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X-ray Fluorescence Used to Characterize the Salt Content of Proteins

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The quantitative measurement of the salt content in solid protein samples was performed using X-ray fluorescence. Linear calibration curves were obtained for chloride, calcium and sulfur using sulfur and chloride as internal standards in the range 1–10 protein molar equivalents. The detection limit was ~0.02 molar equivalents for chloride and less than 0.01 molar equivalents for calcium. X-ray fluorescence thus provides a non-destructive sensitive method of testing the efficiency of different purification methods. Commercial hen egg white lysozyme samples contain from 15 to 46 molar equivalents of chloride, whereas the calcium content remains less than 0.2 equivalents. Deionization on ion-exchange resins is a very efficient tool for removing ionic species since deionized lysozyme samples contain less than 0.34 molar equivalents of chloride. Extensive dialysis against water only partially removes chloride ions, the residual chloride content corresponding to the number of counter-ions necessary to ensure the electroneutrality of lysozyme when dissolved in water.

Keywords: X-ray fluorescence; desalted proteins; chloride; calcium.

1. Introduction

Most protein preparations contain various additives because purification processes usually consist of a series of precipitation and chromatography steps that all include buffer and elution agents. These chemicals are usually salts or weak acids/bases, but can also be a variety of compounds such as divalent cations (Ca, Mg, Fe, Zn *etc.*), protease inhibitors, chelating agents (EDTA) and reducing agents (DTT, β-mercaptoethanol). In an ultimate step, dialysis can remove the excess of salts, but not the counter-ions present in solution for electrostatic compensation of the protein net charge, nor the compounds which are adsorbed.

Some life-science domains, such as crystal growth studies ('crystallogenesis'), require working with ultrapure proteins. Crystallogenesis aims at understanding the influence of various crystallization parameters and at improving crystal growth, which is a limiting step for X-ray crystallographic determination of protein structure and structure/function relationships. The composition of the protein solution has to be precisely known and controlled to lead to reproducible experiments. A high protein concentration is usually necessary to induce crystallization. Contaminants can therefore reach significant concentrations, compete with the chosen crystallization agent, and therefore have to be removed before crystallization. A potential purification method is to use ion-exchange resins, as suggested by Edsall & Wyman (1958) and recently described by Riès-Kautt, Ducruix & Van Dorsselaer (1994), to obtain isoionic protein, defined as a protein solution containing only protons and hydroxide as counter-ions. To address the problem of protein purity control analytically, a multi-element analysis is needed since the nature of the contaminants is not always known. The method also has to be very sensitive because trace analysis is involved. For instance, the presence of one molar equivalent of chloride ions in a 10 mg ml^{-1} protein solution means a chloride concentration of ~18 p.p.m. for a 20 000 Da molecular weight protein.

X-ray fluorescence has been commonly used to achieve elemental determination of very different samples, including biological ones. In particular, this technique was used in the detection of metals in metalloproteins (Cramer *et al.*, 1992; Chance, Pennie, Carman, Legallais & Powers, 1982). The determination of metals in metalloproteins separated by gel electrophoresis has been demonstrated (Stone *et al.*, 1994; Szökefalvi-Nagy, Bagyinka, Demeter, Kovacs & Le Huen Quynh, 1990). Investigations have been performed on vegetable matter (Medina, Greenhut & Lachance, 1978), animal tissues (Wallin, Deinum, Rindby & Lagercrantz, 1980) and human tissues. Preiss, Ptak & Frank (1986) determined the trace-element content of tissues such as those provided by biopsy of aorta. Non-metallic elements in proteins, such as bromide ions in tetragonal crystals of lysozyme, were also detected (Kachalova *et al.*, 1995). As far as light elements are concerned, Hebert (1975) measured

the sulfur content of toxic proteins using non-dispersive vacuum soft X-ray fluorescence spectrometry.

Energy-dispersive X-ray fluorescence spectrometry has been described for the measurement of the concentration of several elements (Fe, Cu, Zn, Br, Se and As) in human serum of healthy adults (Sky-Peck & Joseph, 1981). More recently, the method has been used to measure 16 elements at trace levels in 1 mg of human breast tissues (Rizk & Sky-Peck, 1984). Synchrotron radiation, because of the enhancement of the spectral brilliance of the source, allows detection of concentrations in the range of the p.p.m. for most elements. For example, a spectrum of Friend leukemia cell nuclei (previously incubated with an anti-cancer drug containing 1 μM Au atoms) was recorded and a minimum detectable limit of 60 pg Au was calculated for a 1000 s exposure time (Thellier *et al.*, 1993).

