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STUDIES ON EXTRACELLULAR PROTEASES OF *STREPTOCOCCUS SANGUIS*

PURIFICATION AND CHARACTERIZATION OF A HUMAN IgA1 SPECIFIC PROTEASE

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Summary

Extracellular caseinolytic activity was found in the culture fluid of *Streptococcus sanguis* ATCC 10556 grown in a dialyzed culture medium. This activity was due to multiple proteases that differed in their elution from hydroxyapatite, sensitivity to enzyme inhibitors, specificity and optimum pH.

IgA protease, which splits human immunoglobulin A1 into intact Fc and Fab could be effectively separated from these relatively non-specific proteases and purified to apparent homogeneity in 20% yield by a five-step procedure. Although the bulk of the dextran sucrase activity was separated from the IgA protease, a small amount of sucrase activity remained with the final IgA protease preparation. In polyacrylamide gel electrophoresis at pH 9.5 both activities were located in the single protein band detected in this preparation.

A quantitative method for the assay of IgA protease was developed, based on radial immunodiffusion to quantitate the Fab produced. This was used to follow the specific activity and yield during purification, and to characterize some of the catalytic properties of the enzyme. At an enzyme/substrate ratio of 1 : 400 (w/w) the protease could effect 50% proteolysis of IgA in overnight incubation at 37°C. The optimum activity was at pH 8.0, and 50% inhibition was achieved at $4 \cdot 10^{-4}$ M *o*-phenanthroline or $8 \cdot 10^{-4}$ M ethylene diamine tetraacetate. Concentrations of diisopropyl phosphofluoridate, phenylmethylsulfonyl fluoride, iodoacetate and *p*-chloromercuribenzoate up to 10^{-2} M were

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Abbreviations: IgA, immunoglobulin A; SDS, sodium dodecyl sulfate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid.

without effect on the IgA protease activity. Full reactivation of the chelator inhibited enzyme could be achieved by the addition of Mg^{2+}, Mn^{2+} or Ca^{2+}.

Introduction

"IgA protease" is a proteolytic enzyme that specifically cleaves human immunoglobulin A (IgA) into Fab and Fc fragments. It was first detected in human feces [1] and later *Streptococcus sanguis* was found to be an excellent source of the enzyme [2]. More recently, a similar enzyme activity has also been found in *Neisseria gonorrhea* and *Neisseria meningtides* [3]. Since IgA is an important class of immunoglobulins in mucous secretions [4], and these microorganisms are indigenous to membranes, it is conceivable that this enzyme may play an important role in mucosal immunity.

The remarkable specificity of this enzyme has been previously observed [1—3] and the site of cleavage at a proline-threonine residue in the hinge peptide of IgA1 reported [2,3]. Other immunoglobulin classes, including IgA2 and classical proteolytic substrates such as casein, hemoglobin, and synthetic substrates are not proteolyzed. However, the purity of the preparations used in these investigations has not been established, and one preparation gave three bands in polyacrylamide gel electrophoresis [2]. A major problem in the biochemical study of this enzyme was the inability to quantitate its activity. In the work reported here we have developed a method for the quantitative assay of the enzyme activity, purified it to apparent homogeneity and studied its relation to other enzymes in the culture filtrates of *S. sanguis*. Other proteases which are relatively less specific were also found in the same culture filtrates and were effectively separated from IgA protease. These proteases are referred to here as non-specific proteases. The pH optima and effect of enzyme inhibitors on these enzymes have been studied and compared. Sucrase activity was also found in the purified enzyme, and its relation to the previously recognized dextran sucrase [5] has been investigated.

Materials and Methods

S. sanguis ATCC 10556 was obtained from the American Type Culture Collection. Bacto-tryptose, bacto-yeast extract, casamino acids and brain heart infusion were obtained from Difco Laboratories, Detroit, Mich. Human serum myeloma IgA were isolated as previously described [6]. Antisera were raised in rabbits against Fab γ from human Cohn fraction II for detecting light chains, and against monoclonal IgA for detecting Fc α and Fab α in immunoelectrophoresis.

^{125}I was obtained from New England Nuclear, Boston, Mass. and lactoperoxidase from P-L Biochemicals, Inc., Milwaukee, Wisc.

