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Purification and characterization of urease from leaves of *Trigonella foenum-graecum*

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Abstract: Urease is amidohydrolase enzyme (EC: 3.5.1.5) splits urea into carbon dioxide and ammonia. The enzyme was purified from fenugreek (*Trigonella foenum-graecum*) leaves using ammonium sulphate precipitation (80%), phenyl sepharose, hydroxyapatite and sephadex G-200. The specific activity of the purified urease from sephadex G-200 was 210 U per mg protein with 6.6 % yield. The final fold of purification was 677. The optimal pH of the purified urease was 8.0 whereas the optimal temperature was 40°C. The purified urease exhibited appreciable thermal stability at 60 °C particularly in presence of bovine serum albumin (BSA). Also, the enzyme exhibited appreciable storage stability at 25°C.

keywords: Urease - *Trigonella foenum-graecum*- Specific activity- Storage stability- Purification

1.Introduction

Enzymes are important for use in several industries such as beverage, food, confectioneries production and starch in addition to leather and textile industries processing [1]. Enzymes are the principal point in industrial processes due to their involvement in all features of biochemical conversion [2].

Enzymes applications in industries are widely successful; however numerous factors influence their applications as biocatalysts such as, robustness, promiscuity and the various parameters of the enzyme for its applications [3].

The application of enzymes is frequently inadequate because of their high cost, instability and restricted availability and the problem of recovering the soluble enzyme from its reaction medium [4].

Urease (EC 3.5.1.5) is belonging amidohydrolases, and it is a multimeric complex [5] containing two nickel atoms therefore it is a metallo-enzyme. Urease splits urea into CO_2 and ammonia in presence of water [6]. The enzyme is discovered in plants [7, 8, 9], fungi [10], algae [11], protozoa [12] and bacteria [13].

The objective of the present study was to isolate and purified urease from *Trigonella foenum-graecum* for studying some of its biochemical characteristics.

2. Materials and Methods

Plant material

Trigonella foenum (Fenugreek, family: *Fabaceae*) is the experimental plant used in the present study. The plant seeds were purchased from the Ministry of Agricultural, Egypt.

Seed germination and growth conditions

Germination of seeds was carried out according to the method of El-Shora [7]. Fenugreek seeds were surface sterilized with sodium hypochlorite (10% v/v) for 15 min, and then water-logged for 24h. Later the seeds were germinated in sterilized plastic trays using paper towels which are dampened with distilled water. The trays were then covered and incubated for 72h in dark at 25°C. After appearance of well-grown radicals the germinated seeds were then sustained on plastic

bowls containing 0.2 mM $CaCl_2$ solution. $CaCl_2$ solution was continuously aerated. Seedlings were grown in light at 25°C for 20 days. The leaves from 20- day old plants were gathered and chopped on ice using a razor blade for enzyme extraction.

Enzyme extraction

Urease was extracted from the plant leaves as designated by El-Shora [7]. Leaves were pulverized in 50 mM sodium acetate buffer (pH 7.0) at 4°C. The homogenate was centrifuged at 6000 rpm for 20 min, and then the supernatant was gathered, and represented the crude enzyme extract.

Assay of urease (EC: 3.5.1.5)

Urease assay was carried out according to **[7]**. The reaction mixture contained in 5ml: 100 mM-Tris-HCl buffer (pH 7.0), 2 mM $CaCl_2$, 20 mM urea, and 50 µl of urease preparation. The mixture was incubated for 30 min at 40°C and the enzyme reaction was ended by the addition of 5 ml of 1N H_2SO_4 to the reaction mixture. The mixture was centrifuged at 5000 rpm for 15 min and supernatant was treated with Nessler's reagent and the optical density was recorded at 520 nm. The ammonia produced was determined using standard curve of ammonium sulfate. One unit (U) of the enzyme activity is defined as the amount of urease which produces one µmol of NH_3 in one min standard assay conditions. Specific activity of urease is described as the enzyme unit (U) per mg protein.

Enzyme purification

The crude extract obtained by the extraction method was treated with solid ammonium sulphate (80% saturation) and then centrifuged at 6000 rpm for 20 min. The pellet obtained was re-suspended in a small volume of 50 mM sodium acetate buffer, pH 7.0, and dialyzed and the dialysate was centrifuged at 6000 rpm for 20 min and then poured on to phenyl sepharose column (2× 40 cm), equilibrated with 50 mM acetate buffer (pH 7.0).

