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THE EXTRACELLULAR NUCLEASE ACTIVITY OF *MICROCOCCUS SODONENSIS*

IV. PHYSICAL STUDIES, CHARACTERIZATION AS A GLYCOPROTEIN AND INVOLVEMENT WITH THE CELL WALL

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SUMMARY

1. The molecular weight and $s_{20,w}$ of *Micrococcus sodonensis* nuclease were found to be concentration dependent and extrapolation to zero concentration yielded values of 500 000 and 2.2 S, respectively. The concentration dependence and behaviour of the peak in sedimentation velocity experiments are indicative of an asymmetric molecule.

2. The enzyme was shown to be a glycoprotein consisting of 21% carbohydrate covalently linked to the protein moiety. Glucosamine, glucose, galactose and rhamnose were identified as the component sugars and were present in a ratio of 4:2:1:1. A serine–glucosamine linkage involving at least 80% of the serine residues was demonstrated.

3. Amino acid analyses showed the enzyme to be low in cysteic acid residues and to contain relatively high amounts of serine, threonine, glycine and proline. Unexpected high values for tryptophan were shown to be a result of the presence of amino sugar.

4. The carbohydrate moiety of purified enzyme was shown to be related both chemically and immunologically to the cell wall carbohydrate and active enzyme was found bound in a stable configuration to the cell wall.

5. The possible role of the wall in enzyme formation is discussed.

INTRODUCTION

A large number of extracellular microbial enzymes have been reported, but only a few have been described in detail and none have been characterized definitively

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as glycoproteins, although carbohydrate has been found associated with the protein in some cases[1,2]. EYLAR[3] reported that the majority of mammalian extracellular proteins were glycoproteins and several mammalian nucleases such as ribonuclease B of bovine milk[4] and ribonucleases B, C and D from bovine pancreases[5,6] have been well characterized.

Glycoproteins are defined[7] as conjugated proteins containing one or more heterosaccharides with a relatively low number of sugar residues, lacking a serially repeating unit and bound covalently to the peptide chain. The carbohydrate moiety is usually highly branched and can constitute as much as 80% of the molecule. The sugars most commonly found are D-mannose, D-galactose, L-fucose, D-glucosamine, D-galactosamine and D-neuraminic acid while D-glucose and D-rhamnose occur less frequently. In all glycoproteins so far investigated the linkage of the carbohydrate to protein has been through an amino sugar[8]. There are also certain common physical properties which include polydispersity, a high degree of asymmetry, high intrinsic viscosity and a high molecular charge density[9].

Studies on *M. sodonensis* nuclease have indicated the presence of a carbohydrate moiety which is closely related to that in the cell wall. Although many enzymes have been reported to be associated with the envelope fraction of the bacterial cell, most are in the membrane or periplasmic space (*e.g.* ATPase[1–3]) and can be readily released by osmotic shock. ANRAKU AND MIZUNO[39], isolated a ribonuclease which was associated with the "debris" fraction of *Escherichia coli* and which could be released by treatment with urea. The wall-associated enzymes described previously[10–12] have been for the most part autolytic enzymes which, in contrast to membrane-associated enzymes, are firmly bound (*e.g.* the *N*-acyl-muramyl-L-alanine amidase of *Bacillus subtilis* has been shown to be covalently linked to teichoic acid[12]). The 5'-nucleotidase of *E. coli*[13], previously thought to be located in the periplasmic space[14], and yeast invertase[37,38] have also been shown to be associated with the cell wall.

The present report is concerned with both physical and chemical analyses of the purified *M. sodonensis* nuclease, its characterization as a glycoprotein, and the possible role of the cell wall in enzyme formation.

MATERIALS AND METHODS

Enzyme preparation

Purified *M. sodonensis* (ATCC 11880) nuclease was prepared as described previously[15].

Protein analyses

The technique of LOWRY *et al.*[16] was employed.

Ultracentrifugal analyses

A Spinco Model E analytical centrifuge employing Schlieren optics was used for these studies. Varying concentrations of the purified enzyme in phosphate buffer (pH 7.3), ionic strength 0.1 were prepared. Sedimentation velocity experiments were carried out at 60 000 rev./min and a rotor speed of 12 000 rev./min was used for the Archibald approach to sedimentation equilibrium. The $\bar{v}$ was calculated on the basis

of the carbohydrate and amino acid analyses and a value of 0.713 was used in the calculations of molecular weight and $s_{20,w}$.

Amino acid composition

Samples of purified nuclease containing 0.1 mg of protein were evaporated to dryness and hydrolyzed in 6 M HCl at 100° for 12, 24, 48 and 72 h under reduced pressure. Samples for cysteic acid analysis were prepared according to the method of MOORE[17]. Aliquots were analysed for their amino acid composition using the Spinco Model 120B automatic amino acid analyser. Analysis of the enzyme for tyrosine and tryptophan was carried out spectrophotometrically[18].

