Physical and Chemical Studies of a Low-Molecular-Weight Form of Urease

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1. A new form of enzymically active jack-bean [*Canavalia ensiformis* (L.) DC] urease corresponding to an $S_{20,w}$ value of 11.8 s and a molecular weight of 260 000 was investigated. 2. Conversion of 18 s urease (EC 3.5.1.5) into the 12 s form depends on both low protein concentration and pH. Above pH 5.3 urease exists in the 18 s form and below pH 4.8 in the 12 s form; between these two pH values a 12 s–18 s rapid-equilibrium process is observed. 3. Comparison of the properties of 18 s and 12 s urease indicated no major differences. 4. A survey of other good urease sources revealed that the 12 s form can also be obtained from soya bean (*Glycine soja* Sieb. and Zucc. cultivar Biloxi) and the bacterium *Bacillus pasteurii* (Miguel) Migula, but not from watermelon (*Citrullus vulgaris* Schrad. cultivar Congo). 5. The 12 s forms from jack bean and *Bacillus pasteurii* did not hybridize.

Previous chemical and physical investigations of urease (EC 3.5.1.5) have dealt principally with the 18 s form of the enzyme. Invariably jack bean [*Canavalia ensiformis* (L.) DC] has been used as the enzyme source because of the large amounts of urease present in the cotyledonary material of the plant (Mateer & Marshall, 1916). Jack-bean urease having an $S_{20,w}$ value of 18.6 s (mol. wt. 483 000) was first reported by Sumner and co-workers (Sumner & Gralen, 1938; Sumner, Gralen & Eriksson-Quensel, 1938). Using enzyme of widely varying specific activity, others have confirmed these reports (Kuff, Hogeboom & Striebig, 1955; Creeth & Nichol, 1960; Nichol & Creeth, 1963; Gorin, Fuchs, Butler, Chopra & Hersh, 1962; Gorin & Chin, 1965). During all these investigations small amounts of the polymeric forms of the enzyme were noted, with $S_{20,w}$ values of 26, 33 and 44 s. In addition, Creeth & Nichol (1960) and Nichol & Creeth (1963) observed a minor 12 s urease peak, but were unable to correlate it with the known urease system. Later, Sehgal and co-workers (Sehgal, Tanis & Naylor, 1965; Sehgal & Naylor, 1966) also noted the 12 s species and suggested that it might represent the monomeric form of the enzyme.

Reithel, Robbins & Gorin (1964), using guanidine hydrochloride, determined that there were six sub-units comprising the 18 s urease molecule. These sub-units of mol. wt. 83 000 were not enzymic-

ally active. Further investigations (Gorin & Chin, 1966) demonstrated that, by using pH 3.5 buffer and varying the protein concentration, urease sedimentation coefficients of 4, 6 and 10 s could be observed. Setlow (1952), using deutron bombardment, calculated that there were four or five active sites per 18 s molecule. This agrees with the observation (Ambrose, Kistiakowsky & Kridl, 1951) that 4 g. ions of Ag^+ must bind per mole for complete urease inactivation, but does not agree with the findings of Kobashi, Hase & Komai (1966), who calculated that there were only two active sites from the binding of the specific urease inhibitor octanohydroxamic acid.

Reithel (1963) pointed out that in many cases the preparative molecular weight of an enzyme is larger than its active sub-unit molecular weight. Such a concept is of interest in the case of urease because of its large preparative molecular weight. Thus the objective of the present investigation was to examine urease from a variety of sources to determine if there is a low-molecular-weight form of the enzyme that is active.

MATERIALS AND METHODS

Enzyme preparation. During this investigation crude extracts, partially purified and highly purified enzyme from several sources were examined. Throughout all phases of this work 0.014 M-2-mercaptoethanol was used and all purification steps were carried out at 1–4°.

Crude extracts from seeds were prepared after 24 hr. of soaking and with the seed coats removed. Extraction of the

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Sigma type IV, V and VI urease powders was carried out by making a 2% (w/v) solution. In all cases the extraction buffer was 0.01 M-phosphate buffer (KH_2PO_4 – Na_2HPO_4), pH 7.0, and each extract was clarified in the Servall high-speed centrifuge with the SS-34 rotor (30000g for 30 min.). The watermelon (*Citrullus vulgaris* Schrad. cultivar Congo) and soya-bean (*Glycine soja* Sieb. and Zucc. cultivar Biloxi) seeds were common commercial varieties, and the jack-bean [*Canavalia ensiformis* (L.) DC] seeds were obtained from Mr E. Nelson of Waldron, Ark. 72958, U.S.A.