Urease was eluted from the column by a steep gradient of NaCl from 0.1 to 0.5 M in equilibrating buffer with flow rate of 0.5 ml min^{-1}. The rich fractions with urease were combined and dialyzed over night against 50 mM acetate buffer (pH 7.0). The eluted enzyme from phenyl Sepharose column was loaded onto hydroxyapatite column (2×40 cm). The rich fractions with urease were pooled, concentrated by dialysis as mentioned above and the enzyme was further purified on Sephadex G-200 (2×40 cm) and eluted with a flow rate of 0.5 ml per min. The active fractions were collected and considered as the pure enzyme. In both cases, columns were equilibrated with 500 mM acetate buffer containing 0.5M NaCl. Fractions containing active enzyme were pooled, and concentrated by dialysis using the same buffer.

Estimation of total soluble protein

Total soluble protein in the prepared extract was estimated according to Bradford [14].

Effect of pH on purified urease activity

The influence of the pH of the reaction medium for urease was done with appropriate buffers (0.1 M citrate for pH 3.0 and 4.0, 500 mM phosphate for pH 5.0 and 6.0 and 50 mM Tris–HCl for pH 7.0 – 9.0). The optimum pH obtained from this assay was used in coming experiments

Effect of temperature on purified urease activity

Urease activity was measured at different incubation temperatures in the range 10-60°C. All the other factors affecting the enzyme activity was kept constant.

Thermostability of purified urease at 60°C

To determine the thermostability of purified urease the reaction medium was incubated at 60°C for 100 min. An aliquot of the enzyme solution 50 µl was withdrawn at appropriate intervals time (20 min) to determine the enzyme activity and calculation of the residual activity. The thermostability test was carried out in presence or absence of 10% (w/v) bovine serum albumin (BSA).

Storage stability of purified urease

The storage stability of purified urease was studied by measuring the residual activity after incubation of the purified enzyme in 25 mM

phosphate buffer at pH 8.0 and the enzyme activity was determined every 60 days at 25 °C.

Sodium Dodecyl Sulphate Polyacrylamid Gel Electrophoresis (SDS-PAGE)

The applied method was described by [15], for the separation of protein according to their molecular weights by SDS-PAGE.

3. Results and Discussion

Urease was purified from *Trigonella foenum-graecum* by ammonium sulfate (80%), phenyl Sepharose, hydroxyapatite and Sephadex G-200 (Table 1).

Table 1: The profile of purified urease from *Trigonella foenum-graecum* leaves.

Step of purification	Total protein (mg)	Total activity (U)	Specific activity (Umg^{-1} protein)	Yield (%)	Fold
Crude extract	1017	320	0.31	100	1
Ammonium sulfate (85%)	287	315	1.1	98	3.55
Phenyl Sepharose	38	213	5.6	66	18.1
Hydroxy-apatite	0.50	42	84	13.1	271.0
Sephadex G-200	0.1	21	210	6.6	677.4

The profile of purified urease from Sephadex G-200 is shown in Fig 1.

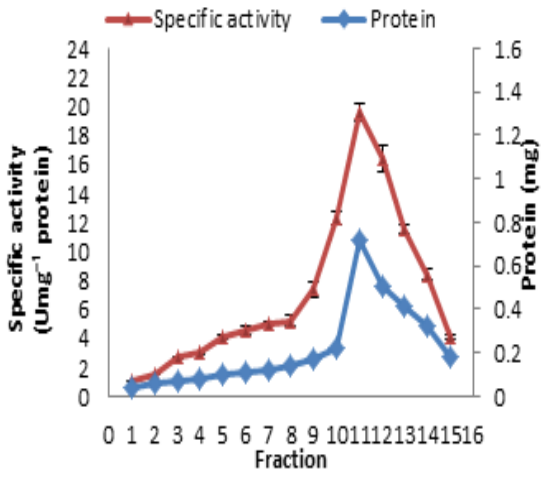

Fig.1: Profile of urease purified fromSephadex G-200.

It was apparent that the specific activity of urease increased gradually up to fraction number 11 value it was 19.6 U mg^{-1} protein, then declined gradually to reach 4.1 U mg^{-1} protein at fraction number 15. The purification process was confirmed by SDS-PAGE (data not shown).

