ACETYLHEXOSAMINE COMPOUNDS ENZYMICALLY RELEASED FROM *MICROCOCCUS LYSODEIKTICUS* CELL WALLS

II. ENZYMIC SENSITIVITY OF PURIFIED ACETYLHEXOSAMINE AND ACETYLHEXOSAMINE–PEPTIDE COMPLEXES

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SUMMARY

Free di-saccharide (N-acetylglucosamine-N-acetylmuramic acid) is released from a purified amino sugar complex, probably tetra-saccharide, by the action of egg-white lysozyme and of a similar enzyme secreted by a *Streptomyces*. The di-saccharide is also released from a purified poly-acetylamino sugar-peptide–di-saccharide compound by the action of the same enzymes on its poly-acetylamino sugar moiety. Differences in the affinity of egg-white lysozyme and of the *Streptomyces* enzyme for their substrates are discussed.

A second bacteriolytic enzyme, also secreted by the *Streptomyces*, liberates free disaccharide from the purified peptide-di-saccharide and poly-acetylamino sugar-peptide-di-saccharide complexes by splitting the bond between the carboxyl group of muramic acid and the amino group of the peptide moiety.

INTRODUCTION

Preliminary experiments showed that the various purified acetyl-hexosamine compounds[1] isolated from the dialysable fractions obtained after lysozyme- or F_1-digestion of *Micrococcus lysodeikticus* cell-walls still contained some enzymically sensitive linkages. The present paper presents the results of an investigation of the action of egg-white lysozyme and of the F_1 and F_2B fractions obtained from bacteriolytic *Streptomyces* culture, on these isolated substrates of low molecular weight.

MATERIALS AND METHODS

Conditions of chromatography and electrorheophoresis, and sprays and color reactions used, are described in the preceding paper[1].

The F_2B *Streptomyces* enzyme was isolated by adsorption at pH 5.5 on an amberlite IRC 50 resin column from 280 L of *Streptomyces* culture filtrate. This

Abbreviations: AG = N-acetylglucosamine; AMA = N-acetylmuramic acid.

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filtrate had already been adsorbed on a column of the same resin at pH 7 in order to prepare the F_1 enzymic fraction[1]. The resin was then washed three times with a *N* KCl solution and treated with 50 ml (per 200 g of resin) of a 4 *N* KOH–*N* KCl solution. The dried product obtained by lyophilization of the dialyzed supernatant was dissolved in 500 ml of 0.1 *M* ammonium sulfate solution and precipitated by salting-out with 1500 ml of saturated ammonium sulfate solution. The insoluble brown material was resuspended in water, dialysed, taken to dryness (1.84 g) and then resuspended in phosphate buffer pH 7, μ 0.05. The supernatant, dialysed and lyophilized (1.38 g), constituted the F_2B enzyme fraction, while the material insoluble in the phosphate buffer (crude F_2A enzymic fraction) was further purified for other purposes. F_2B is entirely water-soluble.

Substrates

The substrates studied were all isolated[1] from digests of cell walls of *Micrococcus lysodeikticus* with either lysozyme or *Streptomyces* enzyme F_1. They fall into four classes: di-saccharide AG–AMA (DS); oligo-saccharide $(AG–AMA)_n$ (OS); di-saccharide-peptide (DSP): and di-saccharide-peptide-poly-saccharide [(AG–AMA)-peptide-AG_{10}-AMA_{10}] (DSPPS). When the origin of a particular substrate is relevant, it will be indicated by the suffix L (prepared after lysozyme digestion) or F_1 (prepared after F_1 enzyme digestion).

Incubation

Each purified small molecular weight compound was incubated under the same conditions: 200 μg of compound + 20 μg of enzyme (lysozyme, F_1 or F_2B) in a final volume of 0.02 ml and an ionic strength (ammonium acetate) of 0.05 (if incubated with F_1 or lysozyme) or of 0.005 (if incubated with F_2B). Incubation for 20 h at 37°.

EXPERIMENTAL

Enzymic degradation of di- and oligo-saccharides

Table I summarizes the changes produced in di- and oligo-saccharides as a result of incubation with lysozyme, F_1 and F_2B. The reaction mixtures after enzymic digestion were chromatographed and the spots were detected with SALTON's reagent[2] and ammoniacal silver nitrate.

