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Note

Purification of ovomucoid by hydroxyapatite chromatography

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It is well known that ovomucoid prepared by the widely used method of Lineweaver and Murray[1] contains several impurities. The major contaminants identified are lysozyme, ovoinhibitor, glycoprotein, conalbumin and ovalbumin[2–4]. As a result, commercial preparations of ovomucoid characteristically exhibit both trypsin- and chymotrypsin-inhibiting activities. Pure ovomucoid is usually obtained by chromatography on ion-exchange celluloses. The low pH employed in this chromatography, however, poses the problem of the stability of sialic acid residues[5]. A method has been described in which the undesirable exposure to low pH is minimized, but not eliminated[6]. We wish to report a very simple purification procedure which involves a single chromatography on hydroxyapatite at pH 6.8 and yields a highly purified ovomucoid.

Drawing on criteria established for protein absorption to and desorption from hydroxyapatite[7,8], the procedure devised was based on the fact that ovomucoid, being a glycoprotein with little structure, should display marginal binding to hydroxyapatite compared to the impurities expected in the system[5]. Furthermore, this procedure makes possible the removal of the inactive non-protein material with maximal ultraviolet (UV) absorption at 260 nm, probably nucleic acid, which does not bind to hydroxyapaptite at all.

MATERIALS AND METHODS

Commercial ovomucoid was purchased from the Worthington (Freehold, N.J., U.S.A.). (lots 01570, 33J800, 570 and 8DA) and Sigma (St. Louis, Mo., U.S.A.) (Type II-0 ovomucoid, lot 127C-8065). Hydroxyapatite was prepared according to the method of Bernardi[9]. *p*-Toluenesulfonyl-L-arginine methyl ester (TAME) and N-benzoyl-L-tyrosine ethyl ester (BTEE) were from Sigma.

Absorption spectra were measured on a Cary Model 118 recording spectrophotometer. Fluorescence emission spectra were obtained with a Hitachi–Perkin-Elmer MPF-3 spectrofluorometer. Circular dichroism (CD) spectra were measured on a Cary Model 60 spectropolarimeter equipped with a Model 6001 CD attachment. The inhibiting capacity of ovomucoid fractions was determined spectrophotometrically: trypsin inhibition was measured from the rate of change in absorption at 274 nm using TAME as substrate, while chymotrypsin inhibition was measured at 256

nm using BTEE as substrate[10]. The enzyme-to-inhibitor ratios were determined according to Davis *et al.*[11].

Fractionation procedure

A 20 × 1 cm column of hydroxyapaptite was equilibrated with 100 ml of 0.001 *M* pH 6.8 sodium phosphate buffer, followed by 100 ml of 0.001 *M* sodium chloride, the flow-rate being kept at *ca.* 30 ml/h. A 1-ml sample of commercial ovomucoid in 0.001 *M* sodium chloride, amounting to 20–60 O.D.-units at 280 nm, was layered on. After two 1-ml washes with the same solvent, the chromatography was carried out as follows. The column was washed with 0.001 *M* sodium chloride until the elutant showed no UV absorption. This was followed then by steps of 0.001 *M* and 0.01 *M* sodium phosphate, pH 6.8 to elute the ovomucoid. The protein impurities remained on the column. If desired, they could be eluted in two steps: 0.5 *M* sodium chloride–0.01 *M* sodium phosphate, pH 6.8, followed by 0.5 *M* sodium phosphate, pH 6.8.

RESULTS AND DISCUSSION

Fig. 1 shows the chromatographic elution patterns of two commercial lots of ovomucoid. The 0.001 *M* sodium chloride washing removes primarily material having UV absorption maximal at *ca.* 260 nm, although some material absorbing at 280 nm may also be eluted. Sometimes this fraction contained trace amounts of ovomucoid, since it showed in such cases a weak trypsin-inhibiting activity. The ovomucoid elutes in phosphate buffer as one peak, if 0.01 *M* phosphate is used. If the elution is carried out in two steps, using 0.001 and 0.01 *M* sodium phosphate, pH 6.8, the ovomucoid emerges in two peaks. The half molar sodium chloride step removes from the column quite pure lysozyme, which is followed by a trace of material having both trypsin- and chymotrypsin-inhibiting activities. In one commercial batch of ovomucoid, this material was found in a significant amount. The half molar phosphate elutes more trypsin- and chymotrypsin-active material.

