Purification and Crystallization of Avidin

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An improved purification, including crystallization, of avidin from hen's-egg white is described. The product bound $15.1 \mu\text{g}$ of biotin/mg, corresponding to an equivalent weight of 16200. The amino acid composition showed an integral number of amino acid residues per 16200g, which suggested that each molecule of avidin (mol. wt. approx. 66000) contained four identical subunits.

The protein avidin from hen's-egg white had previously been obtained in an essentially pure state by adsorption and chromatography on CM-cellulose (Melamed & Green, 1963). It has now proved possible to crystallize the avidin obtained by this method. The present paper describes a shorter and more effective purification procedure incorporating the crystallization step. The specific activity of the product (15 units/mg) was about 10% higher than the best obtained previously and the analytical characterization of the protein has therefore been repeated. This was of particular interest in view of current work on the determination of the amino acid sequence of avidin (de Lange, 1969, 1970). Some properties of the crystals of avidin, including a preliminary X-ray-crystallographic study, are given in the following paper (Green & Joynson, 1970).

MATERIALS AND METHODS

Most of the materials and methods used have been described previously (Melamed & Green, 1963).

Frozen egg white was obtained in 28lb cans from Behr and Mathew Ltd., London S.E.1, U.K. CM-cellulose (Whatman CM11 or CM22, 100g/8lb of egg white) was thoroughly washed as described previously (Melamed & Green, 1963).

Cysteic acid was determined on samples of avidin oxidized with performic acid. The oxidation was carried out by two slightly different methods (Crumpton & Wilkinson, 1963; Moore, 1963), which gave similar yields of cysteic acid. Tryptophan content was calculated from the u.v.-absorption spectrum as described previously (Melamed & Green, 1963), except that the solvent was 6M-guanidinium chloride and the extinction coefficients used were those determined by Edelhoch (1967) for acetyl-tryptophan amide in this solvent. Biotin-binding activity was measured spectrophotometrically by using the dye 4-hydroxyazobenzene-2-carboxylic acid (Green, 1965, 1970).

Purification of avidin. All operations except crystallization were performed at room temperature.

Step 1: adsorption of basic egg-white proteins on CM-cellulose. Frozen egg white (84lb) was thawed, and gently homogenized in 5 litre batches with equal volumes of deionized water with a Silverson model L2R homogenizer. The homogenate was partly clarified by centrifugation (1200g for 20min). After adjustment of the pH to 7.0 with 1M- H_2SO_4 , CM-cellulose filter cake (3kg, 15% dry weight) was added and stirred for 10min. After standing for several hours, the supernatant was siphoned from the CM-cellulose and then combined with further supernatant obtained by centrifuging the residual slurry (1200g for 10min). A further quantity of CM-cellulose (1.6kg) was added and the process repeated. The combined CM-cellulose fractions were suspended in sufficient ammonium acetate (0.05M, pH 7) to give a thick slurry, which was then poured on to a large Buchner funnel (30cm) without any paper and allowed to drain without suction. The filter cake was washed under gravity with ammonium acetate (0.05M, pH 7, 10 litres; 0.05M, pH 9, 15 litres) followed by $(\text{NH}_4)_2\text{CO}_3$ (0.3%, 5 litres). The cake was finally drained by gentle suction and suspended in $(\text{NH}_4)_2\text{CO}_3$ (0.3%, 12 litres).

Step 2: selective elution of avidin. The suspension was degassed and poured into a $9\text{cm} \times 100\text{cm}$ column. During packing the downward flow rate was limited by decreasing the overall head to 40cm. The column was fitted with an upward-flow adaptor and washed overnight with $(\text{NH}_4)_2\text{CO}_3$ (0.4%, 15 litres). (The use of downward flow frequently led to clogged columns.) Elution was then performed by using a stepwise gradient of $(\text{NH}_4)_2\text{CO}_3$ (0.5, 0.6, 0.7, 0.8, 0.9, 1.0 and 3.0%; 1.5 litres of each); the flow rate was 20ml/min. Fractions (300ml) were collected manually. The E_{280} and avidin content of each was measured and the avidin-containing fractions, contained in the first emerging peak, were pooled. In order to regenerate the CM-cellulose for further use, the column was washed with 4% $(\text{NH}_4)_2\text{CO}_3$ in 1M- NH_3 (10 litres) and 0.5M-NaCl (10 litres). At this stage, the column was unpacked and the final washing with 0.05M-ammonium acetate (15 litres) was carried out on a large Buchner funnel, since swelling of the CM-cellulose in the dilute buffer prevented further washing on the column.

Lysozyme (40mg/ml) was eluted by the 3% $(\text{NH}_4)_2\text{CO}_3$ and about 100g was readily crystallized by raising the pH to 10 with NaOH (2M) and adding NaCl (5% by weight).

