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The Mode of Action of *Aspergillus niger* Protease on Diphtheria Antitoxic Horse Serum*

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SUMMARY

1. After digestion of diphtheria antitoxic horse sera (DAHS) by *Aspergillus niger* protease (ANP), elctrophoretic patterns of DAHS showed only one major component (D.G.-component) having a mobility between that of the T-component and of γ-globulin. The D.G.-component after precipitation by half saturated ammonium sulfate had a mobility of -1.5×10^{-5}sq. cm. per volt per second at pH 8.6.

2. The percentage yields of antitoxic activity in the D.G.-component from DAHS rich in T-component were always higher than those from serum containing less T-component.

3. The susceptibility of the T-component to ANP was always less than that of γ-globulin. An increase in rate of digestion of γ-globulin by ANP was observed at a later stage of the digestion.

4. The D.G.-component was obtained from the digestion products of the T-component previously isolated by zone electrophoresis. Therefore it can be stated that the end product of digestion of DAHS by ANP is a T-component modified by the protease.

INTRODUCTION

As shown in our previous report (Amano *et al.*, 1955), a considerable despeciation of the diphtheria antitoxic horse serum was attained by digestion with *Aspergillus niger* protease. Thirty mg N. of the diphtheria antitoxic horse serum globulin (DAHS-GL) digested with ANP did not evoke anaphylaxis in guinea pigs passively sensitized to normal horse serum. In addition, in guinea pigs DAHS-GL digested with ANP could not produce any antibodies to react with normal horse serum, when tested by systemic anaphylaxis and the Schultz-Dale's test. Therefore it can be concluded that antigenicity of DAHS was almost completely altered by digestion with ANP.

However, it is well known that the antitoxic activity of DAHS is distributed in two different serum fractions. The one is the γ-globulin fraction and the other the T-component. These two antitoxins differ in their electrophoretic properties and in their immunological characteristics, as shown by Kekwick and Record (1941). In the previous report, the fraction which gave rise to the digested antitoxin was not examined.

Therefore it seems of interest to study the way in which the serum components of DAHS are modified during digestion with ANP and the behaviour of the two

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types of antitoxin during digestion with ANP.

Materials and Methods

Electrophoresis: Electrophoretic analyses were performed at 4°C using a Tiselius apparatus. Veronal buffer (pH 8.6, $\mu=0.1$) was used for electrophoresis and all samples were dialyzed against the same buffer for 16 hours previously. The relative concentration of each component was calculated from the electrophoretic pattern according to the method of Tiselius and Kabat (1939). The electric conductivity of each sample was also measured at 4°C and the electrophoretic mobility of each component was calculated.

Zone electrophoresis: Zone electrophoresis was performed at 4°C using with a vertical column of bufferd starch, according to the method of Flodin and Porath (1954). Veronal buffer (pH 8.6, $\mu=0.1$) was used for zone electrophoresis.

Total nitrogen estimation: The micro-Kjeldahl analysis was used (Kabat and Mayer, 1948).

Non-protein nitrogen (N.P.N.): An equal volume of 10 per cent trichloracetic acid (TCA) was added to the sample and the mixture was filtered through hard filter paper (Toyo Roshi Company No. 4) after standing for 30 minutes at room temperature. The nitrogen content of the filtrate was estimated by micro-Kjeldahl analysis.

Antitoxin unit (AU) determination: Antitoxin unit of each sample was estimated according to the Ramon's flocculation test.

Aspergillus niger protease (ANP): Partially purified ANP was prepared from extracts of whole cultures of *Aspergillus niger* strain Usami, as described in our previous report. (Amano *et al.*, 1955)

RESULTS

1. Time course of digestion of DAHS with ANP.

The fate of the serum protein fractions was first studied during the digestion of DAHS by ANP. For this purpose, samples taken from the digestion mixture were tested by electrophoresis.

