THE PURIFICATION AND MOLECULAR WEIGHT ESTIMATION OF TELEOST VISUAL PIGMENTS

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1. Introduction

The vertebrate visual pigments, rhodopsin and porphyropsin, are insoluble membrane proteins with retinal and 3-dehydroretinal as their respective prosthetic groups. Purification of these proteins has been restricted to mammalian (bovine and rat) and amphibian (frog) rhodopsins [1]. The insolubility of these membrane proteins has made it difficult to obtain reliable determinations of their molecular weight, the reported values ranging from 26 000 – 40 000 [1–8]. We wish to report the purification of teleost rhodopsin and porphyropsin. These visual pigments may occur together in a single retina so that species containing only one type were used in this study [9]. Rhodopsin was isolated from the guppy, *Poecilia reticulatus,* and porphyropsin from the goldfish, *Carassius auratus.*

2. Materials and methods

All chemicals used were reagent grade. Digitonin, bovine serum albumin, pepsin, trypsin, haemoglobin and soybean trypsin inhibitor were obtained from Sigma Corp.

2.1. *Extraction and purification*

Fish were dark adapted overnight before killing and all subsequent experimental procedures were carried out in dim red light, at room temperature, unless otherwise stated. Eyes from freshly decapitated fish were excised and in the case of the guppy were ground directly in 4% aqueous alum and stored at −20°C. With the goldfish, however, it was found more convenient to remove the eye cups which were homogenised in a similar fashion. Later the thawed homogenates were washed three times with distilled water. The visual pigments were then isolated by two successive two-hour extractions at 4°C, each with 2% digitonin and 10^{-3} M dithiothreitol in 0.066 M phosphate buffer, pH 7.0. Each extraction was followed by centrifugation at 27 000 *g* for 20 min and the two supernatants then combined. In the case of each species visual pigment extracts were then chromatographed on a Sepharose 6B column (0.9 × 30 cm). The eluting buffer was 0.1% digitonin and 10^{-3} M dithiothreitol in 0.066 M phosphate buffer, pH 7.0. The flow rate was 4 ml/h and 1 ml fractions were collected. Aliquots of each fraction were made 0.02 M with neutralized hydroxylamine before reading the absorbance of rhodopsin (500 nm) or porphyropsin (520 nm) and protein (280 nm) on a Unicam SP800 spectrophotometer. The retinal and protein content of both rhodopsin and porphyropsin samples were determined as previously described [10,11].

2.2. *Electrophoresis*

For electrophoresis the purified visual pigment samples were dialysed for 24 h against 8 M urea and 0.1 M mercaptoethanol in 0.01 M phosphate buffer, pH 7.1. Dialysate samples containing 20–50 μg protein were electrophoresed on 7.5% acrylamide gels containing 8 M urea in 0.1 M Tris, pH 9.0. The electrode buffer was 0.0025 M EDTA and 0.0088 M borate in 0.087 M Tris, pH 9.4. Electrophoresis was allowed to proceed for 2 h at 100 V. Gels were stained for protein, carbohydrate and phospholipid using Coomassie brilliant blue [12], periodic-acid Schiff [13] and ammonium molybdate-methyl green [14], respectively.

2.3. *Molecular weight estimation*

Electrophoresis in polyacrylamide gels, containing sodium dodecyl sulphate was carried out according to the method of Weber and Osborne [12]. Before electrophoresis the urea dialysate was further dialysed against 0.1% aqueous mercaptoethanol and, subsequently, against 0.1% sodium dodecyl sulphate and 0.1% mercaptoethanol in 0.01 M phosphate buffer, pH 7.1. Protein standards, which were used to calibrate the gels, were treated in a similar manner. These sodium dodecyl sulphate samples were further used for molecular weight estimations on a calibrated Sepharose 6B column (0.9 cm × 60 cm). The sample was applied in less than 2% of the bed volume and eluted using 0.1% sodium dodecyl sulphate and 0.1% mercaptoethanol in 0.01 M phosphate buffer, pH 7.1.