Step 3: chromatography on CM-cellulose. The combined avidin fractions (7 litres) were acidified to pH 6.0 with acetic acid (50%), degassed and run on to a column (6.5 cm $\times$ 80 cm) of CM-cellulose. The column was eluted with a linear gradient of $(\text{NH}_4)_2\text{CO}_3$ (0.5–4.0% in 5 litres total volume). The avidin that emerged in the first peak (580 ml, 1.5 mg/ml) was concentrated about sixfold by vacuum dialysis.

Step 4: crystallization of avidin. The concentrated avidin solution was acidified to pH 5.0 with acetic acid (2M), and $(\text{NH}_4)_2\text{SO}_4$ (41.8 g/100 ml) was added to give a concentration of 2.5M. The mixture was centrifuged (12000g for 20 min) after standing overnight and the precipitate rejected. $(\text{NH}_4)_2\text{SO}_4$ (9.2 g/100 ml of solution) was added slowly with stirring (final concn. 3.0M). Crystallization (thin plates) usually commenced within a few hours and at this stage a further 10.8 g of $(\text{NH}_4)_2\text{SO}_4$ /100 ml of solution was stirred in (final concn. 3.5M). The crystals were filtered after 24 h at 4°C and washed with a little 3.5M- $(\text{NH}_4)_2\text{SO}_4$. The avidin was redissolved in water (10–20 mg/ml) and the fractionation and crystallization were repeated. The product was dissolved in 2.5 ml of water, dialysed against deionized water and freeze-dried.

RESULTS AND DISCUSSION

The course of a large-scale purification is shown in Table 1. The specific activity of the final product was almost 10% higher than the best obtained previously and corresponds to an equivalent weight of 16200. Since the molecular weight calculated from sedimentation, diffusion and sedimentation-equilibrium measurements was between 66000 and 70000 (Green, 1964), this is consistent with a four-subunit molecule. The nitrogen content (15.5%) and the extinction factor ($E_{1\text{cm}}^{1\%}$ 15.4) were the same as those observed previously. When the earlier preparations of avidin had been tested for homogeneity by chromatography on columns of Amberlite CG-50, two or three active components had been obtained showing slight differences in their amino acid composition. Amino acid analyses were therefore performed on subfractions of crystalline avidin obtained from these columns (Fig. 1). As before, it proved difficult to reproduce

Table 1. *Purification of avidin from frozen egg white (84 lb)*

Stage	Volume (ml)	E_{280}	Protein (g)	Avidin (g)	Specific activity (μg of biotin bound/mg of protein)
Homogenized egg white	74000	—	4200	1.2	0.004
First column effluent	7400	0.86	4.1	0.96	3.5
Second column effluent	580	2.4	0.90	0.83	14.1
First crystals*	40	29	0.75	0.75	15.1†

* It is possible to crystallize the avidin starting from material of specific activity 10, if seed crystals are available.

† Recrystallization gave no further increase in activity. A further 50–100 mg of slightly less pure avidin can be recovered from tail fractions from the columns and from the 2.5M- $(\text{NH}_4)_2\text{SO}_4$ precipitate.

Fig. 1. Chromatography of crystallized avidin on a column (2.5 cm $\times$ 20 cm) of Amberlite CG-50. The avidin (35 mg) was eluted with a linear gradient (---) of sodium borate, pH 10.0, at a flow rate of 9 ml/h. Duplicate chromatograms (—) and (— — —) are shown. Fractions from the chromatogram (—) were pooled and analysed (Table 2): fraction 1, 70–120 ml; fraction 2, 130–190 ml; fraction 3, 191–250 ml.

the exact shape of the elution profile in spite of extensive washing of the column before use. The peaks were only rarely sharp, but the overall profile was reproducible insofar as there were always two major peaks followed by a tail fraction. The fractions from one of the chromatograms (Fig. 1) were combined in three pools and their amino acid compositions were determined (Table 2). Their specific activities were low (13 units/mg), suggesting

some inactivation on the column, but they did not differ significantly from each other.

The analyses of the three fractions were almost identical, except for the uniformly low (95%) recovery shown by fraction 1. The only amino acid to show any significant difference between the fractions was threonine, and even this was a difference of only one residue out of 20. The previous results (Melamed & Green, 1963) suggesting

Table 2. *Composition of avidin fractions from Amberlite CG-50 columns*

The analyses are averages of the results of 24 h and 72 h hydrolyses, except for those for serine, threonine, methionine, cystine and tryptophan. The values for serine and threonine were corrected for destruction by a linear extrapolation from the 24 h and 72 h values. The numbers of residues/subunit have been calculated for an equivalent weight of 16200 (15.1 μ g of biotin bound/mg of avidin). nd, not determined.

