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Note

Isolation of coloured peptides from cataractous lens proteins

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Senile nuclear cataract formation in man is associated with brown pigmentation and opacification in the inner core of the lens. Biochemical studies on cataractous and normal lenses have revealed a number of progressive changes to the lens proteins with the development of senile nuclear cataract. Amongst these are an increase in insoluble protein [1, 2], an increase in the extent of protein cross-linking [2] and a pronounced oxidation of both protein sulphydryl groups [3] and methionine residues [4].

Despite these findings, an understanding of the aetiology of senile nuclear cataract is unlikely to proceed far without a detailed knowledge of the chemical modifications which have taken place in the lens proteins to render them pigmented and insoluble.

This paper describes a method for isolating a purified fraction of coloured peptides from cataractous lens protein as a preliminary to subsequent structure determination of the pigment.

EXPERIMENTAL

Cataractous lenses were obtained from extractions performed at the Repatriation Hospital (Heidelberg, Australia) or from extractions performed at eye camps in India. The latter group of lenses were stored in ethanol. Lenses were classified as Type I—IV on the basis of nuclear colour as described by Pirie

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[1]. Proteolytic enzymes were purchased from Sigma (St. Louis, MO, U.S.A.), and Sephadex G-10 and G-25 from Pharmacia (Uppsala, Sweden). The 250 × 4.5 mm C_{18}, 10 μm particle size, reversed-phase high-performance liquid chromatography (HPLC) column was purchased from Waters Assoc. (Bedford, MA, U.S.A.) and the 75 × 7.5 mm Biogel TSK DEAE 5 PW column was from Bio-Rad (Richmond, CA, U.S.A.).

The outer portions of the lenses including the capsulè were removed by dissection and the nuclei extracted into 0.1 *M* phosphate, pH 7.0, containing 8 *M* urea and 50 m*M* mercaptoethanol. The proteins were carboxymethylated using the procedure of Crestfield et al. [5] except that the Tris buffer was pH 7.5.

Carboxymethylated lens proteins (20 mg/ml in 0.1 *M* ammonium acetate, pH 8.0) were digested with 1% (w/w) of both trypsin and chymotrypsin for 17.5 h at 37°C. One drop of chloroform was added as preservative. Carboxypeptidase Y and aminopeptidase M, each 0.25% (w/w), were then added and digestion continued for a further 6.5 h. The digest was then centrifuged (3000 *g*, 10 min) and a further 0.01% each of carboxypeptidase and aminopeptidase added to the supernatant and the hydrolysis continued for a further 2 h. The digest was lyophilised and redissolved in 0.1 *M* ammonium formate, pH 3.8, for chromatography on Sephadex G-10. Sephadex G-10 (86 × 2.5 cm) and Sephadex G-25 (115 × 1.0 cm) columns were equilibrated in 0.1 *M* ammonium formate, pH 3.8.

Reversed-phase HPLC was performed using a C_{18} column in a Waters liquid chromatograph fitted with a U6K injector (Waters). The solvent programme consisted of a gradient from 5 to 100% acetonitrile in 30 min. Both the aqueous and organic phase contained 0.1% trifluoroacetic acid. The flow-rate was 1 ml/min. The solvent system for ion-exchange HPLC consisted of 20 m*M* Tris, pH 9.0, with a gradient to 0.5 *M* sodium chloride in the same buffer over 60 min at a flow-rate of 1 ml/min.

Thin-layer chromatography (TLC) was performed on cellulose plates (Merck) using the solvent butanol–acetic acid–water (4:1:2, v/v/v).

RESULTS

Extensive hydrolysis of the carboxymethylated Type I and Type IV cataract lens proteins using the enzymes described in Experimental resulted in 75—80% of the protein becoming solubilised after 24 h at 37°C. Neither carboxymethylation nor proteolysis appeared to alter the colour of the cataract lens proteins. A greater proportion (84%) of the carboxymethylated calf protein was solubilised as a result of proteolysis. The insoluble material from nuclear cataract lens proteins was brown whereas that from Type I and calf lens proteins was white.

