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A RAPID METHOD FOR THE DETECTION AND QUANTITATION OF IgA PROTEASE ACTIVITY BY MACROBORE GEL-PERMEATION CHROMATOGRAPHY

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SUMMARY

A rapid assay to detect and quantitate immunoglobulin A1 (IgA1) protease activity was developed by the use of a high-performance gel-permeation chromatography column. The assay measured the disappearance of intact substrate and the emergence of cleavage fragments and the results could be expressed in absolute units. The utility of the assay was demonstrated in the partial purification of an IgA1 protease from a strain of *Haemophilus influenzae*.

INTRODUCTION

Immunoglobulin A (IgA) proteases are a group of extra-cellular bacterial enzymes capable of cleaving human IgA, the principal mediator of specific immunity on mucosal surfaces. Most of the IgA proteases that have been examined in detail cleave one of several prolyl–threonyl or prolyl–seryl peptide bonds located in the hinge region of the heavy chain of IgA subclass 1. Intact Fc and monomeric Fab fragments of IgA1 are results of the enzymatic activity. These enzymes have attracted considerable interest because of their exclusive presence in bacteria that are responsible for important infectious diseases in humans, including meningitis, gonorrhoea and destructive periodontitis[1,2].

Previous methods for quantitation of IgA protease activity have involved electrophoretic separation of cleavage fragments of radiolabelled substrate[3,4], binding of radiolabelled IgA to Fc_α receptors on haemolytic streptococci[5], or demonstration of cleavage fragments by radial immunodiffusion[6] or rocket immunoelectrophoresis[2]. These methods require radiolabelled substrate or incubation periods as long as several days. We have recently described a sensitive enzyme-linked immunosorbent assay (ELISA) method for quantitation of IgA1 proteases[7].

The method described in this communication uses high-performance liquid chromatography (HPLC) technology to detect and quantitate IgA1 protease activity. The method has the advantage that it is extremely fast and allows a partial characterization of enzyme and substrate cleavage fragments.

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EXPERIMENTAL

Enzymes and substrate

Crude preparations of IgA1 proteases were obtained from two strains of *Haemophilus influenzae* (HK 50 and HK 295) by the method described by Higerd *et al.*[8]. The enzyme substrate was a dimeric IgA1 paraprotein (Kah), purified from plasma of a patient with IgA myelomatosis, as described by Mestecky *et al.*[9]. The lyophilized protein was dissolved in a 0.1 *M* sodium phosphate buffer (pH 7.05).

Enzyme assay

Aliquots of IgA1 protease preparation were mixed with IgA1 substrate at a final substrate concentration of 0.8 mg/ml. A 100-μl sample of the reaction mixture was immediately analysed in the assay and served as control for samples analysed following incubation at 35°C for appropriate periods of time.

IgA1 protease activity was measured in an assay monitoring the disappearance of intact IgA1 substrate. Separation of intact substrate and fragments of IgA1 in the reaction mixtures was accomplished by high-performance gel-permeation chromatography in an LKB HPLC system (LKB, Bromma, Sweden) equipped with a TSK-GSWGP column (75 × 21.5 mm I.D.). The mobile phase was 0.1 *M* sodium phosphate buffer (pH 7.05). The flow-rate was 5 ml/min resulting in a separation of the relevant components within 4 min. Eluted proteins were detected spectrophotometrically at 206 nm. The localization of intact IgA, Fc_α and Fab_α fragments was confirmed by ELISA as described previously[10].

One unit of protease activity was defined as the amount of enzyme required to decrease the IgA peak by 0.001 absorbance units (206 nm) per min under the assay conditions. Only the initial linear part of the activity graph was used for activity determinations.

RESULTS AND DISCUSSION

After incubation with IgA1 protease, separation of remaining intact dimeric IgA substrate (MW 335,000) and the cleavage fragments (Fc_α) (MW 135,000) and Fab_α (MW 50,000) could be achieved on the TSK-GSWGP column within 4 min at a flow-rate of 5 ml/min. Fig. 1 illustrates the progressive disappearance of intact IgA and emergence of cleavage fragments with time.

