Stabilization of Lyophilized Porcine Pancreatic Elastase

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Porcine pancreatic elastase, a well-characterized serine protease, has been used as a model to assess the effects of excessive humidity on solid-state stability of the lyophilized protein. Elastase lyophilized without excipients retained full activity immediately after freeze-drying but became denatured upon continued storage at 40°C, 75% relative humidity. The extent of inactivation could be monitored through assays of amidolytic activity, as well as through changes in the circular dichroism (CD) and fluorescence spectra. Differential scanning calorimetry (DSC) was employed as a means of screening potential stabilizing additives; based on the results, sucrose and dextran 40 were selected for further evaluation. Both additives were effective in preventing denaturation. Possible mechanisms for the denaturation and stabilization of elastase are discussed.

KEY WORDS: elastase, stabilization; lyophilization; circular dichroism (CD); fluorescence; differential scanning calorimetry (DSC).

INTRODUCTION

With the increasing importance of proteins as pharmaceuticals, an understanding of the factors which stabilize such macromolecules becomes critical for optimal formulation. Of particular interest is the solid-state stability of proteins, since lyophilization or freeze-drying is often employed to enhance product shelf life. However, exposure of lyophilized proteins to excess moisture has been reported to cause undesirable physical and chemical changes (1–3). While these reports provide information on the chemical decomposition of proteins, less is known about irreversible conformational changes of proteins which result in loss of biological activity and formation of insoluble aggregates. It seems likely that such structural changes of proteins are linked with exposure of specific amino acid side chains to degradation conditions. Therefore, methods for monitoring protein structural changes during the course of inactivation are necessary for optimization of formulation stability.

In this study, porcine pancreatic elastase, an extensively characterized protein (4,5), was chosen as a model for investigating denaturation induced by exposure of the lyophilized enzyme to high humidity conditions. The conformational changes of the enzyme during storage were monitored with spectroscopic techniques and correlated with loss of activity. DSC was used to screen and select various potential additives, which were then evaluated for stabilizing effects on lyophilized elastase; sucrose and dextran 40 were found to be most effective. Possible routes of irreversible denaturation and mechanisms for the observed stabilization are discussed.

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MATERIALS AND METHODS

Porcine pancreatic elastase and its synthetic substrate, *n*-succinyl-(L-alanine)$_3$-*p*-nitroanilide, were obtained from Calbiochem, San Diego, CA. The additives sucrose, dextran (MW 38,000), dextran (MW 81,500), sorbitol, mannitol, lactose, and ascorbic acid were obtained from Sigma Chemical Company, St. Louis, MO; other chemicals were reagent grade, obtained from J. T. Baker, Phillipsburg, NJ. Elastase solutions containing additives were prepared in deionized water containing 0.002% sodium azide; the solution pH was adjusted to 5.0 prior to lyophilization. A solution volume of 0.4 mL was added to 1-mL vials of Flint type I-treated glass. Freeze-drying was carried out with a VirTis 25-SRC lyophilizer attached to a VirTis full cycle programmer (FCP-1), using the following conditions: freeze for 3 hr at −50°C, first drying for 2 hr at −25°C, ramp from −25 to 0°C for 6 hr; hold drying temperature at 0°C for 6 hr; and secondary drying for 6 hr at 25°C. The freeze-dried elastase samples were incubated in desiccators where the relative humidity was controlled by means of saturated salt solutions (6); a control freeze-dried elastase sample was stored with desiccant at −20°C.

For spectroscopic and activity measurements, the lyophilized elastase samples were first reconstituted in 10 m*M* sodium acetate, pH 5.0, to a protein concentration of 1 mg/mL. In cases where insoluble precipitate was observed, centrifugation (4000 $g \times 5$ min) was done to recover the supernatant. Protein concentration was determined using the Bio-Rad protein assay, with detection at 595 nm (7); a standard curve was prepared using the control elastase sample. Residual activity determination of elastase samples was carried out at room temperature using the assay method of Bieth *et al.* (8); activity values were normalized using the appropriate protein concentration values. Polyacrylamide gel electrophoresis (PAGE) was carried out as described by Reisfeld *et al.* (9).