The bacterium *Bacillus pasteurii* (Miguel) Migula (A.T.C.C. 11859) was first cultured on agar slants and later transferred to liquid shake-culture by the method of Larson & Kallio (1954). The cells were ruptured with a 20 kHz ultrasonic generator.

Much of the work was carried out on urease crystallized by the method of Sehgal & Naylor (1966) from Sigma defatted jack-bean meal. The purification scheme employed involved two steps of $(\text{NH}_4)_2\text{SO}_4$ fractionation followed by a step that permitted recovery of insoluble urease. This was followed by one to several steps of crystallization by the method of Dounce (1941).

Enzyme assay. Urease assays were carried out in macro Conway diffusion cells by the procedure of Sehgal & Naylor (1966). One unit of urease is the amount of enzyme necessary to catalyse the transformation of 1 $\mu\text{equiv.}$ of $\text{NH}_3/\text{min.}$ under standardized conditions at $23 \pm 1^\circ$. This international unit may be converted into Sumner units by dividing it by 6.67.

Catalase activity was determined by spectrophotometrically measuring the decomposition of H_2O_2 at 240 m μ (Chance & Maehly, 1962). Initial velocities were obtained and used as a relative measure of catalase concentration within the sucrose density gradient.

Centrifugation. Sucrose-density-gradient centrifugation runs were carried out with linear 20–5% (w/v) gradients. The 29 ml. gradients were layered directly into the Spinco SW 25 rotor buckets. After 12 hr. a sample (1 ml.) was placed on top and the run started (63000g). During the 25 hr. centrifugation run the gradient temperature was not permitted to vary by more than 1° . After centrifugation, the tubes were pierced and unloaded by means of an all-liquid fractionation system with a synchronous motor-powered syringe. Protein concentration and enzymic activities of all 31 fractions were measured, the former by means of the 1 cm. Gilford flow cell. Approximate urease sedimentation coefficients were determined by the method of Martin & Ames (1961) with catalase ($S_{20,w}$ 11.3s) as the standard.

Analytical ultracentrifugation was carried out in the Spinco model E machine at a rotor temperature of 8° .

Gel filtration. A 2.5 cm. $\times$ 0.38 cm. column of Sephadex G-200 (bead form) was packed after the Sephadex had equilibrated with buffer for 5 days. After 2 days of packing, a constant flow rate of 16 ml./hr. could be maintained with a Mariotte flask. The column was used to determine the Stokes radius of the various forms of urease (Siegel & Monty, 1966). Column calibration, by the method of Ackers (1964), was carried out with catalase. The Stokes radius was related to molecular weight by means of the Svedberg equation:

$$M = 6\pi\eta NaS/(1 - \bar{v}\rho)$$

where η is the viscosity of water at 20° , N is Avogadro's

number, a is the Stokes radius, S is the sedimentation coefficient, $\bar{v}$ is 0.73 g./ml. (Sumner & Kirk, 1932) and ρ is the density of water at 20° .

Disc electrophoresis. This was done with the Canalco apparatus and procedures (Canal Industrial Corp., Rockville, Md., U.S.A.). The electrophoretic runs were made at 7° with a protein concentration at 25 $\mu\text{g.}$ applied in 0.2 ml. of large-pore gel solution. The gels, stained with Amido Schwarz dye, were scanned with a Photovolt recording microdensitometer with a special wiping resistor calibrated for this dye.

Reagents. The guanidine hydrochloride used in these studies was twice recrystallized from methanol. It was stored over silica gel and under vacuum. Spectral analysis of a 6 M solution showed an extinction of 0.21 at 225 m μ .

Phosphate-citrate buffer, made according to the Table compiled by McIlvaine (1921), was used in several portions of this study. Because variation of ionic strength accompanies variation of pH with this buffer, both the pH and the molarity of stock reagents used to prepare a particular phosphate-citrate buffer are reported.

RESULTS AND DISCUSSION

Two methods were developed to produce enzymically active 12s urease (mol. wt. 250 000); each gave satisfactory, though different, results. The first method involved dialysis of a urease solution against 0.02 M-phosphate buffer (potassium dihydrogen phosphate–disodium hydrogen phosphate), pH 7.0, for 4 days. The specific activity used was 11 units/mg. Subsequent sucrose-density-gradient analysis showed approximately half of the activity occurring in the common 18s form and half in the 12s form. Dialysis for longer periods did not result in any increase in the amount of 12s material. It was possible to check on the stability of this 12s species, since it could be isolated and re-run on sucrose gradients. Such analysis revealed that, when treated in this manner, the 12s material remained as such and showed no evidence of reverting to the 18s form. This technique has the inherent disadvantage that it appears to apply only to crude urease extracts and will not convert crystalline urease into the 12s form. The reason for this has not been determined.

