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# Investigation of Physicochemical Changes to L-Asparaginase During Freeze–thaw Cycling

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## Abstract

L-Asparaginase derived from *Erwinia chrysanthemi* which is being investigated as an alternative to *E. coli* for the treatment of lymphoblastic leukaemia has been found in our laboratory to lose activity upon exposure to consecutive freeze–thaw cycles. An investigation was undertaken using several techniques to characterize fully the physicochemical changes L-Asparaginase is undergoing during freeze–thaw cycling leading to the loss of its activity.

A total protein assay suggested that the loss of some enzyme activity was a result of protein precipitation. Circular dichroism (CD) studies showed a decrease of α-helical structure with a concomitant increase in β sheet and random coil content, suggesting alterations in the secondary structure leading to unfolding, the first step of denaturation processes. The elution profiles obtained from size-exclusion chromatography (SEC) studies indicated the formation of several species during the process of freezing and thawing. Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) studies showed bands corresponding to 1–3 kDa and 32 kDa, suggesting that some of the species are fragments and shortened monomers resulting from cleavage of monomers. The molecular weight distribution obtained using SEC-linked light scattering indicated a substantial fraction of polydispersed fragments ranging from 900 Da to 3 kDa and a small fraction of aggregates corresponding to 300 kDa.

A scheme was proposed to explain the cascade of events leading to the loss of soluble protein and accompanying loss of enzyme activity. Tetramers of the enzyme dissociate into monomers some of which are cleaved into small fragments. The shortened monomers then aggregate and precipitate.

L-Asparaginase from a variety of sources is able to destroy asparagine-dependent tumours (Kidd 1953; Broome 1961, 1963). Asparaginase derived from *E. coli* is used clinically in the treatment of acute lymphoblastic leukaemia (Hill et al 1967, 1969). The major limiting factor in the use of this species of L-Asparaginase has been the development of hypersensitivity, ranging from mild allergic reactions to life-threatening anaphylaxis which occurs during the primary course of therapy (Capizzi et al 1971; Evans et al 1982). The immunogenicity of this enzyme causes the immune system to develop anti-Asparaginase antibodies which accelerate enzyme clearance leading to a shortened half-life of 2·5 h, thus reducing the effectiveness of the drug (Goldberg et al 1973; Charles & Bono 1981).

To reduce this immunogenicity and overcome the problems associated with it, pegaspargase has been developed wherein the native protein from *E. coli* is covalently modified with poly(ethylene glycol), PEG, with an average molecular weight of 5000 Da. PEG-L-Asparaginase has lower immunogenicity and a longer half-life, 15 days (Ho et al 1986). Allergic reactions in patients with a previous history of allergies to native Asparaginase and other side effects such as pancreatic toxicity (Bertolone et al 1982), hepatic toxicity (Jenkins & Perlin 1987) and coagulation factor abnormalities (Homans et al 1987) are, however, still associated with its use.

Intensive studies over the past few years have demonstrated that some of the complications associated with the *E. coli* form can be prevented by the use of an Asparaginase from *Erwinia chrysanthemi*. Eden et al (1988) have shown that the incidence of allergic reactions to the *Erwinia*-derived molecule is lower, approximately 2 compared with 20% for that from *E. coli*. It is being argued that because the antigenic sites are different for the enzyme from *E. coli* and *Erwinia*, the *Erwinia* enzyme can be used to treat patients allergic to *E. coli* and vice versa (Ohnuma et al 1972; Kay et al 1971). It has also been found that hepatic and pancreatic toxicities are less frequent with the *Erwinia* enzyme, which appears to be less diabetogenic and less antigenic (Ridgway et al 1989). Gugliotta et al (1992) reported that the *Erwinia* enzyme alters the coagulation system less severely than does *E. coli* Asparaginase. The above findings strongly suggest that the *Erwinia* enzyme is a promising alternative to the modified and native forms of *E. coli* Asparaginase.