The assay takes advantage of the ability of the TSK-GSWGP column to separate intact substrate from the cleavage products within less than 5 min. The assay thus allows a very rapid detection of enzyme activity in samples. Furthermore, the activity may be directly expressed in absolute terms (units). The TSK-GSWGP precolumn was chosen for this assay because it has better separation properties than the TSK-SW precolumn; in addition, the larger samples that could be applied to it increased the precision of the quantitative determinations. The following examples illustrate areas for application of the assay.

Crude IgA1 protease preparation from *H. influenzae* HK 50 was fractionated by high-performance gel-permeation chromatography under conditions given in the legend for Fig. 2. In order to monitor the elution of the IgA1 protease, 50 μl of each fraction were incubated with 100 μl of IgA1 substrate (final concentration 0.8 mg/ml)

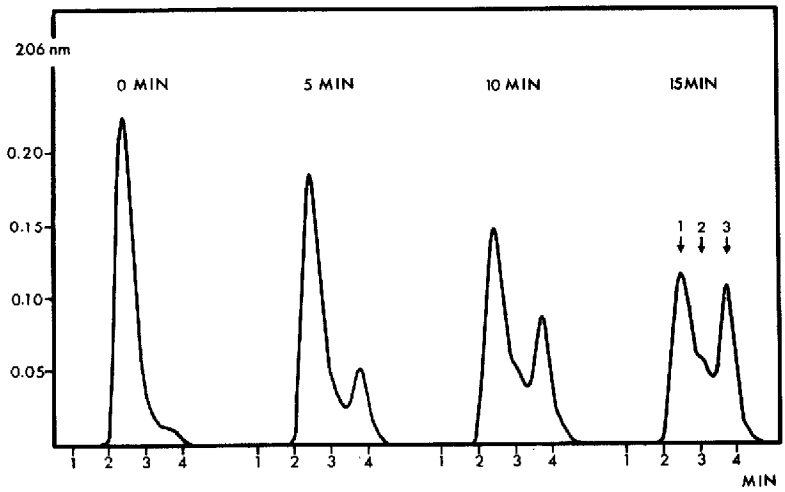

Fig. 1. Progressive cleavage of a dimeric IgA1 paraprotein (Kah) incubated with IgA1 protease from HK 50, as detected in an HPLC assay. Separation of intact substrate IgA1 (1), dimeric Fc_α (shoulder on the IgA1 peak) and Fab_α fragments (3) was accomplished on a TSK-GSWGP column (75 × 21.5 mm) using a flow-rate of 5 ml/min. Eluent buffer was 0.1 *M* sodium phosphate (pH 7.05). Proteins were detected spectrophotometrically at 206 nm.