The other means of forming 12s urease involves adjusting the pH to a value below 4.9. It was observed that this method is dependent on buffer type, pH and ionic strength. However, phosphate-citrate buffer stabilizes urease activity and therefore was used in most of the present experiments. The effect that varying the pH has on the observed sedimentation coefficient is shown in Fig. 1. As the pH is lowered, more of the 12s form exists at any given moment and a lower $S_{20,w}$ value is observed. It is significant that the conversion process occurs over a pH range of only 0.5 pH unit. In the sigmoidal region the decreasing of ionic strength has the effect of lowering the observed sedimentation

coefficient, but not by an amount sufficient to explain the curve entirely in terms of ionic strength. Substitution of 0.02M-sodium acetate buffer at these various pH values resulted in identical sedimentation behaviour. This indicates that more than just the simple titration of a single binding group is involved. Van Holde & Cohen (1962) have noted a similar abrupt dissociation of haemocyanin as the pH was varied. Possibly in urease there are multiple binding sites between two 12s molecules.

The 12s form is active in the pH range 4.5-4.8; however, its activity is only about 5% of that found at pH7.0. The decreased activity at the lower pH is not due to enzyme inactivation, since urease subjected to pH 4.0 for 1 hr. showed complete enzymic recovery when later assayed at pH7.0. This was not found to be the case with acetate buffer at pH4.0.

Further studies of this pH effect were conducted with a Sephadex gel column. Two different pH values were studied, pH4.7 (0.024M-di-

sodium hydrogen phosphate-0.013M-citric acid) and pH 5.6 (0.028M-disodium hydrogen phosphate-0.010M-citric acid), since previous work had shown these two values to represent the 12s and 18s forms respectively. By using such pH values the effect of large differences in pH was minimized. Table 1 shows the results obtained and indicates that urease converted from the 18s into the 12s form undergoes a halving in molecular weight. Also shown in Table 1 are the calculated values for the frictional ratio, f/f_0 , for the two forms. Both values are within the range expected for globular proteins.

A study was undertaken to detect possible inter-conversions within the urease system resulting from pH variation. Fig. 2 shows the separation of twice-crystallized urease on a pH7.0 sucrose gradient. There are two major protein peaks, with all the urease activity being associated with the heavier of the two. Tubes 12-14 were pooled and most of the sucrose was removed by dialysis. The enzyme was then concentrated against Aquacide II (sodium salt of CM-cellulose; Calbiochem, Los

Fig. 1. Variation of the $S_{20,w}$ value of urease with respect to changing pH. The $S_{20,w}$ values were determined by means of the sucrose-density-gradient technique. Catalase was the standard of comparison. Phosphate molarity varied from 0.014M to 0.042M and citrate molarity from 0.018M to 0.005M.

Fig. 2. Sucrose-density-gradient analysis of twice-crystallized jack-bean urease. Gradient contained 0.02M-phosphate buffer, pH7.0. The $S_{20,w}$ value of the urease peak is 18.3s with the catalase standard. The arrow shows the direction of sedimentation. —, Activity; ----, protein.

Table 1. Urease molecular parameters

Material	Elution volume (ml.)	K_d	Stokes radius (Å)	S (s)	Mol. wt.	f/f_0
Blue Dextran 2000						
pH 5.6	64					
pH 4.7	64					
Catalase						
pH 5.6	108	0.37	52*			
pH 4.7	108	0.37				
Urease						
pH 5.6	94	0.25	68	18.3	520 000	1.29
pH 4.7	106	0.36	53	11.8	262 000	1.26

* Sumner & Galen (1938).

Fig. 3. Sucrose-density-gradient analysis of previously isolated 18s urease. Gradient contained 0.02 M-phosphate buffer, pH 7.0.

Fig. 4. Sucrose-density-gradient analysis of previously isolated 18s urease. Gradient contained 0.025 M-phosphate-0.013 M-citrate buffer, pH 4.8.