Like the other known species of Asparaginase, *Erwinia* Asparaginase is a tetramer of four identical subunits, each with a molecular weight of approximately 35 kDa, held together by non-covalent forces. It has been reported that its biological activity is related to the degree of association of its tetramers (Handschumacher 1971). *Erwinia* Asparaginase has been previously reported to be stable to freezing and thawing but unstable to freeze-drying (Hellman et al 1979). We, however, have found that it loses substantial activity upon exposure to freeze–thaw cycling under conditions used in our laboratories (Jameel et al 1995).

The purpose of this paper is to describe the results of a wide variety of techniques used to characterize the physicochemical changes causing the loss of activity of *Erwinia* Asparaginase as

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a result of cycles of freezing and thawing. The rationale behind using multiple freezing and thawing cycles is to accentuate any changes seen during the process. Freezing represents the first portion of the freeze-drying process. It is the effect on the Asparaginase of this part of the process that we seek to understand.

The research reported here is meant to supplement earlier work on the stabilization of *Erwinia* Asparaginase by hetastarch, a hydroxyethylated starch derivative, during freeze-thaw cycling. In that paper (Jameel et al 1995) we described the profound stabilization provided by hetastarch in comparison with that provided by mono and disaccharides. In an attempt to understand the mechanism(s) by which hetastarch provides its stabilizing effect on *Erwinia* Asparaginase, we sought to understand the pathways leading to the loss of activity of the enzyme during freeze–thaw cycling.

## Materials and Methods

*Materials*

L-Asparaginase (L-asparagine amidohydrolase E.C. 3.5.1.1) derived from *Erwinia chrysanthemi* was obtained as a lyophilized powder containing approximately 40% protein (Sigma, St Louis, MO). Solutions of this enzyme were prepared as described in the next section. Tris-(hydroxymethyl)aminomethane (Tris) and Nessler reagent were used as received (Sigma). Protein assay dye reagent for the Bradford assay was obtained as a concentrate (Bio-Rad labs, Life Science Group, Melville, NY) and diluted fivefold before use. Sodium dodecylsulphate (SDS) and acrylamide were obtained from Ultrapure (Gaithersburg, MD). Coomasie Blue and silver stain kits for staining gels were also obtained from Bio-Rad Labs. Tris buffer (0·05 M, pH 8.6) and phosphate buffer (0·05 M, pH 7.3) were prepared from salts purchased from Fisher Scientific (Springfield, NJ). All phases of the freeze-thaw experiments were performed in polypropylene culture tubes (Fisher Scientific).

*Sample preparation*

L-Asparaginase received as the lyophilized powder from Sigma was dissolved in Tris buffer (pH 8·6, 50 mM; 1 mL) and dialysed (Diacell with a 6000 Da cut-off membrane; Instrumed, North Union Bridge, MD) against three to four volumes (75 mL) of the same buffer over 2 to 3 days at 4°C. The dialysed solution was assayed for protein concentration and diluted to concentrations ranging from 0·02–0·25 mg $mL^{-1}$, depending on the requirements of the experimental technique. Diluted L-Asparaginase solutions were frozen at −20°C for 1 day and thawed at room temperature for 30–40 min. To amplify the resulting changes, the freezing and thawing process was repeated up to four times.

*Biological assay*

Samples of frozen and thawed enzyme solutions were diluted to 500 μL yielding a concentration in the range 0·9–$1{\cdot}1 \times 10^{-4}$ mg $mL^{-1}$, mixed with asparagine (0·04 M; 0·5 mL), and made up to 2·0 mL with Tris buffer. After 30 min at 37°C, the enzyme reaction was terminated with trichloroacetic acid (1·5 M; 0·5 mL). The mixture was diluted to 9 mL and Nessler reagent (1 mL) was added. After 10–15 min the absorbance of the ammonia–Nessler complex was assayed at 390 nm.