The aim of the work reported here is to use synchrotron X-ray fluorescence to determine element traces in protein samples. This study focuses on chloride, calcium and sulfur as realistic protein contaminants because they are likely to be used in salts, buffers or salting-out agents in usual protein purification processes. The concentrations of chloride and calcium are measured simultaneously after preliminary determination of calibration curves using sulfur as an internal standard. The two main experimental difficulties consist first in achieving a good resolution to separate the sulfur and chloride signals, and second in determining the sensitivity limit of the method since we aim to measure light-atomic-number elements.

2. Materials and methods

2.1. *Materials*

Hen egg white (HEW) lysozyme was bought from different sources: Miles (batch 017A 363234), Boehringer (batch 1303 2021-90) and Sigma (type L2879, batches 73H7045 and 51H7150). BPTI was obtained from Bayer GmbH. Haemoglobin from human blood, cytochrome c from horse heart (type VI) and α-amylase from *Bacillus Sp.* were purchased from Sigma. Collagenase was purified as previously described (Lecroisey, Boulard & Keil, 1979). $CaCl_2$ (pro-analysis, Merck) and $FeNO_3$ (Sigma) were used without further purification. The water used to prepare the solutions was commercially available deionized and three-times distilled for injectable purposes (Meram).

2.2. *Desalting of proteins*

The experimental conditions for desalting lysozyme were basically those described by Riès-Kautt, Ducruix & Van Dorsselaer (1994). About 100 mg of commercial powder was dissolved in 5 ml of distilled water (pH 5.2) and dialysed against water. The dialysed solution was mixed with 12 ml of a cation-exchange resin (Biorad AG 50W-X8, H^+ form) in a 50 ml syringe. The syringe and its contents were shaken for 1 min and the protein solution was quickly recovered through a 0.22 µm filter. The resin was then rinsed twice with 5 ml of water. The pH of the solution decreased slightly (4.9) due to the exchange of the remaining cations against protons. The anion exchange was performed using 17 ml of an anion-exchange resin (Biorad AG 1-X8 20–50 mesh OH^- form). The resin was rinsed twice with 10 ml of water to optimize the protein recovery. The pH of the final solution was 10.7, which is close to the isoelectric point of lysozyme, 10.9 (Wolbert *et al.*, 1989). The yield of the overall procedure was, on average, 70%. The desalted protein solution was then freeze-dried and kept at 253 K.

2.3. *Preparation of protein samples*

Two different procedures were employed. The first consisted of depositing $\sim$1 mg of freeze-dried powder onto a one-face kapton film. Because of the adhesive properties of this support the preparation of the sample is very simple, but X-ray fluorescence spectra recorded at several locations on the support alone showed that kapton contained some elements, mainly Si, Fe and Ca, non-uniformly distributed on the surface. The contamination level was, however, low enough to allow the use of this support for the determination of the calibration curves. For deionized proteins, samples were deposited on a polypropylene film purchased from Goodfellow (Lille, France). In this case, lysozyme powder was dissolved or suspended (depending on the solubility of the protein) in three-times distilled water to a concentration between 50 and 100 mg ml^{-1}. 5 µl of the solution (or suspension) were deposited on the film and air-dried until the spot became solid. In this procedure, 0.5 mg of protein is required for the analysis.

2.4. *Data recording*

All data were recorded at the X-ray fluorescence station on line D15 of the DCI storage ring (LURE, Orsay, France). The synchrotron radiation incident beam was monochromatized and focused on a 1 mm^2 spot by two mosaic pyrolytic graphite crystals. The selected energy can be continuously tuned between 9 and 21 keV and was chosen to enhance the sensitivity for the elements under study. A 1 mm-diameter collimator was placed 2 cm in front of the sample to eliminate scattered radiation from the monochromator exit window. The sample was placed at 45° from the incident beam. Many samples can be prepared and deposited on the same stand fixed on a sample holder that can be moved in three directions through remote-controlled step motors (Micro-Controle).

