*Pharmaceutical Research, Vol. 7, No. 3, 1990*

*Report*

# The Effect of Compactional Pressure on a Wheat Germ Lipase Preparation

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*Received April 24, 1989; accepted September 19, 1989*

Wheat germ lipase is a relatively homogeneous proteinaceous enzyme known to suffer inactivation by compactional pressure. However, earlier investigators suggested that the inactivation was due to thermal degradation following the application of mechanical energy. A wheat germ preparation (Sigma) was compacted over a range of pressures from 85 to 1800 MPa. The 100-mg compacts were carefully dispersed and dissolved in aqueous 0.05 *M* potassium phosphate buffer, pH 7.4, and the biological activity was determined using triacetin as a substrate. No significant loss of activity occurred up to a pressure of 175 MPa. After this point, a discontinuity was evident with a loss of 30% activity but this loss of activity remained constant over the remainder of the applied pressure range studied. The density/applied pressure relationship indicated that the discontinuity was due to space constraints. The density failed to go higher than a limiting value of approximately 1.2 g $cm^{-3}$, irrespective of the applied pressure. There was an approximately linear relationship between the relative loss of biological activity and density, indicating that the observed loss of biological activity is unlikely to be due to applied thermal energy but more likely to be due to space constraints on the volume occupied by the molecule. However, circular dichroism measurements and SDS-PAGE examination did not reveal any obvious protein structural changes, suggesting that the mechanism involved in activity loss is subtle.

KEY WORDS: lipase; compaction; activity loss.

## INTRODUCTION

Previous investigations in this laboratory have demonstrated a loss in biological activity when proteinaceous enzymes are compacted in a tablet press (1–3). In particular, using jack bean urease as a model of a biologically active proteinaceous drug, Teng and Groves (1) have shown that, at least in this case, the loss of activity is most likely caused by a reduction in the molecular volume producing subtle irreversible changes in the active moiety of the molecule.

In the case of proteinaceous lipases, loss of activity during compaction has been attributed to thermal effects. Fürhrer and Parmentier (4) reported that upto 90% of the mechanical energy used to form a tablet compact was converted into heat. Graf and Sakr (5) found that lipase was readily inactivated by heat in solution. The same group (6) later demonstrated that the addition of diluent and lubricant diminished loss of lipase activity following tablet compaction. The initial loss of activity was therefore attributed to thermal effects that were mitigated in the presence of materials which enabled forces to be transmitted through the compact more evenly. Mechanical denaturation of enzymes was also reported by Hüttenrauch and Keiner (7). Nevertheless, intermolecular space reduction was suggested as a mechanism for protease inactivation by Horikoshi *et al.* (8–10). Volume reduction was also found to be a significant factor in enzyme activity reduction by Nürnberg and Hamperl (11).

The present communication is an examination of the effect of pressure on a commercially available purified lipase preparation over a significantly higher applied pressure range than that used by previous investigators.

## EXPERIMENTAL

### Materials

Materials were as follows: wheat germ lipase (Cat. No. L3001, Sigma Chemical Company, St. Louis, MO), triacetin (Sigma), monobasic potassium phosphate (Fisher Scientific Company), Tris base (Sigma), thymolphthalein indicator stock No. 800-3 (Sigma), and sodium hydroxide, 0.05 *N* (Sigma). All were used as received.

### Equipment

Equipment consisted of a Carver laboratory press, Model 2702, and a water bath at 37°C.

## METHODS

### Preparation of Compacts

Wheat germ lipase powder stored over a desiccator

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0724-8741/90/0300-0247$06.00/0 

prior to use was weighed, and 100 mg placed into a nonlubricated 7-mm flat-faced stainless-steel punch and die set. The compact was formed at a compaction rate of 18.65 cm/min to the required pressure using the Carver press. Tablet weight and dimensions were determined immediately after ejection and the compacts stored at 5°C in a closed container until measurement of activity. Three compacts were made and evaluated at each pressure.

