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Influence of storage time and temperature on the activity of urease

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Enzymes are made of protein, that is why they are sensitive molecules and are affected by storage conditions. A small change in enzyme activity during storage may cause a big error in analysis results. The aim of the study was to evaluate the effects of storage time and temperature on urease activity. Urease solutions were prepared at different activities (from 100 to 2000 U/mL) and stored at room temperature, in the refrigerator (4°C), and in the deep freezer (-18°C and -80°C). Activity measurements were made at regular intervals until 28 days by the modified Weatherburn method. The relative activities of 100-1000 U/mL urease solutions stored at room temperature, 4, -18 or -80°C were 75% and below after 4 days. Twenty-eight days later, for 2000 U/mL urease solutions, only at room temperature, the relative activity was reduced to 37%, while at 4, -18 or -80°C, the relative activities were above 80%. Since urease can be maintained at 4°C for 28 days without significant loss of activity, it has practical importance. Low-activity urease solutions (such as 100-1000 U/mL) should not be stored at -18 or -80°C for short or long term storage, they should be stored at 4°C only for one day.

Keywords: Urease activity, storage time, storage temperature

INTRODUCTION

Urease, (urea amidohydrolase, EC 3.5.1.5) is a multisubunit (homohexamer), nickel-dependent metalloenzyme synthesized by plants, some bacteria and fungi [1]. Urease plays a primary role in nitrogen metabolism in the nature. Its physiological role is to hydrolyze urea by forming ammonia and carbon dioxide. Because of released ammonia, nitrogen is produced during growth of organisms and the medium becomes alkaline [2].

Apart from its natural significance, there are many uses for urease. These include removal of urea from aqueous solutions, determination of urea concentration in aqueous solutions, precipitation of calcium carbonate in geoenvironmental applications, determination of heavy metal ions in aqueous solutions [3-10]. Urease is also used as an anticancer agent and a vaccine antigen, as well as for determining the concentration of urea in biological fluids (blood, urine, saliva) in medicine [11-15].

The storage of enzymes is a quite important issue from both practical and economical points of view. Enzymes undergo denaturation during production, storage, and application. Their activities may change in response to environmental factors such as temperature, pH, chemical agents, autolysis or ionic strength [16]. Storage conditions may vary from enzyme to enzyme because of individual structural differences [17]. However, some basic rules for storage can be mentioned: Enzymes are more stable at low temperatures. They can be kept in a buffer in a 4°C refrigerator for short-term storage. There are different strategies for long-term storage, such as keeping them at –20°C and –80°C in the deep freezer, or under liquid nitrogen at –196°C, and storing in lyophilized or immobilized forms [18,19].

In practice, enzymes that are not to be used immediately, are refrigerated or frozen. During storage, the enzyme stability can change according to its physical state such as being in liquid, lyophilized or immobilized form. Therefore, defining the appropriate storage conditions for each enzyme is very important to minimize pre-analytical errors. The aim of our present study was to examine the effect of storage time and temperature on urease activity. This paper describes some recommendations for storage of urease enzyme.

EXPERIMENTAL

Materials

All chemicals used were of analytical grade from Merck (Darmstadt, Germany), Riedel-de Haën (Seelze, Germany) or Fluka (Buchs, Switzerland). Urease from *Canavalia ensiformis* was purchased from Merck Millipore (Massachusetts, USA). The lyophilized enzyme was stored at −20 °C until use according to manufacturer's instructions. Ultra-pure water (Human Corporation Zeneer Power I) was

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used throughout the experiments. pH determinations were carried out on the HI 2211 pH/ORP-Meter (HANNA Instruments). The absorbance measurements were performed on a Shimadzu UVmini-1240 spectrophotometer.

Methods

Preparation of Urease Solutions and Storage Conditions. Lyophilized urease enzyme (10 kU) was reconstituted in 1 mL of 50 mM phosphate buffer, pH 7.6 to obtain a stock urease solution. It was diluted with appropriate buffer to prepare 100, 200, 300, 400, 500, 1000 and 2000 U/mL enzyme solutions. They were all divided into small volumes and were put into eppendorf tubes. To investigate the effect of storage temperature on urease activity, the tubes were stored at room temperature, in the refrigerator (4°C), in the deep freezer (-18, -80°C) separately. To determine the effect of storage time, urease activity was measured at 0, 1, 4, 7, 11, 14, 17, 21, 24, 28 days. Flow chart for all experiments is shown in Figure 1.