Avidin sample ... Fraction from CG-50 ...	A.6						D.31*	
	1		2		3		Integral residues/ subunit	(mol/ 16200g)
	(g/100g)	(mol/ 16200g)	(g/100g)	(mol/ 16200g)	(g/100g)	(mol/ 16200g)		
Amino acid								
Lys	7.87	8.8	8.40	9.3	8.3	9.2	9	8.9
His	0.94	1.0	0.97	1.0	0.96	1.0	1	1.02
Arg	7.83	7.4	8.47	7.9	8.30	7.7	8	7.8
Asp	10.76	13.2	11.63	14.15	11.25	13.7	14	14.3
Thr	13.15	17.9	14.4	19.5	13.5	18.3	19	19.8
Ser	5.58	8.5	5.97	9.2	5.96	9.2	9	8.9
Glu	8.56	9.5	8.90	9.85	8.96	9.9	10	10.8
Pro	1.09	1.6	1.24	1.75	1.31	1.8	2	2.2
Gly	4.97	10.8	5.21	11.2	5.25	11.25	11	11.2
Ala	2.60	4.8	2.76	5.0	2.76	5.0	5	5.0
Cys	1.20	1.6	1.2	1.6	1.0	1.3	—	1.2
CySO ₃ H†	—	—	2.17	2.07	—	—	2	6.4
Val	4.66	6.5	5.08	7.05	4.95	6.8	7	7.2
Met	1.65	1.8	1.75	1.9	1.68	1.8	2	2.04
Ile	5.78	7.2	6.34	7.8	6.20	7.7	8	7.9
Leu	5.37	6.7	5.81	7.2	5.70	7.0	7	7.1
Tyr	1.20	1.1	1.16	1.0	1.16	1.05	1	1.03
Phe	6.66	6.6	7.25	7.1	7.05	6.9	7	6.9
Amide	nd	—	nd	—	nd	—	(14)§	14.1
Trp‡	nd	—	5.1	4.05	nd	—	4	4.3
Total amino acid residues							126	128**
Total weight recovered	96.0	—	101.7	—	99.7	—	—	—
Theoretical total ¶	105	—	105	—	105	—	—	—
Mean residue weight							112	112
Mannose							(5)§	5.3
N-Acetylglucosamine							(3)§	2.7
Mol. wt. calculated as sum of amino acid and carbohydrate components							15537	15767

* The results for this preparation have been calculated from those of Melamed & Green (1963), by using the new equivalent weight.

† Cysteic acid: average of two determinations by the methods of Crumpton & Wilkinson (1963) and Moore (1963).

‡ Spectrophotometric determination (see the Materials and Methods section).

§ Values assumed to be the same as for the D.31 preparation.

|| Including tryptophan.

¶ 100g of avidin contains 91g of amino acid residues, which would give 105g of amino acids after hydrolysis.

** Assuming two half-cysteine residues, as found for A.6 preparation.

differences in arginine and lysine content were not reproduced. It is still probable that the differences in chromatographic behaviour reflect differences in amino acid composition, but more extensive analyses would be required to establish their nature.

The values for molecules of amino acid/subunit based on the new equivalent weight are all close to whole numbers, and the earlier discrepancies indicating the presence of more than one type of subunit have disappeared. The results therefore suggest that the four subunits of avidin are identical.

The final column of Table 1 shows the values for an earlier preparation of avidin (D.31) recalculated on the basis of the new equivalent weight. The proportions of the different amino acids present were almost exactly the same in the two preparations. The only exception was cysteic acid. The new results showed only a single cystine residue in each subunit, in agreement with the results of Fraenkel-Conrat, Snell & Ducay (1952) and de Lange (1970). The high results of Melamed & Green (1963) were probably due to some ninhydrin-positive contaminant emerging with the cysteic acid at the front of the column. The only other marginally significant differences were the slightly lower amounts (one residue each) of glutamic acid and threonine in the new preparation.

The close agreement between the two sets of analyses suggests that the main impurity present in the earlier preparation was probably avidin-biotin complex, whose removal has increased the specific activity without affecting the composition. This is also consistent with the earlier observation of a small amount of tryptophan that was resistant to oxidation by *N*-bromosuccinimide. Such tryptophan is characteristic of the avidin-biotin complex and was absent from the crystalline avidin (Green &

Ross, 1968). The close approach of the number of amino acid residues/molecule of biotin bound to integral values suggests that the avidin was substantially pure and that the equivalent weight is not far from the true subunit molecular weight.

The total number of amino acid residues in the peptide chain is 126–128, close to the number known to be present in lysozyme, with which avidin shares other features (Green, 1968). The results of de Lange (1970) showed 129 residues, identical with those shown in the penultimate column of Table 2, with the exception of two more threonine residues and one more aspartic acid. However, sequences totalling 40 residues from the *N*- and the *C*-terminal ends showed only one small homology with that of hen's-egg white lysozyme.

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