DSC studies were carried out using a Perkin-Elmer DSC-7 instrument equipped with a Perkin-Elmer 7500 computer. Samples were prepared by dissolving 0.4 mg of lyophilized elastase in 20 µl of buffer and sealing the solution in a volatile sample pan. Samples were heated from 40 to 90°C at a scan rate of 10°C/min. The near-ultraviolet circular dichroism spectra of elastase (240–340 nm) were obtained using a Jasco J-500 spectropolarimeter equipped with a microprocessor; solutions were 1 mg/mL with a 1-cm path length. Fluorescence spectra were analyzed with a Perkin-Elmer MPF-66 spectrophotometer. Elastase solutions of concentration 0.1 mg/mL were excited at 295 nm while scanning the emission spectrum from 300 to 500 nm. As with activity values, spectroscopic parameters were normalized according to their protein concentration in order for direct comparison of spectra.

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RESULTS AND DISCUSSION

Inactivation of Lyophilized Elastase at 40°C, 79% RH

The residual activity of elastase measured immediately after lyophilization was 100%, and full activity could be maintained for up to three weeks when stored at 40°C with dessiccant. However, elastase lyophilized without additives underwent rapid inactivation when stored at 40°C, 79% RH, losing about 70% activity in 2 weeks, followed by further activity loss at a slower rate (Fig. 1). Concomitant with inactivation, these elastase samples also exhibited changes in their physical appearance, i.e., browning and a loss in solubility upon reconstitution manifested by cloudiness, suggestive of aggregate formation. Centrifugation of these samples was needed to separate the elastase solution from undissolved precipitate; attempts to redissolve the precipitate in 6 *M* guanidine–HCl or in a 10-fold molar excess of dithiothreitol were unsuccessful. With the exception of infrared studies conducted on the precipitate (data not shown here), spectroscopic studies and activity assays were carried out on the remaining soluble protein. PAGE conducted on these solutions showed a single band, suggesting that elastase did not undergo any extensive autolysis during storage.

Spectroscopic Studies of Elastase

Further information about the inactivation of elastase could be gained by examining its spectroscopic properties. Previous studies have demonstrated that native elastase undergoes reversible conformational changes in aqueous solution as a function of pH. At pH 5.0 or above, elastase maintains its native conformation, but lowering the pH induces spectral transitions around pH 4.0 and pH 3.0, concomitant with activity loss (10–12). These changes can be followed in the near-uv portion of the CD spectrum (Fig. 2a) and in the fluorescence spectrum (Fig. 2b). For comparison, Figs. 3a and b show the same spectral regions for samples of lyophilized elastase stored at 40°C, 75% RH, for varying amounts of time.

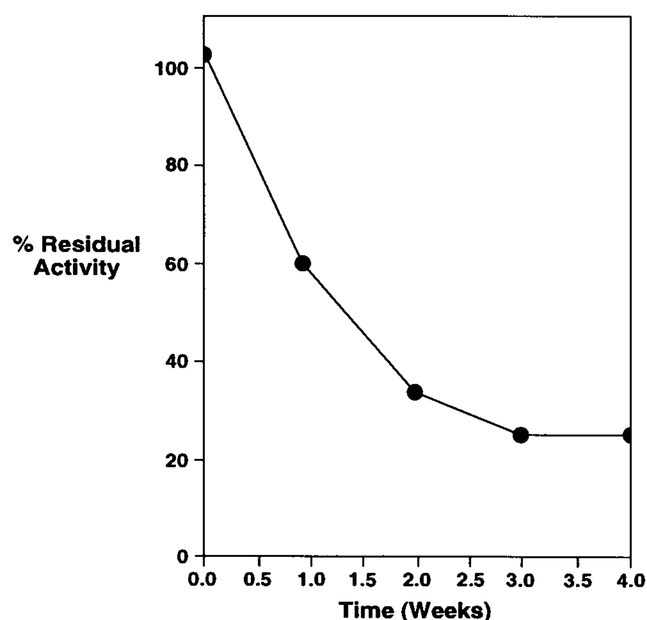


Fig. 1. Inactivation of lyophilized elastase at 40°C, 79% RH.