The X-ray fluorescence spectrum was recorded with an Si(Li) detector of 150 eV energy resolution and 13 mm^2 area, set at 2.7 cm from the sample in the orbital plane of the synchrotron ring and at 90° from the incident beam to minimize scattering from the sample, and thus enhanced the signal-to-background ratio. The detector was under vacuum and isolated by a 7 µm-thick Be foil. Pulses from the detector were transferred to a Tennelec TC 244 amplifier and then processed through a Nucleus Multichannel

Analyzer card set of an IBM PC. The acquisition time was set to 1000 s to improve the statistics for trace-element determination.

The X-ray fluorescence spectra were processed through a specially written program, *WAPI* (Wang, 1986; Wang, Picot, Chevallier, Legrand & Abbas, 1993). This program calculates the area of each peak and gives a list of each element with the area of its $K\alpha$ (or $L\alpha$) line corrected for air absorption and detector efficiency. These areas are directly related to the elemental composition of the sample. The deconvolution procedure is based on a non-linear least-squares algorithm, as suggested by Marquart (1963), which is a combination of the gradient and the Gauss–Newton methods. In the first step the program gives a list of the different elements found in the spectrum. Elements can be added or suppressed from this list which is then used as a first guess for the iteration procedure that continues until convergence is met. The comparison between the raw data and the result of the fit may suggest the addition of new elements to the list for a new set of iterations. At the end the program supplies a table where the area of the $K\alpha$ (or $L\alpha$) line of each element is recorded with the correction for air absorption and detector efficiency.

3. Practical aspects of quantitative analysis

The total X-ray spectrum observed corresponds to the superposition of the characteristic spectra of the atoms present in the sample. Under the experimental conditions described above, analysis of the X-ray spectra allows for the simultaneous detection of any chemical element whose atomic number is above 13. Quantification can be achieved by measuring the peak area of the signal corresponding to a given element and comparing it with a calibration curve previously determined for the same element.

3.1. *Quantitative determination of chloride and calcium*

The calibration was performed simultaneously for both calcium and chloride using $CaCl_2$. Increasing amounts of a calibrated $CaCl_2$ solution were added to 1 ml aliquots of a 2 mg ml^{-1} desalted lysozyme solution. Both solutions were mixed to ensure the homogeneity of the later solid sample. The mixture was then freeze-dried. As the amount of solid deposited on the support was not calibrated, it was necessary to use an internal standard to obtain quantitative information. The internal standard element must fulfil two conditions: first, the resolution from the measured element peak must be sufficient, while remaining within the same energy range; second, its concentration must remain constant in all the samples. Usually, it is necessary to supply the sample with an additional element to use this method. The advantage of using the sulfur signal, from cysteine and methionine residues of the protein, as an internal standard is to preserve the quality of the sample in terms of purity.

Once the calibration curves are established for one protein, *e.g.* lysozyme, they can be used for further proteins with respect to the internal sulfur content of each protein. For example, lysozyme (MW 14305 Da) contains ten sulfur atoms, *i.e.* 2.2% *w/w*, and BPTI (MW 6510 Da) contains seven, *i.e.* 3.4% *w/w*. To deduce the molar equivalent content of an element in BPTI from the calibration curves obtained with lysozyme, the corresponding peak (normalized by sulfur) has to be multiplied by the ratio 3.4/2.2.

The calibration curves are shown in Fig. 1. The ratio of the peak surface of Ca or Cl to the peak surface of protein S are given as a function of the concentrations of calcium and chloride, expressed as molar equivalents of ions per mole of protein. For both elements the calibration curve is linear. For a given concentration the chloride peak area is significantly lower than that of the calcium, as expected from the X-ray fluorescence detection efficiency which is a function of the atomic number (elements of greater atomic number have a greater sensitivity). The calculated calibration line for chloride passes through the origin, confirming that the contribution of the kapton film can be neglected. This is also an indication that desalted lysozyme, used as a matrix for the calibration curve determination, contains only traces of chloride. However, in the case of calcium, contamination of the film can no longer be neglected for a calcium concentration lower than 0.1 molar equivalents.

