*Acta Cryst.* (1996). D**52**, 776–784

# Heterogeneity Determination and Purification of Commercial Hen Egg-White Lysozyme

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*(Received 18 October 1995; accepted 26 February 1996)*

## Abstract

Hen egg-white lysozyme (HEWL) is widely used as a model protein, although its purity has not been adequately characterized by modern biochemical techniques. We have identified and quantified the protein heterogeneities in three commercial HEWL preparations by sodium dodecyl sulfate polyacrylamide gel electrophoresis with enhanced silver staining, reversed-phase fast protein liquid chromatography (FPLC) and immunoblotting with comparison to authentic protein standards. Depending on the source, the contaminating proteins totalled 1–6%(*w*/*w*) and consisted of ovotransferrin, ovalbumin, HEWL dimers, and polypeptides with approximate $M_r$ of 39 and 18 kDa. Furthermore, we have obtained gram quantities of electrophoretically homogeneous [> 99.9%(*w*/*w*)] HEWL by single-step semi-preparative scale cation-exchange FPLC with a yield of about 50%. Parallel studies of crystal growth kinetics, salt repartitioning and crystal perfection with this highly purified material showed fourfold increases in the growth-step velocities and significant enhancement in the structural homogeneity of HEWL crystals.

## 1. Introduction

Over the last few years it has become apparent that protein crystallization is influenced by impurity levels that are widely considered inconsequential for biochemical studies; see, in particular, a series of studies by Giegé and coworkers (Giegé *et al.*, 1986; Lorber & Giegé, 1992; Lorber, Skouri, Munch & Giegé, 1993; Skouri, Lorber, Giegé, Munch & Candau, 1995). Ideally, proteins utilized in crystallization and crystallography studies have uniform physical and biochemical properties. In practice, however, even most modern biochemical preparation techniques, such as high-performance liquid chromatography (HPLC), can only minimize, but not eliminate protein heterogeneity. Hence, proteins must be viewed statistically as populations of molecules defined by limits on a range of commonly held characteristics. For definitive, reproducible crystallization studies, the heterogeneity of protein samples needs to be minimized and quantified. Such quantitative definitions of protein preparations typically require the synergistic application of various bioanalytical techniques, such as electrophoresis, immunoassay, spectrophotometric assays and high-performance liquid chromatography.

Hen egg-white lysozyme (HEWL), E.C. 3.2.1.17 mucopeptide *N*-acetylmuramoyl-hydrolase, has been the protein most widely used in crystallization studies. Despite this central role, the heterogeneity of HEWL has not been adequately defined by modern biochemical techniques. Increased purity of HEWL has been recognized by various investigators as important for crystal growth and further purification was attempted (Durbin & Feher, 1986). Similarly, Abergel, Nesa & Fontecilla-Camps (1991) deliberately introduced protein contaminants into turkey egg-white lysozyme solutions, and found that nucleation and crystal morphology were affected. Monaco & Rosenberger (1993) observed the formation of macrosteps or step bunching on growing HEWL crystal surface at low supersaturations, which is indicative of the presence of large protein impurities. Macrostep formation and growth cessation at low HEWL supersaturations were also more pronounced in a relatively impure HEWL preparation than in a preparation believed to possess greater purity (Vekilov, 1993; Vekilov, Ataka & Katsura, 1993, 1995). These studies also revealed lower normal growth rates and lower tangential (average step) velocities in growth from the less pure solutions.

In this study, we define HEWL purity utilizing highly resolving and sensitive biochemical techniques. In addition, we describe the purification of a commercial HEWL preparation to electrophoretic homogeneity [> 99.9%(*w*/*w*)].

## 2. Materials and methods

HEWL preparations referred to as SG, BM and SK, respectively, were obtained from Sigma Chemical Co. (St Louis, MO, USA), Boehringer Mannheim (Indianapolis, IN, USA) and Seikagaku America, Inc. (Ijamsville, MD, USA). Buffers, reagents, trimethylchlorosilane, ovalbumin (OVA), ovotransferrin (OVT, conalbumin), ovomucoid, rabbit anti-avidin antibody and rabbit anti-ovalbumin antibody were obtained from Sigma. The *Micrococcus luteus* substrate for HEWL

activity analysis was obtained from Boehringer Mannheim. Goat anti-rabbit IgG (HRP conjugate), rabbit anti-HEWL antibody, acetonitrile (ACN), trifluoroacetic acid (TFA) and an octadecyl bonded phase, 5 μm particle size, 300 Å pore size, 12.5 cm length × 0.46 cm internal diameter HPLC cartridge were acquired from Fisher Scientific (Pittsburgh, PA). A Milli-RO/Milli-Q water purification system, Sterivex 0.45 μm and Millex-HV 0.45 μm filters from Millipore (Bedford, MA, USA) were used.

