STUDIES ON MUCOPROTEINS

III. THE ACCESSIBILITY TO TRYPSIN OF THE SUSCEPTIBLE BONDS IN OVINE SUBMAXILLARY GLAND MUCOPROTEIN

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SUMMARY

Ovine submaxillary gland mucoprotein was found to be susceptible to trypsin. The evidence is a marked increase in terminal NH_2-groups and a corresponding rise in filterability through cellulose acetate membranes and an increase in electrophoretic mobility after trypsin treatment. Enzymic removal of the terminal neuraminic acid units of the prosthetic groups (6-α-D-N-acetylneuraminyl (2 → 6) N-acetylgalactosamine) increased the trypsin effect on ovine submaxillary gland mucoprotein by about 45 %. This means that at least one-third of the trypsin-susceptible bonds in ovine submaxillary gland mucoprotein are inaccessible to the enzyme molecule. This figure may represent only a minimum value since any steric hindrance exerted by the remaining N-acetylgalactosamine unit cannot be assessed at present.

The complete loss of virus haemagglutinin inhibitory capacity of ovine submaxillary gland mucoprotein on trypsin action is interpreted in terms of a critical molecular size below which a mucoprotein ceases to act as an inhibitor.

INTRODUCTION

In the first paper[1] of this series it was shown that the prosthetic group of OSM is the disaccharide α-D-N-acetylneuraminyl (2 → 6) N-acetylgalactosamine. OSM contains 58 % protein and 42 % carbohydrate. From the quantitative amino acid analysis[2] an average molecular weight of 100 for the component amino acids (referred to the amino acid residue) may be calculated. On this basis one in 6.4 amino acid residues carries a prosthetic group. The amino acid analysis revealed further that about one in sixteen amino acids is a diamino-monocarboxylic acid; this means that on the average there are 2.5 prosthetic groups to one diamino acid residue. It is known that trypsin cleaves specifically the peptide bond at the carboxyl end of lysine and arginine residues in the polypeptide chain. It was of interest, therefore, to investigate the effect on trypsin action of the prosthetic groups which actually are bulky substituents at the side chains of frequently occurring amino acids. As a test object for studying effects of steric hindrance on proteolytic enzymes OSM offers the advantage

Abbreviations: OSM, ovine submaxillary gland mucoprotein; RDE, *Vibrio cholerae* neuraminidase; NANA, N-acetylneuraminic acid; BSM, bovine submaxillary gland mucoprotein.

that 60 % of the bulk of each individual prosthetic group can be specifically removed by *Vibrio cholerae* neuraminidase[1,3].

EXPERIMENTAL

Materials

OSM was prepared according to GRAHAM AND GOTTSCHALK[1].

Trypsin, twice recrystallized, was a Sigma preparation.

Crystalline RDE was prepared and kindly supplied by Dr. G. L. ADA, The Walter and Eliza Hall Institute, Melbourne.

Indicator viruses were prepared according to standard procedure[4].

Graded cellulose acetate filters were obtained from Membranfilter Gesellschaft Göttingen.

Methods

NANA was determined according to SVENNERHOLM[5] using NANA as standard. Readings were made in a Beckman Quartz Spectrophotometer Model DU (1.0-cm cells).

Quantitative ninhydrin determinations were carried out by the improved method of MOORE AND STEIN[6] using L-leucine as standard.

For the filtration tests the moistened membranes were placed on a sintered glass disc of a filter assembly and compressed between rubber washers. The free area of the membranes was 2.0 cm^2, and a constant pressure of 40 cm water was applied. 10 ml of the assay was placed on the membrane and the time required for the first 5 ml to pass through was recorded. The dilution of the filtrate due to the water content of the membrane was negligible.

Haemagglutinin inhibitory titrations were performed as described previously[1].

RESULTS

Number of peptide bonds split by trypsin

Action of trypsin on native OSM. OSM-trypsin: 42.5 mg OSM was dissolved in 10.0 ml 0.1 *M* phosphate buffer (pH 8.0) and 1.02 mg crystalline trypsin and a drop of toluene added. OSM-blank: prepared as assay, but omitting trypsin. Trypsin-blank: prepared as assay, but omitting OSM.