Carbohydrate analyses

Total neutral sugar content was determined by the indole and anthrone techniques described by ASHWELL[19] and the phenol–H_2SO_4 technique of DUBOIS *et al.*[20]. Total hexose and methyl pentose were assayed by cysteine–H_2SO_4 technique[19] while uronic acid determination employed the carbazole assay described by DAVIDSON[21]. The above assays were carried out on the unhydrolysed enzyme.

Tests for sialic acids were carried out by means of the thiobarbituric acid assay of WARREN[22] on samples hydrolyzed in 0.05 M H_2SO_4 for 60 min at 80°. Estimation of glucose and galactose was done after 3-h hydrolysis of the nuclease in 3 M HCl at 100°. Samples were evaporated to dryness and assayed using the Glucostat and Galactostat reagents of Worthington.

Amino sugars were assayed by the Morgan–Elson technique as described by GHUYSEN *et al.*[23]. The *N*-acetylglucosamine standard and the nuclease were hydrolyzed in sealed tubes in 3 M HCl at 100° for varying times and the assays were carried out after chemical reacetylation of the samples.

Reducing sugars were detected by the Park–Johnson ferricyanide procedure as modified by GHUYSEN *et al.*[23].

Paper chromatography

After hydrolysis of the nuclease in 3 M HCl at 100°, identification of the individual carbohydrate components was carried out by means of descending chromatography in a pyridine–ethyl acetate–water–acetic acid (5:5:3:1, by vol.) solvent system. Alkaline $AgNO_3$ was the location reagent employed and the background was cleared with 5% sodium thiosulfate. ^{14}C isotopes were located by means of a Nuclear Chicago Actigraph III strip scanner.

Diesterase activity

Assays were as described previously[15] using ^{14}C-labeled RNA as substrate.

Serological tests

The Oudin single gel diffusion described by KABAT AND MAYER[24] was employed. Antigen concentration was 1 mg dry weight of cell walls or 1 mg of enzyme protein per ml of solution.

Preparation of antisera

Purified enzyme and purified cell walls (post-trypsin) were employed as

antigens at a concentration of 1 mg/ml (as above). Anti-cell wall and anti-enzyme sera were prepared by intravenous injection of the purified antigens into 2-kg rabbits. After 6 weeks of triweekly injection the rabbits were bled out and the sera retained for serological testing.

Preparation of cell walls of M. sodonensis

Cell walls were prepared according to a modification of the technique of GHUYSEN *et al.*[25]. This procedure is summarized to indicate the state of purification of the various wall preparations used.

(1) Washed whole cells were disrupted in a Servall omnimixer using glass beads, residual whole cells removed by centrifugation and the fragments subjected to a repeated (20 times) washing procedure involving alternate 1 M NaCl and distilled water washes with intermittent brief sonications, until the preparation was free of detectable phosphate (*i.e.* membrane contamination). These are "Crude cell walls".

(2) Walls were then heated to 90° in 0.5 M NaCl for 30 min and then subjected to prolonged dialysis *vs.* water. This material was then extracted 3 times with 90.5% phenol at 70° and then dialyzed *vs.* water for 96 h. These are "Pre-trypsin cell walls".

(3) Walls were then digested at pH 8.25 with 50 μg/ml trypsin for 4 h, washed and dialyzed as described and lyophilized from distilled water. These are "Post-trypsin cell walls".

RESULTS

Ultracentrifugal analyses

Since earlier studies[26] of the *M. sodonensis* nuclease had suggested the possibility of an asymmetric molecule it was necessary to carry out the ultracentrifugal studies at several enzyme concentrations in order to further substantiate this possibility. Fig. 1 shows the Schlieren profile obtained with a 0.25% enzyme solution at three different time intervals. A single homogeneous peak was obtained which was indicative of a high degree of purity.

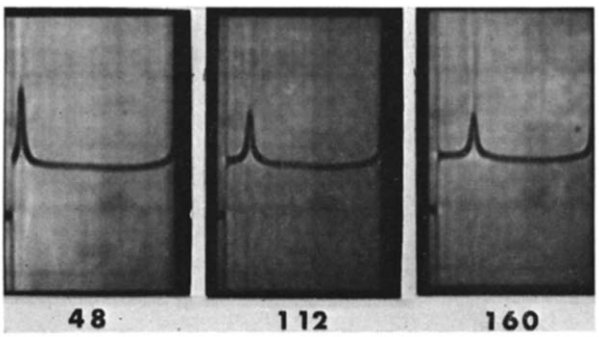


Fig. 1. Schlieren pattern of purified *M. sodonensis* nuclease. A 0.25% solution of purified nuclease in pH 7.2 buffer (I = 0.1) was sedimented at 60 000 rev./min at 20°. Bar angle = 50°. Sedimentation is from left to right. Photos were taken at 48, 112 and 160 min after attaining full rotor speed.