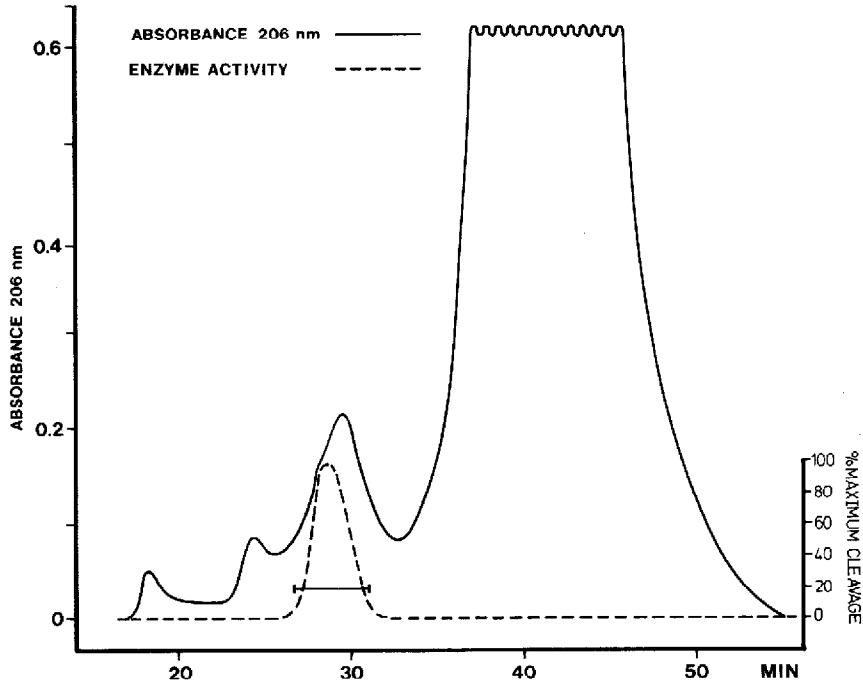

Fig. 2. Separation of 1 ml *H. influenzae* HK 50 protease preparation on a TSK G3000 column (600 × 21.5 mm), equipped with a TSK-GSWGP precolumn (75 × 21.5 mm). Eluent buffer 0.1 *M* sodium phosphate (pH 7.05). Flow-rate was 5 ml/min. Fraction size 1.25 ml, collection of fractions starting 16 min after injection. Active fractions were combined as indicated by the horizontal bar. Proteins were detected spectrophotometrically at 206 nm.

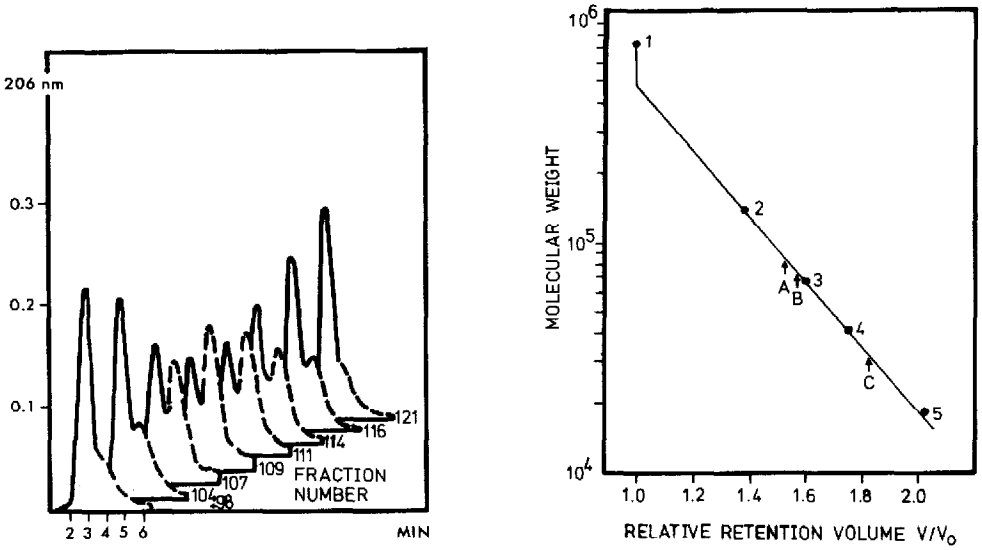

Fig. 3. HPLC analysis of fractions around the IgA1 protease peak from Fig. 2. A 50-μl volume of each fraction was incubated with 100 μl IgA-dimer (1.2 mg/ml) for 5 min. Of each sample 100 μl were applied to a TSK-GSWGP column (75 × 21.5 mm). Flow-rate 5 ml/min. Eluent buffer 0.1 *M* sodium phosphate (pH 7.05). Proteins were detected spectrophotometrically at 206 nm.

Fig. 4. Estimation of the apparent molecular weight of IgA1 proteases from HK 50 and HK 284 by high-performance gel-permeation chromatography on TSK G3000 SWG. Chromatographic conditions as in Fig. 2. The following standard proteins were used: (1) α_2-macroglobulin (MW 725,000); (2) dimeric bovine serum albumin (MW 136,000); (3) bovine serum albumin (MW 68,000); (4) ovalbumin (MW 43,000); (5) myoglobin (MW 17,800). The protease from HK 50 had an apparent molecular weight of 86,000 (A), and the two proteases from HK 295 had apparent molecular weights of 74,000 (B) and 34,000 (C).

for 5 min and then stored at −20°C for subsequent HPLC analysis. Fig. 3 shows the results of the analysis of selected fractions around the IgA1 protease peak (Fig. 2). In Fig. 3 the protease activity is expressed as percentage IgA substrate disappearance relative to the sample that showed the highest degree of IgA cleavage.