### Dispersion of Powder or Compact in Water

Monobasic potassium phosphate (0.05 *M*), aqueous buffer solution, pH 7.4, was warmed to 37°C. Each approximately 100-mg tablet was carefully dispersed in a volume of 500 ml buffer, occasionally stirring until dissolved. A reference solution was prepared from 20 mg of the noncompacted powder dissolved in 100 ml of buffer.

### Assay of Lipase Activity

Lipase activity was analyzed by measuring the rate of hydrolysis of a standard triglyceride, triacetin, and determining the fatty acid by titration with alkali (kit supplied by Sigma). A volume of 3 ml buffered substrate solution (0.33 *M* triacetin in 0.15 *M* Tris base, pH 7.4) at 30°C was pipetted into "test" and "blank" tubes and incubated for 5 min at 37°C. At zero time 1.0 ml of the enzyme test solution was added to the test tube, and the incubation was continued for 60 min. At 60 min 1 ml of the enzyme test solution was added to the blank tube. To each tube was added 4 drops of thymolphthalein indicator solution, and the solutions were immediately titrated with 0.05 *M* sodium hydroxide to a pale blue end point. The difference between the two titers was proportional to the amount of fatty acid liberated by the enzyme.

### Determination of the Total Protein

The amount of total protein in both solutions remaining in the lipase activity assay was then determined by the Lowry procedure (12) using the Sigma kit P5656.

### Circular Dichroism (CD)

Three 100-mg lipase compacts were prepared as previously described at 980 MPa and dissolved in 25 ml dissolution buffer. Solutions were filtered through 0.2-μm membrane filters (Gelman Science Inc., Ann Arbor, MI) and diluted to 4 mg/ml lipase solution with the same buffer before circular dichroic spectra were taken by a Jasco J-40A automatic recording spectropolarimeter (Japan Spectroscopic Co., Ltd., Tokyo). Results are shown in Fig. 4.

### SDS-PAGE

Three 100-mg lipase compacts prepared as for CD analysis were dissolved in 20 ml Milli-Q-water. Solutions were filtered through a coarse filter paper (Scientific Products, McGaw Park, IL). Precipitates were collected and air dried at 5°C, then redispersed in pH 8.3 electrode buffer (13). SDS-PAGE was performed following conditions (8% separation gel with 3% stacking gel) described by Laemmli (13) under reducing conditions. Powder solution, compact solution (filtrate), and precipitate dispersions were diluted with pH 8.3 electrode buffer to required concentrations equivalent to 30 μg of protein and loaded for separation. Results, relative to protein markers of known molecular weight, are shown in Fig. 5.

## RESULTS AND DISCUSSION

Although this investigation is preliminary in nature, some general conclusions about the behavior of this protein preparation during a compaction process may be made. Clearly, valuable information on the deformation mechanism could be obtained using an instrumented press but the use of a simple press enables some data to be obtained over a wider range of applied pressures. It should be noted that the applied pressures (up to 1800 MPa) are considerably in excess of those experienced during a normal tableting operation (15). The scanning electron microscopy of wheat germ lipase pellets suggested a plastic deformation mechanism over the range of applied pressures studied (16). Further data are required to confirm this point since, conceivably, the final compacts could exhibit elastic and viscoelastic expansion at different pressures.

The effect of compactional pressure in the relative activity of wheat germ lipase is shown graphically in Fig. 1. It can be seen that there is no appreciable degradation at pressures of up to 175 MPa but beyond that pressure approximately 30% of the activity is lost. A parallel behavior is evident for the tablet thickness in a similar fashion to that demonstrated earlier for urease (3). Using the Lowry method for total protein this commercial source of enzyme consisted of +97% protein. Whether or not this is "pure" in terms of "lipase" activity is less certain. However, relative to noncompacted protein powder, the total protein content remains essentially constant over the whole pressure range, although biological activity drops off significantly once compacted beyond the critical point. This suggests that, although the protein remains substantially soluble in water, structural changes induced by pressure have resulted in a permanent loss of activity.