The three samples were kept for 5 h at 35° under gentle shaking. At the end of the incubation period the samples were appropriately diluted and their ninhydrin values determined. The experimental readings and their evaluation are given in Table I. The results show that by action of trypsin on 42.5 mg OSM under above conditions 9.63 mmole new N-terminals have been formed or 22.66 mmole/100 mg OSM as compared with 29.3 mmole/100 mg OSM to be expected from the amino acid analysis[2] of OSM, had all susceptible bonds been cloven (see note, Table II).

Action of trypsin on OSM pretreated with neuraminidase (RDE): 20.0 mg OSM was dissolved in 5.0 ml distilled water and the pH adjusted to 6.5 with a drop of 0.1 *N* NaOH; 2 drops of 1 % $CaCl_2$ solution were added.

0.97 mg crystalline trypsin was dissolved in 5.0 ml 0.2 *M* phosphate buffer (pH 8.0). OSM-blank: 1.0 ml of OSM solution plus 1.0 ml 0.2 *M* phosphate buffer.

TABLE I

N-TERMINALS LIBERATED BY TRYPSIN ACTION ON OSM

	Experimental values			*Evaluation of experimental values*	
	Dilution for ninhydrin test	*Extinction*		*N-Terminals in total volume of assay*	*N-Terminals liberated by trypsin per 100 mg OSM*
		*Reading**	*Expressed as N-terminals mM*	*mM*	*mM*
OSM–trypsin	1:10	0.502	0.168	16.80	22.66
OSM–blank	1:5	0.389	0.131	6.55	—
Trypsin–blank	1:1	0.186	0.062	0.62	—
0.2 m*M* leucine standard	1:1	0.596	0.200	—	—

* Corrected for reagent blank.

Trypsin-blank: 1.0 ml trypsin solution plus 1.0 ml distilled water. OSM-trypsin: 1.0 ml OSM solution and 1.0 ml trypsin solution (see below). OSM-RDE-trypsin: To 1.0 ml of OSM solution was added 30,000 units RDE and the assay kept for 18 h at 35°. Then 1.0 ml trypsin solution was added and the digest kept for another 6 h at 35°.

The OSM-blank was kept for 24 h at 35°, the trypsin-blank for 6 h at 35°, the OSM portion of the OSM-trypsin assay for 18 h at 35° and after addition of trypsin solution for another 6 h at 35°. All samples contained 0.1 % toluene. At the end of the trypsin digestion the samples were analysed as above. The results are shown in Table II.

TABLE II

N-TERMINALS LIBERATED BY TRYPSIN ACTION ON NATIVE OSM AND OSM PRETREATED WITH NEURAMINIDASE

	Experimental values			*Evaluation of experimental values*	
	Dilution for ninhydrin test	*Extinction*		*N-terminals in total volume of assay*	*N-terminals liberated by trypsin per 100 mg OSM*
		*Reading**	*Expressed as N-terminals mM*	*mM*	*mM*
OSM–trypsin	1:5	0.489	0.164	1.640	25.10
OSM–RDE–trypsin	1:10	0.297	0.099	1.980	33.60
OSM–blank	1:2	0.385	0.129	0.516	—
Trypsin–blank	1:1	0.178	0.060	0.120	—
0.2 m*M* L-leucine standard	1:1	0.597	0.200	—	—

Note: On calculating N-terminals it is assumed that ninhydrin colour intensities of peptides and standard are the same mole for mole. This assumption may underestimate peptides somewhat[6]; yet, comparison of such figures will scarcely be biased as systematic errors tend to cancel.

* Corrected for reagent blank.

After treatment of OSM with RDE which was shown previously[1] to split off 80 % of the total NANA, a subsequent digestion with trypsin increased the newly formed N-terminals by 33.9 % when compared with trypsin action on native OSM. If a correction is made for the 20 % NANA remaining after RDE treatment, though the relationship may not be strictly linear, the increase will amount to 42.4 %. In a similar experiment an actual increase of 37.8 % or a corrected increase of 47.2 % was found.