The results of the fractionation of five different batches of commercial ovomucoid are shown in Table I, where the yields are based on the percent of the total UV absorption at 280 nm. The two ovomucoid fractions (I and II) were characterized by their UV absorption spectra, fluorescence emission spectra and the inhibiting activity toward trypsin. The UV absorption spectra of commercial ovomucoid and of a typical purified fraction are shown in Fig. 2A. It can be seen that the purification procedure eliminates material absorbing at wavelengths both higher and lower than the spectrum characteristic of ovomucoid and that it removes the shoulder at 289 nm seen in the starting material. The UV spectrum of the purified ovomucoid is characterized by a maximum at 277.5 nm with a shoulder at 280 nm and falls off rapidly between 280 and 290 nm, typical of tryptophan-free proteins[12]. The fluorescence emission spectra of the commercial ovomucoid and a purified fraction are shown in Fig. 2B. A drastic difference is evident. The starting material has a broad emission band, maximal at 335–340 nm, which is characteristic of tryptophan-containing proteins. The purified ovomucoid, on the other hand, displays much weaker fluorescence, maximal at 305 nm, which is typical of proteins that are devoid of tryptophan[13]. These changes in the fluorescence emission spectrum on purification of ovomucoid are similar to those described by Davis *et al.*[11]. All the purified ovomucoid

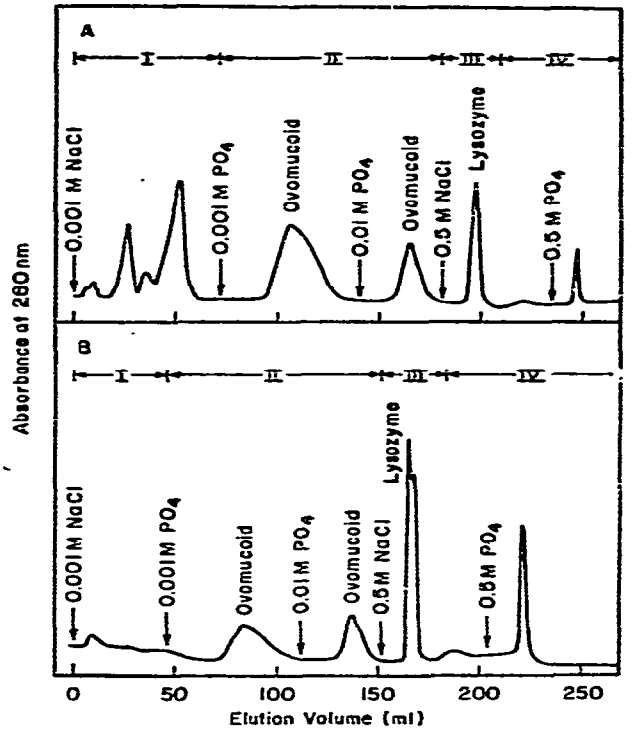

Fig. 1. Elution patterns of two samples of commercial ovomucoid. A = sample 2 of Table I; B = sample 4 of that Table. Zone I consisted on inactive material with maximal UV absorption at 260 nm; zone II contained the ovomucoid factions; zone III was lysozyme; zone IV contained the remainder of the impurities, trypsin- and chymotrypsin-active.

TABLE I

FRACTIONATION OF OVOMUCOID ON HYDROXYAPATITE

Sample	*Ovomucoid (%)*		*Lysozyme (%)*	*Trypsin- and chymotrypsin-active material*	
	I	*II*		*0.5 M NaCl fraction (%)*	*0.5 M PO_4 fraction (%)*
1	39	8	30	trace	13
2	48	12	15	trace	4
3	50	18	3	trace	trace
4	18	12	33	9	13
5	36	4	48	trace	trace

fractions (with the exception of fraction II of sample 4*) exhibited essentially identical UV absorption and fluorescence emission spectra.

The trypsin-inhibiting activity of all fractions was measured. Components I and II of ovomucoid were found to have identical trypsin-inhibiting activity in all

* Fraction II of sample 4 obviously contained a tryptophan-containing impurity as shown by its fluorescence emission spectrum which had a maximum at 308 nm and a shoulder at 330 nm. This fraction had lower trypsin-inhibiting activity than any other fraction. This may be due to the age of sample 4 (ten years in the refrigerator).

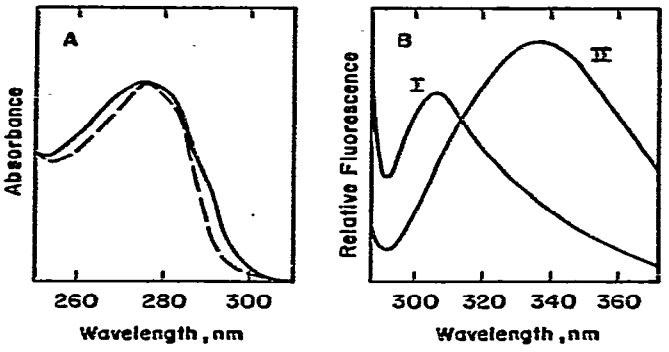

Fig. 2. A, UV absorption spectra of a commercial sample (———) and purified (— — —) ovomucoid. B, Fluorescence emission spectra of purified ovomucoid (I) and starting material (II). The intensity of the purified material (I) is multiplied by a factor of three. Excitation at 278 nm; excitation and emission bandwidths 100 Å; both samples had an absorptivity of 0.12 at 280 nm.