The great majority of the soluble colour in the digest of advanced cataract (Type IV) lens proteins was excluded from Sephadex G-10 and therefore has a molecular weight in excess of 700. This is illustrated by the 340-nm absorbance profile shown in Fig. 1. The UV—visible spectrum of this coloured peptide fraction showed no distinct peak in the visible region, in agreement with the results of Dillon et al. [6]. Most of the 365/440 nm fluorescence which is

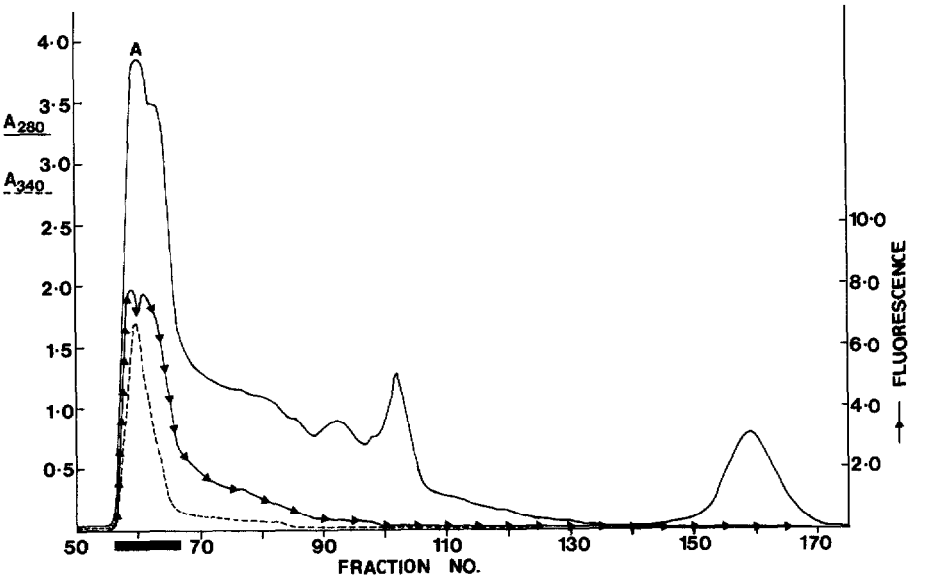

Fig. 1. Type IV cataract protein. Gel chromatography on Sephadex G-10 of the soluble portion from protease digestion of carboxymethylated lens protein (240 mg). Fraction size: 2 ml up to fraction 78, then 5 ml thereafter. Fluorescence: excitation 365 nm, emission 440 nm. Fractions were pooled as indicated.

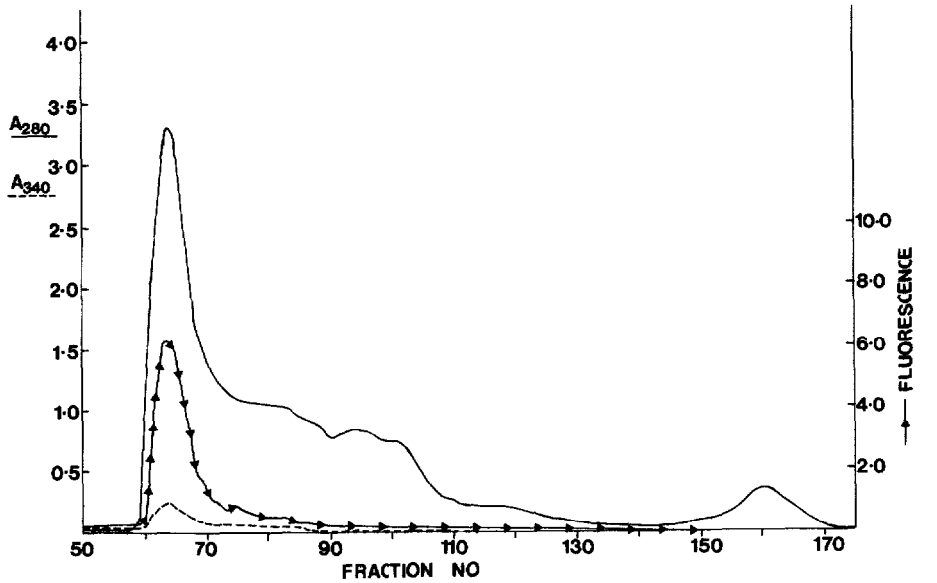

Fig. 2. Type I protein. Gel chromatography on Sephadex G-10 of the soluble portion from protease digestion of carboxymethylated lens protein (240 mg). Conditions as for Fig. 1.

characteristic of adult human lens proteins [7] also eluted at the void volume of the Sephadex G-10 column. The fluorescence maxima and intensities were not markedly affected by changes in pH.