IgA protease assay. Various dilutions of the enzyme were incubated at 37° C for 16 h in 100 μl 0.05 M Tris · HCl buffer (pH 8.0) containing 100 μg monoclonal IgA. The enzyme reaction was stopped by adding 10 μl 0.2 M EDTA, mixing and cooling. Immunoelectrophoresis of samples of the incubation media against an antiserum made against the same monoclonal IgA was performed [7]

to evaluate the extent of digestion (Fig. 1). Complete digestion into Fab and Fc is shown by the crossing of the precipitin lines formed by the two fragments as shown in Fig. 1A. For quantitation of IgA protease activity, radial immunodiffusion [8] in agar containing antiserum to light chains was performed on triplicate 10-μl samples of the incubation media. The diameters of the precipitin rings were measured after 3 days incubation at 37° C by a slide micrometer and a dissecting microscope. By testing mixtures of varying proportions of a completely digested mixture and an undigested IgA solution, it was found that the square of the diameter was directly proportional to the extent of digestion. By including a completely digested and undigested incubation mixture in each radial immunodiffusion plate, it is possible to quantitate the extent of digestion of each sample, and from a standard curve (Fig. 2), the enzyme activity can be determined. One unit of IgA protease is defined as that producing 50% digestion of the IgA protein under the conditions of the assay. For best results, only incubations showing 20—70% digestion are used.

Non-specific protease assay. Non-specific proteases were assayed by the increase in trichloroacetic acid-soluble peptides produced on incubation with casein. Three methods were used to measure the amounts of soluble peptides. For crude preparations, [^{125}I]casein was used, whereas for purer preparations, either the ninhydrin reaction or the absorbance at 280 nm was employed. Absorbance at 280 nm could not be used in the presence of ultraviolet-absorbing materials such as *o*-phenanthroline and EDTA, whereas the ninhydrin method was not suitable in the presence of NH_4^+.

(a) Cold casein assays: For assays, the casein solution (10 mg/ml in 0.2 M NaOH) was diluted with a 0.5 vol. 0.1 M Tris · HCL buffer (pH 7.0) and 200 μl of this mixture were added to 100 μl enzyme solution. After incubation for 16 h, 300 μl 12.5% trichloroacetic acid were added and the absorbance of the supernatant solution read at 280 nm. For the ninhydrin color reaction, 100-μl supernatants were mixed with 100 μl 0.38 M NaOH and 300 μl ninhydrin reagent [9] and the mixture heated in a boiling water bath for 20 min. After cooling, 1.5 ml 50% ethanol was added and the absorbance read at 570 nm. One unit of non-specific protease was defined as that amount of enzyme producing a net change of 1.0 absorbance unit under these conditions.

(b) Radio-iodinated casein method: Casein was iodinated with ^{125}I by the

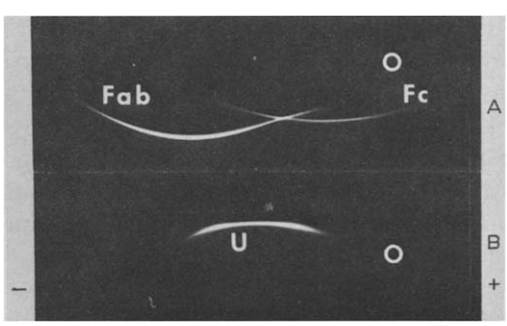


Fig. 1. Immunoelectrophoresis of monoclonal IgA digests showing complete digestion by IgA protease into Fab and Fc (A) compared to the undigested IgA (B).

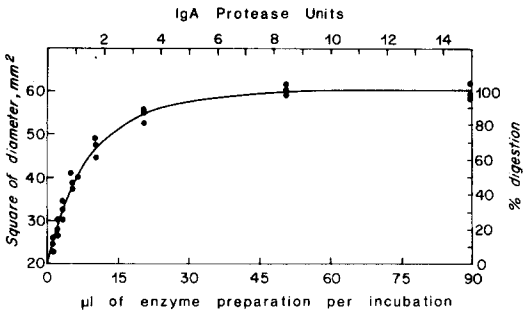


Fig. 2. Standard curve for IgA protease assay. Activity was determined by radial immunodiffusion of 16 h digests of monoclonal IgA1 (100 µg) at 37° C in 50 mM Tris · HCl (100 µl) at pH 8.0. The agar in the immunodiffusion plates contained antiserum specific for light chains. One unit of IgA protease is defined as that producing 50% digestion, the percent digestion being directly proportionate to the diameter square.

lactoperoxidase method [10] and purified by gel filtration on Sephadex G-50 and by repeated precipitation with 5% trichloroacetic acid (neutralized with 0.02 M NaOH) until more than 90% of the radioactivity was acid precipitable. Radiolabelled casein was diluted with unlabelled casein solution to give a 1 mg/ml solution in 0.02 M NaOH and 500 000 cpm/ml. This solution was mixed with 1 vol. 0.2 M phosphate buffer (pH 6.5) and 100 µl was incubated at 37° C for 16 h with 50 µl enzyme solution. After incubation, 50 µl unlabelled casein solution was added followed by 50 µl 25% trichloroacetic acid, mixed and centrifuged for 30 min at 2000 rev./min. Triplicate 50-µl samples of the supernatant solution were added to 0.5 ml water and counted in a gamma scintillation counter (Packard). Standard curves were linear up to a net increase of 1000 cpm and only the values in this range were used. A unit of non-specific protease was defined as that producing a net increase of 1000 cpm in the supernatant solution.