### 2.1. *Polyacrylamide gel electrophoresis and blotting*

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) analyses were carried out with a PhastSystem electrophoresis unit (Pharmacia Biotech, Uppsala, Sweden) utilizing 12.5%T, 2%C homogeneous and 8–25%T, 2%C gels. Coomassie brilliant blue R-250 staining solutions contained 0.1%(*w*/*w*) dye, 10% acetic acid and 20% ethanol (Neuhoff, Stamm & Eibl, 1985). Gels were stained for 1 h, then destained in the same solution-less dye. A silver staining protocol (Heukeshoven & Dernick, 1985) and an enhanced silver staining protocol (Heukeshoven & Dernick, 1988) were used. Both were silver nitrate methods adapted for use in the PhastSystem. Diffusion (Western) blotting to nitrocellulose membranes was accomplished, with the membranes soaked and transferred in 20 m*M* CAPS buffer pH = 11.0, 0.2 *M* NaCl, for HEWL dimer identification (Gianazza *et al.*, 1995). For OVA identification, the membranes were soaked and transferred in water. Both transfers were made by simple diffusion on the PhastSystem separation bed at 343 K. Horseradish peroxidase (HRP) conjugate and 4-chloro-1-naphthol substrate system was used to develop the blots. Rabbit antibodies to HEWL, OVA and avidin were used as primary antibodies with a goat anti-rabbit IgG secondary antibody conjugated to HRP. Gels and blots were scanned for densitometric quantification as suggested by Patton (1995). The OVA protein standard was obtained in two forms (Sigma Chemical Co.). One contained S ovalbumin resulting in two bands (two band) in SDS–PAGE and reversed-phase FPLC, while the other did not (one band). OVA with the single band was used for quantitative analysis, although two-band OVA yielded similar results.

### 2.2. *Fast protein liquid chromatography (FPLC)*

The FPLC system used was a basic Pharmacia unit with a 280 nm fixed-wavelength detector, two pumps and a simple controller/integrator. Cation-exchange FPLC (CIE FPLC) was performed with a HiLoad 26/10 SP Sepharose high performance column. SK (2 g) was purified by dissolving it in 100 ml of 5 m*M* sodium acetate buffer pH = 4.5, passing it through a Sterivex 0.45 μm filter, then loading it onto the CIE column in the same buffer. The column was equilibrated with 200 ml of 20 m*M* CAPS buffer pH = 11.0 and then the HEWL was eluted at 8 ml $min^{-1}$ with a linear salt gradient to 0.2 *M* in 1 h. The HEWL peak eluted at about 0.1 *M* NaCl. Each 8 ml fraction was collected into 0.1 ml of 0.8 *M* sodium acetate buffer to bring the pH of the solution to 4.5. Fractions were stored at 278 K. Aliquots (10 μl) of each fraction were diluted into 1 ml water in test tubes silanized with trimethylchlorosilane and their 280 nm absorbance was measured and converted to HEWL concentration with 2.64 AU = 1 mg HEWL $ml^{-1}$. The same samples were used for specific activity determinations with 10 μl aliquots of each diluted fraction added to a 1 ml reaction mixture containing 3 mg *Micrococcus luteus* substrate 10 $ml^{-1}$ of 40 m*M* sodium phosphate buffer pH = 7.0. Activity was measured by a decrease in absorbance from 1.0 AU at 450 nm. This procedure was similar to that of Shugar (1952) although a more recent study has indicated that higher pH and lower ionic strength yield higher activity (Davies, Neuberger & Wilson, 1969). The older method was used for better comparison to literature activity values. Reversed-phase FPLC (RP FPLC) analyses of HEWL samples were carried out in the same FPLC system, employing a reversed-phase HPLC column with a mobile phase consisting of 0.1% TFA in water (buffer *A*, pH = 2.0) with a gradient to 20% water/ACN with 0.1% TFA (buffer *B*). With a 0.75 ml $min^{-1}$ flow rate the gradient was 0–30% *B* in 5 ml, 30–40% *B* in 20 ml and 40–100% *B* in 5 ml. This gradient was optimal for HEWL resolution.

## 3. Results

### 3.1. *Commercial HEWL preparations*

As indicated by SDS–PAGE (see Figs. 1*a*–1*c*) SG was contaminated by $M_r \simeq 78$, 66, 28 and 18 kDa polypeptide bands. BM was contaminated by $M_r = 66$, 28 and 18 kDa polypeptide bands. An 8–25%T SDS gel (not shown) indicated that polypeptide bands with a $M_r < 14$ kDa and with a $M_r > 78$ kDa were also present in BM. Hence, although overall more pure than SG, BM had a wider molecular weight range of contaminating proteins. SK was contaminated only by $M_r \simeq 39$ (trace), 28 and 18 kDa polypeptide bands. Hence, SK, which was also the most soluble preparation and resulted in the highest recoveries of purified HEWL, was used for further purifications; see §3.3. The SG and BM were more difficult to filter (0.45 μm) than the SK at 20 mg $ml^{-1}$ in 5 m*M* sodium acetate pH = 4.5 and the solutions were turbid.

In addition to the differences in heterogeneity of the three preparations, Figs. 1(*a*)–1(*c*) demonstrate the relative sensitivity of three staning techniques. Loaded with identical samples, gel (*a*) was stained with Coomassie blue, gel (*b*) with a silver stain and gel (*c*) with a silver stain that provided enhanced sensitivity.

The enhanced silver stain was approximately tenfold more sensitive than the normal silver stain and 100–1000-fold more than Coomassie blue staining. However, one sees that many of the protein impurities can be discerned with the relatively insensitive Coomassie blue stain.

