdercus peruvianus*; Inactivation; IMAC

Urease (EC 3.5.1.5; urea amidohydrolase) is a nickel-dependent enzyme [1] that catalyzes the hydrolysis of urea to form ammonia and carbon dioxide. In 1926, jack bean urease (JBU)[1] was crystallized by Sumner [2] and these first crystals of a characterized enzyme demonstrated the proteinaceous nature of the enzymes. JBU exists as monomers, trimers, and hexamers of identical 91 kDa subunits, each containing two nickel ions per subunit [3]. Fishbein, in 1969, concluded that "jack bean seeds contain distinct molecular forms of JBU and the multiplicity of urease isoenzymes, in conjunction with their interconvertibility, may underlie many catalytic complexities of this enzyme" [4].

Recent studies of our group have shown that JBU displays biological activities that are unrelated to its ureolytic property, such as activation of blood platelets, interaction with glycoconjugates, and entomotoxic activity, this latter suggesting that urease(s) may be involved in plant defense [5–8]. Many of these biological activities are modified under conditions in which protein oligomerization takes place, notably the ureolytic and insecticidal properties seem to be greatly influenced by JBU aggregation [9]. Moreover, JBU aggregation is believed to impact the shelf life of diagnostic kits based on this enzyme [10]. Aggregation is also a major obstacle to useable crystal growth [11] and may be the reason of the

* Corresponding author. Present address: Department of Medical Biochemistry, ICB-CCS, Universidade Federal do Rio de Janeiro, CEP 21.941-590, Rio de Janeiro, Brazil. Fax: +55 21 2562 6756.
E-mail address: follmer@bioqmed.ufrj.br (C. Follmer).

[1] *Abbreviations used:* JBU, jack bean urease; DEPC, diethylpyrocarbonate; IMAC, immobilized metal affinity chromatography; IDA, iminodiacetic acid.

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difficulties encountered for growing crystals of JBU capable of diffracting X-rays at low angles [12,13].

In a previous work, we have shown that oligomerization of JBU induced by freezing and thawing cycles causes partial inactivation of its insecticidal properties as well as of its ureolytic activity [9], suggesting that association/dissociation of the protein could play a role in modulation of these activities. Although several studies have addressed the oligomerization behavior of JBU there are still unanswered questions regarding which factors are involved in this process. Elucidation of these factors is a crucial step towards understanding JBU biological properties, not only of its catalytic activity but also the other recently described pharmacological [5] and insecticidal properties [7,8].

The high affinity of JBU for metal ions (e.g., Hg^{2+}, Cu^{2+}, Ni^{2+}, and Zn^{2+} ions) as well as their inhibitory effect [14] upon the enzyme's ureolytic activity, opens the possibility of metals being modulators of JBU activities. Preliminary data from our laboratory have suggested that JBU aggregation could be induced by metal-binding. In this work, we investigated the protein oligomerization induced by metals, the role of some amino acid residues in this process and how this affects JBU's biological activities, notably the ureolytic and insecticidal properties.

Experimental

Protein determination

The protein content of samples was determined by their absorbance at 280 nm. Alternatively, the method of Spector [15] was used.

Polyacrylamide gel electrophoresis

Electrophoresis in non-denaturant 3% polyacrylamide gels (1.5 mm thick) were run at 20 mA for 2–3 h and the gels were stained using silver nitrate.

JBU purification

Urease was isolated from jack bean seed meal based on the method of Blakeley et al. [16] with modifications introduced as in [17]. Briefly, defatted seed meal (50 g) was extracted with buffer A (20 mM sodium phosphate, pH 7.5, 1 mM EDTA, and 2 mM of 2-mercaptoethanol) for 1 h at 4 °C. The meal was removed by centrifugation (30,000*g*, 20 min, 4 °C), and 28 % (v/v) ice-cold acetone (final concentration) was added to the supernatant. After removing the precipitate formed overnight at 4 °C, the concentration of acetone in the supernatant was increased to 31.6% (v/v). The new precipitate was discarded and the supernatant was dialyzed against buffer B (20 mM sodium phosphate, pH 7.5, 1 mM EDTA, and 5 mM of 2-mercaptoethanol). The resulting material was then mixed with 25 mL of Q-Sepharose resin (Amersham-Biotech) in buffer B. After stirring for 30 min, the mixture was filtered and the non-retained proteins were washed out from the resin with 100 mM NaCl in buffer B. Elution of the urease-enriched fraction was achieved adding 300 mM NaCl to buffer B. This material was concentrated with a CentriPrep cartridge (Millipore) and applied into a Superose 6 HR 10/30 gel filtration column (Amersham-Biotech) equilibrated in 20 mM sodium phosphate, pH 7.5, 1 mM EDTA, mounted in a FPLC system. The peak fraction containing urease activity was dialyzed against 20 mM sodium phosphate, pH 7.0, 0.5 M NaCl (buffer C) and then submitted to immobilized metal affinity chromatography using 10 mL of Co^{2+} loaded iminodiacetic acid–Sepharose (IDA–Co^{2+}), equilibrated in buffer C. The aim of the IDA–Co^{2+} step is to isolate a single jack bean urease isoform [17], the major form of urease being recovered in the non-retained fraction. Homogeneity of the purified protein was checked by capillary electrophoresis (data not shown).

Urease activity

The ammonia released was measured colorimetrically [18]. One unit of urease releases 1 μmol ammonia per minute, at 37 °C, pH 7.5. Kinetic parameters were calculated as in [19]. For the inhibitory studies with metal ions, JBU aliquots in 50 mM sodium citrate, pH 7.0, were pre-incubated for 24 h at 4 °C with chloride salts of copper, mercury, nickel, and zinc, then dialyzed against buffer containing 20 mM EDTA before being assayed for residual ureolytic activity.

Insecticidal activity

The insecticidal activity of JBU was evaluated in feeding trials with the cotton sucker bug *Dysdercus peruvianus* (Hemiptera), an economically important crop pest as model insect [8]. JBU was given to the insects by adding the freeze-dried protein (at final concentration of 0.25% w/w) to their diet consisting of cotton seed meal. The toxicity was expressed as survival rate over a period of 20 days. To evaluate the effect of Cu^{2+} on the insecticidal activity of JBU, the protein was incubated for 24 h at 10 °C with 10 μM of $CuCl_2$, dialyzed against 50 mM sodium citrate, pH 7.0, containing 20 mM EDTA, and then tested as above.

Chemical modification of JBU histidines

Histidine residues in JBU were chemically modified was described in [20,21]. Briefly, 2 mL JBU (1.5 mg/mL) in 50 mM sodium citrate, pH 7.0, was mixed with 10 μL of 0.1 M diethylpyrocarbonate (Sigma Chemicals)