Unit L-Asparaginase activity is defined as the amount of enzyme that liberates 1·0 μmol of ammonia from L-asparagine per min at pH 8·6 and 37°C (Yellin & Wriston 1966). All activity results are expressed as a percentage of the initial activity of the unfrozen control solution.

*Protein quantification*

The total mass of protein in solution before and after each freeze–thaw cycle was quantified using the Bradford test, a colorimetric assay with microgram sensitivity (Bradford 1976). Unfrozen enzyme solutions (0·02–0·25 mg $mL^{-1}$) and sequentially frozen and thawed solutions were centrifuged at 800 *g* for 3–4 min. The supernatant liquid (100 μL) was taken and mixed with Coomassie Blue G-250 dye (3 mL). After gently mixing for several minutes the absorbance of these solutions was read at 595 nm. The concentration of the protein in each sample was obtained from a standard curve generated using known concentrations of the dialysed L-Asparaginase.

*Circular dichroism (CD)*

Circular dichroism was employed to monitor and quantify the alterations in the secondary structure of the protein during the processes of freezing and thawing. Enzyme solutions (0·02 mg $mL^{-1}$) either unfrozen or after successive freeze–thaw cycles were scanned from 190–260 nm at a scan speed of 50 nm $min^{-1}$. Triplicate scans were generated against a blank (phosphate buffer, pH 7·3, 50 mM) at room temperature using a spectropolarimeter (Model J-710; Jasco, Tokyo, Japan). Using the corresponding concentration determined from the UV spectrophotometer, the molar ellipticity of the enzyme in each solution was calculated.

*Size-exclusion chromatography*

Size-exclusion chromatography which separates molecules primarily on the basis of hydrodynamic size was used to determine whether aggregation, dissociation or any other disruption of the tetramer had occurred during the freezing and thawing processes. A sample (100 μL) of the unfrozen enzyme solution (0·056 mg $mL^{-1}$) and solutions exposed to consecutive freeze–thaw cycles were analysed using a Biosep-SEC-S2000 column (300 × 7·8 mm). Phosphate buffer (pH 7·3, 50 mM) was used as the mobile phase at a flow rate of 0.5 mL $min^{-1}$. The peaks were monitored at 280 nm with a diode-array detector.

*Light scattering*

SEC-linked light scattering photometry was used for further characterization of the peaks observed by SEC. Unfrozen enzyme solutions and those subjected to consecutive freeze–thaw cycles (0·056 mg $mL^{-1}$) were injected on to the column. To obtain a clear signal the volume injected was varied (100–250 μL) depending on the concentration. The column and assay conditions used were the same as in the SEC studies. After separation in the column, the eluent fractions of the enzyme solutions were passed through a multi-angle laser-light-scattering detector and a refractive index detector to determine both the molecular weights and concentrations of the protein species. A well characterized sample of bovine serum albumin was run to obtain the calibration constants of

the instrument. Using the scattering intensities, the calibration constants and the concentrations of the sample, the molecular weight distribution of each sample was determined.

*Gel electrophoresis*

Unfrozen and freeze–thawed enzyme solutions (100 $\mu$L) of various concentrations (0·022, 0·056 and 0·126 mg $mL^{-1}$) were loaded on to a 10% sodium dodecylsulphate (SDS)-10% polyacrylamide (PA) gel. Pre-stained molecular weight markers ranging from 3 to 300 kDa were used as standards. The gel was run at a constant current of 30 mA until the dye marker had moved to 1 cm from the bottom of the gel. The gel was removed, fixed and stained with Coomasie blue for 1 h. It was de-stained with a mixture of 10% methanol and acetic acid overnight with constant shaking. The gel was stained further with silver stain to reveal the bands that were not clear with Coomasie blue stain.

