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Short Communication

Analysis of lysosomal degradation of fluorescein isothiocyanate-labelled proteins by Toyopearl HW-40 affinity chromatography

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ABSTRACT

Fluorescein isothiocyanate (FITC) was found to have a strong affinity to Toyopearl gel, which is used for gel filtration. FITC-labelled amino acids also showed affinity to Toyopearl gel, their elution from a Toyopearl HW-40 column being retarded. On the other hand, FITC-labelled proteins had no affinity to the gel and were recovered in the flow-through fractions. These findings were applied to the analysis of the degradation of various FITC-labelled proteins by lysosomal enzymes *in vitro*. FITC-labelled degradation products were easily separated from FITC-labelled substrate proteins on a small Toyopearl HW-40 column. Their production increased with the incubation time and was markedly suppressed by the proteinase inhibitor leupeptin. The FITC-labelled degradation product was identified to be mainly lysine with a FITC-labelled ε-amino group by its different elution position to those of lysine with a FITC-labelled α-amino group and other various FITC-labelled amino acids.

INTRODUCTION

Lysosomes degrade various species of proteins sequestered by autophagy and/or heterophagy. As the final degradation products of proteins by total lysosomal proteinases are amino acids, measurements of the release of amino acids are important for examining the total proteolytic activity of lysosomes. Isotope-labelled proteins have been used as substrates, but their use is limited quantitatively and requires facilities to prevent environmental radioactive contamination. Fluorescein isothiocyanate (FITC) has been used for the fluorimetric labelling of proteins and amino acids [1–5]. Protein degradation has been analysed by measurements of acid-soluble materials from fluorescently labelled proteins [6–8]. Acid-soluble materials, however, do not always consist only of free amino acids. We found that FITC has a strong affinity to Toyopearl gel, which is used in gel filtration, and that FITC-labelled amino acids retain affinity to this gel, showing retarted elution from a Toyopearl HW-40 column, whereas FITC-labelled proteins have no affinity to the gel. In this study, we developed a method

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for the analysis of protein degradation based on these differences in interactions of FITC-labelled amino acids and proteins with Toyopearl HW-40 gel. In this way, lysine with an FITC-labelled ε-amino group was identified as the main FITC-labelled degradation product of FITC-labelled proteins by lysosomal enzymes *in vitro*.

EXPERIMENTAL

Reagents

Fluorescein isothiocyanate (FITC) was obtained from ICN Biomedicals, Toyopearl HW-40 (Fine) from Tosoh, Sephacryl S-200, Sepharose 4B and Percoll from Pharmacia, fetuin (from foetal calf serum), myoglobin (from horse heart) and bovine serum albumin (BSA) from Sigma and neuraminidase (from *Arthrobacter ureafaciens*), 4-nitrocatechol sulphate and leupeptin from Nacalai Tesque.

Labelling of amino acids with FITC and Toyopearl HW-40 column chromatography

FITC was dissolved in 0.25 *M* hydrogencarbonate buffer (pH 8.0) and stored at 0°C. This stock solution was stable for at least 5 h. Amino acids were dissolved in water and stored at −20°C until used.

The reaction mixture (0.2 ml) containing 100 nmol of each amino acid, 5 μg of FITC and 0.25 *M* hydrogencarbonate buffer (pH 9.0) was allowed to stand for 1 h at room temperature in the dark. Its pH was then decreased by addition of 0.3 ml of 0.5 *M* hydrogencarbonate buffer (pH 8.0). Samples were applied to a Toyopearl HW-40 column (20 ml bed volume) equilibrated with 10 m*M* phosphate buffer (pH 8.0) containing various concentrations of NaCl. The running buffer was introduced with a peristaltic pump at 0.8 ml/min. Fractions of 2 ml of eluate were collected and their fluorescence intensities were measured with excitation and emission wavelengths of 490 and 520 nm, respectively in a Hitachi Model F-2000 spectrofluorimeter.