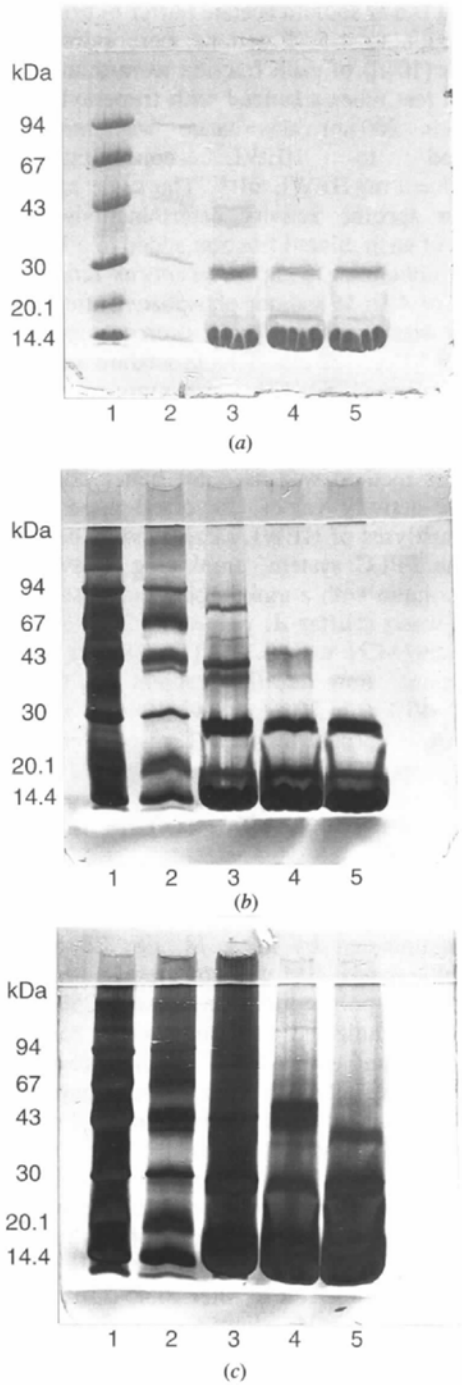



Fig. 1. Comparison of HEWL's and staining techniques in SDS–PAGE. 12.5% T homogenous PhastSystem gels, each lane loaded with 4 µl. Samples applied to each gel (*a–c*) were identical: (lane 1) LMW standards, (lane 2) LMW standards diluted 1:20, (lane 3) SG 10 mg ml$^{-1}$, (lane 4) BM 10 mg ml$^{-1}$, (lane 5) SK 10 mg ml$^{-1}$. Staining: (*a*) Coomassie brilliant blue R-250; (*b*) standard silver stain; (*c*) enhanced sensitivity silver stain.

### 3.2. *Identification and quantification of protein contaminants in HEWL*

The contaminating proteins in the three HEWL samples (Figs. 1*a*–1*c*) were identified by their known presence in egg white, their molecular weight in SDS–PAGE (Figs. 2*a*–2*c*), the binding of specific antibodies in immunoblotting (Figs. 3*a* and 3*b*) and RP FPLC retention times (Fig. 4), all compared to authentic protein standards. The unambiguously identified impurities include OVT (78 kDa), OVA (66 kDa) and HEWL dimer (28 kDa). While the polypeptide found at $M_r = 39$ kDa (lane 5 in Figs. 1*b* and 1*c*) is possibly ovomucoid, the $M_r = 18$ kDa component (lanes 3–5 in Figs. 1*a*–1*c*) was shown not to be avidin. For quantifications of the impurities by scanning densitometry, concentration *versus* optical density curves were established for the SDS gels and immunoblots with authentic standard proteins. The results are summarized in Table 1. In the following, some details are given for the various impurity components.

3.2.1. *Ovotransferrin*. Since no ready source for an antibody to OVT was located, identification and quantification were performed only in SDS gels with reference to an authentic OVT sample, Fig. 2(*a*).

3.2.2. *Ovalbumin*. OVA was identified and quantified by comparison to an authentic OVA standard in SDS gels (Fig. 2*b*) and in immunoblots (Fig. 3*a*). OVA transferred well to nitrocellulose under standard conditions. The quantity of OVA as determined by scanning densitometry of SDS gels and immunoblots differed on average only ±5%.

3.2.3. *HEWL dimer*. This multimer was quantified by comparison to the monomer in SDS gels (Fig. 2*c*). Although HEWL did not transfer well to nitrocellulose even at the high pH = 11.0 chosen to increase hydrophobicity and, thus, nitrocellulose binding (Gianazza *et al.*, 1995), binding to the 28 kDa polypeptide was reproducible in three immunoblots (Fig. 3*b*). In blots developed in the absence of the primary HEWL antibody the 28 kDa bands did not stain indicating that this band was not an endogenous egg-white peroxidase. The BM contained much less dimer than the SG and SK (Fig. 2*c*). According to the manufacturers, the SG and SK were purified by the 'isoelectric method', involving pH values at 9.5–10 (Tallan & Stein, 1953; Hamaguchi & Funatsu, 1959, respectively), while BM was purified by an affinity-chromatography method. We found in CIE FPLC separations (§3.3) at pH = 12.0 and low ionic strength (not shown) that many HEWL fractions assayed by SDS–PAGE contained dimers, tetramers and hexamers.

Table 1. *Summary of identification and quantification of contaminating proteins in commercial HEWL*

| Contaminant | Sigma (SG) Lot 15H7090 | Boehringer-Mannheim (BM) Lot 13618726-94 | Seikagaku (SK) Lot E 94203 |
|---|---|---|---|
| OVT (78 kDa) | 0.2%(*w*/*w*)* 0.04 mol% | None detected | None detected |
| OVA (43 kDa) | 3.8%† 0.8 mol% | 2.2%† 0.5 mol% | None detected |
| 39 kDa (Ovomucoid?) | None detected | None detected | Trace (< 0.1%) |
| HEWL Dimer (28 kDa) | 0.7%* as monomer 0.4 mol% | 0.45% 0.2 mol% | 0.5% 0.3 mol% |
| 18 kDa (not avidin) | 1.0%* as HEWL 0.8 mol% | 1.1% 0.9 mol% | 1.0% 0.8 mol% |
| Total | 5.7%(*w*/*w*) 2.0 mol% | 3.8%(*w*/*w*) 1.6 mol% | 1.5%(*w*/*w*) 1.1 mol% |