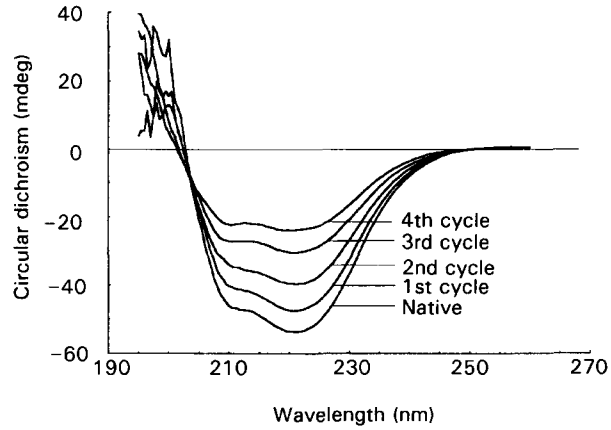



FIG. 1. Circular dichroism spectrum of native L-asparaginase (0·02 mg $mL^{-1}$) overlaid with the spectra obtained after each consecutive freeze–thaw cycle.

## Results and Discussion

Exposure of L-Asparaginase solutions to consecutive freeze–thaw cycles has previously been shown to result in substantial loss of biological activity. It was observed that enzyme solutions subjected to more cycles showed a greater loss in activity (Jameel et al 1995). Hanafusa (1969) reported that similar oligomeric enzymes undergo structural changes often accompanied by reductions in biological activity upon freezing and thawing. Since that time, however, there have been few reports on the results of the stresses of freezing and thawing on proteins. Protein stabilization to freezing and thawing is important for the shipping of protein solutions to extreme climates. In addition, changes in a patient's status might enable clinicians to change the therapy, leaving the pharmacy with an unstable, expensive, reconstituted protein solution. It would be useful to be able to freeze the reconstituted protein solution for later use. Here, we seek to understand the pathways whereby L-Asparaginase is destabilized, so that we might seek appropriate stabilizers.

In this work the concentration of soluble protein in L-Asparaginase solution was monitored as a function of freeze-thaw cycling to determine any correlation between protein precipitation and loss of activity. Table 1 indicates that after the first freeze–thaw cycle there was a substantial loss of activity and a reduction in the concentration of soluble protein. The next two cycles did not further reduce the activity nor the concentration of soluble protein. At the end of the fourth cycle, however, there was again a reduction in activity and protein concentration. The correlation coefficient, $R^2$, between activity remaining and protein concentration was 0·996. This suggests that loss in activity is caused by protein precipitating from the solution. The protein precipitation is, however, secondary to physicochemical changes to the protein. It is the primary process, the physicochemical change, that is of interest to us here.

Table 1. Biological activity and protein concentration of L-asparaginase solution as a function of freeze–thaw cycles.

| Number of freeze–thaw cycles | Amount of initial activity remaining (%) | Protein concn (mg $mL^{-1}$) |
|---|---|---|
| 0 | 100 | 0.11 |
| 1 | 57·4 ± 0·5 | 0·057 |
| 2 | 64·2 ± 0·25 | 0·061 |
| 3 | 61·3 ± 0·48 | 0·060 |
| 4 | 48·4 ± 1·25 | 0·039 |

Mean ± s.d.; n = 3.

The far UV CD spectrum of L-Asparaginase (Fig. 1) indicates the presence of a substantial amount of $\alpha$-helical structure in the native form. With each consecutive freeze–thaw cycle, Fig. 1 shows that the changes in the CD spectrum are progressive. The progressive changes in the spectrum can be attributed to changes in concentration or conformation or both. In order to correct for known changes in protein concentration (Table 1) the data were reported in terms of molar ellipticity (Fig. 2). This clearly shows the progressive loss of $\alpha$-helical structure as indicated by the decrease in the intensity at 222 nm and 208 nm, both of which are characteristic of an $\alpha$-helix. The components of the secondary structure of L-Asparaginase were also quantitatively analysed using a neural network (an optimized self-organizing map algorithm) k2D programme (Andrade et al 1993). This analysis showed a 48% decrease in $\alpha$-helical content, a 14% increase in $\beta$-sheet and a 34% increase in random coil after four freeze–thaw cycles. The CD spectra and their quantitative analysis suggest a significant alteration in secondary structure which might lead to gross unfolding of the protein. As the protein unfolds the hydrophobic groups that were in the interior of the folded form might become exposed leading to a more hydrophobic and less soluble protein. This would explain the reduction in the amount of soluble protein with successive freeze–thaw cycles. The conformational changes indicated by CD might also lead to disruption of the tetramer resulting in monomers which form large aggregates, because of increased hydrophobicity, and precipitate out.