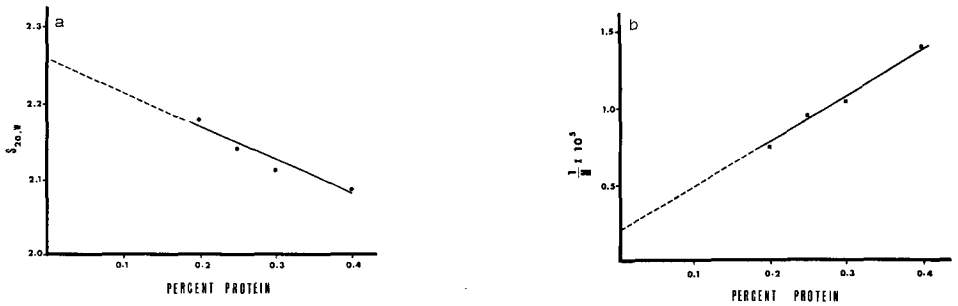


Fig. 2. Concentration dependence of the $s_{20,w}$ and molecular weight of *M. sodonensis* nuclease. Solutions of purified *M. sodonensis* nuclease were prepared in pH 7.2 phosphate buffer ($I = 0.1$). Values were calculated at each concentration and extrapolated to infinite dilution. (a) $s_{20,w}$. Rotor speed 60 000 rev./min. (b) Molecular weight. Rotor speed 12 000 rev./min.

Both the $s_{20,w}$ and the molecular weight varied with the enzyme concentration. Fig. 2a is a plot of the $s_{20,w}$ values obtained in the experiment which, when extrapolated to infinite dilution, gave an $s^{\circ}_{20,w}$ of $2.26 \cdot 10^{-13}$. The Archibald approach[27] to sedimentation equilibrium was employed for molecular weight determinations and the reciprocal of the molecular weight was plotted at each concentration and extrapolated to infinite dilution. As shown in Fig. 2b the extrapolated value obtained

TABLE I

AMINO ACID COMPOSITION OF *M. sodonensis* NUCLEASE

Amino acid	*Moles/100 g protein*	*Moles/mole protein*	*g/100 g protein*
Lysine	0.0226	90.4	3.31
Histidine	0.0083	33.2	1.29
Arginine	0.0164	65.6	2.86
Tryptophan*	0.0151	60.4	3.08
	(0.0065)	(26)	(1.24)
Aspartic acid + asparagine	0.0658	263.2	8.77
Threonine	0.0575	230	6.85
Serine	0.055	220	5.78
Glutamic acid + glutamine	0.0574	229.6	8.45
Proline	0.0441	176.4	5.08
Glycine	0.0704	281.6	5.28
Alanine	0.0834	333.6	7.43
Valine	0.064	256	7.50
Methionine	0.0075	30	1.12
Isoleucine	0.025	100	3.28
Leucine	0.0405	162	5.31
Tyrosine	0.0103	41.2	1.87
Phenylalanine	0.0156	62.4	2.58
Cysteic acid	0.0075	30	0.91
Total			80.75
			(78.91)

* Values in parentheses are calculated on the basis of the amino acid analyser value for tyrosine assuming a tyrosine/tryptophan ratio of 1.7.

was 500 000. The sharpness of the peak in the sedimentation velocity experiment, the concentration dependence and the discrepancy between the $s_{20,w}$ and molecular weight values are all highly indicative of an asymmetric molecule.

Amino acid composition

Amino acid analyses of the purified enzyme were performed as described in MATERIALS AND METHODS. The results are shown in Table I and are the average values calculated from the different hydrolysis times with the following exceptions. The 12-h value was excluded from the glutamic acid results, serine and threonine were extrapolated to zero time and the values for valine and isoleucine were obtained after hydrolysis for 72 h. Tryptophan was determined spectrophotometrically and on the basis of this assay a tyrosine: tryptophan ratio of 1.7 was obtained. The value obtained for tyrosine spectrophotometrically did not agree with the amino acid analyser value, and on the basis of the analyser result for tyrosine, using the ratio of 1.7, the value for tryptophan is reduced by about 50% (Table I).

Carbohydrate analyses

Initial observations had shown a discrepancy between the protein values obtained by the technique of LOWRY *et al.*[16] and total nitrogen and this suggested either that the protein was low in aromatic residues or that something else was present which was contributing to the nitrogen value. There was a darkening and charring of the sample after acid hydrolysis and, although spectrophotometric analyses indicated that aromatic residues were present at relatively normal levels, an abnormally high peak appeared in the tryptophan region during the amino acid

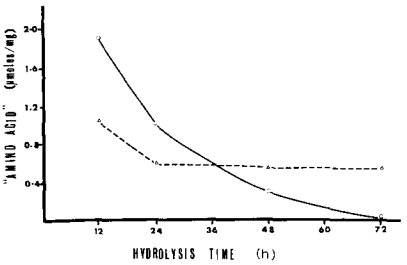


Fig. 3. Effect of time of hydrolysis on apparent concentration of tryptophan and glutamic acid in amino acid analyses of *M. sodonensis* nuclease. ○—○, tryptophan; △— — —△, glutamic acid.