Purification of substrate proteins and their labelling with FITC

Asialofetuin was prepared by treating fetuin with neuraminidase as described [9], except that desialylated fetuin was purified by gel filtration on a Sephacryl S-200 column. L-Lactate dehydrogenase (EC 1.1.1.27) (LDH) was purified from the cytosol fraction of rat liver by the method of Scopes [10]. BSA and myoglobin, obtained commercially, were purified further on a Sephacryl S-200 column. Proteins were labelled with FITC as described [11], and stored at −20°C in the presence of 25% glycerol until used.

Preparation of lysosomes

Lysosomes were prepared from rat liver by Percoll density gradient centrifugation as described [9]. The final precipitate of lysosomes was suspended in 20 m*M* sodium acetate buffer (pH 5.0), mixed with an equal volume of 50% glycerol and stored at −20°C until used. Arylsulphate was assayed with 4-nitrocatechol sulphate as substrate [12]. Protein was determined by the microbiuret method of Itzhaki and Gill [13].

Analysis of degradation of FITC-labelled proteins in vitro

For degradation of FITC-labelled proteins by disrupted lysosomes, the incubation mixture (100 μl) contained 50 m*M* acetate buffer (pH 5.0), 10 m*M* 2-mercaptoethanol, 1 m*M* EDTA, 10 or 20 μg of FITC-labelled substrate protein and disrupted lysosomes containing 50 mU of arylsulphatase and was incubated at 37°C for the indicated times. The reaction was stopped by adding 0.3 ml of 0.5 *M* hydrogencarbonate buffer (pH 8.5) and the mixture was applied to a small Toyopearl HW-40 column (20-ml bed volume) equilibrated with the running buffer described in the text. The running buffer was introduced with a peristaltic pump at 0.8 ml/min. Fractions of 2 ml of eluate were collected and their fluorescence intensities were measured with excitation and emission wavelengths of 490 and 520 nm, respectively.

RESULTS AND DISCUSSION

Interaction of FITC and FITC-labelled compounds with Toyopearl HW-40

FITC and FITC-labelled glycine and BSA were each applied to a small Toyopearl HW-40 column (20-ml bed volume) and their elution positions were compared (Fig. 1). Elution of FITC from the column was much delayed with running buffer containing 0.2 *M* NaCl, but was fast when the running

