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Report

The Effect of Compactional Pressure on Urease Activity

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Jack bean urease is a proteinaceous enzyme, MW approximately 489 kD, readily soluble in water but losing activity when sheared in solution at stresses as low as 2.5 Pa. There is a need for controlled-release forms of many of the new genetically engineered peptide and polypeptide drugs with high specific activities. The simplest form of controlled release would be a sterile compressed pellet of the active component inserted subdermally. However, "activity" may be lost on compaction. Urease can be regarded as a model protein which may lose activity when sheared during compaction in the dry state. Tablets of urease weighing 100 mg were compressed over a range of pressures from 60 to 1750 MPa. No relative loss of activity would be detected following compaction at pressures up to 474 MPa. Above this limiting pressure there was a 50% loss of relative activity, evidently by a compactional effect on the protein quaternary and tertiary structures. No direct relationship was observed between stress (compactional pressure) and inactivation.

KEY WORDS: urease activity; dry-state compaction; protein drugs.

INTRODUCTION

In recent years a number of highly active and specific proteinaceous and polypeptide drugs have been produced by recombinant DNA techniques (1,2). In a number of situations controlled drug delivery techniques would provide considerable advantages for the administration of some of those genetically engineered compounds. These materials may have to be administered at doses several magnitudes higher than is required in nature and more frequently than is convenient or, indeed in some cases, feasible. One example here is bovine growth hormone (BGH), used to promote or prolong the bovine lactation cycle. Daily administration of a convenient dose is extremely difficult and ideally a sterile implantable controlled-release device would be required to deliver BGH at a zero-order rate over a period of a month or longer. Physically a suitable device may be made by direct compaction of the drug under appropriate conditions. Earlier we demonstrated that the water content of a protein powder, soy protein isolate, was critical for the formation of compacts which would release their contents at different release rates (3). However, it seemed possible that a protein or polypeptide drug, critically dependent on tertiary and quaternary structural convolutions in order to exert their often exquisitely sensitive biological activity, might be adversely affected by even the most moderate compactional pressures required to form a pellet or tablet.

In the main, studies of the effects of pressure on protein inactivation have been confined to studies of enzymes dissolved in water (4). At low concentrations of ribonuclease A, for example, denaturation occurred under stresses near 1 atm but was virtually 100% reversible when the pressure was released. Kornblatt and Hoa (5) noted the effects of pressure on rabbit brain enolases up to 3400 bar (or 3.4×10^2 MPa) pressure and found dissociation and inactivation at pressures in excess of 1200 bar (or 1.2×10^2 MPa). Tirrell and Middleman (6) demonstrated the effects of shear rate on the kinetics of urease-catalyzed urea hydrolysis, both reversible and irreversible denaturation (inactivation), occurring under shear. Tirrell (7) showed that hydrodynamic stresses as low as 2.5 Pa (in the absence of EDTA) were sufficient to produce partially reversible inactivation of urease, and if the viscosity was increased by adding glycerol, inactivation was shear stress dependent and reversible up to 21 Pa (in the presence of EDTA). It was suggested that this effect was the result of hydrodynamically induced urease conformational changes (7). Similar effects have been reported for lactic dehydrogenase, catalase, carboxypeptidase, and rennet enzymes (8). However, these studies were carried out by shearing aqueous enzyme solutions. Bello (9) noted that an important contribution to native globular protein stability in water was the contribution of hydrophobic effects which resulted in sequestration of most of the apolar side chains in the protein interior, out of contact with water. In the anhydrous state the hydrophobic stabilization is expected to be minimal (9).

In the present study we have evaluated the effect of compactional pressure on the activity of a crystalline jack bean urease in the *dry state*.