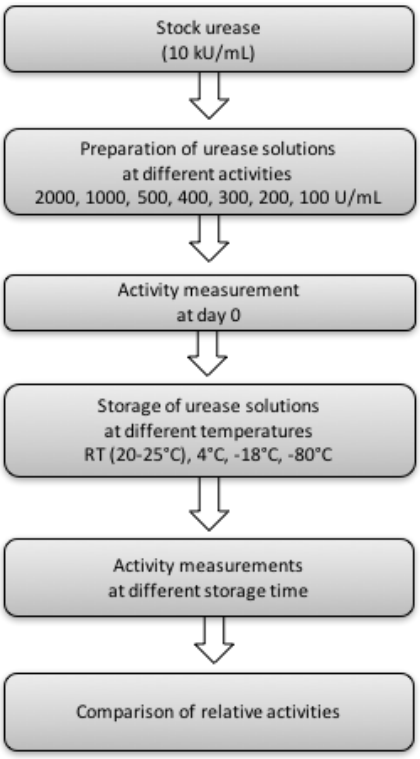

Fig. 1. Experimental flow chart (RT: Room temperature)

Measurement of Urease Activity. The urease activity was measured by a modified Weatherburn method [20]. The reaction was carried out in a tube containing 100 µL of urease solution, 10 µL of 7 mM urea and 1890 µL of 50 mM phosphate buffer, pH 7.6. The reaction mixture was incubated at room temperature for 15 min. 500 µL of phenol reagent and 500 µL of alkaline hypochlorite reagent were then added to the tube, and incubated at 60°C for 5 min. After the tube was rapidly cooled under tap water to room temperature, absorbance was read at 630 nm against reagent blank. The amount of ammonia liberated was calculated using a standard curve obtained from ammonium sulfate. One unit urease activity was defined as the amount of enzyme required to hydrolyze 1 µmol of urea per minute. All measurements were repeated six times. Results were reported as mean ± standard deviation (SD). GraphPad Prism 5 version 5.0a (GraphPad Software, San Diego, CA, USA) was used to calculate means and SDs.

RESULTS

The urease activities of all solutions at zero time were accepted as 100% activity. The relative activities of solutions of 100-1000 U/mL were 75% and below after 4 days for all storage temperatures. Therefore the enzyme activities of urease solutions of 2000 U/mL were also measured.

After storage for 1 day at room temperature, the decrease in the relative activities of 100, 200, 300, 400, 500 and 1000 U/mL urease solutions were 37, 34, 38, 37, 37 and 24%, respectively; at 4°C, they were 17, 17, 17, 15, 15 and 6%; at -18°C, they were 95, 91, 87, 29, 20 and 18%; at -80°C, they were 91, 85, 79, 60, 43 and 22% (Figure 2A-F).

After storage for 4 days at room temperature, the decrease in the relative activities of 100, 200, 300, 400, 500 and 1000 U/mL urease solutions were 29, 28, 25, 30, 28 and 31%, respectively; at 4°C, they were 37, 32, 31, 34, 36 and 29%; at -18°C, they were 96, 94, 89, 79, 48 and 37%; at -80°C, they were 94, 88, 73, 62, 46 and 36% (Figure 2A-F).

As seen in Figure 2 there was unexpected increase in relative urease activities of 100-500 U/mL urease solutions from day 1 to day 4 at room temperature.

At the end of the 14th day, the relative activities of 2000 U/mL urease solutions, kept at all storage temperatures, were 84% and over (the decreases were 16% and below). After day 14 till day 28, only at room temperature, the relative activity was reduced to 37% (the decrease was 63%), while at other storage temperatures, the relative activities were above 80% (the decreases were below 20%) (Figure 3).