Experimental errors were estimated by analyzing a desalted lysozyme sample, to which five molar equivalents of chloride were added and then deposited on a polypropylene film. Reproducibility tests were based on the recording of nine aliquots from this sample, each being counted separately. The relative error on the mean value was ~3%. This represents the sum of the errors introduced into a single measurement by each step of the analyzing procedure. To study further how much instrumental variation or data processing contributes to the counting variability, precision

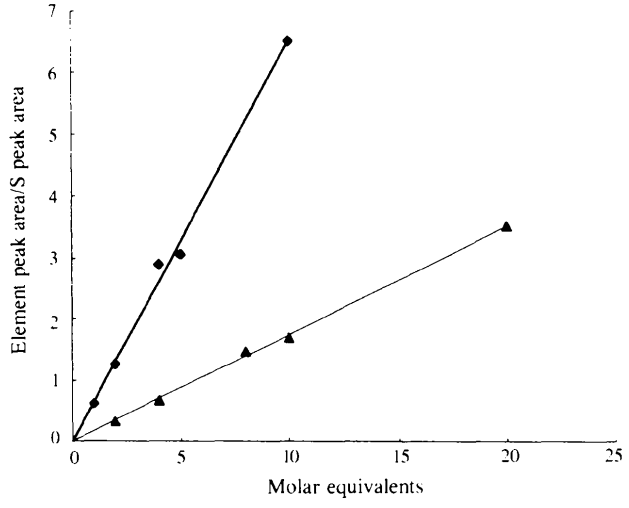

Figure 1
Calibration curves of calcium (♦) and chloride (▲) with lysozyme. Solid protein samples (~ 1 mg) were deposited on a kapton film. The photon energy of the incident beam was 12 eV. The counting time was 1000 s.

data were obtained by counting one single spot six times. The variation in the relative chloride peak area standardized to the sulfur peak area was <0.2%. As a consequence, the greatest source of error was due to the sample preparation rather than being instrument- or procedure-dependent. The same reproducibility test was applied to estimate the experimental error in a protein sample with low ion content. The X-ray fluorescence spectrum of this sample showed that the chloride and calcium peak areas were very similar. However, the relative error was 10% at 0.2 molar equivalents of chloride, and increased to 21% for 0.1 molar equivalents of calcium. The relative error for calcium is thus twice as high as that for chloride, which is rather surprising since the calcium peak is better resolved from the sulfur peak than the chloride peak.

Another important parameter when measuring low concentrations concerns the detection limit of the method. The detection limit depends on the number of X-ray photons detected, the background at the peak under consideration, the counting time and the statistical treatment used. The minimum detection signal for a given element Z can be set to

$$s_m = 3N_B^{1/2}, \quad (1)$$

where N_B is the background signal.

According to the calibration curve used, the detection limit for Z can therefore be expressed in terms of concentration, c_L, as

$$c_L = (1/\Delta)s_m c_F/S_{\text{corr}}, \quad (2)$$

where Δ is the slope of the calibration curve, c_F is the correction factor accounting for the air absorption of the Z signal, and S_{corr} is the sulfur signal, corrected for absorption.

The detection limit calculated using (2) is less than 0.01 molar equivalents for both Cl and Ca for each point of the calibration curve. The sensitivity is thus 13 ng for Cl for a 1000 s acquisition time. For comparison, Hebert (1975) determined the sulfur content of toxic protein samples by non-dispersive vacuum soft X-ray fluorescence and obtained a sensitivity of 50 ng in a 240 s run.

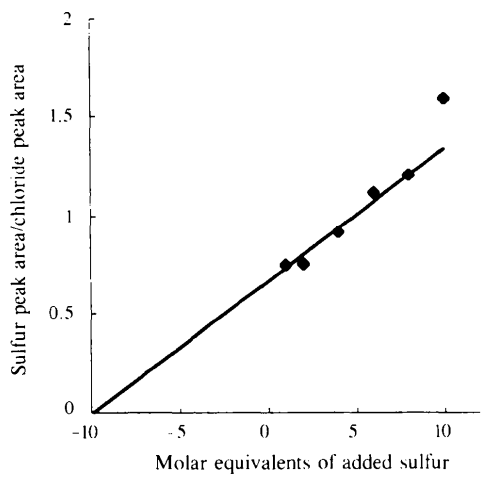

Figure 2
Calibration curve of sulfur with lysozyme. Solid protein samples (~1 mg) were deposited on a kapton film. The photon energy of the incident beam was 10 eV. The counting time was 1000 s.