To monitor changes in the size of the protein as a result of freeze–thaw cycles, size-exclusion chromatography (SEC) was employed. Fig. 3 shows the elution profiles of the native and successively freeze–thawed enzyme solutions. It can be seen that the native tetramer which was not exposed to freezing shows a single peak at 15.5 min. After exposure to two freeze-

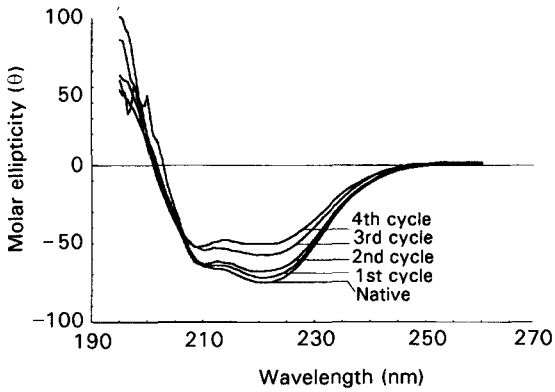



FIG. 2. Circular dichroism spectrum of native L-asparaginase (0·02 mg $mL^{-1}$) overlaid with the spectra of the solution after its exposure to each consecutive freeze–thaw cycle, corrected for concentrations and expressed in molar ellipticity.

thaw cycles the native peak shifted toward higher elution times, suggesting that the size of the protein is being reduced. The split in the peak at the end of the third cycle indicates that the tetramer is undergoing dissociation into two distinct species. At the end of the fourth cycle there appeared three peaks, one near the native peak and the other two at later times. The appearance of peaks at later times (23·9 and 28·8 min) indicates the presence of smalL-molecular-weight protein species, suggesting that some of the dissociated monomers are being cleaved into small fragments. The reappearance of the peak near the native peak could be attributed to re-association of the altered monomers.

Because of interactions between the sample and the column it was not possible in this instance to use molecular weight standards to calculate the absolute molecular weights of the peaks representing these molecules. The molecular weights of these peaks were, therefore, further characterized by two other techniques, SDS-PAGE and SEC-linked light scattering. The results from gel electrophoresis (Fig. 4) were in good agreement with those from SEC. The third, fourth and fifth lanes show the bands resulting from freeze–thawed enzyme samples of various concentrations. At all concentrations, the three samples show four bands. The top band corresponds to a molecular weight of about 35 kDa, indicative of the monomer. The second band corresponds to a molecular weight of about 32 kDa, which is less than that of the monomer, and the third set of bands corresponds to a molecular weights of about 3 kDa and represents small protein fragments. The molecular weights of the fragment(s) located in the fourth band appear to be very small ( < 1000 Da). In the presence of SDS the native tetramer and larger aggregates are reduced to monomers and are expected to result in a single band at 35 kDa. In addition to the expected 35 kDa monomer band, three more bands were seen, suggesting that a fraction of the monomers undergo fragmentation. The third and fourth bands corresponding to molecular weights of 1–3 kDa are small fragments from the monomer. The second corresponds to a 32 kDa protein. This