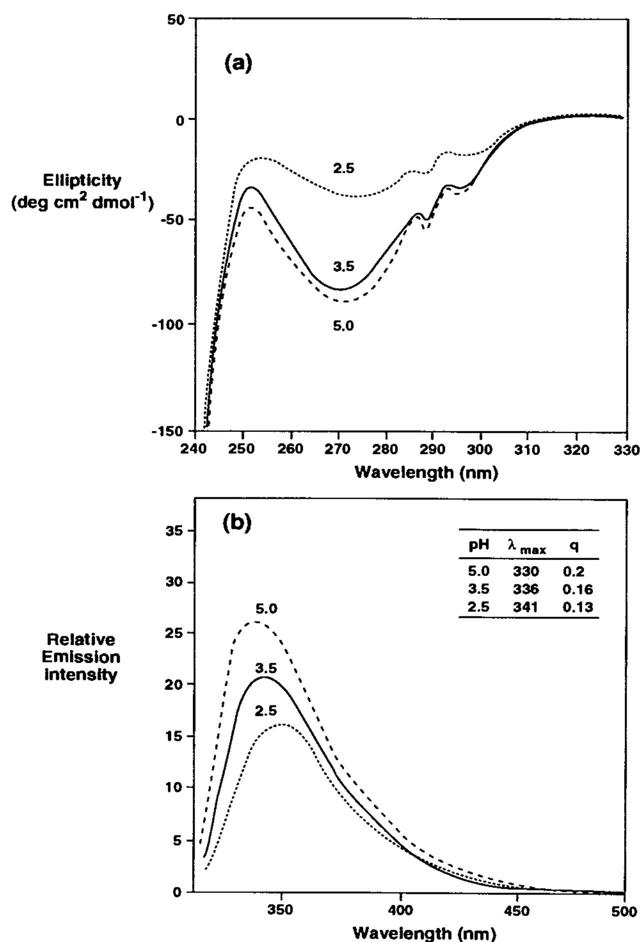


Fig. 2. Spectroscopic properties of elastase at different pH's: (a) near-uv CD; (b) fluorescence. (– – –) pH 5.0, (——) pH 3.5, and (- - - -) pH 2.5.

In the near-uv CD of native elastase (Fig. 2a), a slight loss in ellipticity is visible at pH 3.5, followed by a markedly greater loss at pH 2.5. A similar trend is observed in the fluorescence spectrum (Fig. 2b), where one sees a marked loss in quantum yield as well as an increase in the emission maximum, i.e., a red shift as the pH is lowered. Similar spectral changes occur when lyophilized elastase is stored under high humidity conditions for 2 weeks (Fig. 3). In addition to a decreased quantum yield and a red shift, the fluorescence spectrum of humidified elastase (Fig. 3b) also exhibits a "tailing" effect in the region of 400 to 500 nm. The latter effect is consistent with the aforementioned increasing discoloration of the samples and suggests additional changes are occurring in the structural integrity of the protein.

Note, also, that for native elastase, the denaturation caused by initially lowering the pH can be fully reversed by adjusting the solution pH back to 5.0, although continued exposure to low pH causes increasing inactivation, which is likely to affect the extent of reversibility. This appears to be the case after lyophilized elastase has been continually exposed to 40°C, 79% RH. Even when such samples can be redissolved at pH 5.0, neither the amidolytic activity nor the