The di-saccharide was completely resistant to F_2B, F_1 and lysozyme. The oligo-saccharide was completely resistant to F_2B, slightly sensitive to F_1 and highly sensitive to lysozyme. In the two latter reaction mixtures, only the di-saccharide and a certain amount of undigested oligo-saccharide could be detected. The oligo-saccharide must therefore be a polymer of the di-saccharide. Evidence given elsewhere[3] shows that it is a tetra-saccharide: AG–AMA–AG–AMA.

A quantitative estimation of the di-saccharide released by the actions of F_1 and lysozyme on the tetra-saccharide was made using the MORGAN-ELSON reaction (with the molar extinction coefficient 4870)[1], either directly on the digest itself or on the di-saccharide separated from the undigested tetra-saccharide by chromatography in butanol–acetic acid or by electrorheophoresis at pH 5.5 on Whatman 3MM paper. The same results were obtained estimating the hexosamines liberated from the isolated di-saccharide by 2 *N* HCl hydrolysis (2 h, 100°). The data in Table II show

TABLE I

EFFECT OF F_1, F_2B AND LYSOZYME ON THE PURIFIED AMINO SUGAR AND AMINO SUGAR-PEPTIDE COMPLEXES RELEASED FROM *Micrococcus lysodeikticus* CELL-WALLS BY A PRIMARY DIGESTION BY F_1 AND LYSOZYME

Substrates		*Substances released under the action of*		
		Lysozyme	F_1	F_2B
Di-saccharide (L or F_1)		o	o	o
Tetra-saccharide (L or F_1)		Disaccharide	Disaccharide (trace)	o
*DSP compounds**				
Name	*Preparation*			
F_1I	3	o	o	Disaccharide
F_1IIC	3	(Acetylmuramic acid)	(Acetylmuramic acid)	Disaccharide
F_1I	4a	o	o	Disaccharide
F_1II	4a	o	o	Disaccharide
L I	4b	o	o	Disaccharide
L II	4b	o	o	Disaccharide
*DSPPS compound**				
Name	*Preparation*			
F_1IIB	3	Disaccharide	Disaccharide (trace)	Disaccharide

* See ref. 1.

TABLE II

ESTIMATION OF THE DI-SACCHARIDE RELEASED, UNDER THE ACTION OF F_1, F_2B AND LYSOZYME, FROM THE PURIFIED AMINO SUGAR AND AMINO SUGAR-PEPTIDE COMPLEXES

This estimation is given in percentages calculated by dividing the [number of μmoles of disaccharide liberated $\times$ 100] by the number of μmoles of tetra-saccharide used or by the number of μmoles of di-saccharide bound to the peptide moiety and corresponding to the amount of amino sugar-peptide complex incubated.

Substrate	*Enzyme*	*Percentage of di-saccharide released, estimated by*			
		MORGAN-ELSON *reaction*			ELSON-MORGAN *reaction on the hexosamines isolated from the HCl hydrolysed di-saccharide*
		On the digest itself	*On the di-saccharide separated by*		
			Chromatography	*Electrorheophoresis*	
Tetra-*saccharide*					
(L)	Lysozyme	65	64	62	59
	F_1	11	5	5	6
(F_1)	Lysozyme	54	54	58	57
	F_1	12	8	7	12
*DSP compounds**					
Name *Preparation*					
F_1II 4a			78	75	
L I 4b	F_2B		61	63	
L II 4b			64	63	
*DSPPS compound**					
Name *Preparation*					
F_1IIB 3	F_2B		66	61	

* See ref. 1.

the differences in sensitivity of the tetra-saccharide towards lysozyme (54 to 65 % digestion) and towards F_1 (5 to 12 % digestion).

Action on the amino sugar-peptide complexes

1. Enzyme F_2B released 67 to 78 % of di-saccharide when incubated with DSP complexes as shown both by greater sensitivity to the ninhydrin reagent and by an increase of the positive charges of the peptide residues. Before the enzymic treatment, compound LII B (preparation 4b; Table II)[1] had a migration of 8.5 cm at pH 5.5 (700 V; 270 min; point of application: close to the positive electrode). After F_2B digestion, the electrorheophoresis showed, besides a certain amount of undigested LII B, the presence of the di-saccharide with a migration of 3 cm (*i.e.*, more negatively charged than the original compound) and the presence of the peptide residue with a migration of 14.5 cm (more positively charged). F_1 and lysozyme did not act on DSP complexes since no free di-saccharide was detected. However, with the compound F_1II C (preparation 3; Table II)[1], the butanol–acetic acid chromatograms of the F_1- and lysozyme-digests contained a small amount of a fast moving substance, ninhydrin negative and silver nitrate and *p*-dimethylaminobenzaldehyde positive, which could be free N-acetylmuramic acid.