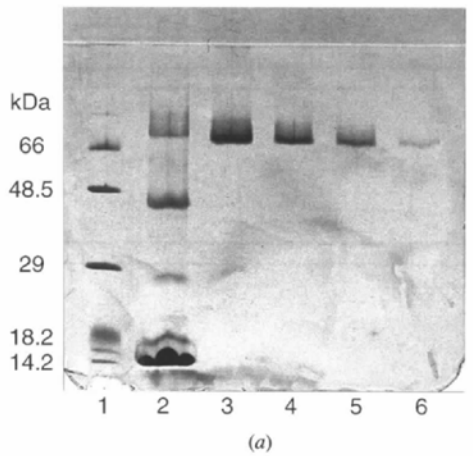
* Determined by comparison to authentic proteins in SDS–PAGE with scanning densitometry. † Determined by comparison to authentic proteins in SDS–PAGE and immunoblots with scanning densitometry.

These multimers appeared to form as a result of high pH and perhaps low ionic strength. Since they did not dissociate in the SDS sample treatment, these multimers were likely to be covalently bound and were not in equilibrium with the monomer.

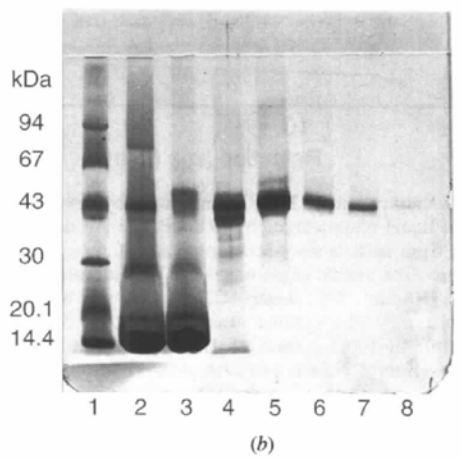
3.2.4. *18 kDa polypeptide.* Antibodies to avidin, an egg-white protein with a dissociated $M_r = 18$–19 kDa in SDS–PAGE and a high $pI \simeq 10.4$, did not bind the $M_r \simeq 18$ kDa polypeptide in immunoblotting (not shown), but did bind authentic avidin. This indicates that the unknown protein was not avidin. For the quantification of this component, the same staining behavior as that of HEWL was assumed.

3.2.5. *39 kDa polypeptide.* The identity of this trace impurity in SK was also not determined. It was not bound by specific OVA antibodies in immunoblots (Fig. 3*a*). The migration distance of this polypeptide was similar to an authentic ovomucoid standard in SDS gels (not shown) and may indicate a barely detectable contamination (< 0.1%) by that egg-white protein.

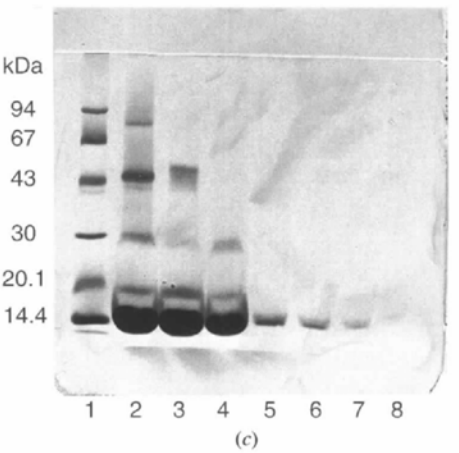
RP FPLC retention times of authentic OVT and OVA were compared to peaks in the three heavily loaded (10 mg injection$^{-1}$) HEWL samples, Fig. 4. One sees the following correspondences: peak 1 in the OVT separation with peak 9 in SG; and peak 2 in the




(*a*)




(*b*)




(*c*)

Fig. 2. Identification and quantification of HEWL impurities by SDS–PAGE with enhanced silver staining. (*a*) Ovotransferrin. Loads: 4 µl. (Lane 1) Sigma LMW silver standards, standard silver staining, (lane 2) SG 10 mg ml$^{-1}$, (lane 3) 0.1 µg OVT µl$^{-1}$, (lane 4) 0.05 µg OVT µl$^{-1}$, (lane 5) 0.025 µg OVT µl$^{-1}$, (lane 6) 0.01 µg OVT µl$^{-1}$. (*b*) Ovalbumin. Loads: 1 µl. (Lane 1) LMW standards diluted 1:20, (lane 2) SG 10 mg ml$^{-1}$, (lane 3) BM 10 mg ml$^{-1}$, (lane 4) 0.5 µg OVA µl$^{-1}$ (2 bands), (lane 5) 0.5 µg OVA µl$^{-1}$ (1 band), (lane 6) 0.1 µg OVA µl$^{-1}$, (lane 7) 0.05 µg OVA µl$^{-1}$, (lane 8) 0.01 µg OVA µl$^{-1}$. (*c*) HEWL dimer. Loads: 1 µl. (Lane 1) LMW standards diluted 1:20, (lane 2) SG 10 mg ml$^{-1}$, (lane 3) BM 10 mg ml$^{-1}$, (lane 4) SK 10 mg ml$^{-1}$, (lane 5) 0.1 µg HEWL µl$^{-1}$, (lane 6) 0.05 µg HEWL µl$^{-1}$, (lane 7) 0.025 µg HEWL µl$^{-1}$, (lane 8) 0.01 µg HEWL µl$^{-1}$.