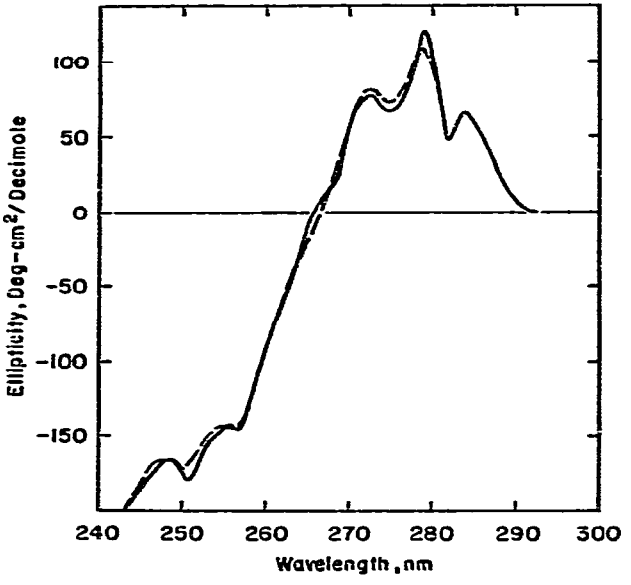

Fig. 3. Circular dichroism spectra of lysozyme eluted from the hydroxyapatite column following removal of ovomucoid (— — —) and of a known sample of pure lysozyme (———), in 0.1 *M* sodium phosphate pH 7.0 buffer.

cases, with the exception of sample 4 in which component II had only 75% of the inhibiting activity of component I. For all five commercial samples investigated, the enzyme-to-inhibitor ratio was 1:1 as expected[11], this number varying by less than 10%*. None of the ovomucoid fractions had any inhibiting activity toward chymotrypsin.

The presently described procedure results in highly purified ovomucoid. Its apparent resolution into two components is probably related to the known hetero-

* Does not include fraction II of sample 4.

geneity of this protein[14–16]. The major protein impurity was found to be lysozyme which was identified by its UV and CD spectra, the lack of inhibitory activity and the ease of its elution from hydroxyapatite with calcium chloride[17]. As is shown in Fig. 3, the lysozyme which emerges from the column is quite pure, since its CD spectrum is essentially identical with that of a standard pure protein. The material which is active toward both trypsin and chymotrypsin emerges at two points: in 0.5 *M* sodium chloride, following lysozyme, and in 0.5 *M* phosphate. The first fraction (0.5 *M* NaCl) consists most probably mainly of ovoinhibitor, since its UV spectrum is similar to that reported by Davis *et al.*[11] for that protein. Its ability to inhibit the two enzymes, however, fell below the molar ratios reported for ovoinhibitor[2,11]. The phosphate fraction is the final wash of the column, since 0.5 *M* phosphate should remove any of the proteins left on the column[7,8,17]. For example, conalbumin and ovalbumin should elute at this point, if present. The low inhibitory activity of this fraction, compared to the 0.5 *M* sodium chloride fraction, as well as its UV spectrum, confirm this.

In conclusion, fractionation on hydroxyapatite affords an easy and convenient one-step method for preparing highly purified ovomucoid. Furthermore, it exposes the protein to the mildest conditions of any currently used procedures.

REFERENCES

1 H. Lineweaver and C. W. Murray, *J. Biol. Chem.*, 171 (1947) 565–581.
2 Y. Tomimatsu, J. J. Clary and J. J. Bartulovich, *Arch. Biochem. Biophys.*, 115 (1966) 536–544.
3 R. E. Feeney, F. C. Stevens and D. T. Osuga, *J. Biol. Chem.*, 238 (1963) 1415–1418.
4 J. Montreuil, B. Castiglioni, A. Adam–Chosson, F. Caner and J. Queral, *J. Biochem. (Tokyo)*, 57 (1965) 514–528.
5 M. B. Rhodes, N Bennett and R E. Feeney, *J. Biol. Chem.*, 235 (1960) 1686–1693.
6 J. G. Davis, C. J. Mapes and J. W. Donovan, *Biochemistry*, 10 (1971) 39–42.
7 M. J. Gorbunoff, S. N. Timasheff and G. Bernardi, *Abstracts, 177th National Meeting of Amer. Chem. Soc., Honolulu, Hawaii, April 1979*, Coll 248.
8 M. J. Gorbunoff, in preparation.
9 G. Bernardi, *Methods Enzymol.*, 21 (1971) 95–97.
10 B. C. W. Hummel, *Can. J. Biochem. Physiol.*, 37 (1959) 1393–1399.
11 J. G. Davis, J. C. Zahnley and J. W. Donovan, *Biochemistry*, 8 (1969) 2044–2053.
12 J. W. Donovan, *Biochemistry*, 6 (1967) 3918–3927.
13 F. W. J. Teale, *Biochemistry*, 76 (1960) 381–388.
14 R. E. Feeney and R. G. Allison, *Evolutionary Biochemistry of Proteins*, Wiley, New York, 1969, pp. 83–208.
15 M. D. Melamed, in A. Gottschalk, (Editor) *The Glycoproteins*, Elsevier–North Holland, Amsterdam, 1966, pp. 317–334.
16 J. G. Beeley, *Biochem. J.*, 117 (1970) 70 P.
17 G. Bernardi, M. G. Giro and C. Gaillard, *Biochim. Biophys. Acta*, 278 (1972) 409–420.