2. The three enzymic systems, lysozyme, F_1 and F_2B liberated a certain amount of di-saccharide from DSPPS. The ninhydrin reactivity of the residual material was unchanged after incubation with F_1 and lysozyme. Coupled with the fact that these two enzymes liberate no di-saccharide from DSP compounds, this indicates that their site of attack on DSPPS must reside in the poly-saccharide $(AGA–AMA)_{10}$ residue. The residual material after treatment of DSPPS with enzyme F_2B showed an increased ninhydrin reactivity. Moreover, the amount of di-saccharide liberated represented 66 % of the di-saccharide moiety present in the untreated compound which is closely similar to the amount of di-saccharide liberated by the same enzyme from DSP complexes.

Therefore, it seems probable that F_2B released the intact di-saccharide bonded to the peptide moiety in the original compounds while the di-saccharide liberated by lysozyme and, to a less extent by F_1, originated from the poly-saccharide residue by breakage of glycosidic bonds.

Differences between egg-white lysozyme and F_1

Despite the similarities between lysozyme and F_1, they can be differentiated as follows:

Thermal denaturation: At a concentration of 2 μg/ml, unheated F_1 and lysozyme reduced the turbidity of a suspension of *Micrococcus lysodeikticus* cell walls incubated under standard conditions[4] to 50 % of its original value after 15 and 5 min of incubation respectively (Fig. 1). The activity of F_1 at a concentration of 10 μg/ml was completely destroyed by heating for 6 min in boiling water bath. With the same thermal exposure, a much more dilute solution of lysozyme (0.1 μg/ml) showed no loss of activity.

Wall acetylation: Various techniques[5] are now available for changing the lysozyme resistance of bacteria and, in the case of *Micrococcus lysodeikticus*, BRUMFITT *et al.*[6] have shown that a greater lysozyme resistance is associated with an increase in the O-acetylated groups of the walls. Complete O- and N-acetylation of the walls entirely

suppressed their sensitivity to lysozyme and very strongly inhibited their sensitivity to F_1. However, by subsequent treatment with 0.1 *N* NaOH for 1 h at 37°, which partially removed the O-acetyl groups, the walls recovered a high lysozyme sensitivity (Fig. 1), while they remained very resistant to F_1: with the latter, a turbidity reduction of 50 % occurred only after 20 h of incubation in presence of 2 μg F_1/ml.

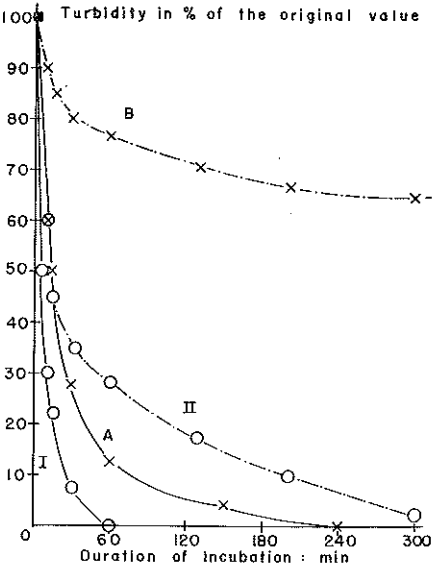


Fig. 1. Lytic action of lysozyme (I and II) and F_1 (A and B) on intact cell walls (I and A) and partially O-acetylated (and completely N-acetylated) cell walls (II and B) of *Micrococcus lysodeikticus*. 2 μg enzyme/ml: 37°; pH 7; μ 0.05; phosphates.

Comparative sensitivity of cells and walls. The sensitivity of *Micrococcus lysodeikticus* cell walls towards F_1 and towards lysozyme was of the same order of magnitude (see above). Conversely, the sensitivity of the intact cells towards the two enzymes was very different. Thus, lysozyme (2 μg/ml) caused a 50 % turbidity reduction in a cell suspension after 5 min of incubation. At the same concentration, F_1 required at least 150 min to produce a similar reduction.