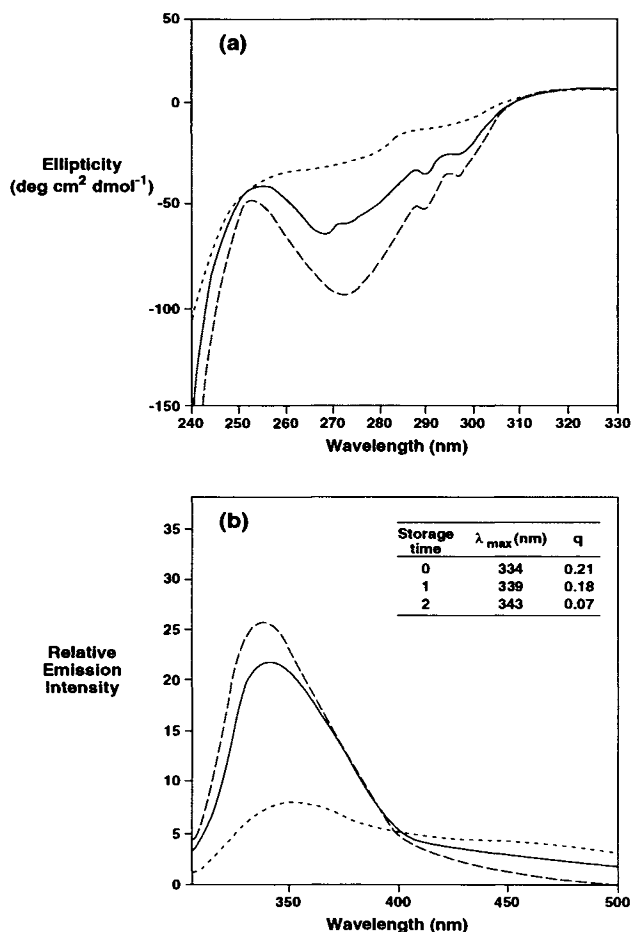


Fig. 3. Spectroscopic properties of lyophilized elastase stored at 40°C, 75% RH, for varying time periods: (a) near-uv CD; (b) fluorescence. (- - -) Initial; (——) 1 week; (----) 2 weeks.

spectral characteristics of the native enzyme are regained; i.e., the alterations in conformation have become irreversible.

The near-uv CD and fluorescence emission spectra are related to the tertiary structure of a protein, in particular the microenvironments of tyrosine and tryptophan residues. There is some additional evidence implicating tryptophan residues in the irreversible denaturation of elastase. Changes similar to the fluorescence emission spectrum of elastase shown in Fig. 2b were observed by Jori *et al.* in the presence of 5 *M* guanidine hydrochloride or when tryptophan residues were selectively modified (11,13); results indicate that tryptophan-26 and tryptophan-164 are on the protein's surface and that their oxidation to *N*-formylkynurenine does not alter enzyme activity or conformation. Tryptophan-83 is partially shielded from the aqueous environment, and its oxidation causes slight changes in activity as well as in tertiary structure. In contrast, tryptophan-12 appears largely buried in the native state, and its photooxidation occurs only after the protein becomes unfolded at pH 2.5, indicating that this residue is important for full enzymatic activity and conformational stability; three other tryptophan residues are apparently also buried and inaccessible to oxidation in the enzyme's native state.

It is therefore speculated that exposure of elastase to 40°C, 79% RH, may induce autooxidation of tryptophyl groups. If such groups reside in hydrophobic sites within the native enzyme, oxidation and resulting changes in polarity would make reversal of unfolding unlikely. The potential involvement of tryptophan residues is also supported by the observed browning of the inactivated samples, as well as the loss of absorption around 284 and 292 nm seen when the uv difference spectrum of native vs denatured elastase is obtained (not shown). However, it should be pointed out that other amino acids may also be susceptible to oxidative processes, e.g., methionine, histidine, cysteine, and cystine. Studies in addition to optical spectroscopy, such as chemical modification and amino acid analysis, would be necessary to confirm or exclude the contributions of these residues to the observed inactivation of elastase.

Selection and Screening of Stabilizing Additives

Based on the pattern of inactivation, the changes in physical appearance, and the spectral alterations, it is proposed that elastase stored at 40°C, 79% RH, undergoes irreversible denaturation after a series of conformational changes. Preventing any of these changes should thus be a means of preserving stability and retarding inactivation. Among reported stabilizers, polyhydric compounds such as sugars have been used successfully to prevent protein unfolding (14,15), and several were chosen for potential stabilization of elastase. Since calcium ions have been reported to stabilize elastase via specific interactions (16), the effect of added calcium chloride was examined. And since amino acid oxidation may play a role in the irreversible inactivation, the effect of ascorbic acid, a common antioxidant, was also tested.

The degree of protection conferred by stabilizers can be screened effectively using DSC (17). While the instrument used for the present studies lacks the sensitivity of microcalorimeters needed to obtain detailed information about protein unfolding, it is useful for qualitative screening of additives based on the peak maximum of the protein denaturation endotherm (T_{max}). An additive expected to stabilize elastase should increase its T_{max} while a destabilizing additive should lower the T_{max}.