OVA separation with peak 8 in SG, peak 14 in BM, and peak 11 in SK. Both authentic standards OVT and OVA contained substantial impurities which limited quantitative analysis by the common HPLC external standard quantification methods. OVA used in the RP FPLC studies contained S-ovalbumin (two band) which may be the later eluting peak in that separation. For other quantitiative analyses of OVA we used OVA without S-ovalbumin (one band), *e.g.* SDS–PAGE. Note the early eluting and relatively hydrophilic peaks in BM, which were not apparent in the other two preparations. Because RP HPLC binds and separates small molecules ($M_r < 1000$) some of the impurity peaks in the chromatograms may be those of small molecules which absorb at 280 nm. Fig. 4 also shows that most of the protein impurities eluted later than the highly hydrophilic HEWL. The HEWL peak(s) was relatively broad because of the gradient selected to yield maximum separation of HEWL from the impurities. SDS–PAGE separations of the HEWL RP FPLC fractions (not shown) indicated that the $M_r \simeq 18$ kDa and the HEWL dimer were not separated from HEWL under these conditions.

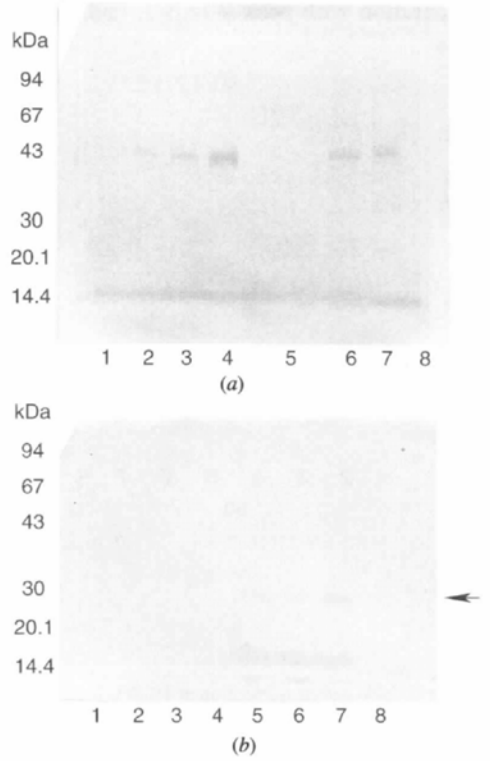



Fig. 3. Identification and quantification of HEWL impurities by immunoblotting (Western blots). (*a*) Ovalbumin. Loads: 1 µl. (Lane 1) 0.01 µg OVA µl$^{-1}$, (lane 2) 0.05 µg OVA µl$^{-1}$, (lane 3) 0.01 µg OVA µl$^{-1}$, (lane 4) 0.5 µg OVA µl$^{-1}$ (1 band), (lane 5) SK 10 mg ml$^{-1}$, (lane 6) BM 10 mg ml$^{-1}$, (lane 7) SG 10 mg ml$^{-1}$, (lane 8) LMW standards diluted 1:20 (*b*) HEWL dimer. Loads: 1 µl. (Lane 5) SK 10 mg ml$^{-1}$, (lane 6) BM 10 mg ml$^{-1}$, (lane 7) SG 10 mg ml$^{-1}$, (lane 8) LMW standards diluted 1:20. Arrow indicates dimer position.

### 3.3. *HEWL purification*

In order to obtain homogeneous HEWL, we separated SK by semi-preparative cation-exchange FPLC (CIE FPLC); see Fig. 5. A gradient separation was used with a mobile phase consisting of 20 m*M* CAPS–NaOH ($pK_a \simeq 10.4$) buffer pH = 11.0. The gradient to 200 m*M* NaCl over 1 h was designed to separate the $M_r \simeq 18$ kDa high p*I* protein and the HEWL dimer from HEWL monomer (p$I \simeq 11.3$) without excessive dilution of HEWL. A loading of 2 g SK yielded a 50% recovery of purified lysozyme, PHEWL. Although the stability of HEWL at pH = 12 has been determined to be adequate over several hours (Shugar, 1952), each fraction collection tube con-