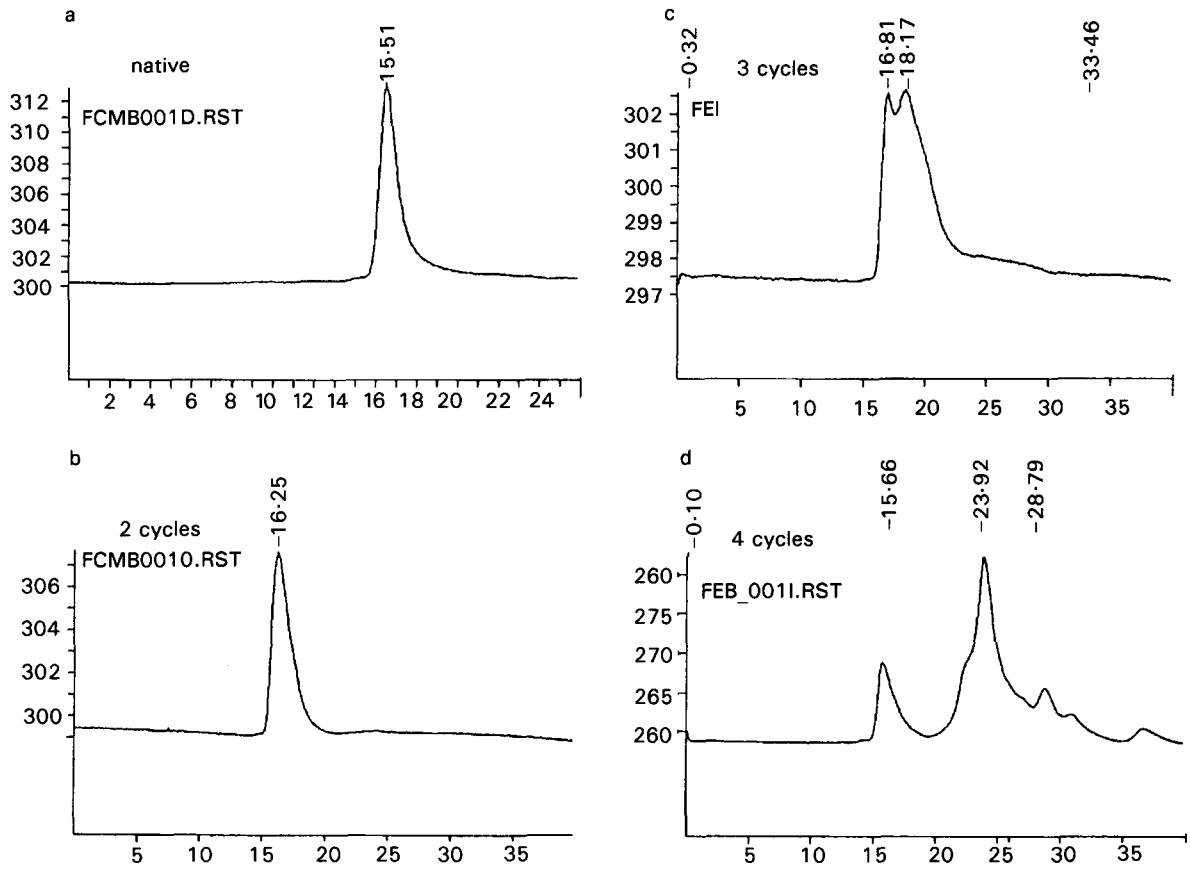



FIG. 3. Size-exclusion chromatograms of L-asparaginase (0·056 mg $mL^{-1}$) before and after exposure to consecutive freeze–thaw cycles.