Angeles, Calif., U.S.A.). When reanalysed on a pH 7.0 sucrose gradient, the enzyme sedimented as a symmetrical 18s peak (Fig. 3), showing that urease has no tendency to dissociate under such conditions. However, if previously isolated 18s urease is reanalysed on a pH 4.8 (0.025 M-disodium hydrogen phosphate - 0.012 M - citric acid) sucrose density gradient, all the activity is recovered in the 12s form (Fig. 4) and no 18s urease is observed. This 12s form can also be dialysed and concentrated as mentioned above. Subsequent analysis on a pH 7.0 sucrose gradient revealed that all the urease activity again resided in an 18s peak. These four experiments, taken together, indicate that the 12s-18s urease system is completely interconvertible.

To determine accurately the sedimentation coefficient of the 12s form of urease, analysis in the Spinco analytical ultracentrifuge was carried out. Crystalline urease was separated on sucrose gradients and the 18s peaks were isolated and pooled. This material was concentrated, by using Aquacide II, and dialysed for 3 days against 0.024 M - disodium hydrogen phosphate - 0.012 M -

Fig. 5. Analytical-ultracentrifuge schlieren peak of jack-bean urease initially isolated as the 18s peak on sucrose density gradients. Sedimentation is from left to right. Protein concentration was 4 mg./ml.; buffer was 0.025 M-phosphate-0.013 M-citrate, pH 4.8. Rotor speed was 42040 rev./min.

citric acid buffer, pH 4.7, at 4°. This dialysis step determined the final pH and removed most of the sucrose. Because of an unknown amount of residual sucrose it is impossible to state the value observed precisely. Fig. 5 shows the peak obtained after 24 min. at running speed. After correction for the effect of temperature, it was calculated that this peak had an approximate sedimentation coefficient of 18s. Similar results were obtained at a protein concentration of 1 mg./ml. In both cases only a single peak was observed.

The effect of guanidine hydrochloride, considered an excellent dissociating agent regardless of the non-covalent forces holding protein chains or sub-units together (Tanford, 1962), was investigated. Initial experiments with this reagent showed that urease activity is unaffected by 0.5 M or 1.0 M concentrations of guanidine hydrochloride. The activity tests were run after 20 min. and again after 2 hr. from the time of mixing. When the guanidine hydrochloride concentration was raised to 2.5 M it was observed that 25% of the urease activity was lost after 20 min. and 65% after 2 hr. Increasing the guanidine hydrochloride concentration to 5.0 M resulted in complete loss of urease activity.

Guanidine hydrochloride, at concentrations up to 1 M with or without 0.1 M-2-mercaptoethanol, produced no observable change in the sedimentation coefficient of the enzyme at pH 5.8 or 7.0; at guanidine hydrochloride concentrations of 2 M and 4 M the activity was irreversibly destroyed. At pH 7.0 0.2 M-guanidine hydrochloride was ineffective in preventing reassociation. The sucrose gradients used for these studies also contained guanidine hydrochloride.

Fig. 6. Disc-electrophoresis separation of the 18s urease peak as isolated by sucrose-density-gradient centrifugation (specific activity 1050 units/mg.). Protein movement is from the cathode to the anode.

Fig. 7. Disc-electrophoresis separation of the 12s urease peak (specific activity 800 units/mg.) with urease initially separated in the 18s form. Protein movement is from the cathode to the anode.

Previous experiments had shown that a combination of crystallization and separation on sucrose density gradients permitted the isolation of relatively pure urease containing up to 1000 units/mg. Work was carried out by analytical disc electrophoresis to check the purity of different urease preparations. Electrophoresis of the 18s peak isolated from sucrose density gradients was carried out with crystallized urease as the starting material. Normal run times (1 hr.) revealed the presence of only two bands near the origin. To separate these two bands further, and to assay them individually, the duration of electrophoresis was extended to 4 hr. Only the heavy band shown in Fig. 6 demonstrated urease activity when eluted. Thus there appears to be at least one protein contaminant. Also analysed was 12s urease isolated from a pH 4.8 sucrose gradient (Fig. 7). Only one band was observed and its position corresponded with the heavy band seen in Fig. 6. By using unstained gels this band was shown to be enzymically active. Because the disc electrophoresis running pH is about 9.5 it is to be expected that identical bands would be obtained since the 12s

material would revert back to 18s urease. These experiments show that very pure urease can be obtained and that 12s-18s interconversions do not affect the electrophoretic mobility of the 18s molecule.

