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SUBSTRATE-INDUCED EVOLUTION OF LYSOZYMES

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Summary

Digestion by five different lysozymes (mucopeptide *N*-acetylmuramoylhydrolases, EC 3.2.1.17) of a soluble uncross-linked peptidoglycan secreted by *Micrococcus luteus* (Mirelman, D., Bracha, R. and Sharon N. (1974) Biochemistry 13, 5045—5053) was investigated. Hen egg-white and human lysozymes converted the peptidoglycan into the disaccharide GlcNAc-$\beta(1 \rightarrow 4)$-*N*-acetylmuramic acid (-MurNAc), the tetrasaccharide (GlNAc-$\beta(1 \rightarrow 4)$-MurNAc)$_2$ and the disaccharide-hexapeptide:

GlcNAc-β(1→4)-MurNAc
|
L-Ala-D-isoGlu(Gly)-L-Lys-D-Ala-D-Ala.

With goose egg-white lysozyme, mainly the disaccharide and the disaccharide-hexapeptide were produced, in approximately equimolar amounts. Papaya lysozyme afforded mainly products without peptide substitution, such as the disaccharide and tetrasaccharide, while T4 phage lysozyme gave only the disaccharide-hexapeptide.

Lysozymes from different sources thus differ in their specificity requirements with respect to peptide substitution on the lactyl moiety of *N*-acetylmuramic acid. This pattern of specificity is discussed in terms which correlate it with the differences in the structures of their presumed natural substrates. We postulate that evolutionary changes in substrate structure may have influenced the development of the active site of lysozyme so that it could function most efficiently with the particular natural substrate encountered by each species.

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Abbreviations: MurNAc, *N*-acetylmuramic acid (2-acetamido-3-*O*-D-carboxylethyl-2-deoxy-D-glucose). All oligosaccharides are $\beta(1 \rightarrow 4)$ linked.

Introduction

Studies of lysozymes (mucopeptide *N*-acetylmuramoylhydrolases, EC 3.2.1.17) from different sources have led to their classification into several groups on the basis of their enzymatic and chemical properties [1—3]. The major group, designated as the *c* type, includes the enzymes which are similar to hen egg-white lysozyme in their amino acid sequence, immunological properties and structure of their active site. This group also includes human lysozyme which resembles the hen enzyme not only in the properties mentioned, but also in its three-dimensional structure [4]. Another group of lysozymes, designated as the *g* type [2], the typical representative of which is goose lysozyme [5—8], is also widespread in birds. Although lysozymes of the *g* type are similar in some of their enzymatic properties to the *c* type enzymes, they differ markedly in their structure and immunological properties from the latter group.

Other bacteriolytic enzymes, characterized as lysozymes because of their ability to lyse cells of *Micrococcus luteus* by hydrolyzing the glycosidic bond between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine of the cell wall peptidoglycan, form a third group and are well separated in their properties from the enzymes mentioned above. These include the lysozymes of T4 phage [9—11] and of papaya [12,13], which also differ from each other.

The availability of a novel lysozyme substrate, a linear peptidoglycan isolated recently in our laboratory from cultures of *M. luteus* cells inhibited by penicillin [14], has provided us with an additional tool for the study of the specificity requirements of lysozymes. This substrate consists of an approximately equimolar mixture of a polysaccharide $(GlcNAc\text{-}MurNAc)_{\approx 40}$ and a peptidoglycan, $(GlcNAc\text{-}MurNAc)_{\approx 40}$ and a peptidoglycan,

$$(GlcNAc\text{-}MurNAc)_{\approx 40}$$
$$\Big|$$
$$L\text{-}Ala\text{-}D\text{-}isoGlu(Gly)\text{-}L\text{-}Lys\text{-}D\text{-}Ala\text{-}D\text{-}Ala$$

both with $\beta(1 \rightarrow 4)$ linkages throughout. Although not homogeneous, examination of the pattern of action of different lysozymes on this substrate has enabled us to delineate more clearly the specificity requirements of the enzymes. Taken together with data in the literature on the presumed role of different lysozymes in nature, our results have led us to propose a new scheme for the evolution of the different lysozyme types based on changes induced in the enzyme's specificity by alterations in the structure of its substrate.