An equal volume of M/5 acetate buffer (pH 3.5) containing 0.5 per cent phenol was added to 300 ml. of DAHS containing 1100 AU. per ml. and the pH of the medium was adjusted to 3.8 with concentrated acetic acid, using a Beckman pH. meter. 240 units of ANP were added to this solution and the mixture was kept at 37°C for 72 hours as described in our previous report (Amano *et al.*, 1955). 50 ml. aliquot of the digestion mixture were taken at 0, 1, 10, 30, 42 and 72 hours, 2 ml. of each were used to estimate non-protein nitrogen and 5 ml. to estimate antitoxin unit after adjusting the pH to 7.6 with 2 N NaOH. The remainder of each digest was adjusted to pH 7.6 with 2 N NaOH and dialyzed against tap water for 3 days and then against distilled water for 3 days. The dialyzed samples were lyophilized. A 2 per cent solution of each sample was made in veronal buffer (pH 8.6, $\mu=0.1$) and dialyzed against the same buffer for 16 hours at 4°C. A control flask containing 25 ml. of the serum (DAHS) and 25 ml. of M/5 acetate buffer (pH 3.5) was also made and kept at 37°C for 72 hours.

The results of electrophoretic analyses are shown in Table 1.

Table I. Electrophoretic study of the course of digestion of DAHS by ANP.

Digestion time (hours)		Electrophoretic properties						
0	Fraction		Albumin	α_1-globulin	α_2-globulin	β_1-globulin	T-component	γ-globulin
	Mobility		−6.5	−5.9	−5.0	−3.9	−2.8	−1.2
	Fraction%		12.8	1.9	13.6	15.7	41.9	14.7
1	Fraction		Albumin	α_1-globulin	α_2-globulin	β_1-globulin	T-component	γ-globulin
	Mobility		−6.3	−5.7	−4.8	−3.6	−2.6	−1.0
	Fraction%		4.3	5.1	18.2	19.6	42.3	10.4
10	Fraction	I-component	II-component		III-component		IV-component	
	Mobility	−9.0	−7.3		−4.6		−2.3	
	Fraction%	4.8	8.2		50.2		36.8	
30	Fraction	I-component			II-component		III-component	
	Mobility	−7.9			−4.4		−2.0	
	Fraction%	8.0			44.9		47.1	
42	Fraction	I-component	II-component	III-component			IV-component	
	Mobility	−9.6	−7.6	−5.2			−1.8	
	Fraction%	7.3	9.3	36.0			47.4	
72	Fraction			I-component	II-component		D.G.-component	
	Mobility			−4.5	−3.3		−1.5	
	Fraction%			17.2	11.7		71.1	

Note: Fraction: Fractions of the digestion products at 0 and 1 hour were designated by the usual names of the serum proteins but fractions of the digestion products after 10, 30, 42 and 72 hours were designated as I-component, II-component.....etc. according to the order of magnitude in their negative charge at pH 8.6.
Mobility: Mobilities are expressed as 10^{-5} sq. cm. per volt per second.
Fraction %: The percentage of the each fraction in the total non-dialysable substances.
D.G.-component: A major fraction in the digestion products at 72 hours.

It can be seen from the table that the number of fractions of serum protein decreased during digestion. An appreciable amount of serum albumin disappeared during the first hour of digestion. After 10 hours, the typical electrophoretic pattern of DAHS was almost completely lost and no serum proteins having mobilities identical with that of serum albumin and that of γ-globulin could be found. After 72 hours digestion of DAHS, about 70 per cent of the non-dialyzable substances consisted of a component having a mobility between that of T-component and that of γ-globulin (D.G.-component). The electrophoretic pattern of the control showed almost no difference from that of untreated DAHS.

The antitoxin unit and non-protein nitrogen content of each digest was estimated at the same time. The data of this experiment are shown in Table II and in Fig. 1.

