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Turbidimetric and HPLC assays for the determination of formulated lysozyme activity

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Abstract

In several studies lysozyme has been employed as a model protein to investigate the effects of formulation factors upon biological activity. The aim of this work was to develop and validate an HPLC technique to assay lysozyme and to compare the results with biological activity determined from a validated turbidimetric assay. The turbidimetric assay was based upon the lytic action of lysozyme on *Micrococcus lysodeikticus* cells, whilst the reverse-phase HPLC assay employed an acetonitrile gradient in 0.1% trifluoroacetic acid. The limits of detection and quantification were 3.84 and 6.24 μg mL^{-1} for HPLC assay, whilst the corresponding values for turbidimetric assay were 1.94 and 3.86 μg mL^{-1}. The methods were used to monitor the loss of enzyme activity after heating. Lysozyme concentrations determined from HPLC peak height were found to correlate ($r^2 = 0.9963$) with those obtained from turbidimetric assay.

Introduction

The advances in biotechnology over recent years have led to the identification and production of a range of peptides and proteins, which have potential to be employed as therapeutic agents. Formulating such proteins in a state that provides chemical and physical stability up to a point of delivery is a more difficult process than formulating traditional small molecular weight drugs (Manning et al 1989). The principal processes utilized for the preparation of powdered protein formulations are spray drying (Broadhead et al 1992) and freeze-drying (Pikal 1991a, b; Franks 1998). These drying processes, however, impose considerable stresses upon the protein molecules, which can result in the degradation of protein structure and loss of the native conformation, leading to a partial or complete loss of biological activity.

Lysozyme is often used as a model protein to study the effects of protein formulation, preparation and manufacture on activity and function (Ghaderi & Carlfors 1997; Remmele et al 1997; Sah 1997; Branchu et al 1999; Tzannis & Prestrelski 1999). It is a low molecular weight (14500 Da) cationic protein with bacteriolytic properties and it is upon the latter that most of the biochemical assay methods are based. Such assays involve the measurement of the degree of lysis induced by the enzyme on microorganisms such as *Micrococcus lysodeikticus* cells, utilizing either turbidimetry, lysoplate assay or immunoassay (Dixon & Webb 1979; Grosowicz & Ariel 1983). The turbidimetric methods are amongst the simplest and quickest to perform and rely upon spectrophotometrical measurement of the clearance rate of turbid suspensions of *M. lysodeikticus* by lysozyme. Whilst some of the methods have monitored the absorbance change in a given time interval

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(Smolelis & Hartsell 1949; Shugar 1952), others have measured the time required to generate a given absorbance change (Jollès & Fromageot 1953; Gorin et al 1971).

For many years the method of choice for the quantitative measurement of lysozyme, when used as a model protein in the development of biopharmaceutical formulations, has been HPLC. The method is highly sensitive and reproducible with a good correlation between enzyme concentration and either peak area or peak height (Thapon & Brulé 1982). Initially, the previously developed methodology involved a gradient mobile phase of acid aqueous and organic solvents. However, the separation process caused partial or complete unfolding of the native structure of lysozyme and resulted in a distorted peak shape or multiple peaks. Consequently, 0.1 % trifluoroacetic acid was added to the mobile phase, which strongly denatures the native structure of proteins, and renders a single and sharp peak of the denatured species (Benedek et al 1984).

Since the preparation, processing and storage of protein formulations can induce a change in the native structure of the protein, this could result in a decrease in the therapeutic activity. Such changes might not be detected when measuring lysozyme utilizing HPLC and consequently the use of such a method could produce erroneous results. Therefore, the aim of this study was to develop a validated HPLC technique to determine lysozyme concentration and to determine whether enzyme concentration correlated with its biological activity obtained by measurement of bacterial lysis using turbidimetry (Gorin et al 1971).

Materials and Methods

Materials

The buffer phosphate salts (ACS reagent grade), trifluoroacetic acid (spectrophotometric grade), acetonitrile (HPLC grade), lysozyme (3 × crystallized, dialysed and lyophilized, Lot 57H7045) and *M. lysodeikticus* (Lot 39H8615) were purchased from Sigma-Aldrich Co. UK. Water purified by an ELGA-Option 3 water purifier was used to prepare all solutions. Absorbance was measured using a Perkin-Elmer Lambda 2 spectrophotometer. The HPLC assay was performed using a computerized HPLC system which included a Perkin Elmer series 200 lc pump, 1022 integrator software, Perkin Elmer series 200 lc autosampler, Applied Biosystems 759A absorbance detector and a Hicrom reverse phase C_{18} column (25 cm × 0.47 cm, 5 μm).