Experimental Procedure

Materials. Hen egg-white lysozyme twice crystallized and salt free was from Worthington. Human leukaemic lysozyme was obtained from Dr. E.F. Osserman, and Embden goose egg-white lysozyme from Dr. R.E. Canfield, both from the College of Physicians and Surgeons, Columbia University. The papaya lysozyme was a gift from Dr. A.K. Allen (Charing Cross Hospital, London). T4 phage lysozyme was isolated by Dr. H.B. Jensen and G. Kleppe (University

of Bergen, Norway). Each of these lysozymes displayed a single band when run on SDS-polyacrylamide gel electrophoresis [15] employing 25 μg protein/gel.

The linear uncross-linked [^{14}C]glycan-labeled peptidoglycan (average mol. wt. 30 000) was obtained by the method of Mirelman et al. [14]. Tritium-labeled disaccharide GlcNAc-MurNAc and tetrasaccharide $(GlcNAc\text{-}MurNAc)_2$ were gifts from Dr. David Chipman (Ben Gurion University of the Negev, Beer Sheba, Israel) [16].

Methods. Enzymatic hydrolysis of soluble peptidoglycan and product analysis by paper electrophoresis, paper chromatography and thin-layer chromatography, were carried out essentially as described [10.11]. Unless otherwise stated assays were done in 10 mM morpholinoethane sulfonic acid buffers, in 0.1 M NaCl. Peptidoglycan (5 nmol) dissolved in water was lyophylized in 1-ml plastic tubes and a solution of lysozyme (50 μl, 0.1 mg/ml) was added. Incubation was at 37°C. Digestion of the tritium-labeled tetrasaccharide ($2.2 \cdot 10^5$ cpm/μmol) was as above.

Results

On digestion with hen egg-white lysozyme, the labeled peptidoglycan gave low molecular weight fragments in which the ratio of unsubstituted to peptide-substituted *N*-acetylmuramic acid residues was approximately 1 : 1 at the three pH values examined (pH 3.4, 5.3 and 7.0) (Table I). Differences were observed, however, in the ratio of disaccharide to tetrasaccharide produced at the different pH values examined. At pH 3.4 and pH 5.3 disaccharide was produced than tetrasaccharide, whereas at pH 7.0 less disaccharide was produced

TABLE I

PRODUCTS OBTAINED AFTER LYSOZYME DIGESTIONS OF THE LINEAR PEPTIDOGLYCAN OF *M. LUTEUS*

For digestion conditions see text. Digestion products were monitored using the procedures discussed in refs. 10 and 11.

Lysozyme source	pH	Time (h)	Products (percent of total counts)				
			Di-saccharide	Tetra-saccharide	Di-saccharide-hexa-peptide	Oligo-saccharide-hexa-peptides	Undigested peptidoglycan
Hen egg-white	3.4	4	28	18	43	—	11
	5.3	4	21	18	50	3	8
	7.0	4	13	29	50	1	7
Human leukaemic	3.4	4	15	27	45	3	10
	5.3	4	15	29	43	4	9
	7.0	4	15	29	41	4	11
Goose egg-white	3.4	4	48	4	40	—	8
	5.3	4	45	7	39	—	9
	7.0	4	42	7	40	3	8
Papaya *	5.3	24	15	16	10	39	20
T4 phage *	7.5	24	2.5	0	28	17	52.5

* Ionic strength 0.02 M. The pH values chosen were those at which these enzymes are known to exhibit optimal activity.

TABLE II

DIGESTION OF ^{3}H-LABELED TETRASACCHARIDE BY VARIOUS LYSOZYMES

Digestion and characterization of products was performed as described [10,11].

Lysozyme source	pH	Products (percent of total counts)		
		Disaccharide	Tetrasaccharide	Material remaining at origin of chromatograms
Hen egg-white	5.2	54	42	4
	6.6	28	72	0
Human leukaemic	5.2	27	63	10
Goose egg-white	5.2	75	21	4
Papaya	5.2	2	94	4
T4 phage	7.5	0	96	4

than tetrasaccharide. The amount of disaccharide-hexapeptide formed (43—50%) in all these experiments was essentially the same; moreover, in all cases digestion of the substrate was almost complete.

Human lysozyme gave the same product ratios at all pH values investigated, the ratio of tetrasaccharide to disaccharide at the different pH values being similar to that found with hen egg-white lysozyme at pH 7.0. With the goose enzyme, the disaccharide and the disaccharide-hexapeptide were the predominant products of digestion and only small quantities of tetrasaccharide were formed. Changes in NaCl concentration in the range of 0.05—0.30 M did not affect the pattern of products formed by the above three enzymes.