True digestion of serum proteins was observed about 60% of protein nitrogen was converted to non-protein nitrogen. The destruction rate of antitoxin was

Table II. Estimations of antitoxic activity and non-protein nitrogen during digestion of DAHS by ANP

Digestion time (hrs)	Antitoxic activity		Non-protein nitrogen	Protein nitrogen*	
	AU./ml.	Recovery %	N.mg./ml.	N.mg./ml.	Residual %
0	550	100	0.30	6.69	100
1	525	81	0.71	6.28	99.5
10	395	72	2.15	4.84	72.3
30	330	63	3.48	3.51	52.4
42	318	58	3.74	3.25	48 6
72	290	53	4.16	2.83	42.3

* Protein nitrogen at each digestion time was calculated by the total nitrogen of the digestion mixture (6.99 mg N.) minus the non-protein nitrogen.

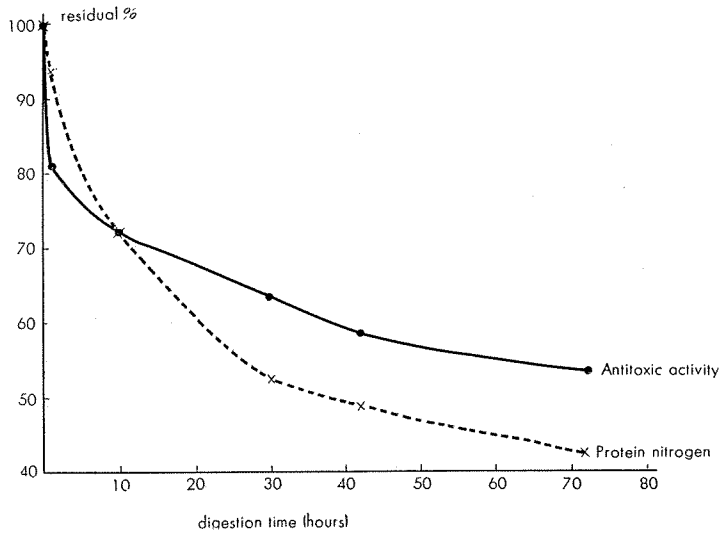

Fig. 1. Antitoxic activity and protein nitrogen during digestion of DAHS by ANP.

rapid at the early stage of the digestion and then decreased. About 50% of antitoxin remained after 72 hours digestion.

2. Electrophoretic properties of DAHS-GL digested with ANP.

In our previous report it was shown that antitoxic activity of the degestion products could be recovered almost completely by fractionation with half saturated ammonium sulfate. A further experiment was performed to see whether the D.G.-component containing the antitoxic activity could also be recovered by fractionation with half saturated ammonium sulfate. The digestion products were further purified with ammonium sulfate as follows.

Three hundred ml. of DAHS digested with ANP were centrifuged at 3000 r. p. m. for 30 minutes to remove insoluble material. Then the pH of the supernatant was adjusted to 7.6 with 2N NaOH and an equal volume of saturated ammonium sulfate was added. The mixture was

kept overnight in a refrigerator. The precipitate was filterd through hard filter paper with super-cell on a Buchner's funnel and washed three times with 100 ml. of half saturated ammonium sulfate. It was then dissolved into as small a volume of water as possible and dialyzed against running tap water for 3 days and then against distilled water for 3 days. The dialyzed solution was lyophilized. The dried material was dissolved in 0.9 per cent NaCl solution and the antitoxin units were estimated.

The purified material was dissolved in veronal buffer (pH 8.6, $\mu=0.1$) at a concentration of 1.5 per cent (w/v) of protein and dialyzed against 20 volumes of the same buffer for 16 hours in a refrigerator. Electrophoretic analysis was carried out using the Tiselius apparatus at 180V. and 10 mA. at 4°C.

The electrophoretic pattern of digested DAHS-GL is shown in Fig. 2.