analysis. This peak decreased with increasing hydrolysis time and at 72 h had disappeared (Fig. 3). Since tryptophan is normally destroyed by 12-h acid hydrolysis. The "tryptophan" peak must be due to some other compound which behaves as tryptophan on the column. The possibility of an amino sugar was considered since glucosamine will peak in the same region as tryptophan in the system and any free glucosamine released by acid hydrolysis would contribute to the tryptophan value. The high value obtained for glutamic acid (Fig. 3) as well as the inability to resolve the serine and glutamic acid peaks in the 12-h hydrolysate could also be attributed to the presence of an amino sugar. Two amino sugar–peptide linkages

which have been demonstrated in glycoproteins involve the hydroxyl group of serine, or the γ-carboxyl of glutamic acid and any amino sugar which was still linked to either amino acid could be responsible for the high value and the "smearing" of the serine–glutamic acid peak.

The enzyme was analysed for the presence of carbohydrate and the individual components were identified by paper chromatography. 100 μl of each hydrolysate (containing about 0.1 mg nuclease) were spotted on Whatman No. 1 paper and developed for 12 and 24 h in the solvent system described in MATERIALS AND METHODS. Standard sugar solutions were run with each chromatogram. Fig. 4a shows a chromatogram which had been developed for 12 h. Three spots were detectable in both the 3- and 12-h hydrolysates, two of which corresponded to glucose and rhamnose. The third spot was subsequently identified as glucosamine. As shown in Fig. 4b, after 24-h development in the solvent system, rhamnose had been eluted from the

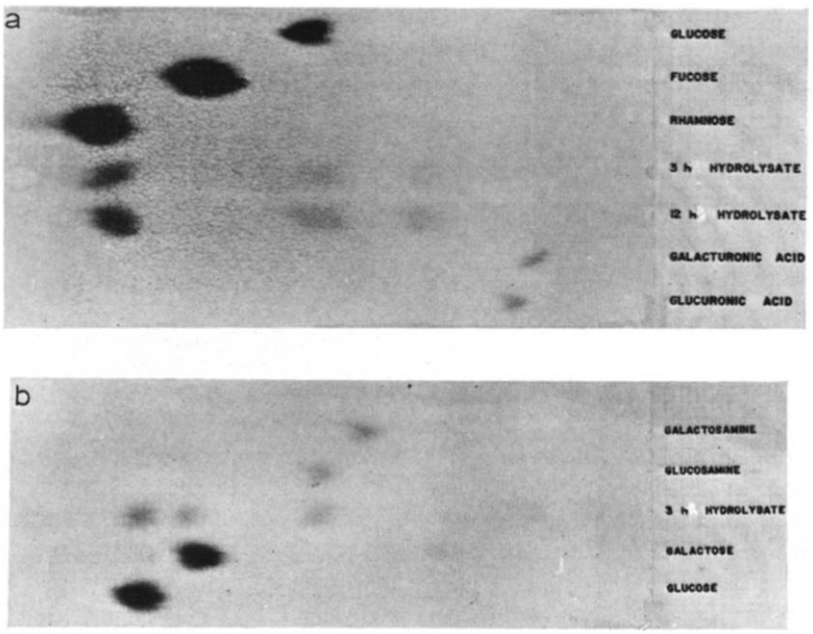


Fig. 4. Carbohydrate composition of *M. sodonensis* nuclease. Samples of purified nuclease were hydrolyzed in 3 M HCl evaporated to dryness and resuspended in water. The hydrolysate was chromatographed for varying times in the pyridine–ethyl acetate–water–acetic acid solvent system and spots were located with $AgNO_3$ reagent. (a) 12-h development, (b) 24-h development.

paper but the apparent single glucose spot was resolved into two, one of which was identified as galactose. The third spot corresponded to glucosamine. The presence of glucose and galactose was confirmed enzymatically by means of the Worthington Glucostat and Galactostat reagents. Further confirmation of glucose and glucosamine was obtained by co-chromatography with ^{14}C-labeled standards. 100 μl of a 3-h acid hydrolysate, containing an estimated 34 and 52 mμmoles of glucose and glucosamine, respectively, were applied to 4-cm Whatman No. 1 filter paper strips together with 1 μl of the ^{14}C-labeled standards (representing about 0.01 mμmole of each) and