PROPERTIES OF UREASE

Urease or urea amidohydrolase is an enzyme crystallized from jack bean (*Canavalia ensiformis*) which acts on nonpeptide C–N bones in linear amides (10). This enzyme is

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notable for its specificity, converting urea to ammonia. In the native state urease appears to be a conglomerate, with a molecular weight of approximately 489 kD (10). Most investigations suggest that only one type of subunit exists in the structure of urease. However, the proposed subunit molecular weight varied between 30 kD (11), 60 kD (12), and 80 kD (13–15). Nevertheless, it will be evident that ureas is a typical biologically active protein with a smallest active unit of 240-kD molecular size (14). Tirrell and Middleman demonstrated the sensitivity of this protein to hydrodynamic shearing forces, suggesting shear-promoted, metal ion-catalyzed oxidation of essential sulfhydryl groups (6,7,16). In other words, hydrodynamic stresses of the order of 2.5 Pa will sufficiently alter the conformation of urease to expose pertinent sulfhydryl groups to irreversible oxidation catalyzed by microsolutes such as ferric ions. However, it required more than 2.5 Pa (in the absence of oxidation catalysts) to distort significantly the geometry of the active site(s). Such distortion was reversible when the hydrodynamic stress, under 21 Pa, was released. Gel electrophoresis suggests that the urease is readily dissociated but associates to polymeric isozymes corresponding to five or six times the molecular weight of the conglomerate (15). These polymeric isozymes are linearly arranged, which may be related to their shear susceptibility. Inhibition of urease by metal ions (such as Fe^{3+}, Ni^{2+}, Zn^{2+}, Ca^{2+}, Co^{2+}, and Cd^{2+}) has been studied by Hughes *et al.* (17). They suggested that metal ions formed insoluble sulfides with sulfhydryl (or mercapto) groups on urease that are essential for urease activity. However, the addition of ethylenediaminetetraacetate (EDTA) eliminated the irreversible inactivation caused by metal ions without direct binding of EDTA to urease. This indicates that metal ions act as an oxidation catalyst of some essential group on the urease molecule, cysteine sulfhydryls being the most likely candidates (16). Metal contamination from the tablet punch-and-die surfaces may complicate the assay, and in the present investigation EDTA was therefore added to remove this source of extraneous interference.

MATERIALS AND METHODS

Materials

Urease (from jack beans, type IV, Sigma lots 55F-7320 and 97F-7130), urea nitrogen standards (10.7 and 53.3 m*M*, Sigma), urease buffer reagent (Sigma), alkaline hypochlorite solution (Sigma), phenol-nitroprusside solution (Sigma), sodium phosphate dibasic anhydrous (Sigma), potassium phosphate monobasic (Fisher), sodium hydroxide (Mallinckrodt), and tetrasodium ethylenediaminetetraacetate (Fisher) were used as received.

Equipment

Equipment was as follows:
Carver laboratory press (Model 2702),
Perkin–Elmer lambda 3B UV/VIS spectrophotometer,
ultrasonic dispersing bath (Sonicor Instrument Inc.), and
JEOL Model JSM-35C scanning microscope

Experimental Methods

Urease Compacts

Urease compacts were prepared by compression of 100 mg urease, fed manually into a 7-mm steel concave punch-and-die set, with a Carver laboratory press at a constant compaction speed of 18.65 cm/min. Both urease and urease compacts were stored in a freezer until required. The weight and thickness of compacts were measured with a micrometer immediately after compaction.

Urease Activity Determination

Principle. Ammonia is released as a result of hydrolysis of urea by urease [detailed mechanisms were proposed by Reithel (10) and Jespersen (18)]. Further reaction of ammonia with hypochlorite and phenol catalyzed by sodium nitroprusside produces indophenol, determined by spectrophotometry (19,20).

Urea Nitrogen Calibration Curve Determination. 1. Urease buffer solution was first prepared by reconstituting urease buffer reagent (10 units of urease) with 30 ml of double-distilled water.

2. Ten-, 20-, 30-, 40-, 50-, 60-, 80-, and 100-fold dilutions of 53.3 m*M* urea nitrogen standard solution were prepared with double distilled water.