Sucrase activity. Mixtures of 100 µl 0.1 g/ml sucrose in *N*-ethylmorpholine · HCl buffer (pH 6.0) and 100 µl enzyme were incubated at 37° C. 25-µl aliquots were removed at 0, 1, 2 and 3 h and diluted with 500 µl 0.02 M NaOH to stop the enzyme reaction. The reducing sugars in these samples were determined by the ferricyanide method [11] using fructose as standard. One unit of sucrase activity was defined as that producing 1 µmol of reducing sugar in 1 h under these conditions.

For identification of the products of the reaction, incubations were continued overnight and 1 vol. absolute ethanol was added. Formation of a precipitate was taken as evidence for the production of dextran. To identify the reducing sugars produced, the supernatant was tested by ascending paper chromatography on Whatman No. 1 paper, using the pad technique and pyridine/ethyl acetate/water mixture [12] for developing the chromatogram and $AgNO_3$ for visualization of the spots [13].

Protein determination. The method of Lowry et al. [14] was used for determination of protein concentration in the different pools. Crystalline bovine serum albumin was used as a standard. Absorbance at 280 or 230 nm was used to monitor the column fractions.

Polyacrylamide gel electrophoresis. Electrophoresis was performed with 5% polyacrylamide gels at pH 9.5 [15]. Staining was carried out with Coomassie Blue [16]. Gels that were sliced by gel slicer model 190 (BioRad) were not stained. Slices of unstained gels were assigned to corresponding bands on stained gels by their R_F values. Enzyme assays were done on slices crushed by a glass rod; otherwise all the conditions were similar to the assays described above.

SDS-polyacrylamide gel electrophoresis was performed in 10% gels as described by Weber et al. [17].

Culture medium. The culture medium consisted of 25 g casamino acids, 22.5 g K_2HPO_4, 0.2 g $CaCl_2$, 75 g glucose and the ultrafiltrate (Amicon PM 10 membrane) of 100 g bacto-tryptose and 50 g bacto-yeast extract solution made up to 7.5 l with water. A few drops of bromothymol blue in 0.5 M NaOH were added as required to give a visible green color.

Growth of S. sanguis. Brain heart infusion media were inoculated with colonies of *S. sanguis* grown on blood agar in 5% CO_2. After 36—48 h growth at 37° C 1.5 ml culture were used to inoculate each 1.5 l of the culture medium and the flasks incubated at 37° C for 48 h. 1 M NaOH was added intermittently to the culture flasks as required to maintain neutral pH.

Preparation of IgA protease. (1) $(NH_4)_2SO_4$ precipitation: Cell-free culture medium was obtained by centrifugation at 7000 rev./min at 5° C for 30 min. 4 g celite and 6 g cellulose powder were added to the supernatant, followed by $(NH_4)_2SO_4$ to 65% saturation and ammonia to maintain neutral pH. After overnight equilibration, the precipitate was collected by centrifugation and kept suspended in 50 ml 65% saturated neutral $(NH_4)_2SO_4$ solution.

(2) Reverse $(NH_4)_2SO_4$ chromatography: The suspension containing celite and cellulose powder was packed into a glass column (15 × 2.5 cm). The column was eluted successively with 40 ml $(NH_4)_2SO_4$ solutions (65—0% in 5% steps) at room temperature. The eluate at each concentration was collected separately and taken to 70% saturation. After overnight equilibration, the precipitate was collected by centrifugation and dialyzed against 25 mM phosphate buffer (pH 6.8). These dialyzed fractions were assayed for protein and enzyme activities. Fractions rich in IgA protease were eluted by 35—20% saturated $(NH_4)_2SO_4$ solutions (Table I).

(3) Hydroxyapatite chromatography: IgA protease-active fractions from two reverse $(NH_4)_2SO_4$ chromatography steps were pooled and applied to a hydroxyapatite (Biogel HT, BioRad Lab) column (175 × 14 mm) equilibrated with 25 mM potassium phosphate buffer (pH 6.8). The column was eluted at 5° C with potassium phosphate buffers (pH 6.8) of increasing molarities as follows: 25 mM, 50 mM, 300 ml gradient to 0.25 M and 200 ml gradient to 0.8 M. The collected fractions were assayed and pooled as shown in Fig. 3. The pooled fractions were concentrated, dialyzed against 50 mM Tris · HCl buffer pH 8.2) and assayed for protein and enzyme activities (Table II).