3. Results and discussion

Vertebrate visual pigments have been previously purified using cetyltrimethyl-ammonium bromide and emulphogene BC-720 as detergents [3,4]. Teleost visual pigments, however, have proved unstable in these detergents and digitonin was selected in preference. In addition, recent spin label studies have shown the integrity of extracted visual pigments to be best preserved using digitonin [15]. The agarose gel elution profile of native teleost visual pigment is shown in fig.1. In replicate experiments both

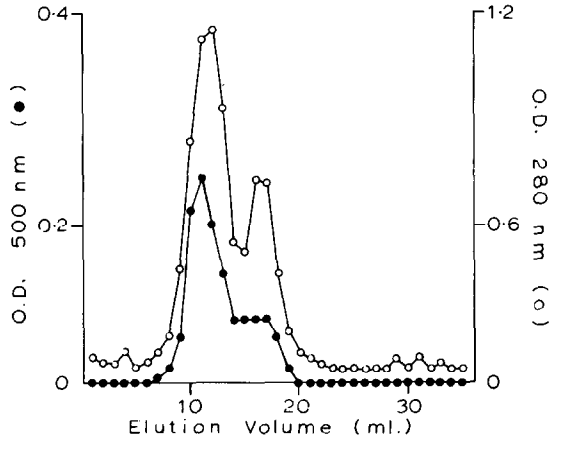

Fig.1. Chromatography on Sepharose 6B of guppy visual pigment extract. A similar profile was obtained from the chromatography of goldfish visual pigment, whose absorbance was read at 520 nm.

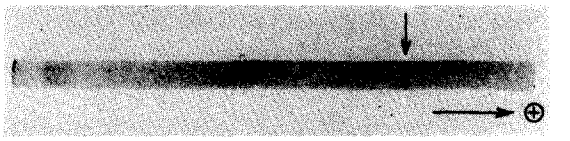

Fig.2. Electrophoresis in polyacrylamide-urea gels of teleost visual pigment. The arrow indicates the position of the visual pigment.

rhodopsin and porphyropsin emerge as single symmetrical peaks with identical elution volumes (11.5 ml). In each case a subsidiary peak, at 19 ml elution volume, contained material absorbing at 280 nm and occasionally a small amount at either 500 nm and 520 nm. This latter peak, however, never exhibited typical visual pigment spectra. In both these purified visual pigments the retinal and 3-dehydroretinal was shown to be bound to the protein in a 1:1 molar ratio.

The chromatographed visual pigments were shown to be homogenous by electrophoresis in 8 M urea (fig.2). This electrophoretic band also reacted positively for glycoprotein and phospholipid. It should be noted that in these urea gels an area near to the origin which stains similarly to the major band was observed. This faint staining is more likely to be

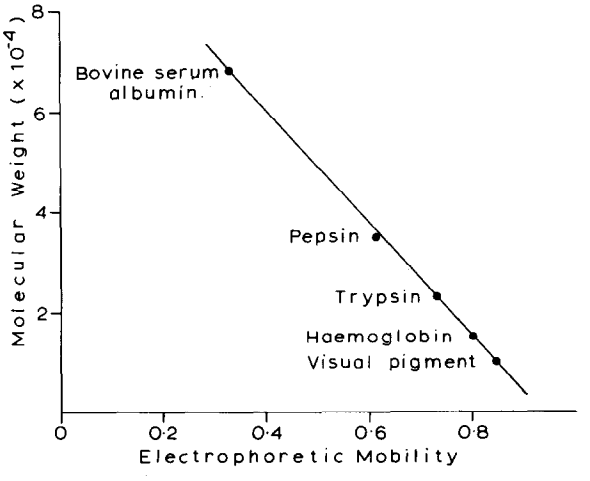

Fig.3. Determination of the mol. wt. of both guppy and goldfish visual pigment on a sodium dodecyl sulphate polyacrylamide gel. The gel was calibrated with the following standard proteins whose mol. wts are given in brackets: bovine serum albumin (68 000), pepsin (35 000), trypsin (23 300) and haemoglobin (15 500). Both teleost visual pigments gave an estimated mol. wt. of 10 000.

an artifact rather than contamination as other electrophoretic techniques do not reveal it.

Electrophoresis in sodium dodecyl sulphate indicates a visual pigment mol. wt. of 10 000 (fig.3). This result is supported by data from sodium dodecyl sulphate agarose gel chromatography. Column chromatography, using denaturing solvents, is not, however, wholly suitable for accurate molecular estimations of membrane proteins [16] and considerable difficulty was experienced in obtaining distinct separations using this technique.

In the presence of their carbohydrate [1] and phospholipid moieties [17,18], these visual pigments resemble the vertebrate rhodopsins already investigated. The mol. wt. of 10 000, however, is the lowest yet recorded. It is unlikely that this low molecular weight is that of a subunit as the electrophoretic mobility of the single band remained unchanged in the presence of strong reducing agents. This electrophoresis was carried out using the digitonin extract in 7.5% acrylamide gels containing 0.5% digitonin (C. M. Regan, unpublished observations). Proteolytic activity may, also, be discounted as inclusion of phenylmethylsulphonylfluoride or soybean trypsin inhibitor in the extraction process did not alter the electrophoretic mobility.

Further characterization of these unique visual pigments is in progress.

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