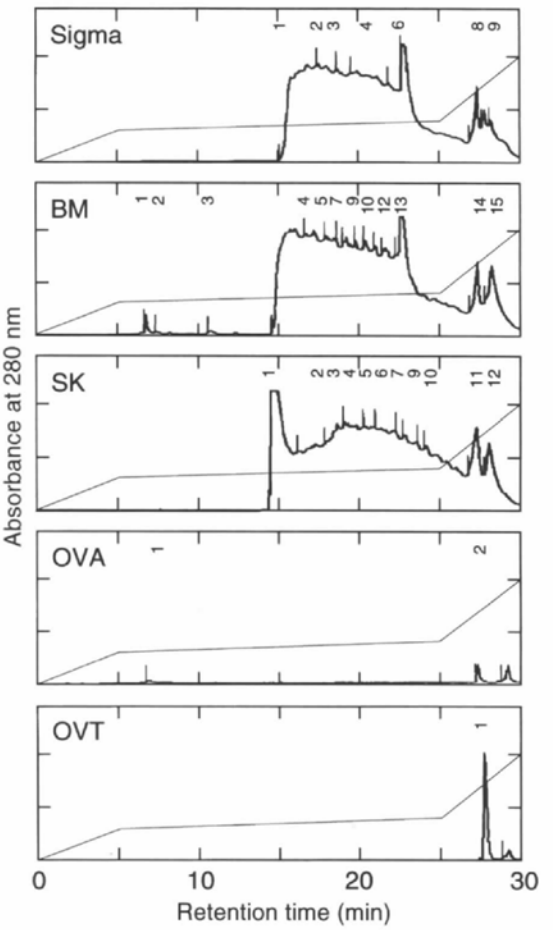



Fig. 4. Identification of HEWL impurities by reversed-phase fast protein liquid chromatography (RP FPLC): An octadecyl bonded phase, 5 µm particle size, 300 Å pore size, 0.46 × 12.5 cm column was used. The mobile phase was 0.1% TFA in water, buffer *A*, and 0.1% TFA in 20% water/ACN, buffer *B*, at flow rate of 0.75 ml min$^{-1}$. The gradient was 0–30% *B* for 5 ml, 30–40% *B* for 20 ml, 40–100% *B* for 5 ml. Detection was at 280 nm with 2.0 AUFS. All three HEWL preparations were injected at 20 mg ml$^{-1}$ in 0.5 ml, *i.e.* 10 mg injected. Authentic proteins OVA (two band) and OVT were injected as 2 mg ml$^{-1}$ in 0.5 ml, *i.e.* 1 mg injected.

tained 0.1 ml of 0.8 *M* sodium acetate to bring the PHEWL to pH = 4.5 as it was collected. Thus, each fraction after collection contained 20 m*M* CAPS–NaOH, 10 m*M* sodium acetate and NaCl. The combined purified fractions 35–39 contained approximately 0.1 *M* NaCl at pH = 4.5.

Attempts to purify SK by using a pH gradient elution from 10.4 to 12.0 in 10 m*M* CAPS/sodium phosphate buffer resulted in 30% recovery. However, when these samples were applied to SDS gels, substantial streaking occurred. This limited the visualization of the 18 kDa and dimer bands. The same pH gradient in CAPS alone caused precipitation of the HEWL. It is noteworthy that SG yielded only a 20%(*w*/*w*) recovery of PHEWL.

### 3.4. *Characterization of purified HEWL*

PHEWL was electrophoretically homogenous in 12.5% T (Fig. 6) and 8–25% T (not shown) SDS–PAGE with a high loading concentration of 10 mg ml$^{-1}$ utilizing a silver stain sensitive to 0.1 ng protein as demonstrated in Fig. 1(*c*). The specific activity of individual column fractions of PHEWL varied from 90–200% of that of SK. Combined fractions of 1 g total protein often resulted in an improvement over SK specific activity; for example, from 144 545 U min$^{-1}$ (SK) to 226 290 U min$^{-1}$ (PHEWL). Because only 1–2% impurity protein was removed from the SK, the improved specific activity was likely due to other factors such as removed inhibitors or enzyme activation.

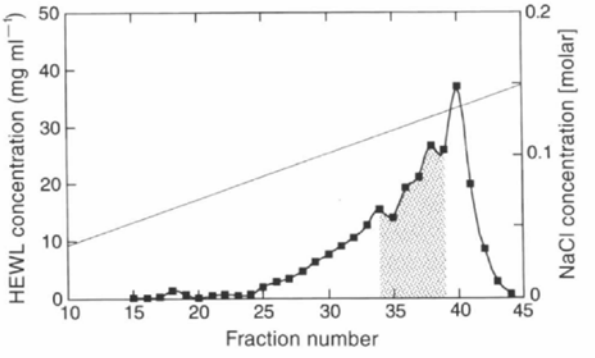



Fig. 5. Purification of SK by cation-exchange fast protein liquid chromatography (CIE FPLC). A linear salt gradient 0–0.2 *M* in 1 h in 20 m*M* CAPS–NaOH buffer pH = 11.0 separated 2 g of SK on a semi-preparative cation-exchange column with approximately 50% recovery of purified HEWL (PHEWL). Fractions (8 ml) were collected into sodium acetate buffer to lower the pH. Aliquots (10 µl) of each fraction (No. 15–45) were diluted with water to 1 ml in silanized test tubes, then absorbance was determined at 280 nm. HEWL concentration was 2.64 AU = 1 mg HEWL ml$^{-1}$. The shaded area represents fractions No. 35–39 which contained PHEWL.

## 4. Impurity effects

In inorganic systems it has long been recognized that the purity of the starting material can be crucial for definitive crystallization studies. In the following we will demonstrate that this applies to HEWL as well.

### 4.1. *Kinetics*

Impurity effects on growth step kinetics are illustrated by Fig. 7; for experimental details of the interferometric technique used see Vekilov, Monaco & Rosenberger (1995), for supplementary kinetics results see Vekilov & Rosenberger (1996). One sees that, in particular at high supersaturations $\sigma$, that typically apply to current crystallization approaches, the growth-step velocity significantly increases with increasing purity of the solution.

### 4.2. *Repartitioning of precipitant*

Strain as well as defects in a crystal often arise from non-uniform chemical composition that results from non-steady precipitant and impurities repartitioning during crystallization. Since lattice defects and strain can limit the X-ray diffraction resolution, an understanding of repartioning phenomena should be of concern in the pursuit of crystal perfection. Hence, we studied the repartioning of NaCl and protein impurities in lysozyme in batch crystallization experiments (Vekilov, Monaco, Thomas, Stojanoff & Rosenberger, 1996). We found that with increasing impurity concentrations pronounced cores formed that were rich in salt and the higher molecular weight proteins. This is illustrated in Fig. 8. The salt uptake per lysozyme molecule in the crystal for low crystallized fractions, corresponding to crystal sizes of $\sim 40\,\mu m$), decreases