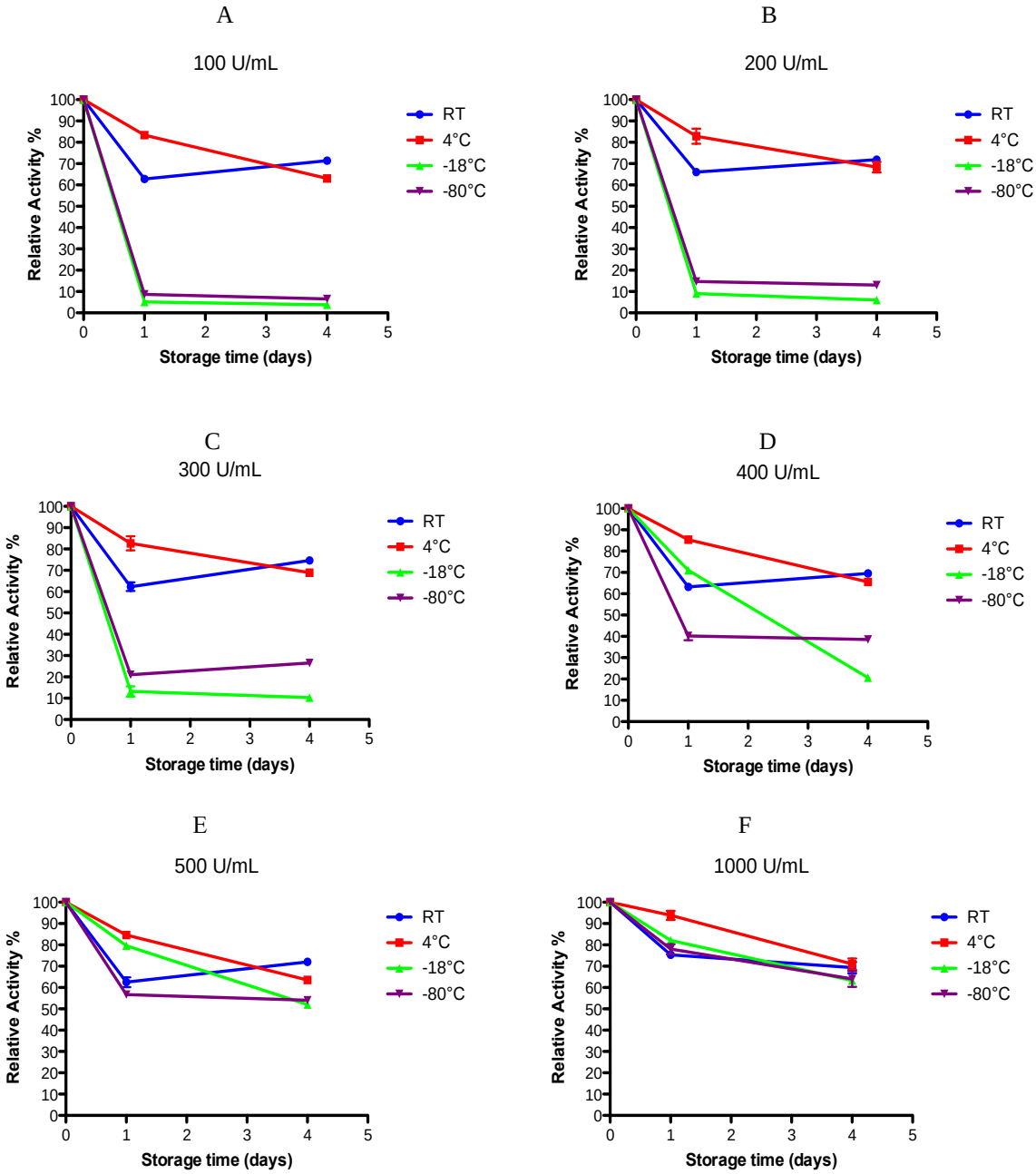

Fig. 2. Effects of storage time and temperature on urease activity. A: 100 U/mL, B: 200 U/mL, C: 300 U/mL, D: 400 U/mL, E: 500 U/mL, F: 1000 U/mL urease solutions. RT: Room temperature