When this profile was compared with that of equivalent weights of Type I protein (Fig. 2) and calf lens protein digests (Fig. 3) several clear differences

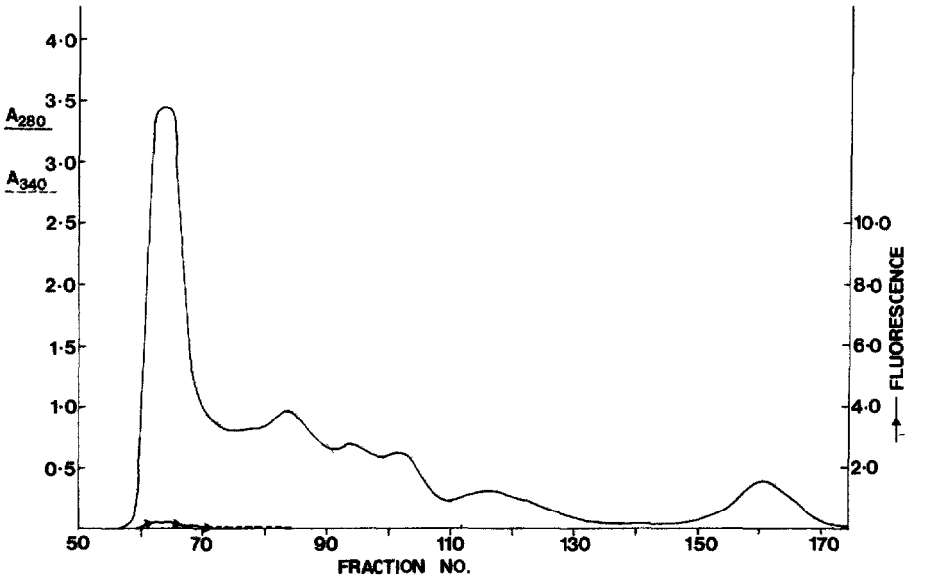

Fig. 3. Calf protein. Gel chromatography on Sephadex G-10 of the soluble portion from protease digestion of carboxymethylated lens protein (240 mg). Conditions as for Fig. 1.

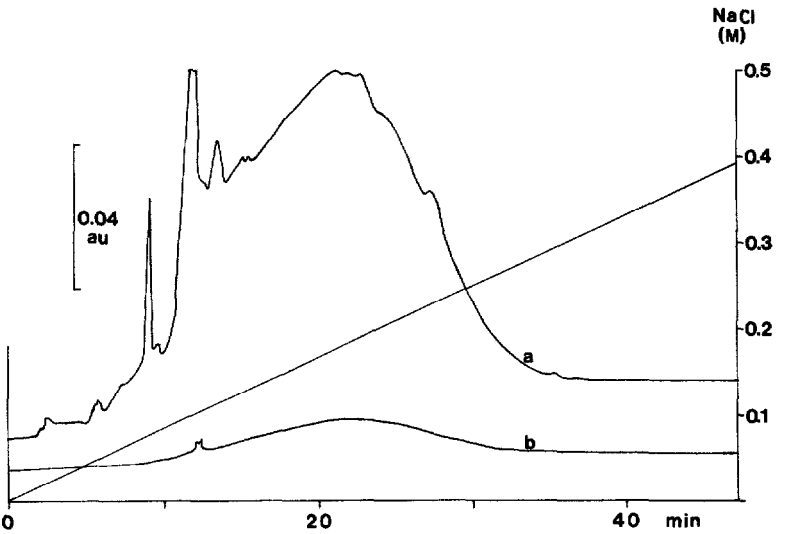

Fig. 4. Ion-exchange (DEAE) HPLC of the excluded peak (A) shown in Fig. 1. (a) Absorbance at 229 nm. (b) Absorbance at 340 nm.

emerged. In the Type I digest a considerable amount of peptide material eluted in the void volume region. This peak contained almost as much fluorescence as in the nuclear cataract digest (see Fig. 1); however, the colour of this peak was considerably less than that of the Type IV digest. This is reflected in the relative sizes of the peaks of 340 nm absorbance in Figs. 1 and 2.