3. To 0.1 ml of each of the diluted urea nitrogen standards was added 0.5 ml urease buffer solution, gently mixed, and stored at 37°C for 10 min. At the end of the incubation period, 1.0 ml of phenol-nitroprusside solution, 1.0 ml of alkaline hypochlorite solution, and 5.0 ml of double-distilled water were immediately added, mixed, and allowed to stand at room temperature for 30 min. Absorbance readings at 570 nm were taken using a Perkin–Elmer lambda 3B UV/VIS spectrophotometer.

4. The linear regression of urea nitrogen concentration and absorbance was $C\ (\mu M) = 0.2109 + 45.30\ A_{570}$ ($r = 0.9998$, $N = 9$).

Dissolution of Urease Powder and Compacts. 1. Phosphate buffer, pH 7.0, was prepared from 0.050 *M* potassium phosphate monobasic and 0.015 *M* sodium phosphate dibasic, as the dissolution medium, using 5 *N* NaOH to adjust the pH to 7.00 ± 0.01.

2. Control: urease powder, 20 mg, dissolved in 100 ml, pH 7.0, phosphate buffer. Test: urease compacts, 100 mg, added to 500 ml, pH 7.0, phosphate buffer and stirred occasionally until dispersed. Both control and test dispersions were sonicated for 10 min.

3. A 0.5 *M* EDTA solution (0.2 ml) was added to the control solution and 1.0 ml of the same EDTA solution was added to the test solution to make a final EDTA concentration of 1 m*M*. Two samples of 0.2 ml were taken from each EDTA/urease solution.

To each sample 0.5 ml of 10.7 m*M* urea nitrogen standard was added, mixed gently, and stored at 37°C for 10 min. Immediately after the incubation period, 1.0 ml of phenol-nitroprusside, 1.0 ml of alkaline hypochlorite, and 5.0 ml of double-distilled water were added, mixed, and stored at room temperature for 30 min before taking absorbance readings at 570 nm. The average absorbance reading of two samples was applied to the urea nitrogen calibration curve to

Table I. Effect of Compactional Pressure on Urease Activity

Compaction pressure (MPa)[a]	Relative activity of urease compacts (%)[a,b]	Effectiveness[c]
61.61 ± 6.70	104.5 ± 3.9	–
107.84 ± 6.70	102.7 ± 19.3	–
233.14 ± 3.35	97.0 ± 55.1	–
473.91 ± 0.00	51.2 ± 2.7	+ ($P \leq 0.005$)
944.00 ± 6.70	45.6 ± 9.7	+ ($P \leq 0.005$)
1753.45 ± 33.40	57.8 ± 2.4	+ ($P \leq 0.025$)

[a] Mean ± SD; $N = 3$.
[b] Relative activity was the ratio of urease compact activity vs mean urease powder activity (control) times 100%. Mean ± SD; $N = 3$.
[c] Effectiveness of compaction was analyzed from analysis of variance of two groups, which were the urease activity before and after compaction. (−) No significant difference between the activity before that and after compaction; (+) a significant difference between the activity before and that after compaction.

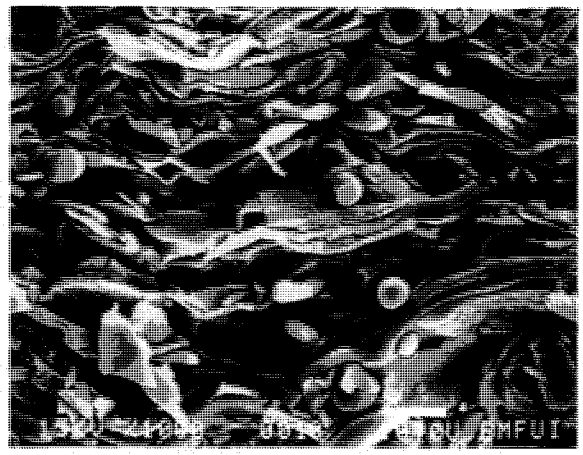

Fig. 2. Cross section of urease compact compressed at 57.79 MPa for 30 sec.

obtain the amount of urea nitrogen released, and the activity of urease determined.