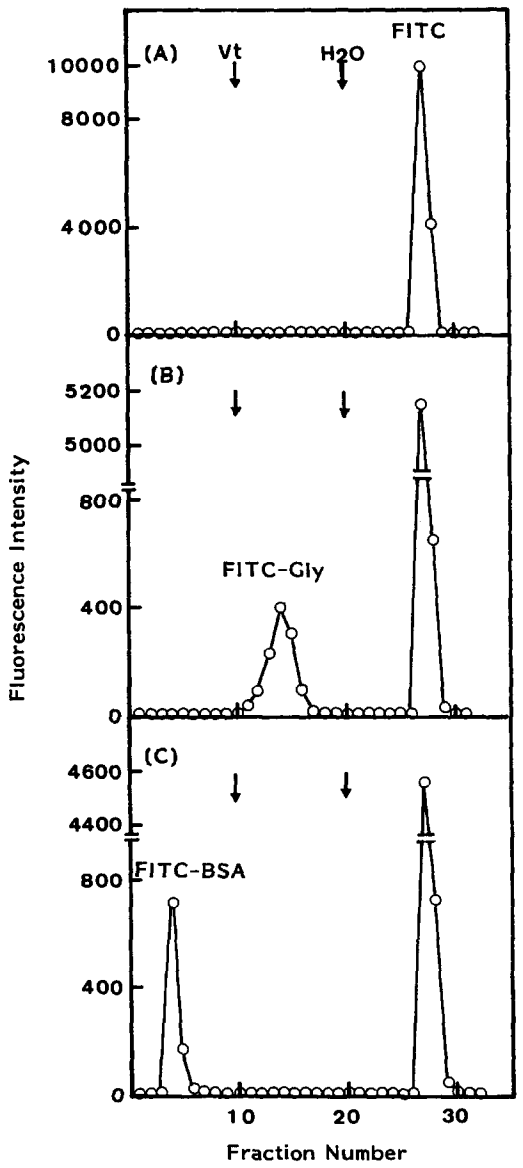


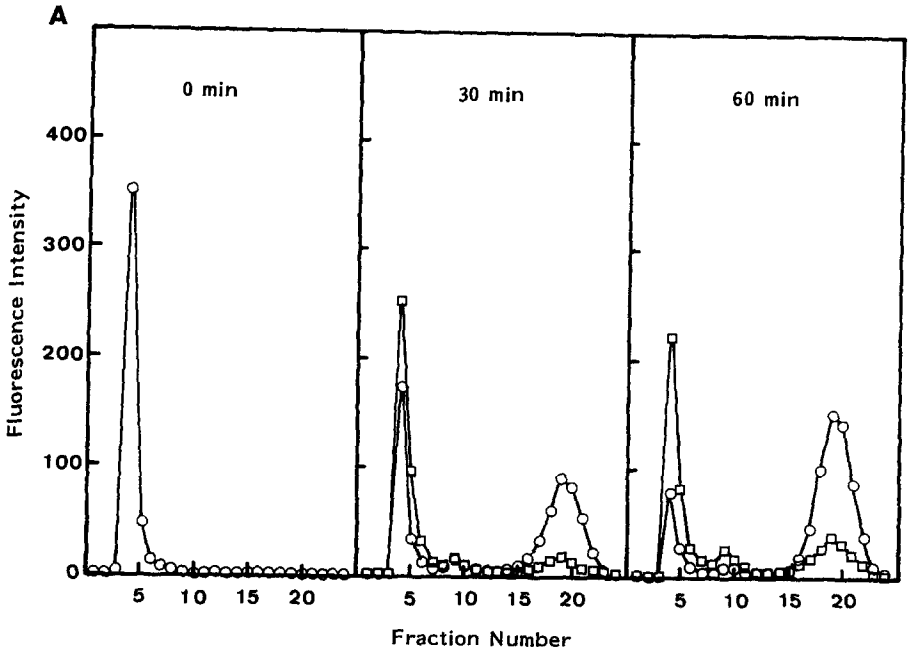
Fig. 1. Comparison of the elution behaviours of FITC and FITC-labelled glycine and BSA on a Toyopearl HW-40 column. Glysine (100 nmol) and BSA (100 μg) were each treated with FITC (5 μg) in 0.2 ml of 0.25 *M* hydrogenbicarbonate buffer (pH 9.0) for 1 h at room temperature. Solutions of FITC (5 μg) in 0.2 ml of 0.25 *M* hydrogencarbonate buffer (pH 9.0) and FITC-treated glycine and BSA were each mixed with 0.3 ml of 0.5 *M* hydrogencarbonate (pH 8.0) and applied to a small Toyopearl HW-40 column (20-ml bed volume) equilibrated with 0.2 *M* NaCl–10 m*M* phosphate buffer (pH 8.0). Materials were eluted with the same running solution and deionized water, both introduced at 0.8 ml/min with a peristaltic pump. Fractions of 2 ml of eluate were collected and their fluorescence intensities were measured with excitation and emission wavelengths of 490 and 520 nm, respectively. The elution profiles of (A) FITC, (B) FITC-labelled glycine and (C) FITC-labelled BSA are shown. The arrows labelled Vt and H_2O show the positions of the bed volume and the change of the running solution to deionized water, respectively.

buffer was changed to deionized water (Fig. 1A) as FITC has a strong affinity to Toyopearl HW-40 in the presence but not the absence of salt. FITC-labelled glycine was eluted much faster than FITC but had affinity to the gel, its elution being retarted with running buffer containing 0.2 *M* NaCl (Fig. 1B). FITC-labelled BSA had no affinity to the gel and was mainly eluted in the flow-through fractions (Fig. 1C). These results show that FITC-labelled amino acids can easily be separated from FITC-labelled proteins on a small Toyopearl HW-40 column. Therefore, we next applied these findings to the analysis of the degradation of FITC-labelled proteins by lysosomal enzymes *in vitro*.