Fig. 2 shows that it is possible to assess the sulfur content of lysozyme by measuring the area of the sulfur peak normalized by the area of chloride as an internal standard. Although sulfur and chloride have successive atomic numbers, the resolution was sufficient to obtain a linear calibration curve, provided that the concentration of sulfur added to the protein standard remained less than 10 molar equivalents. Above this limit, the calibration curve is no longer linear: the chloride signal becomes too small to be correctly resolved from that of sulfur. From the intersection of the linear domain with the x axis, it can be checked that the protein matrix, *i.e.* lysozyme ($C_{613}H_{951}N_{193}O_{185}S_{10}$), contains ten S atoms, which confirms the reliability of the calibration curve.

4. Results and discussion

4.1. *X-ray fluorescence of lysozyme*

Fig. 3(*a*) shows a typical spectrum of commercial lysozyme. The largest peak on the right-hand part of the spectrum close to 10 keV is due to Rayleigh and Compton scattering of the sample. The argon peak is due to the air path travelled by the X-ray beam. Because of the absorption of the signal for the lightest elements in the air path, sulfur is the only element of the protein matrix whose signal is detected. The most important difference between the desalted (Fig. 3*b*) and commercial protein (Fig. 3*a*) concerns their chloride content: desalted lysozyme contains only traces of chloride. Because of the depletion in chloride, the argon peak is much better resolved in the desalted lysozyme spectrum. As shown in Fig. 3(*b*), and also in each desalted protein batch we tested, the sample contains Ca, Fe, Zn and Cu traces. Comparison with the polypropylene spectrum indicates that these elements are also detected in the support alone but to a much lesser extent. As Fe is the most important protein contaminant after desalting, its concentration was determined by comparison with a protein sample to which 0.2 molar equivalents of Fe had been added. From the Fe peak areas (normalized to sulfur peak area used as an internal standard) it was calculated that deionized lysozyme contains less than 10^{-3} molar equivalents of Fe.

4.2. *Results for commercial protein samples*

The amounts of chloride and calcium contained in lysozyme samples were calculated using the calibration curves. Lysozyme batches of three different commercial sources were tested without any further purification. The salt contents are known to differ from one source to another, and also from one batch to another. Table 1 shows that

each of these samples contains between 3.6 and 10.2% *w/w* of chloride. A 10.2% *w/w* chloride content corresponds to 46 mol of chloride for 1 mol of lysozyme. In other words, a 10 mg ml^{-1} lysozyme solution contains 32 m*M* of chloride. It must be recalled that the high-purity-grade lysozyme is three-times crystallized, obviously as a chloride salt.

When dissolved in distilled water, the pH of commercial lysozyme (batch 1) is measured between 5 and 6, corresponding to 9–11 counter-ions per protein molecule for electrostatic compensation, whereas ~15 molar ionic equivalents were found in this batch. As a consequence, additional cations must be present in the sample as counter-ions of chloride ions in excess. These counter-ions are not Ca^{2+} since the amount of calcium is less than one equivalent in all the samples. A significant potassium signal was detected in the lysozyme sample from batch 1 and

Table 1
Calcium and chloride content of commercial proteins.

Results are the mean of two acquisitions. Experimental error is estimated from reproducibility tests.

	Chloride *w/w* (%)	Chloride (molar equivalents)	Calcium (molar equivalents)
Lysozyme			
Batch 1	3.5 ± 0.1	14.7 ± 0.5	0.21 ± 0.04
Batch 2	4.4 ± 0.1	18.7 ± 0.6	0.17 ± 0.03
Batch 3	6.5 ± 0.2	28.1 ± 0.8	0.05 ± 0.01
Batch 4	10.2 ± 0.1	46.3 ± 4	0.033 ± 0.006
α-Amylase	0.030 ± 0.003	0.87 ± 0.03	20.6 ± 4.2
BPTI	7.0 ± 0.2	13.8 ± 0.4	0.33 ± 0.06
Haemoglobin	–	<0.011*	0.17 ± 0.04
Cytochrome c	–	<0.028*	0.19 ± 0.05
Collagenase	4.3 ± 0.1	32 ± 1	0.09 ± 0.02

* Chloride content is below the detection limit.