Physical changes following trypsin action on OSM

Filtration experiments. OSM-assay: 50 mg OSM was dissolved in 10.0 ml 0.1 *M* phosphate buffer (pH 8.0). OSM-trypsin assay: the same as above but with addition of 1.0 mg crystalline trypsin.

Both assays, after addition of a drop of toluene, were kept at 35° for 18 h with gentle shaking. The samples were then filtered through the cellulose acetate membranes (see METHODS) and the filtrates analysed for NANA. Table III shows that at 120 mμ average pore diameter the trypsin treated OSM passed the membrane with an efficiency exceeding 440 times that of native OSM. Even at 200 mμ, a pore size not significantly retaining the OSM-trypsin digest, the filtration efficiency of the native OSM was only 0.01 (or less) of that of the trypsin treated material.

TABLE III

FILTRATION RATES OF NATIVE AND TRYPSIN TREATED OSM

Assay	*Filter average pore diameter mμ*	*50 % filtration time min*	*NANA concentration as % of original*	*Filtration efficiency* × 10²*
OSM	120	⩾ 300**	44.4**	0.116
OSM	200	92.6	69.6	0.79
OSM	300	9.69	79.7	6.68
OSM–trypsin	120	2.59	83.3	51.1
OSM–trypsin	200	1.14	Not analysed	
Phosphate buffer	120	1.54		
Phosphate buffer	200	1.05		
Phosphate buffer	300	0.81		

* Calculated according to the formula

$$\frac{\text{50 \% filtration time of solvent}}{\text{50 \% filtration time of assay}} \times \frac{\text{NANA concentration of filtrate}}{\text{NANA concentration of input}}$$

** 1.6 ml passed the filter in 265 min; NANA determination was carried out on this aliquot.

Dialysis through cellophane: When an OSM solution (concentration range 0.5–1.0 %) was dialysed at 2° exhaustively against 10 volumes of distilled water and the dialysate lyophilized, analysis of its NANA content and dry weight determinations showed that less than 0.05 % of the mucoprotein escaped from the cellophane bag (cellulose casing of circumference 4.7 cm, Visking Corporation). In corresponding experiments with trypsin-treated OSM (0.25–1.0 % OSM in 0.1 *M* phosphate buffer, pH 8.0, and crystalline trypsin in 2 % concentration referred to OSM dry weight, 18 h at 36° in the presence of 0.1 % toluene) 0.7–1.6 % of the total NANA appeared in the dialysate.

15.0 ml of 0.6 % aqueous OSM solution (pH adjusted to 6.5) was pretreated with 60,000 RDE units in the presence of 0.1 % $CaCl_2$ and toluene at 35° for 18 h. After exhaustive dialysis and addition of an equal volume of 0.2 *M* phosphate buffer (pH 8.0) the mixture was digested with trypsin as above and dialysed against 10 volumes of distilled water. 45 % of the trypsin treated material was recovered in the dialysate. Of 90 mg casein digested with crystalline trypsin under similar conditions, 51 % were rendered dialysable.

Paper electrophoresis: 200 mg OSM was dissolved in 10 ml 0.1 *M* ammonium acetate–NH_4OH buffer, pH 8.6. 0.5 ml of above solution was taken as OSM solution. To 9.5 ml of above solution was added 4.0 mg crystalline trypsin. Both assays were kept at 35° for 18 h. At the end of the digestion period 50 μl of each assay was applied to Whatman No. 4 paper strips (10 × 5 cm), previously equilibrated against the buffer. Electrophoresis was carried out for 8 h at 20° in a potential gradient of 10 V/cm at 18 mA. After 8 h the strips were dried at 80° and cut across their width into 5 mm segments. Each of these and a paper-blank were submitted to the procedure of NANA determination. The results in terms of extinction are given in Fig. 1.