Electron Microscopy

Samples were mounted and gold–palladium coated before examination in the scanning mode.

DISCUSSION

Unlike the studies by Tirrell and Middleman (6,7,16), it is not feasible to determine the effect of shearing forces on activity during the shearing process. The results obtained here (Table I) are evidently due to irreversible changes in the structure induced by dry shear. These forces are seven to eight orders of magnitude higher than the shear forces needed to induce irreversible changes in the solution state. The situation encountered when the enzyme is in solution is different since there is no linear relationship between compactional pressure and inactivation in the solid state. There is a statistically significant ($P < 0.001$) difference between the mean of the first three and the last three sets of data from Table I, indicating that, beyond 500 MPa, there is a 50% loss of activity. From a practical point of view this suggests that pellets of this enzyme could be successfully prepared by slow compaction at pressures below 250 MPa without any significant loss of activity.

From Fig. 1, the abrupt change of slope between 250 and 500 MPa suggests a phase transition process, i.e., from a native state to a denatured state. Although completely soluble prior to compaction, some parts of the system are rendered insoluble on reconstitution since the dispersion is slightly cloudy. However, this slight opacity or insolubility is insufficient to account for the overall observed loss of activity.

Measurement of the tablet thickness (Fig. 1) suggests that interparticular spaces are being reduced at pressures up to around 250 MPa. This is also evident from the electron photomicrographs (Figs. 2 and 3), in which a reduction of pore volume is seen.

There is currently some disagreement about the actual mechanism involved in loss of activity of a proteinaceous enzyme caused by compaction. Most previous studies have

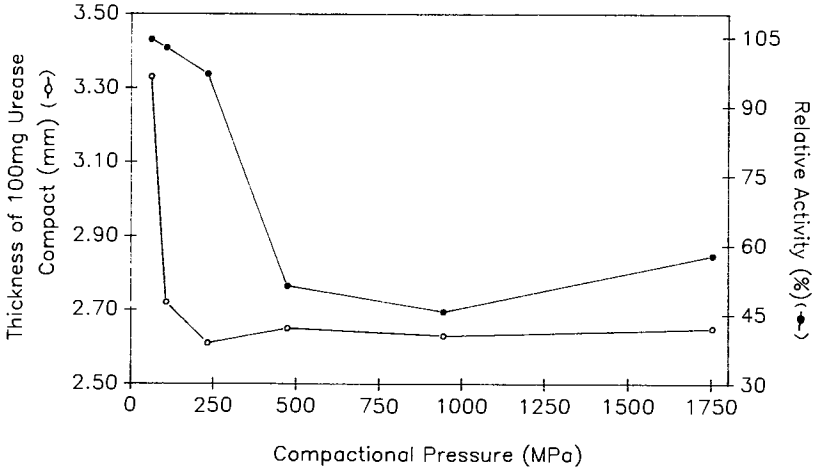

Fig. 1. The effect of increasing compactional pressure on 100 mg urease as measured by compact thickness (open circles) and relative activity (compressed/uncompressed × 100; filled circles).

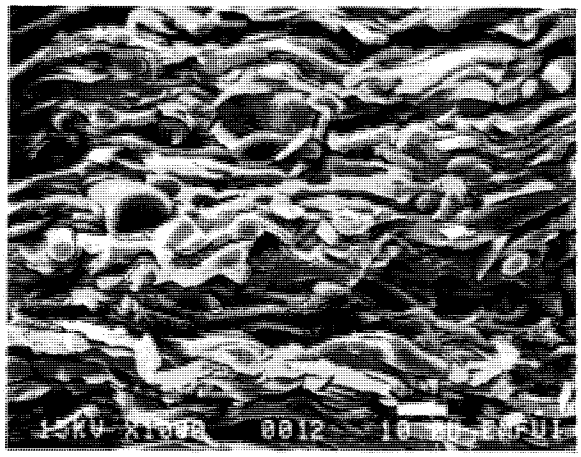

Fig. 3. Cross section of urease compact compressed at 1733.8 MPa for 30 sec.