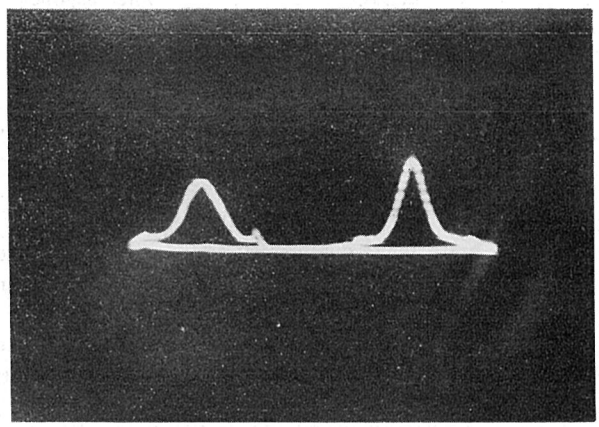

Fig. 2. Electrophoretic pattern of D.G.-component.
The experiment was carried out in a Tiselius apparatus at 180V. and 10 mA, at 4°C. Veronal buffer (pH 8.6, $\mu=0.1$) was used in this experiment and the electrophoretic pattern was photographed after 35 minutes.

It can be seen that the D.G.-component thus obtained is almost homogeneous but it is slightly contaminated by a component moving faster toward the anode at pH 8.6. The mobility of the D.G.-component was calculated from the rate of migration of the pattern and the electric conductivity measured at 4°C. as -1.5×10^{-5}sq.cm.per volt per second.

More than 95 per cent of the antitoxic activity of digestion products was found in the D.G.-component which had a purity of 246 AU. per mg N.

3. Recovery of antitoxic activity from the two types of DAHS differing in the T-component content.

During the experiments on the digestion of DAHS by ANP, the authors became aware of a tendency for a higher per-centage yield of antitoxic activity when a more potent antitoxic serum was used for the experiment. In addition, in preliminary experiment we have shown that the more potent antitoxic sera has a higher concentration of the T-component. Therefore the antitoxin in the T-component may be more resistant to enzymic hydrolysis of ANP than the antitoxin in the γ-globulin.

To study this possibility the recovery of the antitoxic activity was compared after digestion of two batches of antitoxic sera containing different concentrations of T-component. The one contained 36.4 mg per ml. of T-component and 1600 units of antitoxin per ml. The other contained 15.7 mg per ml. of T-component and 320 units of antitoxin per ml. The digestion procedure for each batch of DAHS was the same as described above.

The results are summarized in Table III.

Table III. The recovery of antitoxic activity from two batches of antitoxic sera having different concentration of T-component.

Antitoxic serum	AU./ml.	T-component mg./ml.	Recovery of antitoxic activity %		
			Original serum	After digestion (72 hrs.)	D. G. component
DAHS-I	320	15.7	100	30.2	25.6
DAHS-II	1600	36.4	100	6.25	59.4

From these results it can be stated that the antitoxin of the T-component is more resistant to digestion by ANP than that of the γ-globulin.

4. The susceptibility of the two types of antitoxin to ANP.

The following experiment was performed to elucidate the results of above experiment in which the antitoxin of the T-component is more resistant to enzymic hydrolysis than that of the γ-globulin. In this experiment, T-component and γ-globulin were separated by zone electrophoresis and at the same protein concentration were digested by ANP. The residual antitoxic activity of digest was compared in the course of the digestion. Non-protein nitrogen formed during hydrolysis was also estimated in this experiment.

DAHS-GL was prepared by fractionation with half saturated ammonium sulfate from a DAHS contaning 1100 AU. per ml. 250 mg. of DAHS-GL were dissolved in 3 ml. of veronal buffer at pH 8.6 (μ=0.1) and applied to a starch column (3.5×30cm). Zone electrophoresis was carried out at 500V. and 20 mA. at 4°C. for 18 hours. 1.6 ml. aliqouts of eluate were collected in a fraction collector and the protein concentration of each estimated by a Zeiss refractometer.

The electrophoretic patterns of the T-component fraction and of the γ-ghobulin fraction thus obtained are shown in Fig. 3.

The T-component fraction contained a small quantity of β_1-globulin, but did not contain γ-globulin. The γ-globulin fraction contained only a small quantity of the T-component.