Preparation of buffers

Phosphate buffer (0.1 M, pH 6.2) was prepared by dissolving 10.37 g NaH_2PO_4 H_2O and 3.12 g Na_2HPO_4 in 1 L water.

Preparation of the lysozyme solution

Stock solutions, containing approximately 0.95 mg mL^{-1} lysozyme, were prepared in phosphate buffer and stored in the refrigerator at 4°C. The concentration of the enzyme was estimated from the absorbance at 280 nm, taking the value of the specific absorptivity (1 mg mL^{-1}, 1-cm cell) as 2.6 (Gorin et al 1971). A standard solution (1000 μg mL^{-1}) was freshly prepared in phosphate buffer just before an experiment. Measured samples of this standard were diluted with phosphate buffer to produce a concentration series (10–40 μg mL^{-1} for bioassay and 20–100 μg mL^{-1} for HPLC assay).

Procedure for bioassay

The preparation of suspensions of *M. lysodeikticus* and assay procedure were based upon a previously published but unvalidated method (Gorin et al 1971). This assay entailed measuring the time (min) required for the absorbance of the substrate to decrease by 0.05 units ($t_{0.05}$). A 100-μL sample of each enzyme concentration (10, 15, 20, 30 and 40 μg mL^{-1}) and 3.00 mL bacterial suspension were placed into a cuvette, and quickly mixed with a small spatula, and then the absorbance recorded over time. From the change of absorbance with time, $t_{0.05}$ was determined and a standard curve constructed by plotting the reciprocals of $t_{0.05}$ (min^{-1}) vs enzyme concentrations of standard solutions (μg mL^{-1}). Six calibrations were performed over 30 days.

Reverse-phase HPLC assay

A 50-μL sample of enzyme solution was applied to the column at 25°C and eluted at a flow rate of 0.5 mL min^{-1} in a gradient of acetonitrile in aqueous 0.1 % trifluoroacetic acid using the procedure summarized in Table 1, and detected at a wavelength of 220 nm. A linear gradient of acetonitrile was maintained for the first 40 min so as to elute intact and degraded lysozyme (Figure 1). The remainder of the elution procedure (Table 1) was carried out to remove minor contaminants to ensure non-interference with subsequent enzyme assays and to equilibrate the column back to the starting gradient. Calibration curves were constructed for peak area and peak height (mV) against lysozyme concentration of standard solutions (20–100 μg mL^{-1}) with six

Table 1 Standard elution cycle of HPLC: the eluting phase (acetonitrile: 0.1% trifluoroacetic acid) was modified as a function of time.

Time (min)	% Acetonitrile in eluting solvent
0–40	10–40%
40–45	40%
45–50	40–80%
50–55	80%
55–60	80–10%
60–70	10%

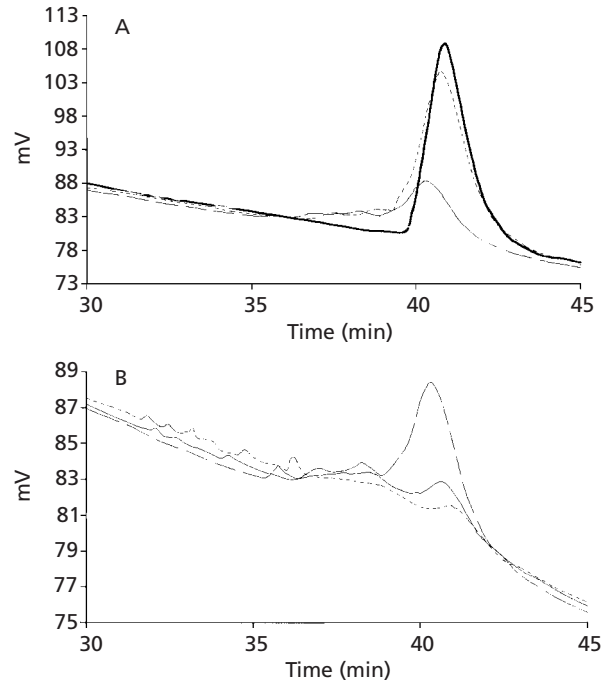

Figure 1 The HPLC chromatograms of original and boiled lysozyme. A. —, Original (50 μg mL^{-1}); …, boiled for 10 min; -----, boiled for 20 min. B. -----, Boiled for 20 min; —, boiled for 30 min; …, boiled for 45 min. For all boiled samples, the concentration before heating was 100 μg mL^{-1}.

replicate samples for each lysozyme concentration. Baseline sensitivity, peak area and peak height were determined automatically by the system software and confirmed by manual over-ride. A total of three calibrations were performed over seven days.