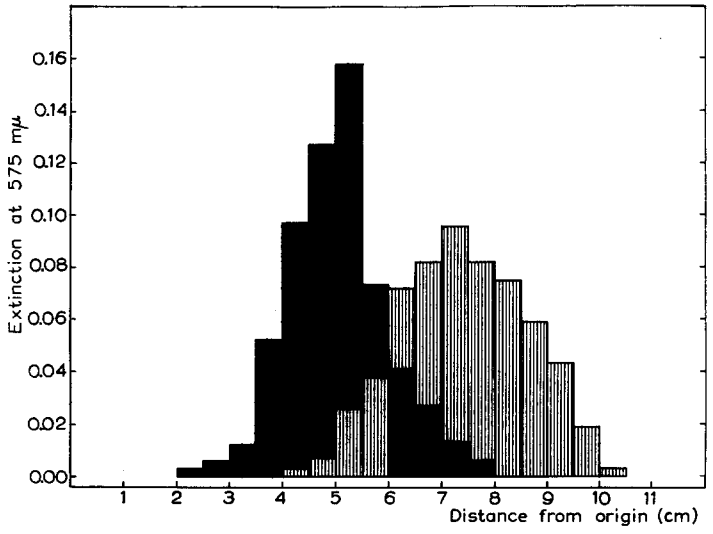

Fig. 1. Paper electrophoresis of native (black columns) and trypsin-treated (hatched columns) OSM.

Evaluation of the data presented in Fig. 1 shows that the areas under the two curves fall within 2 % of each other. The average mobility of native OSM is $1.67 \cdot 10^{-5}$ $cm^2/V/sec$ and that of trypsin-treated OSM $2.39 \cdot 10^{-5}$ $cm^2/V/sec$. The distribution of the patterns does not differ significantly from the normal distribution. The standard deviations of the curves are $\pm$ 0.31 and $\pm$ $0.45 \cdot 10^{-5}$ $cm^2/V/sec$ respectively, *i.e.* the scatter of the OSM–trypsin curve is 45 % greater than that of the OSM curve. In a similar experiment on cellulose acetate strips the mobility of the OSM–trypsin assay approximated that found on paper strips, whereas native OSM did not move from the site of application.

Loss of biological activity of OSM on trypsin action

OSM is the most potent influenza virus haemagglutinin inhibitor prepared so far. Its inhibitory titres against two different strains of influenza virus in the indicator state before and after trypsin action are shown in Table IV. As may be seen, the inhibitory titres of OSM against PR8 and Lee indicator viruses were reduced after 16 h digestion with trypsin 88,000 and 15,500 times respectively. The reduction in titre was accompanied by a complete loss of viscosity; no measurable amounts of free NANA were present in the lyophilized dialysate of the OSM–trypsin assay. In

this context it may be mentioned that the inhibitory titres of OSM against the same indicator viruses were reduced after exhaustive treatment with *Vibrio cholerae* neuraminidase only 172 and 53 times respectively; in this case the reduction in titre was accompanied by the release of an average of 80% of the total NANA content (GRAHAM AND GOTTSCHALK[1]) and also by a considerable decrease in viscosity (GOTTSCHALK AND THOMAS[7]).

TABLE IV

INHIBITORY TITRES PER MILLIGRAM OSM AND THEIR REDUCTION BY TRYPSIN

Conditions of test: 20 mg OSM was dissolved in 15.0 ml 0.1 *M* phosphate buffer (pH 8.0). 2.0 ml of the solution was taken as OSM control, to another sample of 2.0 ml was added 1.0 mg crystalline trypsin. Both assays were kept at 35° in the presence of 0.1% toluene and gently shaken. Inhibitory titre is defined as the dilution factor at which 0.25 ml of the test solution prevents 3 out of 4 haemagglutinating doses of a particular indicator virus from aggregating fowl red blood cells.

Indicator	*OSM*	*OSM-trypsin*	
		*0.25 h**	*16 h**
PR8 – i	$1.76 \cdot 10^6$	$5.35 \cdot 10^2$	< 20
Lee – i	$3.11 \cdot 10^5$	$1.65 \cdot 10^2$	< 20

* Incubation period.