The results in Fig. 2 indicate that there was permanent increase in the activity of urease up to pH 8.0 which the optimal one. After, the optimal pH the activity decreased progressively with the increase the pH and it reached 20.4 units per mg protein. The optimal pH of urease from soybean leaves was 6.5 [16] and the optimal pH of urease from pigeon pea was 7.3 [17]. Also, urease from mulberry exhibited an optimal pH of 9.0 [18].

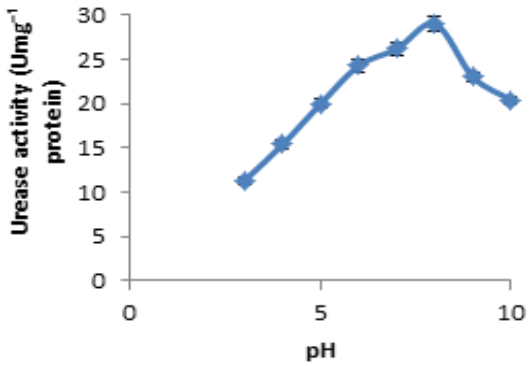

Fig. 2: Effect of pH on the activity of the purified urease activity.

Higher acidic or basic pH inhibited urease activity due to the alteration ionization state of the enzyme, modification of its surface, and dissociation of its subunits then distraction the ES complex. Excessive pH produces denaturation of urease protein through various ways. Firstly, it can affect the ionization of the ES complex. Secondly, it can influence the substrate ionization and so influence the connecting of the enzyme to its substrate [19]**.** Thirdly, extreme pH can change the structure of enzyme protein which modifies the stability of the enzyme. Fourthly, it can change the ionization of the different active residues in the enzyme molecules which disturbs the enzyme affinity to its substrate [20]**.**

The effect of incubation temperature on purified urease was investigated at 10, 20, 30, 40, 50 and 60°C. The obtained results are shown in Fig 3.

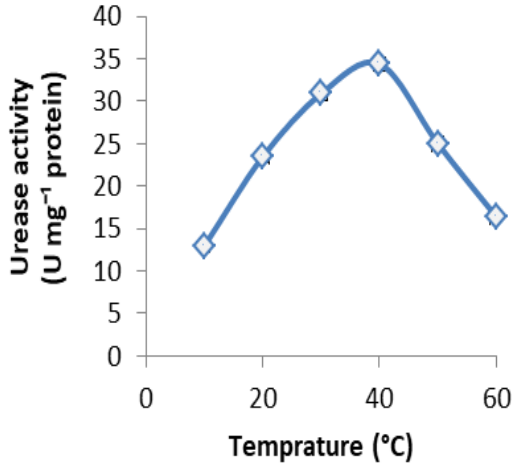

Fig. 3: Effect of incubation temperature on the purified urease activity.

These results indicate that the enzyme activity increased depending on the incubation temperature up to 40°C. The activity was 34.4 U mg^{-1} protein at 40°C which seems likely to be the optimum one. After 40°C the activity of the enzyme declined gradually and reached 16.4 U mg^{-1} protein at 60°C.

The enhancement of urease reaction by temperature outcomes in enhancing of the inherent energy of the reaction medium and much of enzyme molecules can get the activation energy for initiating of its reaction. However, the escalation of the reaction due to temperature at particular point turns out to be equivalent to the reduction in the rate of reaction due to destruction of the tertiary structure [21]. The enzyme activity at this point is described as optimum temperature [22].

The purified urease exhibited appreciable Thermostability at 60°C (Fig. 4) particularly in presence of 10% w/v BSA. This implies that BSA prevents the disruption of protein structure of the enzyme [23]. In support, BSA protected of the enzymes from thermal inactivation such as nitrate reductase [24], glucose oxidase [25] and catalase [26].

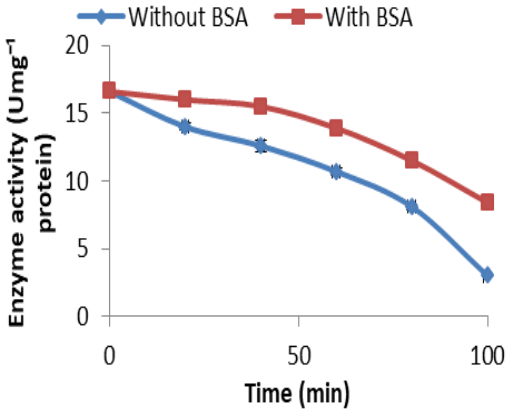

Fig. 4: Thermostability of purified urease at 60 °C in presence and absence of BSA 10% (w/v).

