Bacterial immunoglobulin a proteases monitored by continuous Spectrophotometry

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IgA proteases were estimated in a turbid aqueous two-phase system with 10% polyethylene glycol-Tris buffer, where IgA spontaneously concentrates in microscopic spherical particles (< 1 μm). After enzymatic cleavage of IgA into Fab_α and Fc_α fragments, these fragments are soluble and decreasing turbidity is observed. The reaction may be followed by conventional spectrophotometry. In this manner, IgA proteases may be estimated in 10 min. Examples of the utility of the method are given with results from inhibitor studies, estimation of K_m and purification of IgA protease from *Haemophilus influenzae*.

Immunoglobulin A *IgA protease* *Polyethylene glycol* *Virulence* *Haemophilus*

1. INTRODUCTION

Several pathogenic microorganisms that attack the human organism through mucosal surfaces share a common feature in their ability to secrete extracellular proteases with extreme substrate specificity. Human IgA1 (from serum) and secretory IgA1 (S-IgA) are the only known substrates. After cleavage of a single Pro–Thr or Pro–Ser peptide bond in the hinge region Fab_α and Fc_α fragments appear as reaction products (review [1–3]).

As the IgA proteases and their reaction products may be important virulence factors it is desirable to purify larger amounts of these enzymes from different microbial sources. The possible use of IgA proteases as antigens in vaccine production [2] underlines the importance of such purification projects.

Various procedures for the determination of IgA-proteolytic activities have been developed [1,2], but they are more or less time-consuming or require special equipment. None allow a continuous monitoring of the reaction because no chromogenic substrate analog has been found.

The present method now offers continuous and rapid (automatic) monitoring by means of conventional spectrophotometry, based on the observation that IgA spontaneously forms spherical microparticles when added to 10% polyethylene glycol (PEG). The particles confer a certain turbidity to the mixture, which is constant for hours. When IgA protease is added there is cleavage of IgA into Fab and Fc fragments, that are soluble in the system and the turbidity eventually clears completely. The rate of decreasing turbidity is thus a measure of enzyme activity.

2. MATERIALS AND METHODS

2.1. *Substrates and enzymes*

IgA1 was purified from plasma of patients (Kah and Mor) with IgA myelomatosis as described by Mestecky et al. [4]. A final separation with HPLC on a TSK G3000 SWG column (LKB, Bromma, Sweden) was used to obtain dimeric IgA1.

IgM (Hal) from a myelomatosis patient and colostral S-IgA were kindly donated by Dr J. Mestecky, University of Alabama in Birmingham. The identity of the immunoglobulins was checked by an ELISA technique with antibodies against α and μ heavy chains [5].

Bacterial IgA proteases were prepared from strains of *Haemophilus influenzae* (HK 50,

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HK 295, HK 393) by the method of Higerd et al. [6] using chocolate agar as growth medium.

2.2. *IgA protease assay*

Concentrated, buffered solutions of immunoglobulins (10 mg/ml) were added to a buffer with 0.1 M Tris-HCl and 10% (w/v) PEG (PEG 6000 used throughout) pH 7.2. Final concentrations of IgA were kept below 50 μg/ml in a total volume of 600 μl. The immunoglobulins were pipetted directly into the PEG buffer and the mixture gently swirled or agitated with a spatula. An opalescent appearance occurred without any delay. The proteases were added in volumes of less than 20 μl to 600 μl IgA/PEG buffer in each cuvette to avoid significant dilution of the PEG. Readings were taken in a Beckman DU8 spectrophotometer at 5 min intervals at 400 nm and 37°C. Protein concentrations were estimated by the method of Lowry et al. [7]. Chemicals were of analytical grade.

3. RESULTS

The appearance of dimeric IgA1 (Kah) (M_r 335000) in 10% PEG was observed microscopically as shown in fig.1. As seen in the figure spherical particles (droplets) of diameter <1 μm were formed. The particle size was estimated from comparisons with standard latex particles of 0.12 and 1.1 μm (Unisphere, Serva, Heidelberg). The particles formed a stable suspension at unit gravity (fig.2). By centrifugation at 20000 $\times$ g they could be sedimented. The sediment was shown to contain virtually all the A_{280nm} absorbing material. The particles formed spontaneously (i.e. without any mechanical means) and readily redissolved when the PEG concentration was lowered.

Linearity between IgA concentration and absorbance at 400 nm was observed when the absorbance was below 0.3. Absorbance changes from a typical assay are shown in fig.2, where IgA proteases from various strains of *H. influenzae* were examined. It is seen that the turbidity completely cleared within 30 min for the most active protease preparations. The IgA proteases were not precipitated by 10% PEG since no additional turbidity was produced on protease addition. Preincubation of proteases in 10% PEG did not affect their activity. The reaction could also be restarted after termination by addition of more IgA to the reaction mixture.