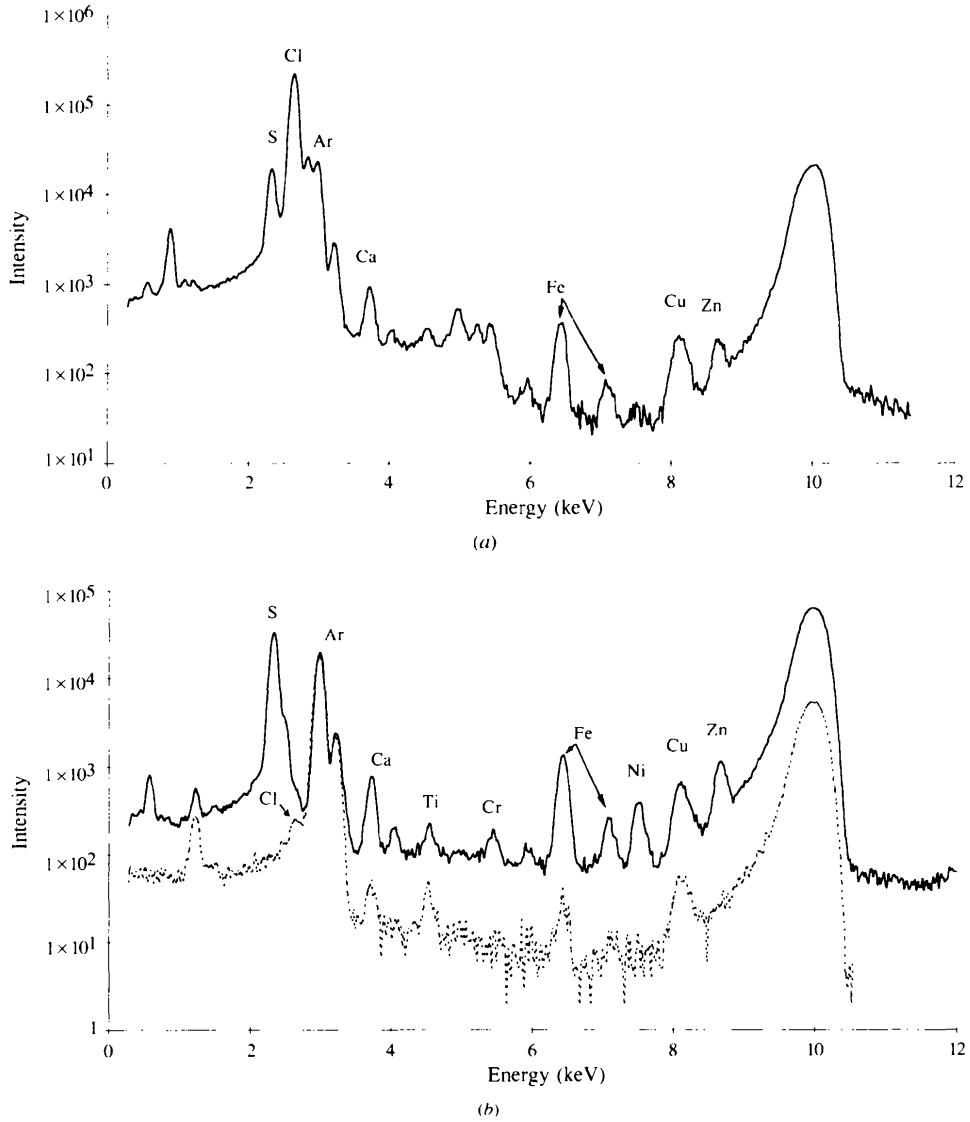

Figure 3
X-ray fluorescence spectra. (*a*) Commercial lysozyme on polypropylene film. (*b*) Desalted lysozyme on polypropylene film (solid line) and polypropylene film at the same position before the protein sample had been deposited (dotted line). The counting time was 1000 s. The photon energy of the incident beam was 10 keV.

Table 2
Calcium and chloride content of desalted proteins.

Results are the mean of two acquisitions. Experimental error is estimated from reducibility tests to 20% for both Cl and Ca. The detection limit was less than 0.02 molar equivalents for Cl and 0.004 molar equivalents for Ca. The counting time was 1000 s. The photon energy of the incident beam was 10 keV.