As noted by Teng and Groves (1), the effect of a temperature rise induced mechanically by compactional forces on the powder mass on the loss of biological activity cannot be entirely ruled out. However, the rate of compaction used

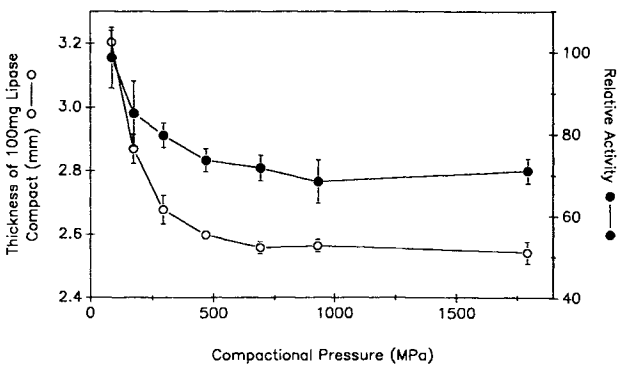


Fig. 1. The effect of compactional pressure on the relative biological activity of wheat germ lipase, and compact thickness. Error bars ±1 standard deviation.

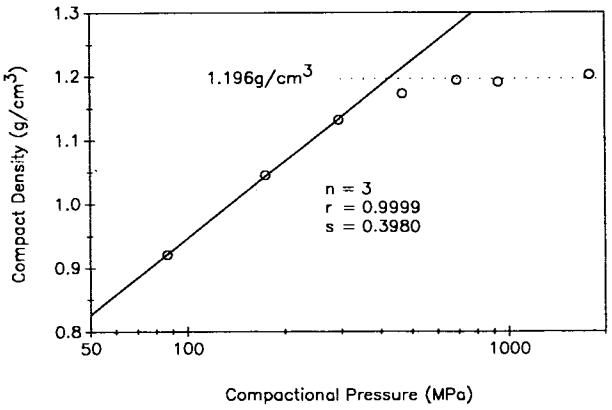


Fig. 2. Lipase compact density as a function of compactional pressure.

here is likely to be slow enough to allow the overall temperature increase in the system to be dissipated throughout the bed. If the enzyme were truly heat labile, compaction at a slow rate would allow the protein to be exposed to an increased temperature-induced degradation for a longer period of time than would be experienced during a rapid compaction process. However, the net temperature increase would be lower. Application of rapid or slow compaction rates had no significant effect on the degradation of urease (16). Furthermore, as noted above, no significant loss of activity occurred until pressures of 175 MPa were exceeded. Again, this suggests that thermal degradation is not a major factor in the observed loss of activity at higher pressures. Local increases in temperature due to disruption of adjacent asperitic surfaces, as discussed by Pilpel *et al.* (14), will occur but a loss of 30% of the total activity solely by surface interactions would not appear to be plausible.

Since the tablet diameters are fixed by the diameter of the punch, it is possible to calculate the solid density of the compact by measuring the compact dimensions. When plotted against applied pressure (Fig. 2), the cause of the discontinuity becomes evident since the compact density apparently cannot rise above a value of approximately 1.196 g $cm^{-3}$. This limiting or critical pressure may correspond to a minimum volume into which the protein molecule can be compacted. However, it should be noted that there are currently no independent data for determining whether or not

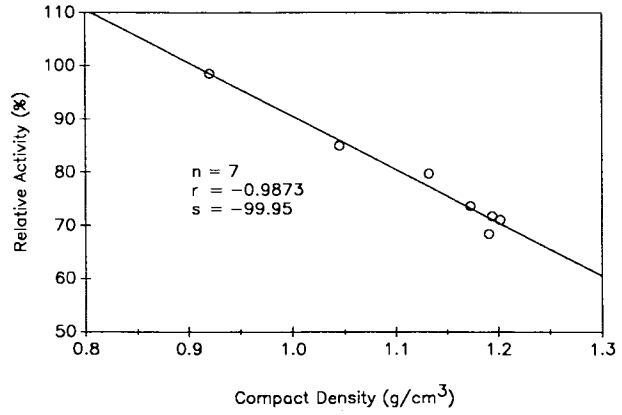


Fig. 3. Lipase relative activity as a function of compact density.