Analysis of degradation of FITC-labelled proteins using a Toyopearl HW-40 column

Using a small Toyopearl HW-40 column (20-ml bed volume), we examined the degradations of various FITC-labelled proteins by disrupted lysosomes *in vitro*. Fig. 2A and B show results for the degradation of FITC-labelled asialofetuin by disrupted lysosomes. FITC-labelled degradation products, elution of which from the column was much delayed, were clearly separated from undegraded or partially degraded FITC-labelled compounds eluted in the flow-through fractions. Their formation increased with time during incubations and was suppressed strongly by leupeptin.

The FITC-labelled degradation product of FITC-labelled proteins by disrupted lysosomes seems to be mainly lysine, the ε-amino group of which is labelled with FITC, but Fig. 1A gives no direct evidence. We therefore compared the elution positions of the FITC-labelled degradation product and lysine and other various amino acids all labelled with FITC. The elution profile of FITC-labelled lysine shows that it contains two components, one major and one minor (Fig. 3A). As the pK_a values of its α-amino and ε-amino groups are 8.95 andf 10.53, respectively, its α-amino group should mainly be labelled with FITC. Hence the main component should be lysine with an FITC-labelled α-amino group. The FITC-labelled degradation product of FITC-labelled asialofetuin was eluted in the same fractions as the minor component of FITC-labelled lysine (Fig. 3B). Therefore, the minor component should be lysine with an FITC-labelled ε-amino group. The amino terminal



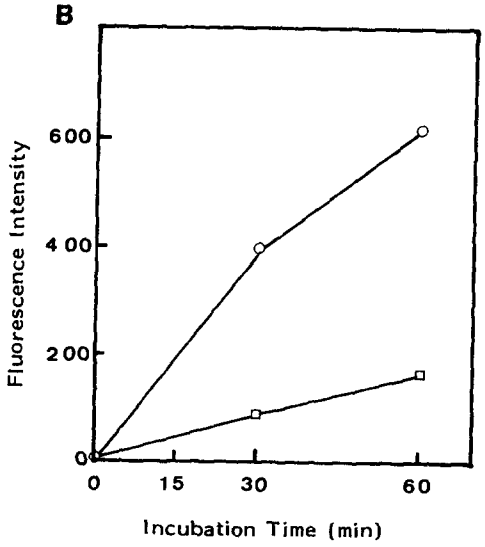


Fig. 2. (A) Analysis of degradation of FITC-labelled asialofetuin on a Toyopearl HW-40 column. FITC-labelled asialofetuin (10 μg) was incubated with disrupted lysosomes in the (□) presence or (○) absence of leupeptin (100 μg) for the indicated times at 37°C, then its degradation was analysed using a Toyopearl HW-40 column (20-ml bed volume) equilibrated with 0.2 *M* NaCl–10 m*M* phosphate buffer (pH 8.0). Fractions of 2 ml of eluate were collected and their fluorescence intensities were measured as described under Experimental. (B) Degradation of FITC-labelled asialofetuin by disrupted lysosomes with time. Eluate fractions 16–23 were pooled and their total fluorescence intensity (□) with and (○) without leupeptin was plotted against incubation time.