	Calcium (molar equivalents)	Chloride (molar equivalents)
Lysozyme		
Batch 1		
Purification 1	0.10 ± 0.02	0.17 ± 0.04
Purification 2	0.089 ± 0.018	0.042 ± 0.008
Batch 2		
Purification 1	0.015 ± 0.003	0.006 ± 0.003
Purification 2	0.036 ± 0.007	0.16 ± 0.03
Purification 3	0.023 ± 0.005	0.046 ± 0.009
Purification 4	0.026 ± 0.005	0.34 ± 0.07
BPTI	0.16 ± 0.03	<0.28*

* Chloride content is below the detection limit.

batch 2, but none from batch 3. For the latter case, the counter-ion is probably Na^+ but this element has an atomic number too low to be detected by X-ray fluorescence.

Table 1 also shows the calcium and chloride content of various proteins. Two of them, cytochrome c and haemoglobin, are free from both chloride and calcium. On the other hand, collagenase, purified from *Hypoderma lineatum* by ion-exchange chromatography using a gradient of NaCl, followed by a dialysis step against distilled water (Lecroisey, Boulard & Keil, 1979), contains more than 30 molar equivalents of chloride.

4.3. *Comparison of the chloride and calcium content of dialysed and desalted proteins*

The efficiency of dialysis as a tool to remove ions was examined. After dialysis of commercial batch 1 lysozyme against distilled water, the chloride content of the protein sample decreases from 15 to 10 molar equivalents. Thus, only a third of the chloride that could migrate through the dialysis membrane was removed. At this pH (between 5 and 6), lysozyme has about ten positive charges, as calculated from its amino-acid composition and the values of the pK of the charged side-chains (Patrickios & Yamasaki, 1995). The detected chloride could thus be the counter-ions necessary to ensure the electroneutrality of the solution.

Dialysed lysozyme was further purified by an ion-exchange step to yield desalted lysozyme, as referred to hereafter. Table 2 gives the chloride and calcium content of desalted lysozyme from two different batches. Each batch was divided into several samples. The purification procedure, *e.g.* dialysis followed by ion exchange, was performed on each sample separately. From X-ray fluorescence measurements, the chloride content of desalted lysozyme varies from 0.006 to 0.34 molar equivalents, which corresponds to 16–900 p.p.m, respectively. Since the protein contained up to 10 molar equivalents after the dialysis step, the ion-exchange step removed more than 97% of the chloride ions in the protein sample. Even for the less efficient purification, the chloride content of desalted lysozyme is low enough to prevent any interference in crystallization experiments.

The calcium content did not significantly decrease from the dialysed to desalted lysozyme. It remained ~0.03 molar equivalents in any tested batch.

The experimental error was estimated from the standard deviation of the mean of six independent acquisitions of the same desalted lysozyme sample. The relative error was ~20% for both the chloride and the calcium contents. Such a large imprecision could be explained by a technical difficulty: solutions of desalted lysozyme in water at a concentration higher than 40 mg ml^{-1} could not be achieved. The protein was not dissolved but dispersed in water, which could induce a heterogeneity between the different spots of the same protein sample deposited on the polypropylene film. However, it could be noticed that the relative error in the chloride content of desalted lysozyme (~20%) was lower than the standard deviation of the mean chloride molar concentration of the six different desalted samples: 0.114 ± 0.124, which seems to indicate that the reproducibility of the desalting procedure could be improved.

For BPTI, a desalting step was performed without previous dialysis, which did not affect the quality of the purification since less than 0.3 molar equivalents of chloride and less than 0.2 molar equivalents of calcium were detected (Table 2).