been carried out on tablet formulations containing enzyme(s), diluent, and lubricant. Graf *et al.* (21) found the loss of amylase and lipase activity to be diminished by the presence of lubricants, suggesting that these materials facilitated the transmission of force throughout the compact. This implied that loss of activity was due to thermal inactivation; the same authors demonstrated earlier that the two enzymes were indeed inactivated by direct heat (22). Graf and his colleagues (23–25) have also demonstrated the effects of diluents and lubricants on activity, some direct interactions between components being detected. Fürhrer and Parmentier (26) reported that 90% of the mechanical energy in a tablet compression process is converted to heat and the remainder is transformed to internal energy, mostly remaining in the tablet. In our present work excessive thermal inactivation appears unlikely, although at a local level, particle to particle, heat is undoubtedly generated at the surface of the particles. For example, the heat produced during compaction should be dissipated during the slow compaction process (18.65 cm/min) and is unlikely to be enough to denature significantly the protein in the interior of the powder particles. Furthermore, there was no significant effect produced by holding the pressure for various times, (Table II), or by compressing at different speeds (Table III), and there was no evident linearity between compactional pressure and loss of activity (Fig. 1). This last observation, albeit over a much wider range of pressures, is contrary to the observations by Graf *et al.* (21) and Horikoshi *et al.* (27,28). The latter group reported proteinase inactivation as a function of shear, suggesting that intermolecular spaces between particles were reduced. Hüttenrauch and Keiner (29) explained loss of enzyme activity in terms of mechanical denaturation, although it is not clear what the authors actually meant by this term. More recently, Nürnberg and Hamperl (30) have evaluated the effect of compactional pressure up to pressures of 55 MPa on the activity of α-amylase and concluded that thermal and tribomechanical stress conditions did not account for the observed loss of activity. They found that volume reduction of the molecule was a significant factor in this case. Although our conditions were in excess of those used by these workers, we believe the volume factor to be applicable in our situation. Structural changes on the macro- and microscales are apparently induced by the application of compactional stress over a wide range of pressures.

Table II. Relative Activity (%) of Urease Compacts Held at Various Holding Times (N = 3)*

Holding time (sec)	Mean relative activity (%)	SD
1	33.47	5.80
10	35.43	7.78
30	31.63	3.90
60	34.07	10.25
Compactional pressure	1792 MPa	
Compactional speed	18.65 cm/min	

* Statistically there is no significant difference between the means according to a single classification analysis of variance.

Table III. Relative Activity (%) of Urease Compacts Prepared at Different Compaction Speeds (N = 3)*

Compaction speed (cm/min)	Mean relative activity (%)	SD
18.65 cm/min	35.97	6.05
104.65 cm/min	37.53	7.91
Final compactional pressure	1792 MPa	
Holding time	30 sec	

* Statistically there is no significant difference between the two means according to a single classification analysis of variance.

Quaternary and tertiary structural elements are distorted enough to expose hydrophobic surfaces, "denaturing" the protein. Some of the mercapto groups are very reactive and may be involved in exchange reactions with disulfide groups, causing polymerization (31). These mechanisms may also be responsible for the incomplete solubility of some of the urease after compaction and subsequent partial inactivation. The denatured state of the protein is a completely different material than the native state and may have a higher resistance to shear. It would therefore act as a shear barrier for the native protein inside the compact.

Failure of the enzyme to lose additional activity at pressures well in excess of those required to collapse substantially the voids in the tablet may indicate that space around and within each molecule has been reduced to the point where no further collapse is possible. Since the particles are in random orientation, the collapsed moieties may simply be protecting others from damage.

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