Validation of methods

Weighted linear regression was employed. Precision of each assay was evaluated by determining the intra-run and inter-run relative standard deviation (RSD) of five different concentrations of the standard solutions and the measured stock solutions at three concentrations (10, 25 and 40 μg mL^{-1} for the bioassay and 20, 50 and 100 μg mL^{-1} for HPLC). The accuracy was calculated from a comparison of the concentration of the stock solutions determined from the standard curve with the corresponding nominal value. The accuracy was therefore expressed as mean percentage of lysozyme (i.e. calculated value/nominal value $\times$ 100%) recovered in the assay.

The limit of detection (LOD) and limit of quantification (LOQ) were calculated from the equations $LOD = S_{blank} + 3\ S_{(y/x)w}$ and $LOQ = S_{blank} + 10\ S_{(y/x)w}$, where S_{blank} was the signal of blank and $S_{(y/x)w}$ was the standard deviation of the predicted y-value from the weighted regression line (Miller & Miller 1993).

Treatment of HPLC data

The concentration of 30 different solutions was measured using HPLC by determining peak area and peak height. The statistical comparison was carried out using a paired Student's *t*-test.

Analysis of heated lysozyme by bioassay or HPLC assays

Samples of phosphate buffer (2.85 mL, 0.1 M, pH 6.24) were placed into a series of 10 mL glass vials, which demonstrated negligible protein-binding potential, because there was no detectable loss of lysozyme activity after storage at 4°C for up to two months (data not shown). The vials were capped and transferred to a boiling-water bath for 30 min to ensure that they were equilibrated to the required temperature. Samples (150 μL) of lysozyme solution (20 mg mL^{-1}) were measured into the vials to give a final concentration of lysozyme of 1 mg mL^{-1}. The samples were then incubated in the boiling water for 10, 20, 30 and 45 min. The vials were immediately cooled in iced water and then stored in a refrigerator (4°C). The heated samples were mixed by vortexing and centrifuged to remove the insoluble aggregates before assay. The concentrations and activities of all supernatants were determined by HPLC peak area, HPLC peak height and turbidimetric assays. The relative activities were expressed as the percentage of the original concentration. A statistical comparison between the concentrations derived from peak height and bioassay was performed using a paired Student's *t*-test.

Results and Discussion

Turbidimetric assay

The enzyme concentrations were shown to be inversely proportional to the $t_{0.05}$ values. In the measured range of enzyme concentration (10–40 μg mL^{-1}), regression analysis gave correlation coefficients (r^2) $\geqslant 0.99$. The repeatability, obtained by the intra-run precision at all concentrations, was determined by calculating RSD, and was $\leqslant 3.45\%$. The reproducibility (RSD of the inter-run) was found to be $\leqslant 4.40\%$ (Tables 2 and 3). The accuracy ranged between 97.5 and 102.9 %.

The mean $t_{0.05}$ (n = 6) of the suspensions for self-clearing (without lysozyme) to occur was 299.8 min, and was defined as $t_{0.05}$ of the blank. The LOD and LOQ were obtained from the mean calibration equation: $y = 0.0398x - 0.0409$ and $S_{(y/x)w} = 0.0109$. Thus, using non-rounded values, the LOD was calculated to be 1.94 μg mL^{-1} and the LOQ was 3.86 μg mL^{-1}.

The factors that affect the enzymatic efficiency of lysozyme and thus its bioassay have been reported previously (Gorin et al 1971; Jollès et al 1974; Grossowicz & Ariel 1983) and include pH, temperature, ionic strength and the method of preparation of the substrate. However, in these studies lysozyme was prepared in phosphate buffer (0.1 M, pH 6.2) and hence reaction conditions remained constant and should not have affected the determination of lysozyme. In addition, the method of preparation of substrate was identical for all samples. Temperature was found to affect the enzymatic efficiency of lysozyme in these experiments, the $t_{0.05}$ at 20°C of 10 μg mL^{-1} lysozyme being approximately 200s, whilst the $t_{0.05}$ at 30°C was approximately 100 s. However, the determination of relative enzyme activity, linearity of standard curve and RSD of intra- and inter-run were not influenced by temperature.