(4) DEAE-cellulose chromatography: IgA protease-active pools of the hydroxyapatite column were combined and applied to a DEAE-cellulose (DE-52, Whatman) column (160 × 9 mm) equilibrated with 50 mM Tris · HCl buffer (pH 8.2). The column was eluted with a 200 ml linear gradient of 0—0.15 M NaCl followed by a 300 ml gradient of 0.15—0.8 M NaCl in 50 mM

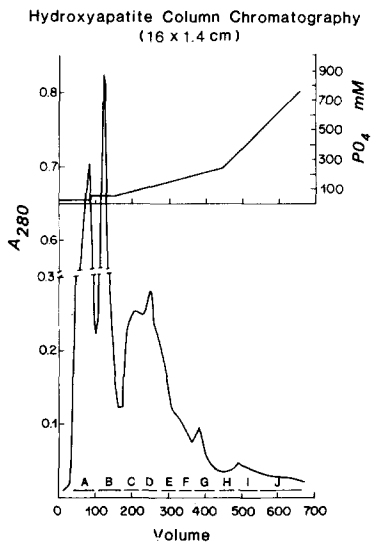
TABLE I

PROTEIN AND ENZYME CONTENT OF THE FRACTIONS OBTAINED BY REVERSE $(NH_4)_2SO_4$ SALTING OUT CHROMATOGRAPHY OF 65% SATURATION PRECIPITATE OF 7.5 LITERS OF THE CULTURE MEDIUM

Saturation of the eluent (%)	Protein (Folin-Lowry method) (mg)	Non-specific protease		IgA protease (units)	Sucrase (units)
		Radioactivity (units)	Ultraviolet (units)		
65	0.6	0	0	0	5
60	2.7	5	0.5	0	8
55	4.8	6	0.6	0	8
50	6.8	10	3.2	0	8
45	9.2	34	5.1	100	12
40	12.2	72	8.9	500	12
35 *	12.6	162	20.5	1720	28
30 *	10.4	126	16.2	1420	26
25 *	10.5	154	15.0	1250	54
20 *	7.8	150	16.5	800	70
15	3.4	35	3.2	140	56
10	2.8	16	1.6	110	38
5	7.2	24	1.1	80	16
0	38.6	128	3.8	240	776

* These fractions were pooled together and applied to the hydroxyapatite column (Fig. 3).

Tris · HCl buffer (pH 8.2). The collected fractions were assayed for IgA protease, pooled as shown in Fig. 4 and then concentrated and assayed for protein and enzyme activities (Table III).



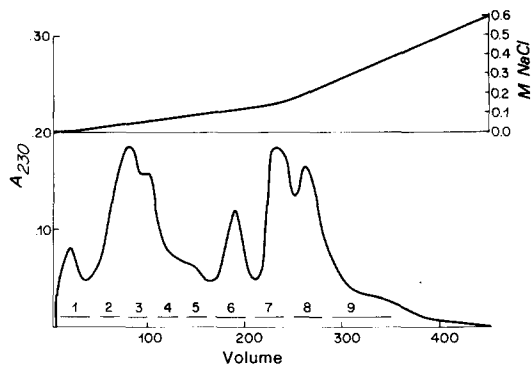


Fig. 3. Chromatographic pattern of the reverse ammonium sulfate IgA protease pool of Table I on a hydroxyapatite column (175 X 14 mm) eluted with potassium phosphate buffer, pH 6.8, of increasing molarity as shown in the top curve. Horizontal bars at the bottom indicate the pooled fractions A—J.

Fig. 4. Elution pattern of the hydroxyapatite IgA protease pool of Table II on a DEAE-cellulose column (160 X 9 mm) with 50 mM Tris · HCl buffer, pH 8.2, and a gradient of NaCl as shown in the top curve. Horizontal bars at the bottom indicate the pooled fractions 1—9.

TABLE II

PROTEIN CONTENT AND ENZYME ACTIVITIES OF THE HYDROXYAPATITE CHROMATOGRAPHY POOLS SHOWN IN FIGURE 3

Pool	Protein (Folin-Lowry method) (mg)	Non-specific protease		IgA protease (units)	Sucrase (units)
		Ninhydrin (units)	Ultraviolet (units)		
A	18.1	47	8.0	0	1
B	19.0	152	17.4	0	7
C	12.1	140	22.0	0	7
D	14.6	126	18.0	100	15
E	9.9	112	20.4	1000	62
F *	3.0	32	9.6	2500	75
G *	2.7	51	18.0	3100	48
H *	1.0	10	2.4	1300	15
I	1.2	8	3.0	800	10
J	0.8	8	2.0	100	6

* These pools were combined and applied to the DEAE-cellulose column (Fig. 4).