1 gm. of the T-component and the γ-globulin were each dissolved in 100 ml. of N/10 acetate buffer at pH 3.5 and the pH was then adjusted to 3.8 with glacial acetic acid. 0.2 ml. of the ANP solution containing 20 units were added to each protein solution. Digestion of both fractions with ANP was carried out at 37°C. Two control tubes were also incubated, the one containing 1 per cent of the T-component buffered at pH 3.8, the other 1 per cent solution of γ-globulin at pH 3.8. There was no appreciable change in either control except for a slight loss of antitoxic activity.

8 ml. aliquots of the digestion mixture were taken at 0, 3, 12, 18, 30, 42 and 72 hours.

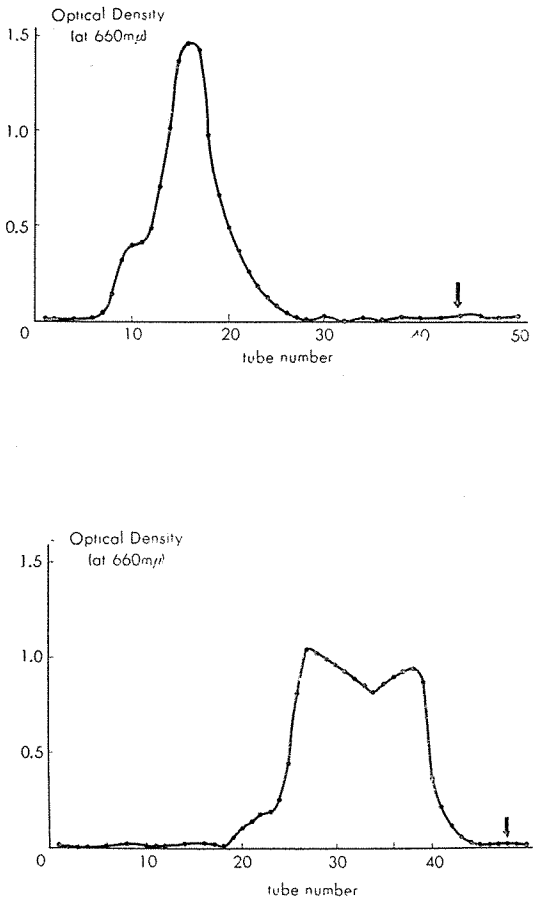

Fig. 3. Electropherograms of the T-component fraction and of the γ-globulin fraction.

Forty mg. of each sample was dissolved in 2 ml. of veronal buffer using a 3×30cm. starch column. After 18 hours electrophoresis at 18 mA (500 V.) and 4°C, the material was displaced from the column and collected in 1.6 ml. aliquots in a fraction collector. The distribution was determined according to the method of Lowry et al. (1951) using Folin-Ciocalteau's phenol reagent. To show the starting point of the material, dextran had been mixed with the sample and after the run each fraction was tested with Anthrone reagent.

The dextran emerged at the point indicated by the arrows.

Table IV. Susceptibilities of T-component and γ-globulin to ANP digestion.

Digestion time (hours)	T-component				γ-globulin			
	N.P.N.*1 mg./ml.	Prot N.*2 %	AU./ml.	AU. remaining %	N.P.N.*1 mg./ml.	Prot N.*2 %	AU./ml.	AU. remaining %
0	0.06	100	210	100	0.08	100	110	100
3	0.58	65.6	168	80	0.62	64.9	60	55
6	0.71	52.0	132	63	0.79	53.8	58	53
12	0.80	51.0	122	58	0.80	53.2	54	49
18	0.82	49.7	122	58	0.87	48.7	50	45
30	0.83	49.1	124	59	1.10	33.5	32	29
42	0.85	47.7	122	58	1.32	20.0	18	16
72	0.86	47.0	120	57	1.43	12.3	9	8

*1 N.P.N.....Non-protein nitrogen.

*2 Prot. N. = total nitrogen — non-protein nitrogen.

Total nitrogen of T-component solution.....1.57 mg. N. per ml.

Total nitrogen of γ-globulin solution.....1.62 mg. N. per ml.

Non-protein nitrogen and the antitoxin units of each sample were estimated as described above. The antitoxin unit determination was performed after adjusing the pH to 7.6 with 2 N NaOH.