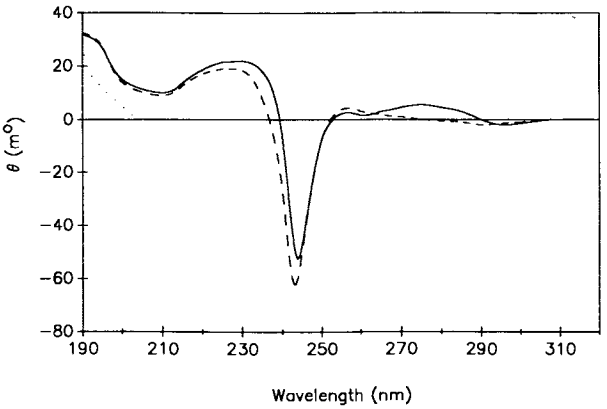


Fig. 4. Circular dichroism spectra of wheat germ lipase solutions before and after the compaction of the enzyme (for conditions see text). Dotted line, spectrum of buffer only; solid line, uncompacted enzyme solution; dashed line, solution of compacted enzyme.

this limiting density is the "true" density of the proteinaceous material. The experimental value is certainly consistent with the density of other proteins [e.g., denatured egg albumin $d_{25}^{25}$ = 1.035 (17)], but clearly, the value obtained will be conditioned by the amount of associated water at the molecular level. This aspect is currently under investigation.

Taking the same data and plotting compact density against relative activity (Fig. 3) suggests that the discontinuity evident in Fig. 1 may be a function of scale. The linearity of this plot ($r = -0.9873$, $n = 7$) is strongly suggestive of a loss of activity due to a decrease in volume since mass is

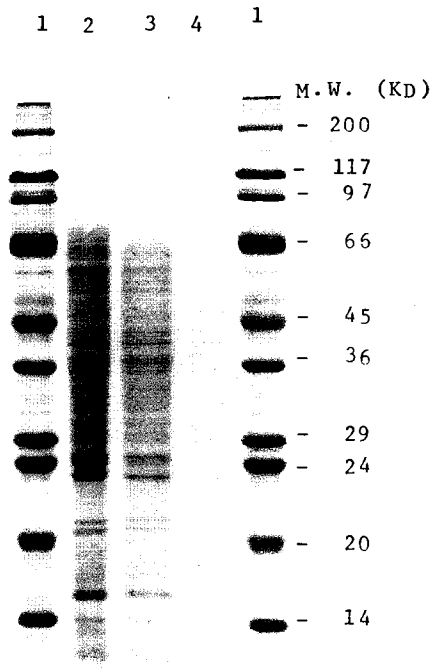


Fig. 5. SDS/PAGE chromatograms of wheat germ lipase before and after compaction (for conditions see text). Channel 1, proteins of known molecular weight as markers; Channel 2, uncompacted enzyme solution; Channel 3, compacted enzyme solution; Channel 4, dispersion of filtrate from the solution of compacted protein.

essentially constant. This is in agreement with the data of Teng and Groves for urease (3), Horikoshi *et al.* for protease (8–10), and Nürnberg and Hamperl for α-amylase (11).

The question therefore arises as to what is the effect produced on the lipase molecule by a reduction of volume during compaction. Denaturation of a protein molecule could ultimately result in a reduction of water solubility. This was noted experimentally here but not to a marked degree, even at the highest pressures used.

However, circular dichroism of enzyme solutions (Fig. 4) showed few differences before and after compaction. Similarly, SDS/PAGE chromatography (Fig. 5) of reconstituted protein after compaction, as well as the redispersion of insoluble material collected from the reconstituted protein solution, showed substantially no differences between each other or the original uncompacted protein solution. These results indicate that, once again, changes induced in a compacted biologically active protein are subtle enough to reduce activity without producing any significant structural changes in the case of wheat germ lipase. Reduction of solubility is evident but quantitatively the amounts precipitated are relatively small and certainly insufficient to account for the observed loss of activity.

## ACKNOWLEDGMENTS

We would like to thank Ms. Kyonghee Son for her help with the SDS-PAGE chromatography and Professor Charles Woodbury for valuable discussions on the interpretation of CD. We are also grateful to the Chancellor of the University of Tabriz, Iran, for the leave of absence granted to H.M.Z. Funding for this project was provided by the University of Illinois at Chicago.

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