HPLC assay

There was a single peak in the HPLC chromatogram (Figure 1) for aqueous untreated samples of lysozyme and the retention time was found to be 39.88 ± 0.33 min (n = 60). Regression analysis, in the measured range of enzyme concentration (20–100 μg mL^{-1}), gave correlation coefficients (r^2) for both peak area and peak height methods $\geqslant 0.999$. The intra-run and inter-run precision for peak area method was $\leqslant 3.06\%$ and $\leqslant 2.47\%$, respectively, while the intra-run and inter-run precision for peak height was $\leqslant 3.65\%$ and $\leqslant 3.45\%$, respectively. The accuracy was examined by analysing the stock solution at 20, 50 and 100 μg mL^{-1}. The mean recoveries $\pm$ s.d. (n = 3 × 3) were 99.55 ± 1.20, 99.22 ± 1.68 and $100.39 \pm 2.27\%$ for peak area and 98.7 ± 1.75, 99.40 ± 2.20 and $100.25 \pm 1.87\%$ for peak height, respectively. The LOD and LOQ of peak area were found to be 3.91 and 5.68 μg mL^{-1}, whilst the LOD and LOQ using peak height were 3.83 and 6.24 μg mL^{-1}. It has been reported previously that the HPLC assay of lysozyme is more sensitive than the bioassay (Thapon & Brulé 1982). However, this study showed that the LOD and LOQ values for HPLC analysis were higher than those obtained from the adapted bioassay.

The concentrations of 30 different lysozyme solutions were measured by using peak area and peak height from HPLC chromatography. There was no significant difference ($P = 0.8887$; paired t-test) between the concen-

Table 2 Intra-run data for determination of lysozyme.

Concn (μg mL^{-1})	Bioassay (n = 6)		Concn (μg mL^{-1})	HPLC area (n = 6)		HPLC height (n = 6)	
	Determined concn ± s.d. (μg mL^{-1})	Accuracy		Determined concn ± s.d. (μg mL^{-1})	Accuracy	Determined concn ± s.d. (μg mL^{-1})	Accuracy
10	10.02 ± 0.13		20	20.23 ± 0.41		20.77 ± 0.61	
10*	9.75 ± 3.45	97.51	20*	19.76 ± 0.44	98.81	19.19 ± 0.37	95.97
15	14.98 ± 0.42		30	29.50 ± 0.57		29.95 ± 0.13	
20	19.96 ± 0.19		40	40.01 ± 1.06		40.69 ± 0.9	
25*	24.69 ± 0.55	98.76	50*	49.55 ± 0.99	99.10	48.96 ± 1.97	97.92
30	29.54 ± 0.81		60	59.75 ± 1.08		60.47 ± 2.19	
40*	41.15 ± 0.56	102.88	100	101.70 ± 2.42		100.70 ± 2.30	
40	40.96 ± 1.05		100*	101.52 ± 3.06	101.52	101.37 ± 2.49	101.37

*Stock samples.

Table 3 Inter-run data for determination of lysozyme.

Concn (μg mL^{-1})	Bioassay (n = 36)		Concn (μg mL^{-1})	HPLC area (n = 18)		HPLC height (n = 18)	
	Determined concn ± s.d. (μg mL^{-1})	Accuracy		Determined concn ± s.d. (μg mL^{-1})	Accuracy	Determined concn ± s.d. (μg mL^{-1})	Accuracy
10	10.27 ± 0.23		20	20.13 ± 0.29		20.42 ± 0.57	
10*	9.82 ± 0.44	98.21	20*	19.91 ± 0.24	99.55	19.74 ± 0.35	98.7
15	15.02 ± 0.33		30	29.32 ± 0.58		29.64 ± 0.53	
20	20.13 ± 0.28		40	40.17 ± 0.82		40.44 ± 0.79	
25*	24.88 ± 0.63	99.52	50*	49.61 ± 0.84	99.22	49.67 ± 1.10	99.40
30	29.92 ± 0.41		60	59.85 ± 0.84		60.17 ± 1.51	
40*	40.71 ± 0.88	101.78	100	101.82 ± 2.47		101.03 ± 2.18	
40	41.07 ± 0.41		100*	100.39 ± 2.27	100.39	100.25 ± 1.87	100.25

*Stock samples.

trations of enzyme determined using peak area with those determined using peak height (data not shown).