Results are shown in Table I. Among the excipients tested, sugars were found to raise the T_{max} of elastase. Dextran 40 (MW 38,000) showed the greatest increase compared to elastase alone, followed by sorbitol, lactose, and sucrose. The effect of sugar concentration was examined using sucrose and dextran 40 as models. As indicated in Fig. 4, the thermostability of elastase increases as the sugar concentration is raised. In the case of dextran, the molecular weight may also affect the degree of stabilization. Although no attempt was made to systematically study this particular effect, dextran 40 appeared to be superior to dextran 80, suggesting that there may be an optimal chain length of the polymer in facilitating its stabilization of the protein. As expected, the addition of calcium chloride also increased the T_{max} of elastase, although the presence of both calcium chloride and sucrose did not provide enhanced stabilization compared with sucrose alone, indicating the lack of a synergistic effect. In contrast to the other additives, ascorbic acid appears to have a destabilizing effect, as evidenced by a low-

Table I. Effective of Additives on the DSC Denaturation Temperature (T_{max}) of Elastase

Additive[a]	T_{max} (C°)
None	66.0
Ascorbic acid, 5 m*M*	63.6
Mannitol, 20%	69.8
Sorbitol, 20%	69.7
Lactose, 20%	69.6
Polyvinylpyrrolidinone, 20%	67.0
Dextran 80, 20%	71.8
Dextran 40, 20%	74.3
Dextran 40, 40%	79.1
Sucrose, 20%	68.7
Sucrose, 40%	71.9
Sucrose, 60%	73.6
Calcium chloride, 10 m*M*	69.6
Sucrose, 20%, + calcium chloride, 10 m*M*	67.9

[a] All samples contain elastase in 10 m*M* sodium acetate, pH 5.0; additive concentrations are given in either m*M* or % (w/w).

ering of the T_{max}. Based on the DSC data, sucrose and dextran 40 were selected as excipients for further evaluation in lyophilization and storage.

Stabilization of Lyophilized Elastase by Sucrose or Dextran

As indicated in Fig. 5, freeze-dried elastase containing sucrose or dextran as an excipient was stable at 40°C, 79% RH, storage. At up to 4 weeks of storage, residual activity was at least 80%, while elastase lyophilized alone lost 75% of its activity in 3 weeks. The enhanced stabilization was also monitored by CD and fluorescence (Fig. 6). While the latter shows a slight loss of quantum yield and red shift of the emission maximum, i.e., comparable to the pH 3.5 conformation, no further change is seen. Likewise, the CD spectral data indicate a slight loss of ellipticity compared to the control, but it is clearly less pronounced than that produced at

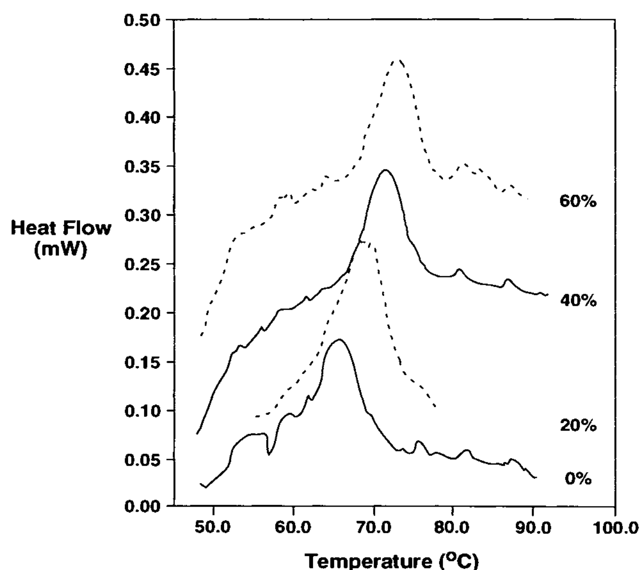


Fig. 4. Effect of sucrose concentration on thermostability of elastase.

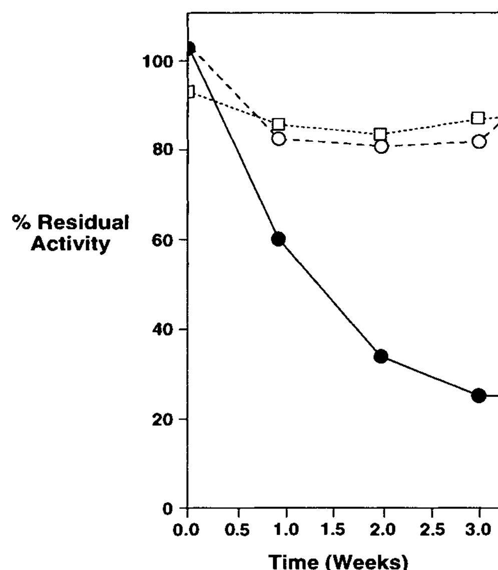


Fig. 5. Residual activity of lyophilized elastase in the presence of additives: (—●—) no additives; (--□--) 10% sucrose; (–○–) 10% dextran.