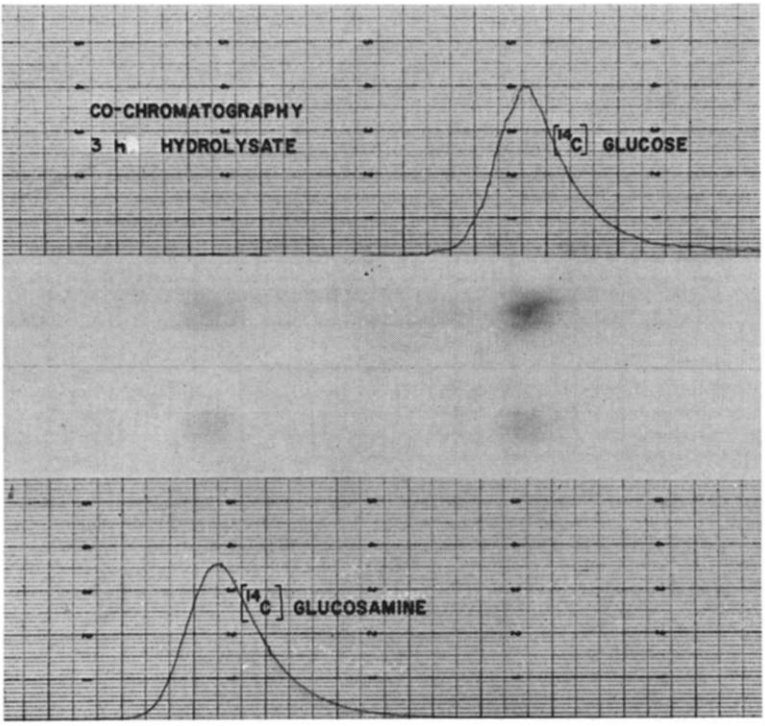


Fig. 5. Co-chromatography of a 3-h acid hydrolysate of *M. sodonensis* nuclease with ^{14}C-labeled glucose and glucosamine. The chromatogram was developed for 24 h in the pyridine–ethyl acetate–water–acetic acid solvent system and spots were located with $AgNO_3$ reagent. Radioactivity was detected by means of a Nuclear Chicago Actigraph III strip scanner.

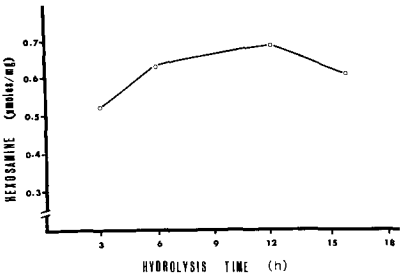
the strips developed for 24 h. As shown in Fig. 5, the spots previously identified as glucose and glucosamine co-chromatographed exactly with the respective radioactive standards. All of the sugars identified in the purified enzyme were also present in purified cell walls of *M. sodonensis*. Rhamnose, glucose, galactose and hexosamine were found in a 1:4:0.5:8 ratio with glucosamine accounting for approximately half of the hexosamine (K. G. JOHNSON, unpublished data).

The individual carbohydrate components of the enzyme were quantitated as described in MATERIALS AND METHODS and the results are given in Table II. Glucose,

TABLE II

CARBOHYDRATE COMPOSITION OF *M. sodonensis* NUCLEASE

Component	*μmoles/mg protein*
Total neutral sugar	0.750
Glucose	0.355
Galactose	0.170
Rhamnose	0.190
Hexosamine	0.678



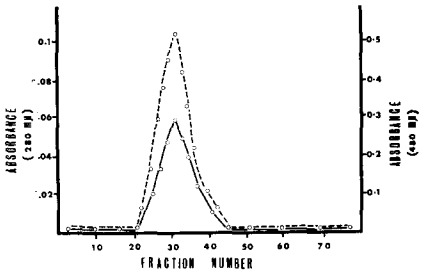


Fig. 6. Effect of hydrolysis time on hexosamine release from *M. sodonensis* nuclease.

Fig. 7. Gel filtration of *M. sodonensis* nuclease after treatment with 8 M urea. The enzyme was dialyzed for 2 h against 8 M urea and applied to a 2.5 cm × 32 cm column of Sephadex G-100. Protein was detected by measuring $A_{280\ m\mu}$. Color development at 480 mμ with the indole technique was a measure of the carbohydrate. ○—○, protein; ○— — —○, carbohydrate.

galactose and rhamnose accounted for 95% of the total neutral sugar. No sialic acids nor uronic acids were detected either chromatographically or chemically. The value for hexosamine was obtained after 12-h hydrolysis which gives maximum release of hexosamine (Fig. 6). It must be emphasized however, that this can only be a minimum value since it was apparent from the amino acid analysis (Fig. 3) that some amino sugar was still bound to the amino acid (*i.e.* serine or glutamic acid). However with longer hydrolysis time degradation of free hexosamine occurred more rapidly and the net result was a decrease in the hexosamine value.

The release of reducing power with acid hydrolysis of the enzyme was determined. A glucose standard was employed and the standards and test samples

TABLE III

REDUCING POWER OF *M. sodonensis* NUCLEASE

Hydrolysis time (h)	*Reducing power* (mμmoles/mg protein)*
0	25
1	1500
3	1415

* Relative to glucose.

were hydrolyzed in 3 M HCl for the indicated times. As shown in Table III, hydrolysis for 1 h under these conditions is sufficient to release all of the reducing power.