Fig.1. Microscopy (dark field) of dimeric IgA1 (Kah), 0.5 mg/ml, in 10% (w/v) PEG, 0.1 M Tris-HCl (pH 7.2). 200 $\times$ magnification.

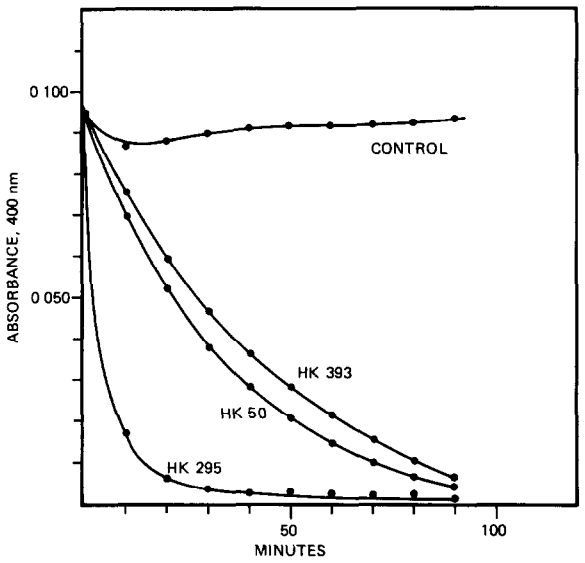

Fig.2. Cleavage of IgA1 by extracellular IgA proteases from *H. influenzae*. The reaction mixture contained 600 μl buffer with 10% PEG, 0.1 M Tris-HCl (pH 7.2) and 30 μg IgA1. The reaction was initiated by addition of 2 μl enzyme preparation from strains HK 50, HK 295, and HK 393.

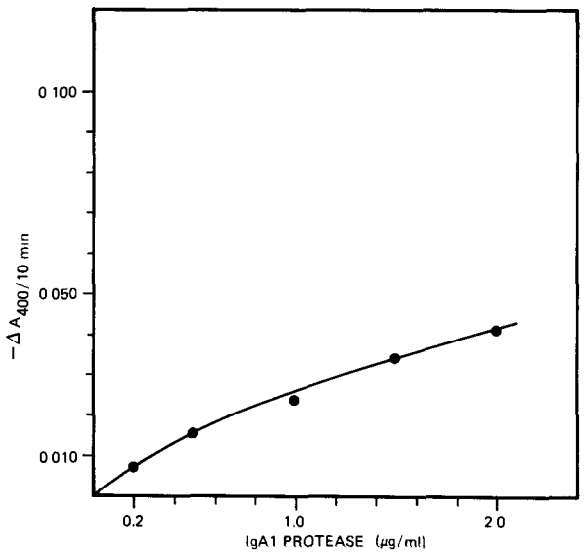

Fig.3. Initial reaction rates vs enzyme concentration. The rates were measured from initial slopes as illustrated in fig.2 at a substrate concentration (dimeric IgA1) of 50 μg/ml. The IgA protease preparation was from *H. influenzae* HK 295.

IgG and IgM also caused turbidity in PEG buffer, but were not affected by the IgA-specific proteases from *Haemophilus*.

3.1. *Initial rates vs enzyme concentration*

The relationship between these parameters is shown in fig.3. A slight curvature of the expected straight line is apparent. It is therefore necessary to use enzyme dilutions in the same concentration range, when comparisons are made.

3.2. *Michaelis constant*

With a protease preparation from *H. influenzae* HK 295 and dimeric IgA1 (Kah) as substrate the Michaelis constant was determined as shown in fig.4. It is seen that a $K_m = 0.8 \times 10^{-6}$ M is obtained. This value is of the same order of magnitude as found for other IgA1 proteases [8,9].

3.3. *Inhibitor studies*

A series of metal and organic cations were examined for inhibitory activity. Zn^{2+}, Cu^{2+} and Al^{3+} were completely inhibitory to the reaction at 50 μM. Ca^{2+}, Mg^{2+}, Mn^{2+}, and Fe^{2+} in concentrations up to 1 mM did not affect the reaction.