Comparison of the results of partially denatured lysozyme samples from three assays

In initial studies, some samples of lysozyme were heated at 75°C for 10 min and complete recovery of lysozyme from the glass vials was achieved by using HPLC area assay, although there was some loss of biological activity (data not shown). After heating in a boiling-water bath, some insoluble aggregates of degraded lysozyme were formed in the sample vials. Samples of supernatants containing the soluble fraction of protein were subsequently subjected to HPLC and turbidimetric analyses. Additional peaks with a shorter retention time than the original intact lysozyme appeared in the chromatogram and in addition the lysozyme peak diminished in size after heating (Figure 1). Most of the loss in lysozyme concentration might be due to the degradation, aggregation and subsequent removal from solution. The HPLC assay of the supernatant showed that boiling had also induced soluble fragments of the degraded protein. The amounts of lysozyme determined using the peak area method were found to be higher than those determined from peak height (Figure 2). The differences observed might have been a consequence of degradation of lysozyme to fragments, which differ only slightly in structure to the original enzyme. Such fragments may not be resolved using this method of HPLC. The data from peak height correlated well with the results from the bioassay (Figure 3), although the concentration determined by HPLC was significantly greater than that observed in the bioassay ($P < 0.0001$). This is conceivable because the disturbed secondary structure can

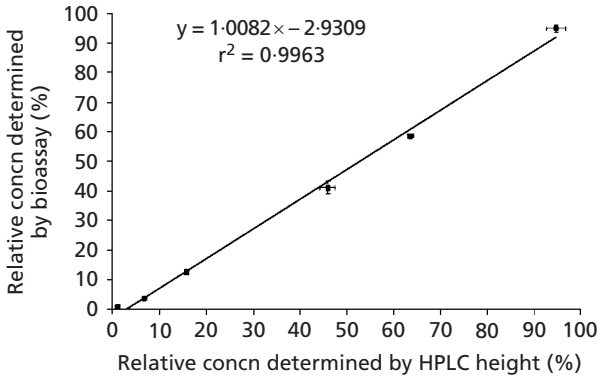

Figure 3 Correlation between the concentration of the boiled lysozyme determined by HPLC peak height and by bioassay (mean ± s.d., n = 3).

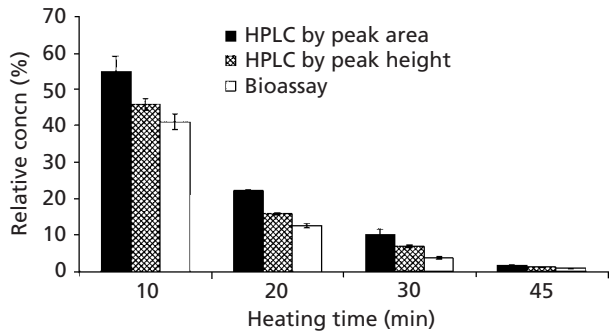

Figure 2 The preserved activity of lysozyme at a concentration of 1.0 mg mL^{-1} after heating in a boiling water bath (relative activity ± s.d., n = 3).

result in the loss of bioactivity, even though the chemical structure does not change. The soluble fraction of the boiled lysozyme samples therefore appeared to contain various forms of lysozyme: intact, active lysozyme eluting at around 37% acetonitrile; partially inactive lysozyme with an intact primary structure, but a disturbed secondary and tertiary structure, also eluting at around 37% acetonitrile; and inactive, fragmented eluting at various positions slightly to the left of the intact peak. These data suggested that the use of HPLC to determine formulated lysozyme concentration did not necessarily produce a true value for the concentration of active enzyme. Changes in the secondary and tertiary structure of lysozyme during the preparation, processing and storage of protein formulations would not necessarily be detected using HPLC and thus any decrease in the biological activity as shown in the bioassay may not be apparent.

In conclusion, the turbidimetric bioassay provided a simple, rapid and reproducible method for quantitatively determining the bioactivity of formulated lysozyme. For the HPLC method the measurement of peak area and peak height provided a reproducible method for measuring lysozyme concentration. However, the peak height method was preferred when the protein sample was partially degraded despite a significantly higher concentration being determined in comparison with that obtained from the bioassay. Having established a correlation between the two methods, either the turbidimetric bioassay or the HPLC peak height assay could be used to measure the initial concentration/purity of the formulated protein. Nevertheless, the turbidimetric assay was found to have a lower limit of detection than HPLC and was the method that provided a definitive indication of biological activity.

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