Attempts to separate the carbohydrate moiety from the intact protein by various methods such as dialysis, ion exchange, gel filtration, precipitation, high salt concentration and treatment with urea were unsuccessful. Fig. 7 shows the results of one such experiment in which the enzyme was dialysed against 8 M urea and applied to a column of Sephadex G-100. As shown, the carbohydrate remained associated with the protein peak. The inability to separate the two moieties by any

of the above techniques, coupled with the fact that prolonged acid hydrolysis was required to release all the amino sugar was indicative of a stable covalent linkage rather than ionic or hydrogen binding.

The results of the amino acid analysis suggested the possibility of an amino sugar linkage to serine or glutamic acid. Following the procedure of CARUBELLI *et al.*[28] the purified enzyme was hydrolyzed in 0.5 M KOH and the increase in absorption at 241 mμ was measured. The sugar was released by β-carbonyl elimination and the α-aminoacrylic acid derivative of serine, which was formed concurrently, absorbs strongly at this wavelength. Assuming a molar extinction coefficient of 5300 (ref. 7) for the aminoacrylic acid derivative, 3 mμmoles of the derivative were formed in 1 h. From the amino acid analyser data the total amount of serine present in the digest sample was 3.65 mμmoles. Thus a minimum of 80% of the serine residues are involved in the protein–carbohydrate linkage.

TABLE IV

DISTRIBUTION OF "BOUND" *vs.* "FREE" NUCLEASE IN A GROWING CULTURE OF *M. sodonensis*

Age of cells (h)	*Activity (units*/mg dry whole cells)*	
	"Crude" cell walls	*Culture supernatants*
12	1.31	307
18	0.84	248
24	0.56	241

* 1 unit is 1 mμmole of nucleoside released per h.

Detection of nuclease in cell walls

Attempts were made to determine whether the enzyme or its precursors could be detected in the cell envelope. Two methods were employed: (a) demonstration of activity and (b) imunological cross-reactivity. Samples of "crude" walls were prepared from 12-, 18-, and 24-h cultures of *M. sodonensis* and assayed for diesterase activity by measuring the relase of label from ^{14}C-labeled RNA soluble in 0.2%

TABLE V

DIESTERASE ACTIVITY IN ISOLATED CELL WALL FRACTIONS OF *M. sodonensis*

Fraction	*Activity* $\times 10^3$ *(units*/mg dry wt. fraction)*
"Crude"	43.8
"Heated crude"	3.7
"Phenol extracted"	15.0
"Pure walls"	—

* 1 unit is 1 μmole acid-soluble nucleoside per h.

uranyl acetate in 10% trichloroacetic acid. Table IV shows the relationship of "bound" to "free" activity in cells of different ages. Cell wall fractions at various stages of purification were also assayed for enzyme activity and the results are given in Table V. As the data indicate, enzyme activity is present in the wall in detectable amounts and is retained throughout the purification procedure until the final trypsin treatment which completely destroys all activity.

Standard tube precipitation tests showed a cross reaction between anti-cell wall serum and purified nuclease with no loss of enzyme activity (*i.e.* the activity was retained in the precipitate). Since anti-wall serum had been prepared from trypsin-treated walls which contained little protein the cross-reaction was obviously with the carbohydrate moiety. This was further substantiated by the fact that anti-

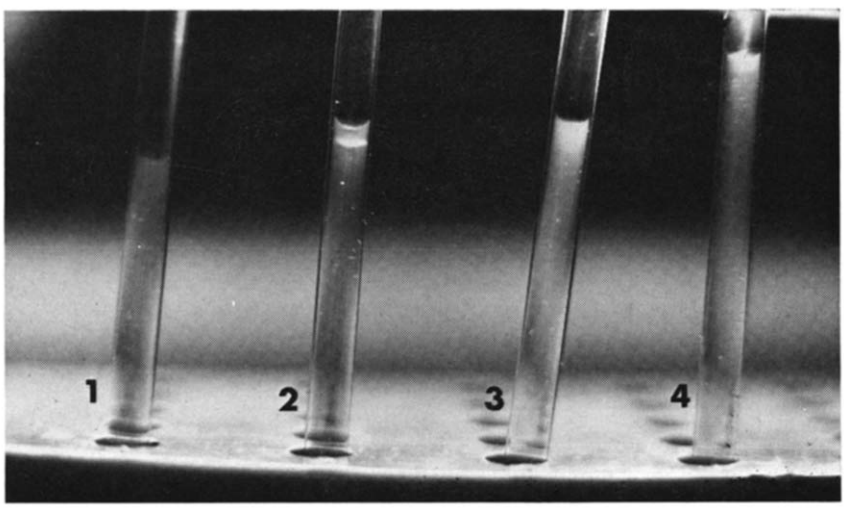


Fig. 8. Oudin single gel diffusion for cross-reactivity of nuclease and purified cell wall of *M. sodonensis*. Antisera were incorporated into the agar and the solidified columns overlaid with antigen or buffer. Tube 1, anti-cell wall serum + buffered saline; Tube 2, anti-cell wall serum + nuclease; Tube 3, control serum + nuclease; Tube 4, control serum + buffered saline.

enzyme serum (primarily anti-protein) inactivated the enzyme while showing only a feeble cross-reaction with pure cell walls which had been treated with trypsin.