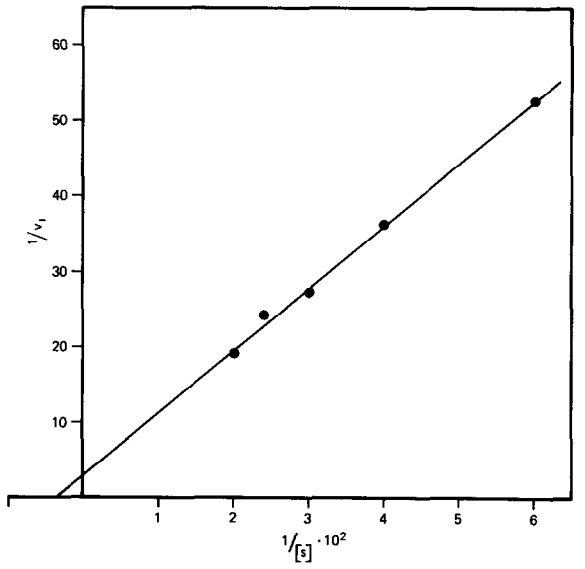

Fig.4. Double-reciprocal plot of initial reaction rates vs substrate concentration. The rates were measured from initial slopes as illustrated in fig.2. The enzyme concentration (total protein) was 2 μg/ml. The straight line was drawn after linear regression ($y = 821.6 \times 3.27$). [S] was measured in μg/ml, and V_i as $-\Delta A_{400\,nm}/10$ min. A K_m value of 0.8×10^{-6} M was obtained.

Cetyltrimethylammonium bromide (1 mM), chlorhexidine [1,1′-hexamethylenebis(5-[4-chlorophenyl]biguanide)] (5 mM) and protamine sulfate (10 μg/ml) were completely inhibitory to the reaction at the concentrations given in parentheses. The inhibition that was observed with metal and organic cations may be a false negative result, because controls with immunoelectrophoresis [2] of the reaction mixtures still showed protease activity.

The IgA proteases were also completely inhibited by SDS (1 mM) but not by conventional non-ionic, cholate-derived or zwitterionic detergents.

From previous studies [10] bathocuproine disulfonate (BCDS) is known as an efficient inhibitor of the IgA proteases from *H. influenzae*. The present assay allowed better quantitation of the BCDS-induced inhibition, shown in fig.5. The inhibition was complete at 20 mM BCDS. Among other metal chelators, EDTA, 2,2′-bipyridyl, Tiron (4,5-dihydroxy-1,3-benzenedisulfonate) (all at 50 mM) and desferrioxamine (10 mM) were without inhibitory effect. Cyanide, azide, and fluoride (20 mM) also produced no inhibition.

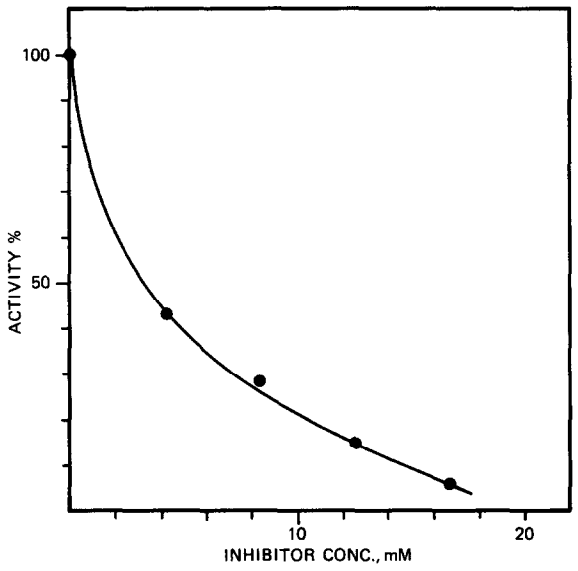

Fig.5. Bathocuproine disulfonate as inhibitor of IgA proteases from *H. influenzae*. Enzyme activities were measured as decsribed in fig.2. The inhibitor was added to the enzyme 1 h prior to substrate addition.

An enzyme preparation from *Streptococcus sanguis*, SK 1, was completely inhibited by 1 mM EDTA when assayed in the present system.

3.4. *Enzyme purification*

Our assay allows rapid and quantitative localization of IgA proteases from purification studies. As an example, fig.6 shows fractionation of *H. influenzae* HK 393 extracellular proteins on an HPLC column (TSK G3000 SW, 7.5 × 600 mm). A clear separation in 2 peaks with IgA protease activity appears. The peaks eluted with relative elution volumes, V_e, of 1.53 and 1.67. Compared to the elution pattern of standard proteins the 2 IgA proteases may be attributed M_r values of 105000 and 80000. The appearance of 2 activity peaks was expected from studies on the antigenic structure of IgA proteases from HK 393 and other strains of *H. influenzae* [11].