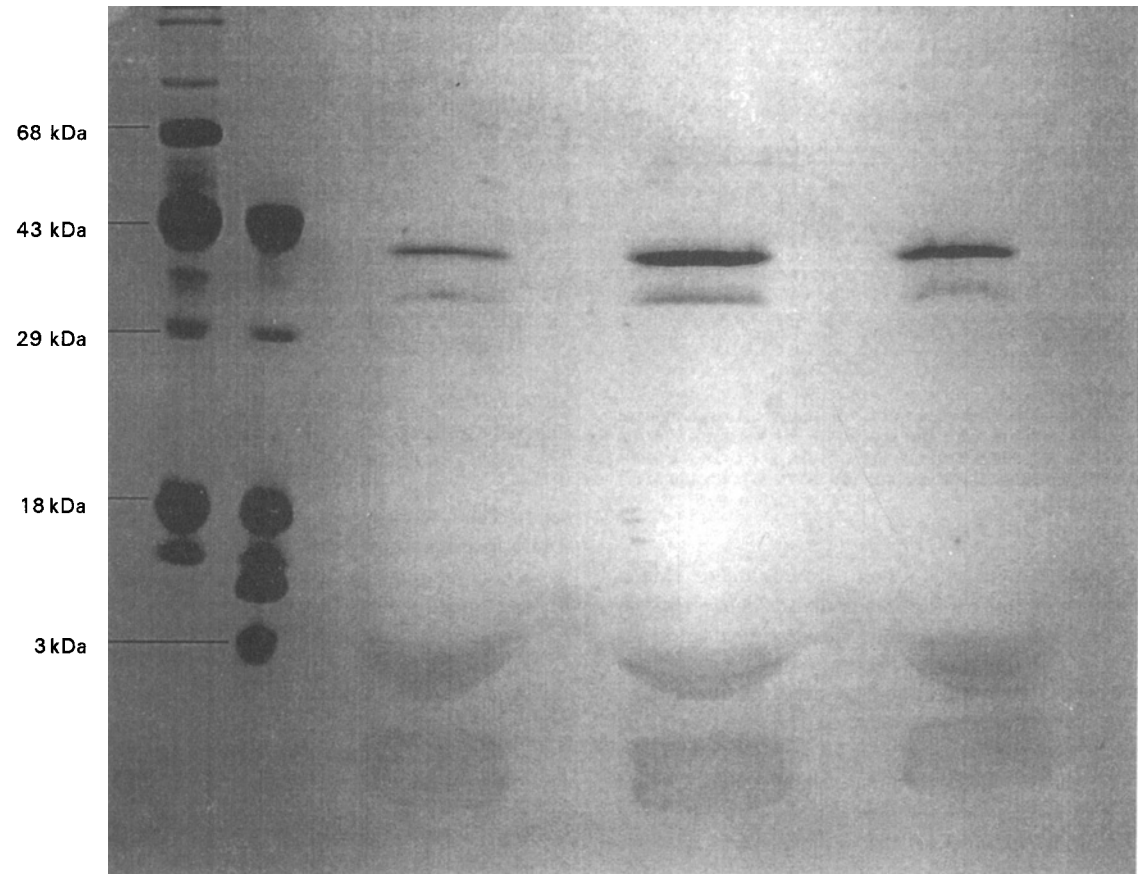



FIG. 4. Polyacrylamide gel (SDS) of L-asparaginase after four freeze–thaw cycles: first lane, high–molecular–weight standards; second lane, low–molecular–weight standards; third lane, L-asparaginase (0·022 mg $mL^{-1}$); fourth lane, L-asparaginase (0·126 mg $mL^{-1}$); fifth lane, L-asparaginase (0·056 mg $mL^{-1}$).

represents monomers that have been shortened by fragmentation.

Use of SDS-PAGE gave no information about the aggregates. The first band might have resulted from dissociation of either the native tetramer or higher order aggregates. To distinguish which form was dissociating, SEC-linked light scattering was employed. The native sample (before exposure to freeze–thaw cycling) showed a single peak with average molecular weight of 138 kDa which closely approximates the reported value of 140 kDa for *Erwinia* Asparaginase (Fig. 5). An enzyme solution subjected to repeated freeze–thaw cycles resulted in the molecular weight distribution shown in Fig. 6. There is a substantial fraction of fragments corresponding to molecular weights of 1–3 kDa and a small fraction of aggregates corresponding to a molecular weight of 300 kDa. This suggests that the monomer band seen on SDS-PAGE results from aggregates and that the fragments are the result of cleavage of the monomer.