Fig. 8 shows the results obtained in an Oudin single gel diffusion test. Sera were incorporated into the gel and the columns overlaid with antigen or buffered saline. As shown in the figure, a single band of precipitation appeared in the tube containing enzyme and anti-cell wall serum.

DISCUSSION

From the ultracentrifugal data it is apparent that *M. sodonensis* nuclease is an asymmetric molecule. Myoglobin, a globular protein has an $s_{20,w}$ of 2.0 S and a molecular weight of 16 900 (ref. 29), but the $s_{20,w}$ of 2.2 S and molecular weight of 500 000 obtained for *M. sodonensis* nuclease resemble the results obtained for myosin

which is an asymmetric molecule. KIELLEY AND HARRINGTON[30] found that the $s_{20,w}$ and molecular weight of myosin varied with the concentration, and an $s^{\circ}_{20,w}$ of 6.4 S and a molecular weight of 619 000 was obtained by extrapolation to infinite dilution. The same concentration dependence is noted with *M. sodonensis* nuclease albeit the asymmetry may well be a result of the presence of the carbohydrate since glycoproteins have certain properties such as high molecular charge density, high intrinsic viscosity, polydispersity and a high degree of asymmetry which distinguish them from simple proteins. These properties, particularly in molecules of high molecular weight and high viscosity, can cause many difficulties in physico-chemical analyses.

Aside from the anomalous results which were later explained by the presence of amino sugar, the amino acid composition was not unusual, although there is a relatively high level of serine, threonine and glycine residues, a characteristic observed in several mammalian glycoproteins[31]. The presence of high levels of proline is also of interest since it is incompatible with an extensive α-helical configuration and is indicative of the presence of β-structure[32]. Cysteic acid and methionine are low, as is noted also in micrococcal nuclease and *B. subtilis* ribonuclease[33]. POLLOCK[34] suggests that a characteristic of bacterial exoenzymes is their lack of cysteine. However, the protease of Myxobacter AL-1 (ref. 2) was found to contain 2 moles of cysteine per mole of enzyme so that the complete absence of cysteine is not universal amongst exoenzymes. The determination of total protein in a glycoprotein is not straightforward and many difficulties exist which limit the accuracy of the different methods. It will be noted from the amino acid data that the recovery of the amino acids was not quantitative. This was thought to be due to the unreliability of the protein determination according to LOWRY *et al.*[16] since the color intensity with this method varies for different proteins. It was subsequently demonstrated in this laboratory that glucosamine can give a positive Lowry and therefore in a glycoprotein might contribute significantly to any value obtained by this method.

From the data it is evident that *M. sodonensis* nuclease is a glycoprotein containing about 21% carbohydrate covalently bound to the protein moiety. The carbohydrate components have been identified as glucosamine, glucose, galactose and rhamnose in a ratio of 4:2:1:1. In all glycoproteins studied, the linkage of the carbohydrate to the peptide has invariably been through an amino sugar. Four types of linkages have been reported: (a) an *N*-acyl-glycosylamine linkage involving the amide-N of asparagine; (b) *O*-glycosidic linkages involving the hydroxyl groups of serine and threonine; (c) a glycosidic ester bond between *N*-acetylhexosamine and the β- or γ-carboxyl groups of aspartic or glutamic acids; (d) an amide linkage between the carboxyl group of *N*-acetylmuramic acid and the α-amino group of L-alanine. The data obtained in the analysis of *M. sodonensis* nuclease clearly indicated the presence of a glucosamine–serine linkage as the major bond between carbohydrate and protein. The release of amino sugar (identified as glucosamine) proceeded slowly with acid hydrolysis and at 12 h was not yet complete (Fig. 3). The amino–sugar complex was present in the glutamic acid–serine region and with longer hydrolysis time was hydrolyzed with the release of acid, as evidenced by the resolution of the peaks in that region. Glucosamine is thus established as the sugar involved and the data suggest the involvement of serine and/or glutamic acid in the carbohydrate–protein bond. Further support for a serine linkage was given by the alkaline hydrolysis which indicated that β-elimination had occurred with the formation of the amino-

acrylic acid derivative[28]. Since at least 80% of the serine residues (440 mμmoles/mg protein) are involved, this accounts for 65% of the total glucosamine residues. It is not established that all the glucosamine is involved so it is only possible to state that a serine–glucosamine linkage represents a major portion of the binding. Other linkages may also exist such as a linkage through the γ-carboxyl of glutamic acid and GOTTSCHALK AND MURPHY[35] produced evidence for the coexistence of both types of linkage (serine and glutamic acid) in bovine submaxillary gland protein.