DISCUSSION

As was to be expected from the amino acid analysis of its protein moiety, OSM was found to be susceptible to trypsin. The chemical and physical evidence on this point would appear to be unambiguous. On the chemical side there is the marked increase in the number of terminal NH_2-groups and on the physical side there is a corresponding rise in filterability through cellulose acetate membranes and an increase in electrophoretic mobility after trypsin digestion, although the overall net negative charge of OSM at pH 8.6 cannot rise by more than 10% on splitting the susceptible bonds. These results would indicate fragmentation of the native OSM molecule on trypsin action.

The extent to which these fragments separate cannot be accurately assessed on these data alone. Thus it is conceivable that fragments are held together by linkages other than peptide bonds; and the physical methods applied provide only compound measures of molecular weight and axial ratio. That in fact reduction in molecular weight is the major contributor to the observed physical effect of trypsin on OSM is proven directly by independent evidence. Work in progress by Mr. M. B. SMITH, Physical Chemistry Unit, Division of Food Preservation, C.S.I.R.O., Sydney, clearly shows that the molecular weight of native OSM (about $1 \cdot 10^5$) is greatly reduced on trypsin digestion (to about $9 \cdot 10^3$). The latter figure also accounts for the very small degree of dialysability (through cellophane) of the OSM–trypsin digest.

The main outcome of the present work is the demonstration that the multiple prosthetic groups distributed along the polypeptide chain provide steric hindrance for the close approach of the active centre of trypsin to the potentially susceptible bonds. The finding that after enzymic removal of the terminal NANA unit of the prosthetic group the trypsin effect on OSM increased by about 45% would indicate that at least one-third of the susceptible peptide bonds are inaccessible to the enzyme

molecule because of the screening effect of the prosthetic groups. It is quite possible that an even larger proportion of the susceptible bonds is inaccessible to trypsin since the remaining unit (N-acetylgalactosamine) of the prosthetic group may still exert some steric hindrance. The reduction in size of the prosthetic group and the more extensive action of trypsin after pretreatment of OSM with neuraminidase render the products of trypsin digestion dialysable through cellophane.

It is an inference from these data that the diamino-monocarboxylic acid content of OSM, as reported earlier[2], is too low. On the assumption that after removal of NANA all susceptible bonds are split by trypsin the total diamino acid content is higher by 12% than the value given by the MOORE AND STEIN technique. However, the true value may be still higher for the reason given above and because the ninhydrin value of the terminal NH_2-group of a peptide may be somewhat lower than the corresponding value of the leucine standard used.

The biological activity of OSM is reduced to practically nil by trypsin, as already observed by MCCREA[8]. Since trypsin is without any effect on the linkages joining NANA to N-acetylgalactosamine and N-acetylgalactosamine to the polypeptide chain, this loss in biological activity can only mean that one condition to qualify as influenza virus haemagglutinin inhibitor is a critical size of the molecule. The evidence suggests that a molecular weight of 10^4 is already below this limit. The fact that treatment of OSM and BSM with *Vibrio cholerae* neuraminidase invariably leaves a residual titre of considerable magnitude[1] is apparently due to the remaining 20% and 28% of NANA respectively which for reasons to be discussed in a later paper cannot be removed.

REFERENCES

[1] E. R. B. GRAHAM AND A. GOTTSCHALK, *Biochim. Biophys. Acta*, 38 (1960) 513.
[2] A. GOTTSCHALK AND D. H. SIMMONDS, *Biochim. Biophys. Acta*, 42 (1960) 141.
[3] A. GOTTSCHALK, *Biochim. Biophys. Acta*, 23 (1957) 645.
[4] J. D. STONE, *Australian J. Exptl. Biol. Med. Sci.*, 27 (1949) 337.
[5] L. SVENNERHOLM, *Acta Soc. Med. Upsaliensis*, 61 (1956) 75.
[6] S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 211 (1954) 907.
[7] A. GOTTSCHALK AND M. A. W. THOMAS, *Biochim. Biophys. Acta*, in the press.
[8] J. F. MCCREA, *Biochem. J.*, 55 (1953) 132.