The results are shown in Table IV and in Fig. 4.

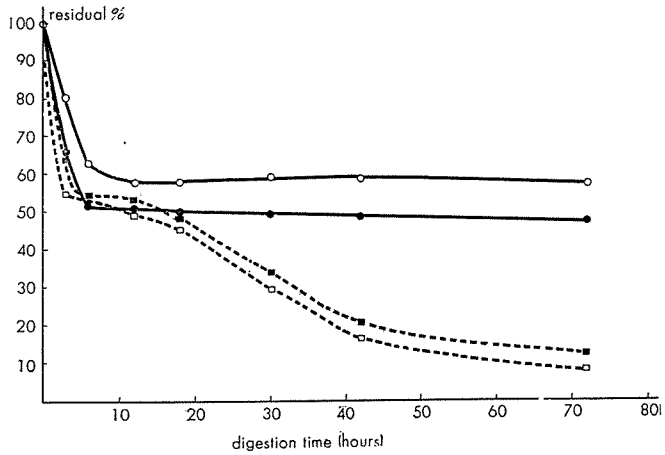

Fig. 4. Susceptibilities of T-component and γ-globulin to ANP digestion.
○——○ Antitoxic activity remaining in the T-component fraction.
●——● Protein nitrogen remaining in the T-component fraction.
□·····□ Antitoxic activity remaining in the γ-globulin fraction.
■·····■ Protein nitrogen remaining in the γ-globulin fraction.

Both fractions were digested at a similar rate for about 18 hours. In the later stage of digestion, the non-protein nitrogen and antitoxin units of the T-component remained constant, but the digestion rate of the γ-globulin fraction increased.

The digestion products of the T-component were fractionated by zone electrophoresis. Again the D.G.-component was found to be the major fraction, with a mobility of -1.5×10^{-5}sq.cm.per volt per second.

From these results it can be stated that the T-component was more resistant to enzymic hydrolysis by ANP than γ-globulin and that the D.G. -component was derived from the T-component by the treatment.

DISCUSSION

It is a well known fact that horse antitoxic serum cotains several protein fractions. It could be presumed that each fraction would be split into numerous fragments having different mobilities by the enzymic hydrolysis. When such sera are digested by some protease and the electrophoretic patterns are taken in the course of hydrolysis, it is possible that numerous fractions would be detected. However experimental results were much simpler in the case of the hydrolysis of horse antitoxic serum by ANP. After 72 hours only one major fraction could be detected by electrophoresis and this was termed as the D.G. -component.

The D.G.-component had a mobility of -1.5×10^{-5} sq.cm.per volt per second which is between the mobility of the T-component and that of γ-globulin. In this experiment it is evidenced that the D.G.-component was derived from the T-component and not from γ-globulin, because the T-component is much more resistant to ANP disgestion than γ-globulin and because the D.G.-component could be produced from the T-component previously isolated by zone electrophoresis.

When horse antitoxic serum was digested by pepsin by the method of Pope (1939), the major fraction obtained had a mobility quite similar that of γ-globulin as shown by Anderson (1955) and by Amoureux & Yeu (1951). Kekwick, Knight, Macfarlane and Record (1941) gave conclusive evidence for the hypothesis that the major product was derived from the T-component by peptic digestion. Their evidences consisting of two results. First percentage yields of antitoxic activity in the major fraction was high when the antitoxic serum rich in T-component was digested by pepsin and low when antitoxic serum rich in γ-globulin was used for. Second the *in vivo/in vitro* antitoxic ratio of the digestion product was very close to that of the undigested antitoxin in the T-component.

Though the *in vivo/in vitro* antitoxic ratio of the D.G.-component was not compared with that of the undigested T-component or with that of γ-globulin in the present study, the D.G.-component was shown to be produced by ANP treatment from T-component.

It can be stated from our results and the results of peptic digestion that the T-component is more resistant to proteases at an acidic pH. The reason for this is not yet known. The authors assume that γ-globulin would be more easily denatured at an acidic pH and the protein structure of the T-component has less affinity to pepsin and ANP.

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