4.4. *Ion replacement*

In previous work, Riès-Kautt & Ducruix (1989) pointed out that the ability of different anions to crystallize lysozyme can be classified following the reverse order of the Hofmeister series. Among the tested ions, it was shown that thiocyanate was the most efficient in reducing the solubility of lysozyme. To test the displacement of Cl^- by SCN^-, successive dialysis steps of lysozyme were achieved against 0.1 *M* KSCN (Fig. 4). X-ray fluorescence measurements performed after this treatment did not detect chloride in the protein sample: all of them had been displaced from the protein vicinity to the bulk of the

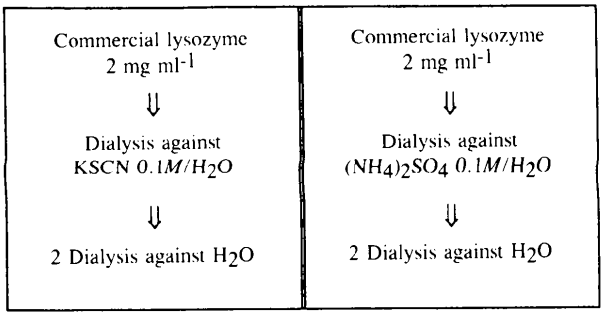

Figure 4
Dialysis procedure of lysozyme samples containing chloride against thiocyanate and sulfate.

solution in the presence of thiocyanate. In other words, chloride ions have been exchanged against thiocyanate, whose affinity for lysozyme is therefore significantly higher than for chloride, although the equilibrium constant of the association of lysozyme with thiocyanate was found to be too low to be precisely determined by a classical equilibrium dialysis method (Guilloteau, 1991).

The crystallization of lysozyme in the presence of sulfate ions also presents some particular features. Indeed, desalted lysozyme was shown to crystallize at pH 8 in the presence of 3–9 molar equivalents of sulfate (Riès-Kautt, Ducruix & Van Dorsselaer, 1994), whereas lysozyme is known to resist crystallization with ammonium sulfate at pH 4.5, which is the most commonly used among crystallizing agents. Ion-spray mass spectroscopy experiments (Riès-Kautt, Ducruix & Van Dorsselaer, 1994) showed non-covalent adsorption of sulfate ions on the protein. In order to test the relative affinity of sulfate and chloride for lysozyme, the same dialysis procedure as described above (Fig. 4) was performed with sulfate. X-ray fluorescence shows that chloride was also eliminated from the sample. Sulfate ions, at a concentration of 0.1 *M*, thus seem to have a greater affinity for lysozyme than chloride ions. This is in agreement with SAXS measurements of lysozyme solutions at pH 4.5 in the presence of various salts (Ducruix, Guilloteau, Riès-Kautt & Tardieu, 1996), showing that the efficiency of anions to bring the repulsive protein–protein interactions towards attractive ones follows the sequence $SCN^- > NO_3^- > SO_4^{2-} > Cl^-$.

5. Concluding remarks

X-ray fluorescence techniques have been used in the determination of the quantitative content of inorganic traces in solid protein samples. It has been shown that the purification of lysozyme by a deionization method decreases the chloride content of the protein to less than one molar equivalent. By comparison, supplied commercial samples were found to contain up to about 50 times more chloride. This needs to be taken into account when undertaking crystallization experiments because a better knowledge and control of the solution composition can certainly improve the reproducibility of the crystallization experiment. Furthermore, the type and the concentration of the protein counter-ions may induce polymorphism, as illustrated by the crystallization of lysozyme in the presence of nitrate. When dialyzed lysozyme is crystallized with $NaNO_3$, triclinic crystals are observed for $NaNO_3$ concentrations higher than 0.2 *M* (Guilloteau, Riès-Kautt & Ducruix, 1992) while tetragonal crystals grow below 0.1 *M* $NaNO_3$ (Guilloteau, 1991). Indeed, at 0.1 *M* $NaNO_3$, lysozyme concentrations up to 360 mg ml^{-1} are required, implying a presence of 250 m*M* counter-ion (Cl) and 50 m*M* acetate from the buffer which compete with only 0.1 *M* $NaNO_3$. If nitric acid is used to bring desalted lysozyme to pH 4 instead of using acetate buffer, monoclinic crystals are obtained over the whole range of $NaNO_3$ concentration (Retailleau, Riès-Kautt & Ducruix, 1996).

X-ray fluorescence experiments have shown that extensive dialysis against water does not remove all chloride ions from a lysozyme solution. The measured chloride content was shown to correspond to the amount necessary to ensure the electroneutrality of the protein solution. Chloride was eliminated from the protein solution by dialysis in the presence of thiocyanate or sulfate. Further experiments are required to measure the concentration of these ions in solution and thus to evaluate their relative affinity for lysozyme.

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