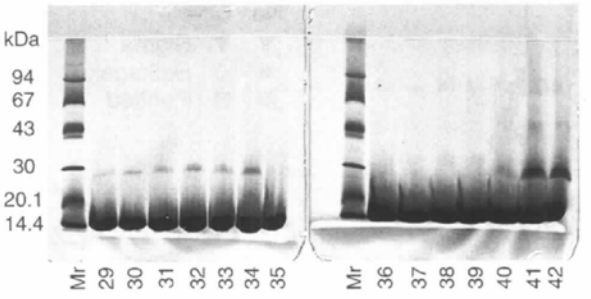



Fig. 6. SDS–PAGE analysis of fraction purity obtained in cation exchange fast protein liquid chromatography (CIE FPLC). Fractions 29–42 of Fig. 5 were analyzed on 12.5% T homogenous PhastSystem gels with an enhanced silver staining system. Lane loading was 1 µl with concentration of protein ranging from about 5 mg ml$^{-1}$ for fraction No. 29 to a maximum of 15 mg ml$^{-1}$. Fractions more concentrated than 15 mg ml$^{-1}$ (No. 36–41) were diluted with water to that concentration to prevent excessive overloading. Fraction numbers are indicated at the bottom of the lanes. First lane in each gel: LMW 1:20.

by a factor of three when higher purity lysozyme is used. X-ray topography of lysozyme crystals grown from as received SG material also revealed highly strained cores (Vekilov *et al.*, 1996; Stojanoff, Siddons, Monaco, Vekilov & Rosenberger, 1996).

### 4.3. *Crystal quality*

Beyond causing strained cores, as shown above, high impurity levels in HEWL solutions result in other degradations of crystal quality. As we have found earlier, temperature changes as small as 0.1 K lead to striations, associated with the accumulation of dislocations or microinclusions (Monaco & Rosenberger, 1993). This is further illustrated in Fig. 9(*a*), where heavy striations were formed in a crystal grown from SG solution in response to $\Delta T$'s of 1 K. However, when PHEWL is used, temperature variations as large as 6 or 12 K induce only faint optical inhomogeneities, Fig. 9(*b*). Furthermore, in contrast to Fig. 9(*a*), no growth sector boundaries can be detected in this crystal at the locations where growth layers on (110) and (101) faces meet.

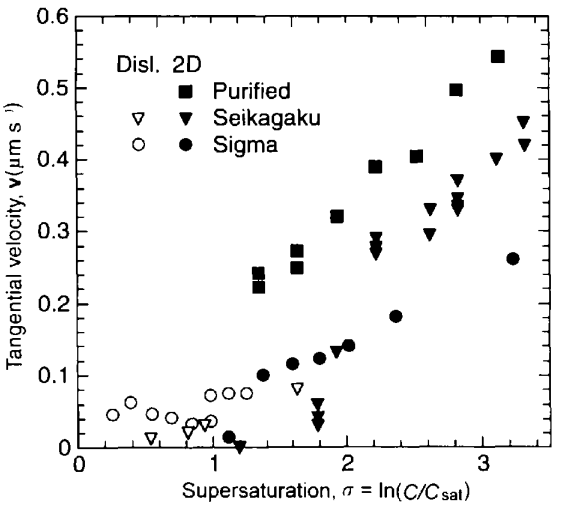



Fig. 7. Dependence of growth step velocity on supersaturation $\sigma$, measured at the edges of HEWL (110) faces growing from Sigma, Seikagaku and purified HEWL solutions.

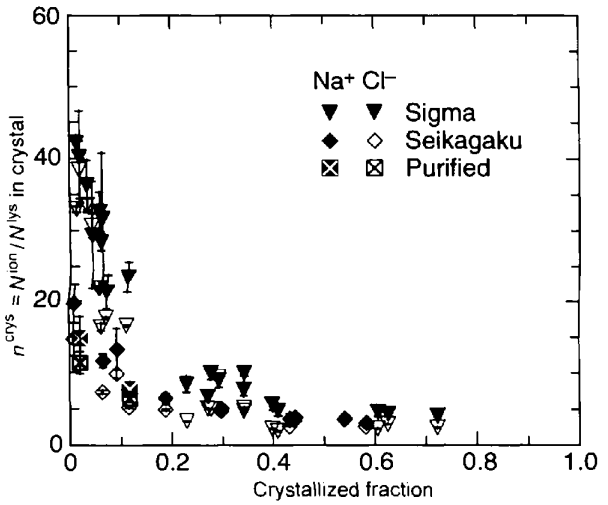



Fig. 8. Ratios of $Na^+$ and $Cl^{-1}$ ions to lysozyme molecules in the crystal, $n^{crys}$, as functions of crystallized fraction, for three solution purity levels: Sigma, Seikagaku, and purified Seikagaku HEWL. Note that $n^{crys}$ decreases with crystallized fraction, and is lower for the higher purity materials.

To establish whether the lower defect contents in the crystals grown from higher purity solutions affects their suitability for molecular structure determinations, diffraction studies were performed (Stojanoff *et al.*, 1996). Crystals were grown in X-ray capillaries (Rosenberger, Howard, Sowers & Nyce, 1993) from SG and SK solutions under otherwise identical conditions. While the SG-grown crystals diffracted only to about 2.0 Å, the crystal grown from the purer SK solutions diffracted to the limit of the experimental setup of 1.55 Å.