The thermostability of an enzyme is manipulated by two factors. The first factor is the enzyme primary structure which offers a compacted structure that is not denatured by changes of the surrounding environment. Also, disulfide bridges offer a high challenge to thermo-inactivation and denaturation. The second factor is represented by divalent cations which can play a role in the stabilization of the enzyme [27].

The thermostability of an enzyme depends on the medium of enzyme production and the extraction method. Improvement of enzyme reaction medium is essential for reducing the cost of the process [28].

Studying the storage stability of purified urease at 25°C (Fig. 5) indicated that enzyme expressed appreciable. However, the enzyme activity declined after 40 days. This could be attributed to autolysis of urease as in case of other enzymes [29].

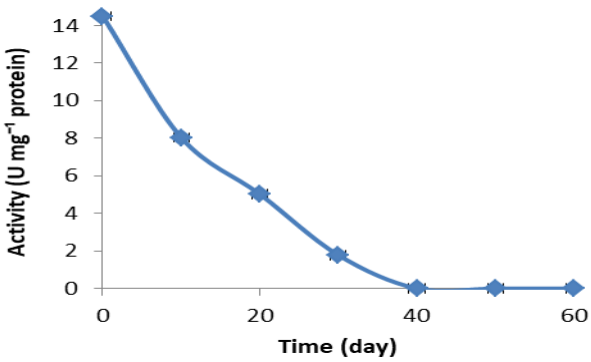

Fig. 5: Storage stability of the purified urease at 25 °C.

Conclusion:

Urease from *Trigonella foenum-graecum* was purified with appreciable schedule and the enzyme exhibited appreciable thermostability stability at 60 °C in presence of BSA and storage stability at 25 °C.

References

1 Toghueo, R. M. K., & Boyom, F. F. (2021). Endophyte enzymes and their applications in industries. Bio-prospecting of Plant Biodiversity for Industrial Molecules, pp 99-129.

2 Robinson, P. K. (2015). Enzymes: principles and biotechnological applications. Essays in Biochemistry, 59, 1–41.

3 Adrio, J. L., & Demain, A. L. (2014). Microbial enzymes: tools for biotechnological processes. Biomolecules, 4(1), 117-139.

4 El-Hefnawy, M. E., Sakran, M., Ismail, A. I., & Aboelfetoh, E. F. (2014). Extraction, purification, kinetic and thermodynamic properties of urease from germinating *Pisum Sativum* L. seeds. BMC Biochemistry, 15(1), 1-8.

5 Hassan, S. T., & Žemlička, M. (2016). Plant-derived urease inhibitors as alternative chemotherapeutic agents. Archive der Pharmazie, **349**(7), 507-522.

6 Vitolo, M. (2022). Notes on urea hydrolysis by urease. *J. World Journal of Pharmacy and Pharmaceutical Sciences*, **11(3)**, 96-135.

7 El-Shora, H. M. (2001). Properties and immobilization of urease from leaves of *Chenopodium album* (C3). Botanical Bulletin of Academia Sinica, **42**(4), 251-258.

8 El-Shora, H. M., & Ali, A. S. (2016). Plant growth regulators induced urease activity in *Cucurbita pepo* L. cotyledons. Acta Biologica Hungarica**, 67**(1), 53-63.

9 Magomya, A. M., Yebpella, G. G., Okpaegbe, U. C., Agu, M. O., & Kefas, K. (2022). An enzymatic method for the determination of urea in aqueous samples using crude urease from *Citrullus lanatus* Seeds. *Journal of Chemical Society of Nigeria*, **47(4)**, 679 – 687.

10 Arumugam, N., Shanmugam, M. K., & Thangavelu, P. (2022). Purification and anticancer activity of glutaminase and urease-free L-asparaginase from novel endophyte *Chaetomium* sp. Biotechnology and Applied Biochemistry**, 69**(5), 2161-2175.

11 Sacristán, M., Millanes, A. M., Legaz, M. E., & Vicente, C. (2006). A lichen lectin specifically binds to the α-1, 4-polygalactoside moiety of urease located in the cell wall of homologous algae. Plant Signaling & Behavior, **1**(1), 23-27.