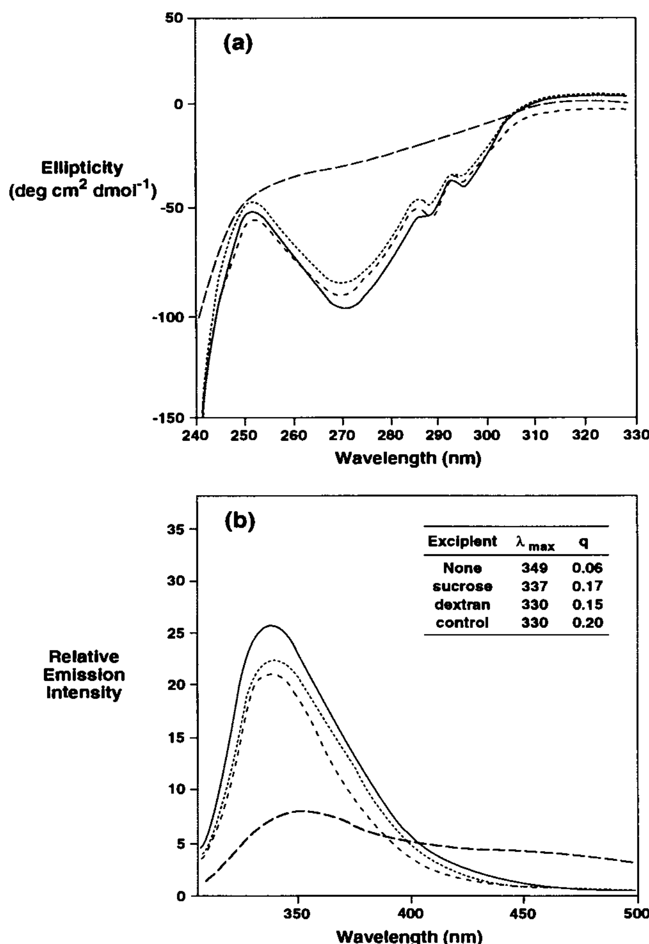
pH 2.5, i.e., the denatured form. Both sucrose and dextran appear to stabilize elastase to the same extent, although from the standpoint of the freeze-dried cake appearance, sucrose is preferable.

The role of sucrose or dextran in stabilizing elastase can be explained in two ways. The first possibility is that these additives restrict conformational changes by forming hydrogen bonds with surface groups on the protein. Such interactions would tend to preserve the native conformation as well as to protect buried tryptophyl groups from exposure to oxidation or other adverse modifications. The relatively small spectroscopic changes observed in the presence of sucrose or dextran may reflect alterations of the surface-accessible tryptophyl groups; recovery of most of the elastase activity was observed as expected, since oxidation of such residues has been demonstrated not to cause significant inactivation of elastase, in contrast to the buried interior tryptophyl residues (13).

The second possible explanation for the role of these stabilizers is related to the prevention of protein aggregation. There is some evidence that such aggregation may be a general phenomenon of protein inactivation in the solid state (3,18,19). The presence of stabilizers may aid in preventing formation of insoluble aggregates and/or preventing chemical modification of critical amino acid residues. In this hypothesis, elastase will still undergo conformational changes, but in the presence of stabilizers the changes are reversible so that the native conformation can be recovered. To maintain the reversibility, competition from destabilizing factors such as tryptophan oxidation must be overcome. A possible means of accomplishing this would be to add compounds that would function as antioxidants in the presence of elastase.