3.5. *Further potentials of the IgA/PEG system*

It is possible to obtain kinetic data on the reaction between thiol compounds and disulfide

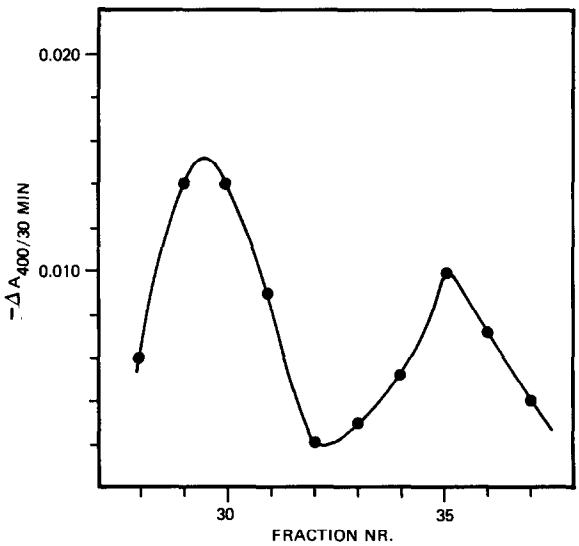

Fig.6. Fractionation of IgA proteases from *H. influenzae* HK 393 on a HPLC-column, TSK G3000 SW (7.5 × 600 mm). The flow rate was 0.5 ml/min. The relative elution volumes, V_e, corresponding to the two peaks were calculated to be 1.53 and 1.67, corresponding to M_r values of 105000 and 80000. Activities were assayed as described in fig.2 with 20 μl from each fraction.

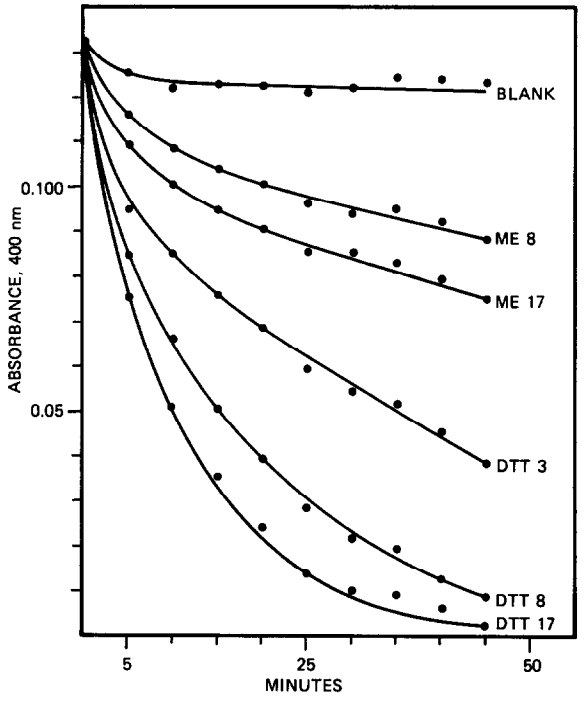

Fig.7. The reaction between mercaptoethanol (ME) or dithiothreitol (DTT) with IgA (dimeric) in 10% PEG. The concentration of ME was 8 or 17 mM, while DTT was 3, 8 or 17 mM.

bridges in IgA with the spectrophotometric method as shown in fig.7. This figure illustrates reactions between 2-mercaptoethanol or dithiothreitol and dimeric IgA. When disulfide bonds between the J-chain or between light and heavy chains are reduced, the reaction products are soluble in PEG buffer, and the turbidity decreases. The reaction with dithiothreitol is much faster than that with mercaptoethanol at equimolar concentrations.

The assay may be used with other proteins and proteases as well. With catalase as a turbidity-causing protein it could be demonstrated that the preparations from *Haemophilus* were free of non-specific proteases.

4. DISCUSSION

The present IgA protease assay offers advantages such as continuous monitoring (i.e. estimation of initial reaction rates), use of simple spectrophotometric equipment [12] and no special demands for immunologic or radiolabeled reagents. With the concentration of polyethylene glycol used only larger molecules like dimeric IgA or S-IgA will cause turbidity, whereas smaller proteins such as monomeric IgA, Fab and Fc fragments remain in solution. The spontaneous formation of uniform spherical particles (fig.1) indicates formation of an aqueous two-phase system [13]. The present demonstration of a K_m value in accordance with estimations obtained by other methods [8,9] suggests a free, non-limiting diffusion of substrate and enzyme between the 2 phases. Polyethylene glycol is remarkable in its apparent absence of chemical interactions with proteins [14]. It is important, of course, to avoid false negative or positive results by including appropriate controls.

Exploitation of the turbidity caused by larger protein molecules in PEG can be extended to other proteins (e.g. catalase) as substrates of various proteases. In this way, protease studies may be performed as easily as with chromogenic peptide substrates (in preparation).

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