Influence of the culture medium. The composition of the medium in which *Micrococcus lysodeikticus* is grown can also preferentially affect the sensitivity of the isolated cell walls to F_1. The same strain NCTC 2665 was incubated, with shaking, under the same conditions, in five different media, each of them containing 0.5 % of yeast extract, supplemented as follows: medium 1: Bacto-peptone 5 %, NaCl 1 %; medium 2: casamino acids 2 %, NaCl 1 %; medium 3: Trypticase Soy Broth 3 %, glucose 2 %; medium 4: casamino acids 2 %, glucose 1 %; medium 5: casamino acids 2 %, fructose 1 %. In each case, the walls isolated from the harvested cells showed an equal sensitivity to lysozyme (see above) whereas the walls obtained from media 1, 2, 3 were three times more sensitive to F_1 than those obtained from media 4 and 5. It must also be pointed out that the *Micrococcus lysodeikticus* cell walls used throughout these studies, had the same sensitivity towards lysozyme as has been previously found[4], but were more sensitive to the present F_1 preparation than would have been expected from the experiments recorded two years ago[4]. At that time a 50 % turbidity reduction was obtained after 120 min of incubation in presence of 10 μg F_1/ml; this compared with 15 min for a similar reduction in the presence of 2 μg/ml of the present F_1

preparation. A sample of the first enzymic preparation which was stored for two years in dry state, exhibited the same activity on the present preparation of cell walls as the present F_1 preparation. Therefore, the observed variations must be due to some differences in the nature of the two batches of *Micrococcus lysodeikticus* cell walls.

DISCUSSION

Enzymic nature of egg-white lysozyme and F_1

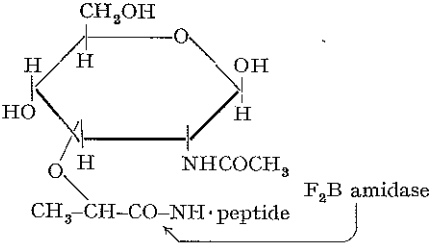
The splitting of the tetra-saccharide (AGA–AMA–AGA–AMA) into two moles of di-saccharide (AGA–AMA) proves that lysozyme has an N-acetylhexosaminidase action. The same weak, but readily detectable, action of F_1 on the tetra-saccharide indicates that this enzymic preparation contains an egg-white lysozyme-like N-acetylhexosaminidase.

However, lysozyme and F_1 polysaccharidase are two different proteins and have a different affinity for the sensitive glycosidic bond contained in the tetra-saccharide. As previously suggested[7], the di-saccharide is the end-product of lysozyme digestion of *Micrococcus lysodeikticus* cell walls. On the other hand, the end-products of the F_1 digestion are both di- and tetra-saccharides. In this latter case, the di-saccharide found in the dialysable fraction is not formed to any significant extent by further digestion of the tetra-saccharide, but rather by the destruction of a more complex amino sugar chain of the walls.

The dialysable fraction obtained by digestion of cell walls of *Micrococcus lysodeikticus* with F_1 or lysozyme does not contain any free di- or tetra-saccharides if incubation is stopped as soon as a complete clarification of the suspensions has occurred. The first action in the dissolution of the walls is therefore concerned with the breakage of glycosidic bond liberating large soluble fragments from which the di- and tetra-saccharides are subsequently split off.

Enzymic nature of F_2B

One of the key linkages in the construction of the bacterial cell wall is postulated to be an amidic linkage between the carboxyl group of muramic acid and an amino group belonging to a peptide chain. This hypothesis was strengthened by the discovery that the transfer of the peptides and muramic acid into the macromolecular structure of the walls is mediated by uridine diphosphate acetylmuramic acid nucleotides to which is linked either an alanine residue or a small peptide of glycine, glutamic acid and alanine[8–10]. It can be concluded from the experiments performed with the small purified DSP compounds that F_2B splits the linkage between the peptide and di-saccharide residues. As this action increases the positive charges of the peptide residues, it seems very probable that F_2B is an amidase acting on the linkage:



The F_2B action on intact *Micrococcus lysodeikticus* cell walls was also examined. In terms of turbidity reduction, F_2B was certainly less active than lysozyme or F_1. A concentration of 20 μg F_2B/ml reduced the turbidity of a wall suspension to 50 % of its original value after 150 min. The same turbidity reduction occurred after 5 and 15 min with 2 μg of lysozyme and of F_1, respectively. The dialysed F_2B-digested fraction contained some strongly ninhydrin positive materials which were resolved into several small peptides and free alanine by two dimensional chromatography. These peptides have the same characteristics as the peptide residues obtained after F_2B action on the purified small DSP complexes. The free alanine must be considered as an N-terminal alanine group (previously detected in the intact cell walls[11]). The dialysable fraction after digestion with F_2B enzyme, probably does not contain any poly-saccharide material, for no di- or tetra-saccharides could be detected directly or after a further incubation of this dialysable fraction with F_1 or lysozyme. Conversely the non-dialysable material was rich in MORGAN-ELSON positive substances from which lysozyme and F_1 released both di- and tetra-saccharides. These observations suggest that in the intact walls, no fragments as simple as a di- or tetra-saccharide exist in a lateral or terminal position to a peptide chain or between two such peptide chains. If the walls have first been treated by F_1, a further incubation of the non-dialysable material with F_2B enzyme releases some free di- and tetra-saccharides. At the same time, the reactivity of the whole mixture to ninhydrin increases, and by paper chromatography, the formation of a number of ninhydrin positive substances of low molecular weight can be shown. The di- and tetra-saccharides liberated by such treatment with F_2B enzyme must be regarded as small lateral or terminal branches linked to peptide chains which are held together in the intact walls by a complex poly-saccharide bridge susceptible to destruction by F_1.

Streptomyces *bacteriolytic enzymes*

The bacteriolytic activities of *Streptomyces* sp. are due to numerous different exo-enzymic systems. The existence of a lysozyme-like enzyme[12–15] was previously suspected because of the great similarity between the lytic spectrum of some *Streptomyces* culture filtrates and that of egg-white lysozyme. The N-acetyl-hexosaminidase activity of the F_1 preparation confirms this hypothesis but shows, however, that F_1 and egg-white lysozyme are two different proteins, each with a particular enzymic affinity. Other lysozymes produced by *Sarcina* sp.[16] and *Bacillus* sp.[17,18] are known, but there is insufficient information in the literature to conclude whether they are identical to the egg-white lysozyme or to the polysaccharidase F_1.

Streptomycetes in addition secrete one other bacteriolytic poly-saccharidase, isolated by MCCARTY[19] and characterized by a lytic spectrum quite different from that of lysozyme or of F_1. MCCARTY's enzyme indeed is inactive on *Micrococcus lysodeikticus* but very active on streptococcal cell walls. On these latter walls, F_1 carried out only a slow and incomplete digestion, reducing the turbidity of a suspension of a trypsin-treated group A streptococcal cell walls to 17 % of its original value after 20 h of incubation in the presence of 20 μg enzyme/ml. It is likely that MCCARTY's enzyme is different from F_1 and lysozyme not only in its affinity but also in its specificity. We have also shown that some *Streptomyces* cultures contain at least two enzymes producing lysis of pneumococci[20] and another enzyme[21] producing lysis of streptococci

—which may be identical to McCARTY's poly-saccharidase. As yet, nothing is known about their mode of action.

The experiments carried out with the F_2B fraction in the present studies strongly suggest the existence of a *Streptomyces* amidase acting on the muramic acid-peptide linkage. It was previously observed[4] that the digestion of various gram positive cell walls by *Streptomyces* bacteriolytic preparations, obtained by a similar technique to that used for the preparation of F_1 and F_2B, was accompanied by a release of small peptides and free alanine and/or glycine and that at least two different enzymic systems, called for this reason actinolysopeptidases I and II[22], were involved in these phenomena. Of course, the F_2B amidase could explain the liberation of these ninhydrin positive materials, but F_2B is inactive on *Bacillus megaterium* (K-M) and on *Staphylococcus aureus* cell walls. Moreover, F_2B seems unable to release the poly-saccharide structure from the purified small compound DSPPS. It is therefore possible to conceive the F_2B enzyme as an amidase with a very strict specificity controlled by the structure of the neighbouring poly-saccharide and peptide chains. On the other hand, besides its polysaccharidase activity on *Micrococcus lysodeikticus* cell walls, F_1 actually releases from *Bacillus* sp. and *Staphylococcus* sp. cell walls some free alanine and small peptides. In these circumstances, the F_1 preparation must be heterogeneous and must contain, in addition to the poly-saccharidase, one or several "peptidases", in the broadest sense, specifically different from the F_2B amidase.

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