TABLE III

PROTEIN CONTENT AND ENZYME ACTIVITIES OF THE DEAE-CELLULOSE CHROMATOGRAPHY POOLS SHOWN IN FIGURE 4

Pool	Protein (mg)	Non-specific protease (ultraviolet units)	IgA protease (units)	Sucrase (units)
1	0.20	1.8	20	2.0
2 *	0.45	1.5	950	19.0
3 *	0.65	2.0	3150	60.0
4 *	0.50	1.3	700	15.2
5	0.27	3.2	200	8.0
6	0.50	1.8	120	4.2
7	0.80	1.2	50	1.8
8	0.75	1.2	50	1.8
9	0.27	0.8	0	1.5

* These pools were combined and applied to the Sephadex G-200 column.

(5) Sephadex G-200 gel filtration: IgA protease-active pools were mixed and concentrated by ultrafiltration and applied to a Sephadex G-200 (Pharmacia) column (110 × 2 cm). The column was eluted with 0.1 M NH_4HCO_3 solution and the fractions collected were assayed for IgA protease. Active fractions were pooled, concentrated and assayed for protein and enzyme activities. These fractions were the highly purified IgA protease preparation. No significant change in activity was noticed when stored at 5° C for up to 2 months.

Results

Purification of IgA protease

The five steps used in preparation of IgA protease led to more than a 100-fold purification of the enzyme in a 20% yield. A summary of the results achieved in each step of a typical experiment are shown in Table IV.

The reverse $(NH_4)_2SO_4$ chromatography (Table I) was the most efficient way

TABLE IV

PURIFICATION OF *STREPTOCOCCUS SANGUIS* IgA PROTEASE

Step	Fraction	Volume (ml)	Total IgA		Total protein (mg)	Specific activity (units/mg)	Total sucrase (units)	Total non-specific protease (ultraviolet units)
			units	%				
1	65% saturated $(NH_4)_2SO_4$ precipitate of 15 l culture	100	18 500	100	470	39.4	8200	360
2	Reverse $(NH_4)_2SO_4$ chromatography pool	40	10 300	55.7	83	124.1	355	136
3	Hydroxyapatite chromatography pool	20	6 900	37.3	7	985.7	138	30
4	DEAE-cellulose pool	6	4 800	26.2	1.6	3031.3	102	5
5	Sephadex G-200	3	3 700	20.0	0.9	4111	65	—

to remove the sucrase activity (>95%) as well as most of the colored material in the 65% $(NH_4)_2SO_4$ saturation precipitate. Sucrase activity appeared in two peaks (Table I), both synthesizing dextran precipitable with 50% alcohol and producing a mixture of glucose and fructose with marked preponderance of the latter.

Hydroxyapatite chromatography removed most of the inactive proteins (10-fold increase in specific activity) and the non-specific proteases from the IgA protease (Table II). However, non-specific protease activity was detected, corresponding to the peak of IgA protease, and sucrase activity was eluted slightly earlier than the IgA protease peak. In DEAE-cellulose chromatography (Table III) the sucrase and IgA protease activities were superimposed, whereas some of the non-specific protease activity was separated. After Sephadex G-200 chromatography the non-specific protease activity was not detectable in the concentrated IgA protease pool, but there was a close association of the sucrase activity with IgA protease. Paper chromatograms of sucrose incubated with the purified enzyme showed both glucose and fructose with some preponderance of the former. No dextran formation was detected.

Polyacrylamide gel electrophoresis

Electrophoresis of the IgA protease pool from DEAE-cellulose showed 4—5 bands, whereas in the final preparation only one band was detected (Figs. 5A and 5B) and IgA protease and sucrase activities were localized only in slices of gels corresponding to this band. Gel electrophoresis of this final preparation in the presence of SDS showed two very close bands near the top of the gel (Fig. 5C) indicating a molecular weight near 100 000.

Specificity of IgA protease

The pure IgA protease was tested for its proteolytic activity on various human monoclonal immunoglobulin preparations of different classes, normal human serum Cohn fraction II, normal human colostrum IgA as well as rabbit IgG and canine secretory IgA. 10 or 50 units of the pure enzyme were

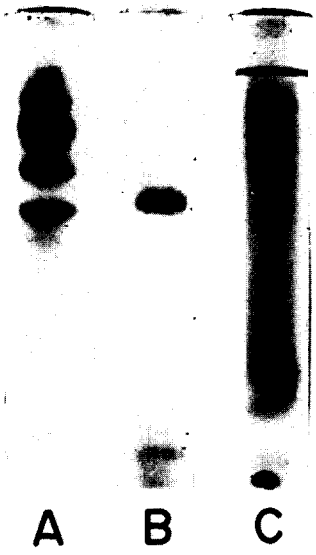


Fig. 5. Polyacrylamide gel electrophoresis of IgA protease pool of DEAE-cellulose chromatography (A) and of Sephadex G-200 gel filtration (B) in 5% gels at pH 9.5, and of the Sephadex pool in 10% gels in the presence of sodium dodecyl sulfate (C).