*Conclusions*

Scheme 1 was generated on the basis of the above findings. The native tetramer first undergoes alterations in secondary structure as seen from CD studies. This partially unfolded form dissociates into species of lower molecular weight as seen from SEC studies on repeatedly frozen and thawed samples. The dissociation from tetramer to monomer is not uncommon. L-Asparaginase monomers undergo further alteration, however. SDS-PAGE and SEC-linked light scattering show that the monomers are cleaved, leaving shortened monomers and fragments. The shortened monomers or denatured monomers, or both, re-associate to form the aggregates seen by use of

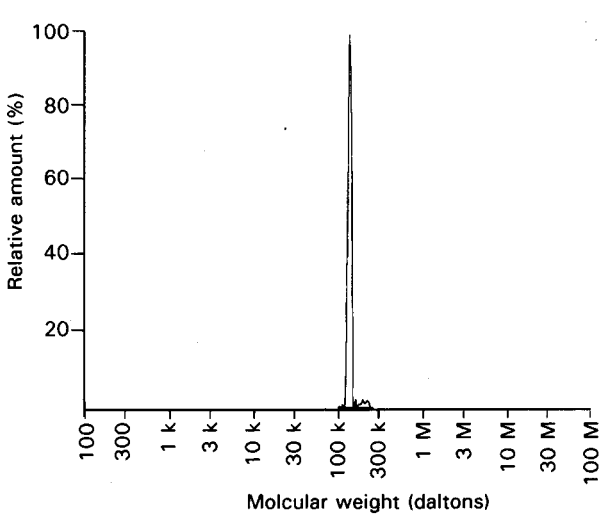



FIG. 5. Molecular weight distribution of native L-asparaginase (0·056 mg $mL^{-1}$) determined by SEC-linked light scattering.

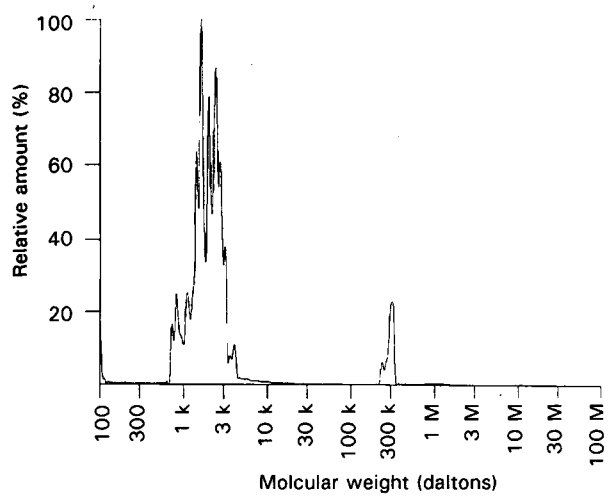
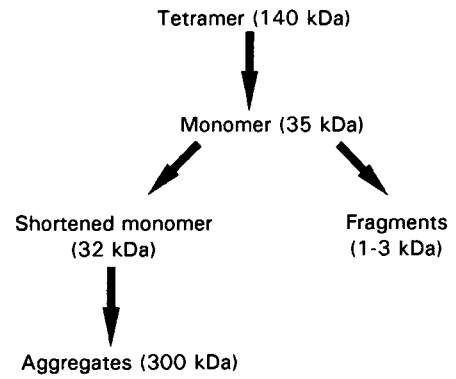



FIG. 6. Molecular weight distribution of L-asparaginase (0·056 mg $mL^{-1}$) after four freeze–thaw cycles determined by SEC-linked light scattering.




SCHEME 1. Pathways leading to loss of activity of L-asparaginase.

SEC-linked light scattering. As seen from the total protein assay, most of the aggregates precipitate out of solution.

Thus, the ultimate cause of the loss of L-Asparaginase activity as a result of repeated freezing and thawing is the precipitation of insoluble aggregates. The insoluble aggregates, however, are formed by a cascade of events. The significance of the fragmentation to the activity of the enzyme will be the subject of future communication.

*Acknowledgement*

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