12 Zhang, X., Xiong, Z., Li, M., Zheng, N., Zhao, S., & Wang, J. (2022). Activity-and enrichment-based metaproteomics insights into active urease from the rumen microbiota of cattle. *International Journal of Molecular Science s*, **23(2)** , 817.

13 Ghorbanzadeh, N., Ghanbari, Z., Farhangi, M. B., & Rad, M. K. (2022). Zinc bioremediation in soil by two isolated L-asparaginase and urease producing bacteria strains. Applied Geochemistry, 140, 105271.

14 Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, **72**(1-2), 248-254.

15 Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680-685.

16 Ângelo, E. A., da Cruz, T. M., Júnior, J. R. P., Zanzarin, D. M., Rodrigues, F. A. V., Pilau, E. J., ... & Seixas, F. A. V. (2021). Purification and characterization of embryo-specific soy urease (Glycine max) and its antifungal potential against *Paracoccidioides brasiliensis*. Eclética Química, **46**, 41-52.

17 Das, N., Kayastha, A. M., & Srivastava, P. K. (2002). Purification and characterization of urease from dehusked pigeonpea (*Cajanus cajan* L.) seeds. Phytochemistry, **61**(5), 513-521.

18 Hirayama, C., Sugimura, M., Saito, H., & Nakamura, M. (2000). Purification and

properties of urease from the leaf of mulberry, *Morus alba*. Phytochemistry, **53**(3), 325-330.

19 Ramos, M. D. N., Milessi, T. S., Candido, R. G., Mendes, A. A., & Aguiar, A. (2022). Enzymatic catalysis as a tool in biofuels production in Brazil: Current status and perspectives. Energy for Sustainable Development, **68**, 103-119.

20 Ghorbanzadeh, N., Ghanbari, Z., Farhangi, M. B., & Rad, M. K. (2022). Zinc bioremediation in soil by two isolated L-asparaginase and urease producing bacteria strains. Applied Geochemistry , **140**, 105271.

21 Rezaei, P. F., Ghanbari, S., & Mahdavinia, G. (2023). Purification and immobilization of exo-polygalacturonase using nanomagnetic beads. Process Biochemistry, **132**, 180-190.

22 Krajewska, B., van Eldik, R., & Brindell, M. (2012). Temperature-and pressure-dependent stopped-flow kinetic studies of jack bean urease. Implications for the catalytic mechanism. *JBIC Journal of Biological Inorganic Chemistry*, **17**, 1123-1134.

23 El-Shora, H. E., & Ali, A. S. (2011). Changes in activities of nitrogen metabolism enzymes in cadmium stressed marrow seedlings. *Asian Journal of Plant Sciences*, **10(2)**, 117-124.

24 Yamaya, T., Solomonson, L. P., & Oaks, A. (1980). Action of corn and rice-inactivating proteins on a purified nitrate reductase from *Chlorella vulgaris*. Plant Physiology, **65**(1)**,** 146-150.

25 El-Shora, H. M., Metwally, A. M., Mohammed, R. M., & Sabry, A. S. (2014). Stability and immobilization of glucose oxidase from *Cladosporium oxysporum*. *Journal Environmental of Science*, **2**, 253-266.

26 Costa, S. A., Tzanov, T., Carneiro, F., Gübitz, G. M., & Cavaco-Paulo, A. (2002). Recycling of textile bleaching effluents for dyeing using immobilized catalase. Biotechnology Letters, **24**, 173-176.

27 Nezhad, N. G., Rahman, R. N. Z. R. A., Normi, Y. M., Oslan, S. N., Shariff, F. M., & Leow, T. C. (2022). Thermostability engineering of industrial enzymes through structure modification. Applied Microbiology and Biotechnology, **106**(13-16), 4845-4866.

28 Wu, H., Chen, Q., Zhang, W., & Mu, W. (2023). Overview of strategies for developing high thermostability industrial enzymes: Discovery, mechanism, modification and challenges. Critical Reviews in Food Science and Nutrition, **63**(14), 2057-2073.

29 Deere, J., De Oliveira, R. F., Tomaszewski, B., Millar, S., Lalaouni, A., Solares, L. F., & Halling, P. J. (2008). Kinetics of enzyme attack on substrates covalently attached to solid surfaces: influence of spacer chain length, immobilized substrate surface concentration and surface charge. Langmuir, **24**(20), 11762-11769.