Although the configuration of the carbohydrate cannot be finally determined from the data presented, some idea can be obtained from the reducing power data. Firstly, the carbohydrate must be highly polymerized since few reducing groups are available in the unhydrolyzed sample. Secondly, although glucosamine is not maximally released until 12 h of hydrolysis, all the reducing power is released by 1 h; therefore, glucosamine cannot be linked to the peptide in a C-1 position. And finally, a minimum size of 56 monosaccharide units and minimum molecular weight of about 11 000 can be assigned to the carbohydrate data. Since the enzyme is about 20% carbohydrate one can then calculate a minimum molecular weight of 44 000 for the peptide. On the basis of the amino acid analysis and assuming 1 cysteic acid residue per peptide, a minimum molecular weight of 10 817 is obtained. However, if there were 4 cysteic acid residues this value would be 43 268 which is in surprisingly close agreement with figures obtained from the carbohydrate calculations. It is emphasized that the calculations are based on reducing power, which is relative to glucose, and that this value may vary a bit depending upon which sugar is in fact at the reducing end of the polymer.

The role of the carbohydrate in the activity of the enzyme is not yet known since it has not yet been possible to remove the carbohydrate moiety leaving the protein intact. It is likely, however, that its role is structural rather than functional since antisera which cross-reacted with the carbohydrate moiety had no effect on activity. PLUMMER AND HIRS[5] found that the protein moiety of ribonuclease B was identical to that of ribonuclease A and both were biologically active. They concluded, therefore, that the carbohydrate moiety of ribonuclease B is non-functional. The role of carbohydrate in *M. sodonensis* nuclease might merely be to impart stability to an extracellular enzyme which must be able to survive variations in environment.

The discovery of the association of *M. sodonensis* nuclease with the cell walls, an enzyme not involved in wall synthesis, suggests that the wall itself might have some hitherto unsuspected function. The nuclease activity is retained in the wall throughout rigorous purification techniques such as drastic alterations in salt concentration, heating and phenol extraction. Although free enzyme is inactivated after 5 min at 55° (ref. 36), when bound to the cell wall it withstands 30 min at 90°. The enzyme which remains, therefore, must be bound in a stable configuration to the wall which enables it to withstand conditions under which it would normally be destroyed. Although activity is lost with trypsin treatment, antigenicity is retained and the cross-reaction between anti-cell wall serum and enzyme further supported the existence of enzyme in the wall itself. Since the wall anti-serum was prepared from virtually protein-free walls the cross reaction is obviously with the carbohydrate moiety of the enzyme. This is borne out by the fact that no inactivation of the enzyme occurs in the presence of anti-cell wall serum. Conversely, with anti-enzyme serum (which is predominantly anti-protein) there is inactivation of the

enzyme but little or no visible cross agglutination with cell walls. It is likely that the carbohydrate moiety, while being an antigenic determinant in the wall, loses much of its antigenicity in the free state (*i.e.* bound to free enzyme). The similarities in enzyme and cell wall carbohydrates further support the relationship.

A comparison of the *M. sodonensis* nuclease with the yeast invertase described by GASSONI AND LAMPEN[37] and LAMPEN[38] shows some significant similarities. (1) Both are glycoproteins and the sugar moiety of enzyme reflects a polysaccharide component of the corresponding cell wall (mannan in the yeast system). (2) Both are large molecules. (3) In both glycoproteins, the sugar–protein link involves serine residues. (4) Both enzymes are intimately associated with the parent cell wall. LAMPEN[38] suggests the possibility of a phosphodiester link between enzyme and wall mannans with the yeast system. In the case of *M. sodonensis* nuclease, however, less than 1% of the total activity is associated with the wall (*i.e.* 99% free), while only 10–30% of the yeast invertase is found free in the supernatant.

From the data presented it can be concluded that the nuclease of *M. sodonensis* is present in the cell wall itself rather than being associated with the membrane or periplasmic space. The exact nature of the relationship cannot be determined at the present time but several possibilities exist: (a) the enzyme may be completely synthesized at the cell surface and released; (b) inactive precursors may be transported through the membrane, assembled at the surface and released as active molecules; (c) the protein moiety may be synthesized elsewhere and simply attached to a carbohydrate portion at the cell surface. Whatever the mechanism, it is apparent that there is no accumulation of the active molecule but rather a formation (or activation) and an immediate release, since the amount liberated per unit cell far exceeds that contained in the walls themselves. Further studies are currently underway in an attempt to determine the exact location of the enzyme or its precursors in the wall.

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