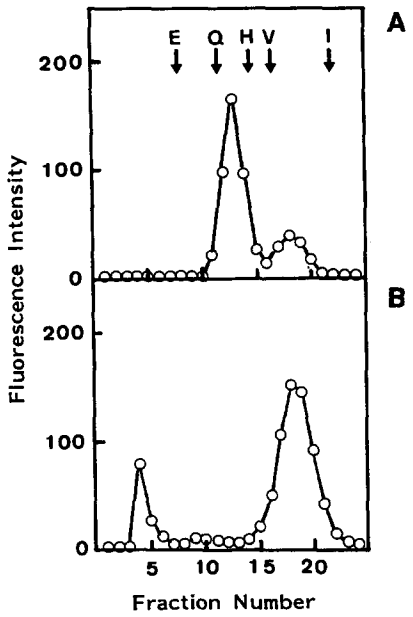


Fig. 3. Comparison of the elution profiles of FITC-labelled lysine and degradation products of FITC-labelled asialofetuin. Amino acids (100 nmol each) were treated with FITC (5 μg) as described under Experimental. They were then mixed with 0.3 ml 0.5 *M* hydrogencarbonate (pH 8.0) and applied to a Toyopearl HW-40 column (20-ml bed volume) equilibrated with 0.2 *M* NaCl–10 m*M* phosphate buffer (pH 8.0). Elution profiles of (A) FITC-labelled lysine and (B) FITC-labelled degradation products of FITC-labelled asialofetuin by disrupted lysosomes (60 min degradation shown in Fig. 2) are compared. The arrows show the elution positions of the peak of various FITC-labelled amino acids (one-letter symbols).

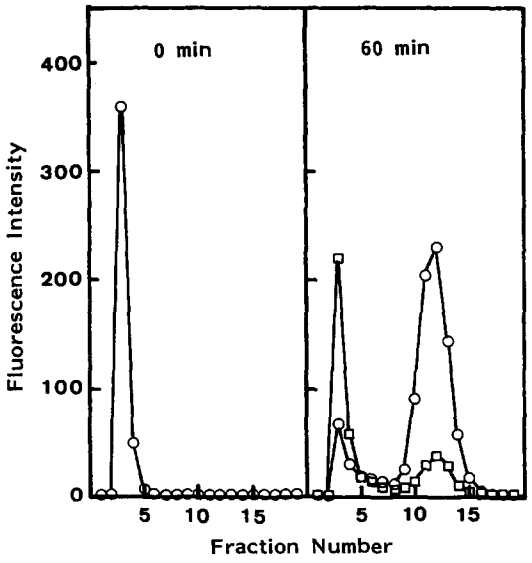


Fig. 4. Degradation of FITC-labelled LDH by disrupted lysosomes. FITC-labelled LDH (10 μg) was incubated with disrupted lysosomes in the (□) presence or (○) absence of leupeptin (100 μg) for the indicated times at 37°C, then the degradation was analysed with a small Toyopearl HW-40 column (10-ml bed volume). Other details as in Fig. 2.

residue of fetuin is isoleucine [14] and may be labelled with FITC. However, the elution positions of FITC-labelled isoleucine and various other amino acids were different from that of the FITC-labelled degradation product (Fig. 3A), the FITC-labelled degradation product being mainly lysine with an FITC-labelled ε-amino group. We also analysed the degradation of FITC-labelled LDH, myoglobin and BSA by disrupted lysosomes using a Toyopearl HW-40 column. In each instance lysine with an FITC-labelled ε-amino group was the main degradation product identified. A smaller column of Toyopearl HW-40 (10-ml bed volume) was available for separating FITC-labelled degradation products from undegraded FITC-labelled substrate protein eluted in the flow-through fractions (Fig. 4).

Toyopearl HW-40 has been used for gel filtration. However, the separation of FITC-labelled compounds on Toyopearl HW-40 is based not on the principle of gel filtration, but on differences in the affinities of the compounds to the gel. The gel filtration effect was negligible, if present at all, because of the small size of the column used (10–20-ml bed volume), the fast flow-rate of the running buffer (0.8 ml/min) and the large fraction size (2 ml per fraction). The presence of salt in the elution solution was necessary for affinity of FITC to Toyopearl HW-40. The affinity of FITC to Toyopearl HW-40 is completely lost on elution with deionized water, suggesting the hydrophobic interaction of FITC with the gel. This method is similar to a method of Sephadex G-25 affinity chromatography that we recently developed for analysing the degradation of FITC-labelled proteins [11], but it gives a better separation of the FITC-labelled amino acids because the differences in the affinities of different amino acids to Toyopearl HW-40 are greater than those to Sephadex G-25. Further, lysine with an FITC-labelled ε-amino group can be clearly separated from lysine with an FITC-labelled α-amino

group on a Toyopearl HW-40 column but not on a Sephadex G-25 column. The Toyopearl HW-40 column chromatography described here is very simple and also inexpensive, because a small column is effective and it can be used repeatedly after simple washing with deionized water.

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