## 5. Discussion

The above work shows that commercially available HEWL, the enzyme most widely used in fundamental protein crystallization studies, contains several protein impurities at the %(*w*/*w*) level. This is not surprising, since most proteins obtained from commercial suppliers are more than 5–10% heterogeneous. In the case of HEWL, these contaminants are primarily due to the purification protocols used by the manufacturers, *e.g.* the 'isoelectric method' (Hamaguchi & Funatsu, 1959; Tallan & Stein, 1953). The high pH used in these and even most recently applied procedures (Awadé, Moreau, Mollé, Brulé & Maubois, 1994) does not allow the reduction of high p*I* impurities (18 kDa, dimer), and appears to facilitate the formation of covalently bound HEWL dimers. High p*I* proteins are co-purified and potentially enriched in these HEWL purification protocols.

There were, of course, earlier attempts to characterize and purify HEWL. Unfortunately, most purity determinations have been based on the insensitive Coomassie blue staining; compare Figs. 1(*a*), 1(*b*) and 1(*c*). It has been accepted in the biochemistry community that silver staining is the best method for 'determining the absolute purity of a protein' (Wirth & Romano, 1995). However, in order to fully utilize their sensitivity, silver staining protocols have to be meticulously executed. For instance, on comparison of Fig. 5 in Skouri *et al.* (1995) with our Figs. 1(*b*) and 1(*c*), which are all to characterize the same grade of SG HEWL, one sees that relatively high impurity concentrations (which were even detected by our Coomassie blue staining, Fig. 1*a*) can remain undetected. Note that Skouri *et al.* (1995) also obtained bands for the OVA,

HEWL dimer (their $M_r \simeq 26$ kDa) and the $M_r \simeq 18$ kDa impurity, although they did not attempt to identify the nature of these polypeptides.

Furthermore, it needs to be emphasized that the mere comparison of impurity band positions with those of commercial molecular weight standards in SDS–PAGE is typically not sufficient to identify a heterogeneity. Comparison with authentic protein standards, and identification with specific antibodies (immunoblotting), is required to make definitive assignments. A particular example of how misleading a mere band position comparison can be, is given by the 18 kDa component in our gels. According to its $M_r$, its high pH elution in CIE HPLC and its known presence in hen egg white, this band should represent avidin. Immunoblotting, however, showed that it is not.

For quantifications of impurities, careful bracketing with authentic protein standards combined with quantitative scanning densitometry on the same gel is required. Even under most careful execution of PAGE and staining, the reproducibility from gel to gel is too limited to allow accurate quantification of a band with standards scanned on a different gel (Patton, 1995).

In purification efforts based on HPLC/FPLC, the importance of proper column selection is often underestimated. Most commonly, 300 Å pore size columns are selected for gel filtration protein separations because of their ability to separate proteins with a wide range of molecular weights, 10–300 kDa. This pore size, however, does not yield the best separations for the 14–80 kDa protein impurities present in HEWL samples, In our experience, 100 Å pore size columns with a separation range of 5–80 kDa would separate HEWL dimers from the monomer, while 300 Å pore size columns would not.

In closing we suggest that, in general, as emphasized by Giegé and coworkers almost ten years ago (Giegé *et al.*, 1986), considerably more work on the purification and characterization of preparations is required to enable fundamental studies based on 'crystallography grade proteins.'

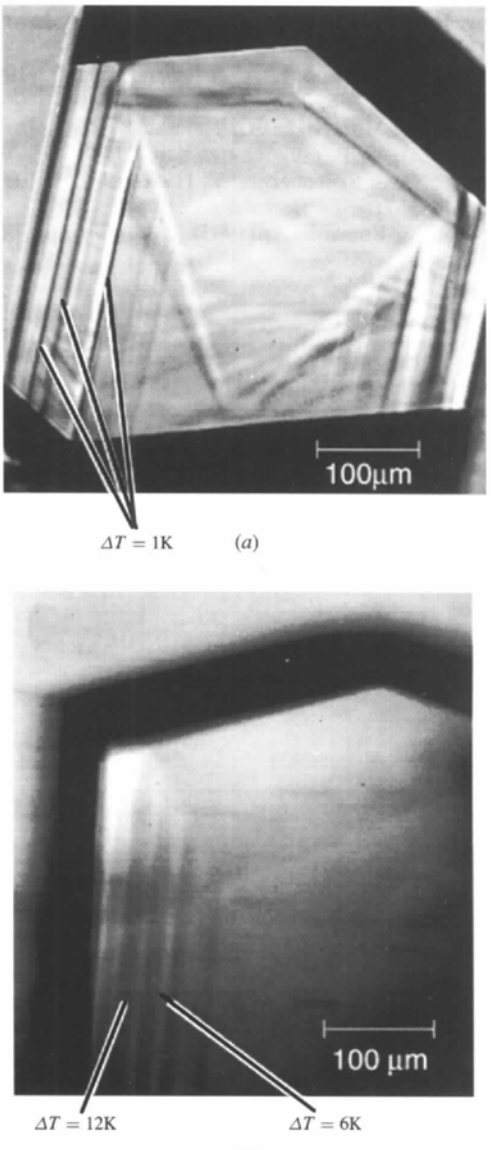



Fig. 9. Striations (structural inhomogeneities) in lysozyme crystals grown from (*a*) Sigma and (*b*) purified HEWL solutions. Transmission differential interference contrast microscopy images. Temperature steps to induce inhomogeneities are indicated. Growth from purified solution results in much weaker striation formation in response to larger temperature/supersaturation changes, and in the absence of growth sector boundary decoration.

We thank M. Muschol for numerous stimulating discussions, as well as V. Stojanoff and D. P. Siddons for permission to cite results prior to their publication. L. Carter expertly prepared the graphs. Research support was provided by the National Aeronautics and Space Administration under Grants NAG8-950 and NAG8-1161.

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