In the case of the calf lens digest, again a large peak was observed at the void volume of the Sephadex G-10 column; however, in this case no 340 nm absorbance and negligible fluorescence were associated with the excluded peak.

When the excluded peak (Fig. 1, peak A) from the nuclear cataract digest was rechromatographed on Sephadex G-25 a broad peak was obtained whose elution position relative to that of known peptides suggested an average molecular weight in the region of 2000—3000 daltons.

When the coloured peak (Fig. 1, peak A) from the nuclear cataract digest was examined by ion-exchange HPLC (Fig. 4) once again a very broad peak of 229 nm absorbance was observed. This was mirrored by the absorbance profile at 340 nm. Using reversed-phase HPLC of peak A, the broad peak of 229 nm absorbance was also found to be coloured and fluorescent (Fig. 5). A similar chromatogram was obtained with longer retention time for the peptide, using methanol as organic solvent. This profile was not altered by further prolonged (18 h, 37°C) digestion with either pronase (at pH 8.0) or pepsin (at pH 3.8). Amino acid analysis of reversed-phase HPLC fractions revealed that all the protein amino acids were present with aspartic acid and glutamic acid at roughly equal concentrations being predominant and accounting for approximately 29% of all the amino acids.

When fractions from the HPLC columns were collected and examined by TLC, yellow-coloured, white fluorescent bands were obtained which corresponded to the patterns of colour and fluorescence seen in the original digest (data not shown) suggesting that the modified peptides had not been altered as a result of the various chromatographic procedures.

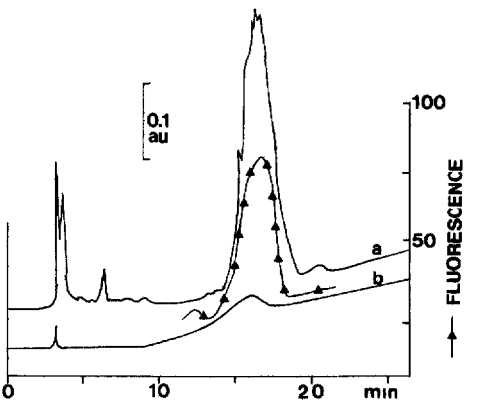

Fig. 5. Reversed-phase (C_{18}) HPLC of the excluded peak (A) shown in Fig. 1. (a) Absorbance at 229 nm, (b) absorbance at 313 nm. Fluorescence: excitation 365 nm, emission 440 nm.

DISCUSSION

The aim of the work described in this paper was to determine if the colour associated with senile nuclear cataract lenses could be isolated from digests of the lens proteins in a form suitable for structure elucidation. The results indicate that pigmented peptides can be isolated apparently free from other peptide material using a combination of gel chromatography and ion-exchange or reversed-phase HPLC. However, this material is of apparently high molecular weight and is resistant to further degradation by proteolytic enzymes. Thus the purified coloured peptides will have to be subjected to additional chemical hydrolysis methods in order to obtain fragments for structure determination.

Whilst extensive protease digestion does not appear to lead to the release of a single low-molecular-weight coloured derivative, the results from a variety of chromatographic systems suggest rather that the coloured peptides produced are characterised by a substantial degree of heterogeneity. This is indicated by the broad peaks obtained in HPLC as well as Sephadex G-25 chromatography. A similar broad coloured peptide peak was obtained previously on gel filtration of cataract proteins following extensive digestion with pronase [8].

A protease-resistant peptide fraction with an apparent molecular weight of approximately 3000 has also been obtained by digestion of cataract proteins with trypsin and *Staphylococcus griseus* protease [6].

The profile of the calf lens proteins showed no colour and essentially no fluorescence. However, the Type I digest was found to contain an amount of fluorescence not appreciably less than that observed in the Type IV digest although the amount of colour was markedly decreased. We have not been able to separate the yellow colour from the fluorescence in any chromatographic step attempted on the cataract peptides, suggesting that they may be associated with the same structural moiety.

The coloured peptides purified from the digests of cataract lens protein using the techniques described are being used at present for raising antibodies as well as for structure determination of the pigment.

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