Several experiments were undertaken to establish possible differences between the 12s and 18s forms of urease. It was observed that sodium dodecyl sulphate (3.5 mM) destroyed all enzymic activity at pH 4.8 and 5.6 within 15 min. This reagent had no effect at pH 7.0. Enzymic activity at pH 4.8 and 5.6 was unaffected by hexadecyltrimethylammonium bromide (2.7 mM), heat (50° for 10 min.) or treatment with trypsin (1:5.4 weight ratio, for 20 min.). The pH 4.8 activity was assumed to correspond to the 12s form and the pH 5.6 or 7.0 activity to the 18s form. In all cases the activity was measured at the pH indicated.

The long-term stability of dilute solutions of urease at pH 4.8 and 7.0 maintained at 4° was investigated. Samples were removed periodically and assayed at pH 7.0. At both pH values used for storage there was a gradual decrease of enzymic activity with time. After 30 days of standing, the

Table 2. *Sources of urease and their $S_{20,w}$ values*

The $S_{20,w}$ values are based on an $S_{20,w}$ value of 11.3s for catalase (standard).

Material	Gradient pH	$S_{20,w}$ (s)
Jack-bean seeds	7.0	12.0, 18.5
Sigma defatted jack-bean meal	7.0	12.7, 19.0
Sigma type V urease	7.0	12.7, 18.7
	4.8	12.1
Crystallized jack-bean urease	7.0	18.3
	4.8	11.8
Soya-bean seeds	7.0	18.0
	4.8	13.3
Watermelon seeds	7.0	18.5
	4.8	18.5
<i>Bacillus pasteurii</i>	7.0	11.8
	4.8	12.1

solution at pH 7.0 had lost 67% of its activity, whereas the solution at pH 4.8 had lost only 50%. These data indicate that conversion into the 12s form results in no rapid loss of enzyme activity.

During this work several sources of urease were investigated in determinations of the sedimentation coefficient under different conditions. These data are shown in Table 2. Neutral extracts of jack-bean seeds, defatted jack-bean meal and Sigma type V urease all showed two peaks with most of the activity occurring as the 18s peak. Enhancement of the amount of 12s urease, in all three cases, could be achieved by dialysis against 0.02M-phosphate buffer (potassium dihydrogen phosphate-disodium hydrogen phosphate), pH 7.0; however, the percentage of total activity in this form varied with the source and was highest in the Sigma type V urease. Crystallized jack-bean urease showed no 12s component when dialysed as described above. However, adjustment of the pH to 4.8 resulted in complete conversion into the 12s form, as shown in Table 2. Unpurified soya-bean urease and Sigma type V urease behave in a manner identical with that of the crystallized jack-bean urease with respect to the pH-dependence of their sedimentation coefficients.

On the other hand, urease obtained from soaked watermelon seeds did not show the characteristic pH-dependence. Both at pH 7.0 and 4.8 only the 18s form could be detected. Also, dialysis of watermelon urease for 4 days, under the conditions specified above, did not result in conversion of any detectable amounts of activity into the 12s form.

It is proposed that the 12s form of jack-bean urease is the result of an equal split, on a molecular-weight basis, of an 18s molecule. Relevant to this point is the finding by Kobashi *et al.* (1966) that

there are two active sites per 18s molecule. This could mean, but not necessarily, that there is one active site per 12s molecule.

One of the most interesting sources of urease investigated was from *Bacillus pasteurii*. Enzyme from this bacterial source showed only a single peak at both pH 4.8 and 7.0 that corresponded to a sedimentation coefficient of 12s. Because of the somewhat unusual behaviour of the *Bacillus pasteurii* urease, an attempt was made to combine a molecule of bacterial urease with a molecule of 12s jack-bean urease and thereby form a hybrid 18s urease molecule. Excess of jack-bean urease at pH 4.8 was added to a solution of bacterial urease also at pH 4.8. The pH was slowly adjusted to pH 7.0 by the addition of dilute sodium hydroxide. This neutralized urease solution was subsequently analysed on a pH 7.0 sucrose gradient. Two urease peaks were observed, one corresponding to 12s urease, the other to 18s urease. Since the amount of activity comprising the 12s peak was approximately equivalent to the amount of activity of the control (an equivalent amount of bacterial urease analysed in the absence of jack-bean urease), it was concluded that no combination of the jack-bean urease and bacterial urease had occurred. Inasmuch as the bacterial enzyme showed no tendency to combine with jack-bean urease it can be assumed that some basic difference exists between ureases from these two sources.

The several experiments conducted to demonstrate differences between the 18s and 12s forms were without success. Stability to pH and resistance to heat and trypsin inactivation are properties attributable to both species of urease. Also, the fractional-ratio data of Table 1 indicate no large amount of urease unfolding as the result of existing in the 12s form.

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