The molecular weight of IgA1 proteases from two strains of *H. influenzae* was determined by comparison of the position of the eluted protease with that of five selected reference proteins applied to the HPLC columns (Fig. 4). With this method an apparent molecular weight of 86,000 was obtained for the IgA1 protease from *H. influenzae* HK 50.

We have previously demonstrated IgA1 protease activity with two distinct specificities in *H. influenzae* strain HK 295. The presence of two separate IgA1 proteases was further indicated by inhibition experiments using specific enzyme-neutralizing antibodies[11]. Detection of enzyme activity in eluates from an HPLC separation of a crude protease preparation from this strain accordingly demonstrated two separate peaks of activity (Fig. 5). The molecular weights of the two IgA1 proteases were found to be 74,000 and 34,000, respectively (Fig. 4). Under the experimental conditions more IgA1 cleaving activity was present at the lower molecular weight.

The assay was used to determine the degree of purification of *H. influenzae* HK 50 protease subjected to HPLC separation on the TSK G3000 SWG column. Of

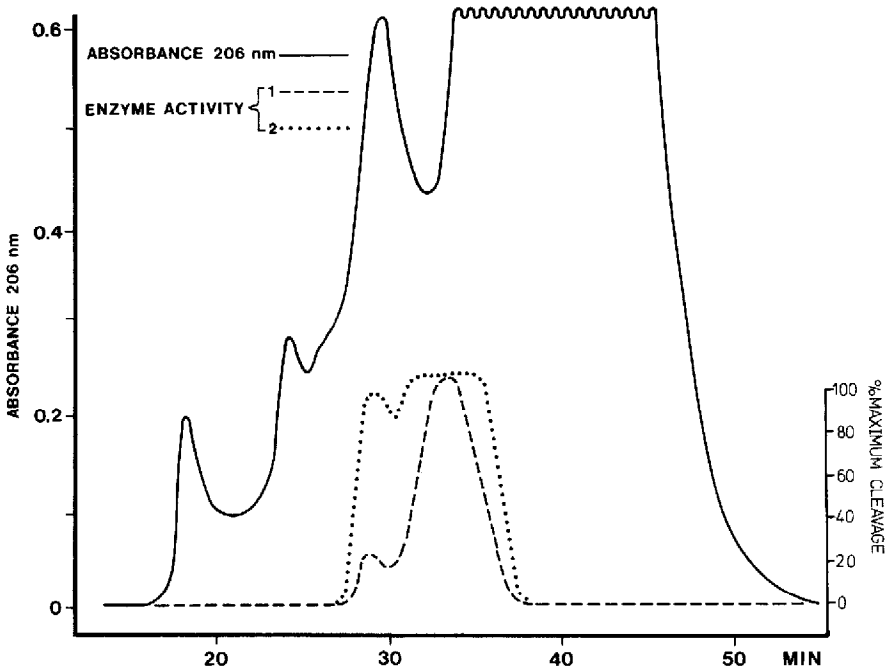

Fig. 5. Separation of 1 ml HK 295 protease solution on a TSK G3000 SWG column (600 × 21.5 mm), equipped with a TSK-GSWGP precolumn (75 × 21.5 mm). Conditions as in Fig. 2. (1 – – – –), Incubation of 50 µl from each fraction for 5 min. (2 · · · · ·), Incubation of 50 µl of ech fraction for 15 min.

TABLE I

PARTIAL PURIFICATION OF *H. INFLUENZAE* HK 50 PROTEASE

Protein was determined according to the method of Lowry *et al.*[12].

IgA1 HK 50 protease	*Protein (mg)*	*Total units*	*Units/mg*	*Purification (×)*
Crude preparation	2.10	8075	3845	1
TSK G3000 SWG pool	0.029	7431	256,241	67

the enzyme activity present in the crude protease preparation 92% remained after fractionation, and a 67-fold degree of purification was achieved by this single step (Table I).

The rapid assay used here to detect and quantitate IgA1 protease activity may be applied to any enzyme for which the substrate and the cleavage products can be separated on the HPLC column.

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