employed to digest 100 μg of the immunoglobulin. Immunoelectrophoresis with specific antisera was used to detect proteolysis. Eleven preparations of monoclonal human IgA1 were hydrolyzed completely to Fab and Fc fragments at both concentrations of the enzyme. An IgA2 protein was not cleaved at all. Secretory IgA from human colostrum was only partially split at both concentrations of the enzyme. Human IgG, IgM, IgD and IgE as well as rabbit IgG and canine secretory IgA were not affected. Casein was not digested by the pure IgA protease using the three assay methods described, even at ratios of enzyme to substrate more than 40 times that needed for complete digestion of IgA to Fc and Fab.

Optimum pH of IgA protease and non-specific proteases

The effect of pH on the activity of pure IgA protease and the non-specific protease of pools B and D of the hydroxyapatite column (Table II) was studied using 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid (HEPES), 25 mM Tris, as the buffer at pH 5—9 in the IgA protease and unlabelled casein assays. IgA protease showed an optimum at pH 8, and the non-specific proteases in both pools showed a broad optimum between pH 6 and 8.5 when the absorbance at 280 nm was used (Figs. 6A and 6B).

Effect of inhibitors on IgA protease and non-specific proteases

This was studied using pure IgA protease in the standard assay system and the non-specific proteases of pools B and D (Table II in the ninhydrin assay method with 50 mM HEPES/25 mM Tris (pH 6.5) as the buffer. The inhibitor was incubated with the enzyme at room temperature for 10 min before adding the substrate. As shown in Table V, both the IgA protease and the non-specific

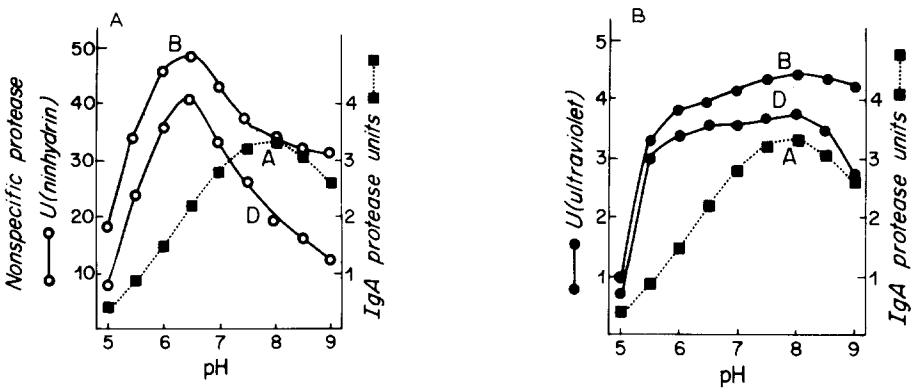


Fig. 6. (A) Optimum pH of IgA protease (curve A) and of non-specific proteases of pools B and D of the hydroxyapatite chromatography (curves B and D, respectively), as determined by the ninhydrin method. (B) The same as A but the non-specific proteases are determined by the ultraviolet method.

protease were inhibited by EDTA and *o*-phenanthroline. However, there was higher residual activity of IgA protease at low concentrations of the chelators suggesting stronger binding of the metal. At higher concentrations, the residual activity of non-specific proteases became relatively constant indicating the presence of a chelator-insensitive protease besides the chelator-sensitive one in each of the pools. Marked inhibition of the non-specific proteases was

TABLE V

EFFECT OF VARIOUS INHIBITORS ON IgA PROTEASE AND NON-SPECIFIC PROTEASES OF POOLS B AND D OF HYDROXYAPATITE COLUMN (Table II)

Inhibitor	Concentration (mM)	Residual activity (%)		
		Non-specific proteases		IgA protease
		B	D	
o-Phenanthroline	0.17	41	54	77
	0.50	40	22	45
	1.67	14	24	17
	3.33	16	15	5
	6.67	17	24	0
EDTA	0.33	40	85	73
	1.00	33	21	42
	3.33	30	17	21
	10.00	37	20	9
	33.33	35	18	5
Diisopropylphosphofluoridate	1.0	82	62	98
	10.0	37	50	95
Phenylmethylsulphonyl fluoride	1.0	80	84	95
	10.0	84	71	97
p-Chloromercuribenzoate	1.0	84	83	95
	10.0	24	19	97
Iodoacetamide	1.0	94	79	100
	10.0	99	78	97

noticed with *p*-chloromercuribenzoate and diisopropylphosphofluoridate at relatively high concentrations (10 mM), whereas IgA protease was unaffected.

The presence of EDTA did not affect the sucrase activity of pure IgA protease, its mobility in polyacrylamide gel electrophoresis, or its elution volume on Sephadex G-200.