Effect of Ascorbic Acid on Elastase Stability

However, as shown in Fig. 7, the addition of ascorbic acid accelerated the inactivation of lyophilized elastase;



Excipient	λ_{max}	q
None	349	0.06
sucrose	337	0.17
dextran	330	0.15
control	330	0.20

Fig. 6. Spectroscopic properties of lyophilized elastase after 3 weeks at 40°C, 79% RH: (a) near-uv CD; (b) fluorescence. (——) Control; (———) no additives; (----) 10% sucrose; (---) 10% dextran.

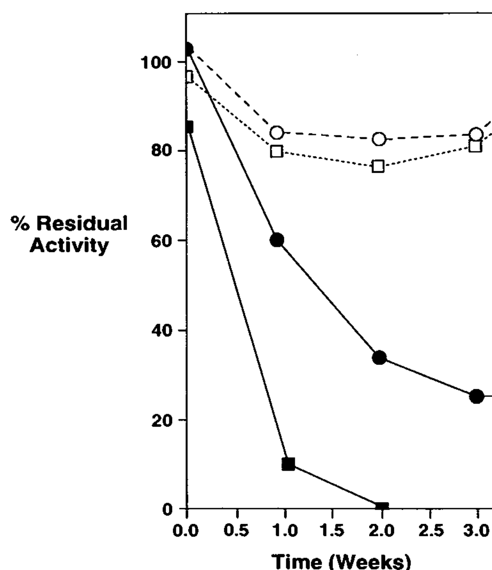


Fig. 7. Effect of additives on lyophilized elastase at 40°C, 79% RH: (—●—) no exipients; (–○–) 10% dextran; (—■—) 5 m*M* ascorbic acid; (--□--) 10% dextran + 5 m*M* ascorbic acid.

when lyophilized in the presence of 5 m*M* ascorbic acid, elastase lost 15% of its original activity immediately after lyophilization but lost 90% of its activity after 1 week at 40°C, 79% RH. Note that while ascorbic acid is frequently used as a pharmaceutical antioxidant, its success will depend on several factors including concentration, temperature, oxygen content of headspace, and concentration of metal ions (20). Obviously in the present situation, ascorbic acid is not functioning as an effective antioxidant; in fact, it is more likely that it encourages undesirable chemical reactions by release of degradation products. Another possibility is that ascorbic acid has a destabilizing effect on the solution conformation of elastase conformation, which is consistent with the observed lowering of the T_{max} in thermal denaturation studies (Table I). This could induce partial unfolding and aggregation without amino acid oxidation.

A similar result, i.e., accelerated inactivation, was observed by Townsend *et al.* when ribonuclease A was lyophilized in the presence of ascorbic acid (18). Also, one cannot discount the effect of trace metal ions, which have been shown to promote oxidation of tryptophan and histidine residues in the presence of ascorbic acid (21). In the case of ribonuclease A, headspace flushing with argon or nitrogen reduced the extent of degradation, suggesting that oxygen may be involved in the inactivation process. However, in the case of elastase, our preliminary studies of headspace flushing suggest that this does not have a significant effect on the course of inactivation. Further work is ongoing to determine the influence of the various environmental factors in lyophilized elastase inactivation and to identify more definitively the critical amino acid residues(s) involved in the denaturation process.

Note also that inclusion of dextran was found to stabilize lyophilized elastase from the adverse effects of ascorbic acid, as shown in Fig. 7. Elastase containing both ascorbic acid and dextran maintained most of its activity up to 3 weeks, which was similar to that containing dextran alone. This suggests that additives such as sucrose or dextran stabilize elastase by preventing conformational changes. While the rate of tryptophan oxidation could be faster in the presence of ascorbic acid, the sugars would stabilize elastase by keeping tryptophan(s) necessary for full activity in hydrophobic regions not available to oxidation.

One must also consider that what is effective as a stabilizer in solution may not be applicable to the lyophilized state due to the changes in concentration, pH, etc., that can occur during the freeze-drying process. This is quite apparent in the case of calcium chloride, which does stabilize elastase in solution but, when lyophilized, yields a collapsed cake devoid of activity. This result is due in large part to characteristics of calcium chloride, i.e., a low glass transition temperature (−109°C) as well as a low eutectic melting temperature (−59°C), which make this compound unsuitable as an additive for freeze-drying (22). It should also be pointed out that while calcium ions do raise the T_{max} of elastase in solution, their presence appears only to delay the onset of aggregation rather than prevent it from occurring (23).

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