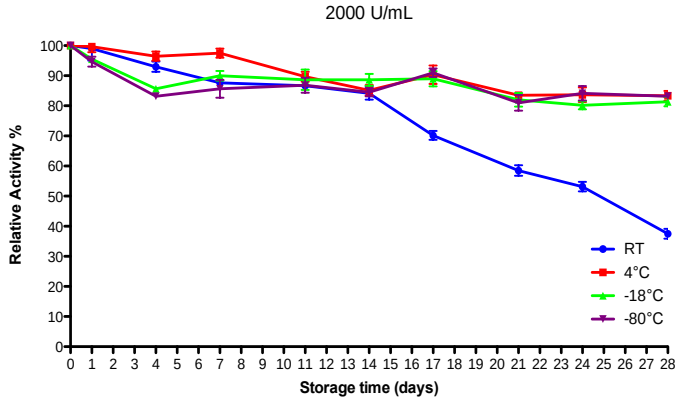

Fig. 3. Effects of storage time and temperature on 2000 U/mL urease solutions. RT: Room temperature

DISCUSSION

Temperature, pH, chemical agents, autolysis and ionic strength can affect enzyme structure and activity by causing unfolding, aggregation or covalent changes. For multimeric enzymes subunit dissociation is known to be an inactivation process [16]. Urease is a cysteine-rich enzyme and over time can aggregate through the formation of intermolecular disulfide bonds. Sumner *et al.* suggested that urease solution is inactivated on storage and the deactivation is caused by some oxidation reactions of sulfhydryl groups [21]. On the basis of previous investigations, urease aggregation and precipitation usually follows when it is stored in some conditions such as high protein concentrations, high temperature, absence of reducing agents, low pH or presence of salts. It is believed that exposed hydrophobic clusters of protein can cause aggregation [22].

It is known that denaturation of proteins during freezing is related to the physical and chemical changes of the local environment around the protein molecules during the process [23]. Follmer *et al.* showed that freezed sodium phosphate buffer induces crystallization of a component salt and a resulting shift of the local pH surrounding the protein. The acidification of medium, which leads to acidic residues' protonation, can decrease the electrostatic repulsion of the negative charges and thus could lead to aggregation of urease [22]. This may explain the drastic decrease in relative urease activity at low concentrations when stored at -18 and -80°C in our study. In contrary, at a high enzyme concentration such as 2000 U/mL, the decrease was not too big. This resistance of urease to damage during freeze-thawing might be due to high initial enzyme concentration [18].

An aqueous environment also introduces a significant risk of microbial contamination and proliferation, which can cause change in the enzyme activity in liquid products [24]. Urease is a microbial enzyme produced by some bacteria and fungi [25]. The unexpected increase in relative urease activities of 100-500 U/mL urease solutions from day 1 to day 4 at room temperature may be attributed to microbial contamination because of using non-sterilized eppendorf tubes and buffer solution [26].

The recommendation of the manufacturer for storage of urease that was used in this study is up to 2 months at –20°C after reconstitution. However, there is a need for more descriptive information on the effect of enzyme concentration on its activity. Our results clearly showed that urease stability during storage is closely related to its concentration and urease is more stable at high than at low concentration.

In this study, it was observed that the high-concentration urease solution can be stored for a month at low temperatures such as -18°C and -80°C. This is an expected result but similar results were also obtained during storage at 4°C. This stability of urease at 4°C is attractive for practical applications.

Jack bean (*Canavalia ensiformis*) urease was used in our study. In the literature, although urease used in another study was of bacterial origin, when it was stored at room temperature and -70°C, an activity decline, which was similar to the findings of our study, was detected [27]. Urease used in another study was also of fungal origin and when it was stored at -20 and -80 °C, there was a similar decrease as our results in enzyme activity after 28 days [28].

CONCLUSION

The current study highlights the importance of defining the appropriate storage conditions for urease enzyme. Since urease enzyme with high activity can be maintained at 4°C for almost a month without significant loss of activity, it has practical importance. Low-activity urease solutions (such as 100-1000 U/mL) should not be stored at -18 or -80°C for short- or long-term storage; they should be stored at 4°C only for one day.

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