Reactivation of IgA protease

IgA protease inhibited by 20 mM EDTA for up to 60 days could be reactivated almost quantitatively by dialysis against 5 mM $CaCl_2$, $MgCl_2$, $MnCl_2$ or $ZnCl_2$ (with $ZnCl_2$ some precipitation of the IgA substrate was observed, preventing accurate quantitation).

Discussion

The present work shows that IgA protease could be purified by a five-step procedure to apparent homogeneity as judged by polyacrylamide gel electrophoresis at alkaline pH (Fig. 5B) and that more than 100-fold purification was achieved. The yield was 20% (Table IV), and the product is highly active in splitting human IgA; 1 mg cleaved 200 mg IgA into Fca α and Fab α under the conditions used in the assay of the enzyme. A previously published purification procedure [2] yielded a product that showed three bands in polacrylamide gel electrophoresis. No quantitative data were given in this procedure. No other known proteolytic enzyme has been found to produce an intact Fc α from IgA except the IgA protease of *N. gonorrhea* and *N. meningitides*, which to date has not been purified [3] and trypsin at high temperature in the presence of 1.0 M NaCl and 1 mM Ca^{2+} which has been described recently [18]. In high temperature trypsinolysis, both IgA1 and IgA2 are digested, the Fab is usually destroyed and trypsin has to be used at a ratio of 4% (w/w) which is much higher than the amount of pure *S. sanguis* IgA protease needed to effect a similar extent of proteolysis (0.6% for 80% proteolysis). This reflects the high specific activity and specificity of the *S. sanguis* enzyme.

The results of the present study of the specificity of pure IgA protease confirms and extends previous findings with crude preparations [2,19]. Thus, canine secretory IgA, human IgD as well as all the other classes and subclasses of human immunoglobulins, except IgA1, are resistant to IgA protease. Also, rabbit IgG, which has a Pro-Thr bond in the hinge region [20], similar to the susceptible bond in human IgA1 [19], was found to be undigested by IgA protease, although the same region is suscepticle to pepsin and papain proteolysis. This suggests that the specificity of IgA protease does not depend only on exposure of the susceptible region to the attacking enzymes, or on the primary sequence in the immediate neighborhood of the susceptible bond, but it may also be affected by long range factors from other parts of the hinge region. It should be noted that cleavage of interchain disulfide bonds of IgA did not appear to affect its digestibility by IgA protease (unpublished data).

No proteases, other than IgA protease, have previously been recognized in *S. sanguis* culture fluids, although some proteolytic activity against casein and salivary mucin has been recently detected in bacterial fractions by Cowman et al. [21]. As *S. sanguis* requires certain amino acids for growth [21,22], the pres-

ence of extracellular proteases of broader specificity than IgA protease could be essential for bacterial nutrition. In the present work, an extracellular protease activity towards casein could be demonstrated in various fractions of the culture fluid proteins. This non-specific protease activity appears to be due to several enzymes as evidenced by: (1) multiple peaks in the hydroxyapatite and DEAE-cellulose chromatography pools (Tables II and III); (2) the ratio of proteolytic activity on caseins as measured by various methods differs from one pool to the other (Tables I and II) suggesting somewhat different specificities among the pools; (3) the difference in pH optimum of the same pool depending on the assay used (ninhydrin or ultraviolet, Figs. 6A and 6B); (4) the inhibition of the non-specific protease activity of pools B and D of the hydroxyapatite by different inhibitors, and also the extent of the inhibition being different among the pools (Table V). Thus, a metal chelator-sensitive protease, a serine protease and a thiol protease (or more than one of each) appeared to be present in the culture medium of *S. sanguis*.

Dextran sucrase (EC 2.4.1.5) purified from the culture fluid of *S. sanguis* was found to produce fructose as the only reducing sugar from sucrose [5]. Later work has suggested that other glycosyltransferase as well as invertase-like enzymes are produced by *S. sanguis*. The 'sucrase activity' referred to in the present work includes all of these activities. After the hydroxyapatite chromatography step, the ratio of IgA protease to sucrase activities of the IgA protease pool became 50 (see Table IV) and remained around this value during the DEAE-cellulose and gel filtration steps. This sucrase appears to be of the invertase type since glucose and fructose were released in approximately equal amounts and no dextran synthesis could be demonstrated in these pools. Apparently the sucrase activity is intimately associated with IgA protease as seen from their distribution in DEAE-cellulose pools (Table III), on gel filtration, the presence of one band in polacrylamide gel electrophoresis (Fig. 5B) of the final preparation and the demonstration of both activities in slices corresponding to this band. The two bands seen in SDS-polyacrylamide gel electrophoresis of the final preparation (Fig. 5C) may be the result of dissociation of the two components: the protease and the sucrase.

Whether the sucrase is a contaminant or it is an essential component of the IgA protease cannot be definitely established at the present time. However, it is interesting to consider a possible function of the sucrase in connection with the remarkable specificity of IgA protease. It has been shown [26] that human IgA1 has a characteristic group of five *o*-glucosidically linked oligosaccharide units in the hinge region. By binding to any of these saccharides or by hydrolyzing the terminal galactose of the disaccharide units, the sucrase could theoretically help bring the associated protease in closer proximity with the susceptible bond in the hinge region. The possibility that IgA protease activity is produced when a certain sucrase is associated with a non-specific metallo protease may be fruitful to explore. Similar examples of associations with concomitant changes in specificity have been previously shown in the lactose synthetase system [27], direct lytic factor with phospholipase A [28], sigma factos with DNA-dependent RNA polymerase [29,30] and Factor VIII with Factor IXa and Factor V with Factor Xa in blood coagulation [31].

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References

1 Mehta, S.K., Plaut, A.G., Calvanico, N.J. Tomasi, Jr., T.B. (1973) J. Immunol. 111, 1274—1276
2 Plaut, A.G., Genco, R.J. and Tomasi, Jr., T.B. (1974) J. Immunol. 113, 289—291
3 Plaut, A.G., Gilbert, J.V., Artenstein, M.S. and Capra, J.D. (1975) Science 190, 1130—1105
4 Tomasi, Jr., T.B. and Grey, H.M. (1972) Prog. Allergy, 16, 81—213
5 Carlsson, J., Newbrun, E. and Krasse, B. (1969) Arch. Oral Biol. 14, 469—478
6 Tomasi, Jr., T.B. and Bienenstock, J. (1968) Adv. Immunol. 9, 1—96
7 Scheidegger, J.J. (1955) Int. Arch. Allergy Appl. Immunol. 7, 103—110
8 Hermans, J.F. (1971) in Methods in Immunology and Immunochemistry (Williams, C.A. and Chase, M.W., eds.), Vo.. III, pp. 213—224, Academic Press, New York
9 Moore, S. (1968) J. Biol. Chem. 243, 6281—6283
10 Marchalonis, J.J. (1969) Biochem. J. 113, 229—305
11 Park, J.T. and Johnson, M.J. (1949) J. Biol. Chem. 181, 149—151
12 Smith, I. (1965) in Chromatographic and Electrophoretic Techniques (Smith, I., ed.), 2nd edn., Vol. 1, pp. 246—260, Interscience Publishers, New York
13 Wheat, R.W. (1966) Methods Enzymol. 8, 60—78
14 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
15 Maurer, H.R. (1971) in Disc Electrophoresis and Related Techniques of Polyacrylamide Gel Electrophoresis (Maurer, H.R., ed.), pp. 44—45, Walter de Gruyter, New York
16 Chrambach, A., Reisfeld, R.A., Wyckoff, M. and Zaccari, J. (1967) Anal. Biochem. 20, 150—154
17 Weber, K., Pringle, J.R. and Osborn, M. (1972) Methods Enzymol. 26, 3—27
18 Calvanico, N.J. and Tomasi, Jr., T.B. (1976) Immunochemistry 13, 203—208
19 Plaut, A.G., Wistar, Jr., R. and Capra, J.D. (1974) J. Clin. Invest. 54, 1295—1300
20 Smyth, D.S. and Utsumi, S. (1967) Nature 216, 332—335
21 Cowman, R.A., Perrella, M.M., Adams, B.O. and Fitzegerald, R.J. (1975) Appl. Microbiol. 30, 374—380
22 Carlsson, J. (1972) Arch. Oral Biol. 17, 1327—1332
23 Carlsson, J. and Elander, B. (1973) Caries Res. 7, 89—101
24 Newbrun, E. (1971) Caries Res. 5, 124—134
25 Arnett, A.T. and Mayer, R.M. (1975) Carbohydr. Res. 42, 339—345
26 Baenziger, J. and Kornfeld, S. (1974) J. Biol. Chem. 249, 7270—7281
27 Brew, K., Vanaman, T.C. and Hill, R.L. (1968) Proc. Natl. Acad. Sci. U.S. 59, 491—497
28 Klibansky, C., London, Y., Frenkel, A. and De Vries, A. (1968) Biochim. Biophys. Acta 150, 15—23
29 Travers, A.A. and Burgess, R.R. (1969) Nature 222, 537—540
30 di Mauro, E., Snyder, L., Marino, P., Lamberti, A., Coppo, A. and Tocchini-Valenti, G.P. (1969) Nature 222, 533—537
31 Davie, E.W. and Fujikawa, K. (1975) Annu. Rev. Biochem. 44, 799—829