The papaya enzyme gave a different pattern of digestion. This enzyme acted on the peptidoglycan very slowly, extensive digestion (80%) after 24 h being observed only under rather specific conditions of pH and ionic strength (pH 5.0, 0.02—0.04 M). The major low molecular weight products formed after 24 h digestion were the disaccharide (15%, based on amount of substrate) and tetrasaccharide (15%) with relatively small amounts of disaccharide-hexapeptide, and high amounts of larger glycopeptides. In the case of T4 phage lysozyme the peptidoglycan was broken down exclusively to the disaccharide-hexapeptide and no disaccharide or tetrasaccharide were produced (Table I).

Incubation of the tritium-labeled cell wall tetrasaccharide with the various lysozymes (Table II) revealed that goose egg-white lysozyme cleaved it to disaccharide even to a larger extent than did hen egg-white lysozyme and human lysozyme. In contrast, the T4 and papaya enzymes had no activity on this substrate. Since the disaccharide did appear in the digest of the peptidoglycan by the papaya enzyme, it must be assumed that this lysozyme can only digest cell wall fragments larger than the tetrasaccharide.

Discussion

Our data, in conjunction with previous findings reported in the literature [1—3] clearly demonstrate that the enzymes studied can be classified according to their specificity requirements. On the one hand, hen egg-white and human lysozymes, both *c* type enzymes, appear to act equally well on peptide substituted or unsubstituted peptidoglycan. These enzymes act also

on chitin oligosaccharides, although slower than on the linear peptidoglycan. Goose lysozyme, a *g* type of enzyme [2,7,8] has a similar specificity of action on the linear peptidoglycan as the above enzymes, but does not act on chitin oligosaccharides nor is it inhibited by the latter [3,8,17]. Furthermore, in contrast to the *c* type enzymes which are capable of both hydrolysis and transglycosylation, the goose enzyme acts only as a hydrolase. On the basis of these observations, we suggest that the active site of goose egg-white lysozyme is less extended than that of the hen or the human enzyme and is also probably deeper so that interaction may take place with the lactyl group of muramic acid and perhaps with the peptide side chain(s) as well. The specificity requirements of the T4 phage enzyme are much more stringent. Recent data have shown that a single *N*-acetylmuramic acid residue is sufficient for the interaction with the T4 enzyme and that the enzyme apparently has additional binding sites for a peptide side chain [11]. This may also be true for the autolytic lysozyme isolated from *Escherichia coli* that only digests glycosidic linkages next to peptide-substituted *N*-acetylmuramic acid residues (Schwarz, U., private communication).

The specificity of the papaya enzyme seems quite distinct from that of the other enzymes studied. In contradistinction to the high activity of this enzyme on chitin-oligosaccharides [12], it cleaved the peptide-substituted peptidoglycan very poorly (Table I). Papaya lysozyme appears therefore to bind poorly to *N*-acetyl-D-glucosamine residues that are substituted by a lactyl group on the C-3 carbon.

Although the substrate used by us is not homogeneous, and consists of an approximately equimolar mixture of glycan and peptidoglycan [10,14], this should not affect our conclusions on the specificity of the enzymes examined, since these are based on the pattern of digestion as revealed by the nature of the products formed. Some of the results were also confirmed with the aid of a peptidoglycan labeled by [^{14}C]alanine in its peptide moiety [10]. It should further be noted that repeated attempts to fractionate the substrate, either by conventional techniques or with the aid of antibodies against the peptide moiety of the peptidoglycan, were not successful.

TABLE III

SUBSTRATE SPECIFICITY REQUIREMENTS OF LYSOZYMES

Digestibility of substrate: +++, well-digested; ++, fair; +, poor; —, not-digested. Substrate types: I, peptide-substituted glycan; II, unsubstituted glycan; III, $(GlyNAc)_n$ = oligosaccharides (n = 3—6) isolated from chitin; data on their digestion by the various enzymes are reviewed in refs. 3 and 9.

Lysozyme source	Type	Substrate Types			Presumed main natural substrate (type)
		I $(GlcNAc\text{-}MurNac)_n$ | peptide	II $(GlcNAc\text{-}MurNAc)_n$	III $(GlcNAc)_n$	
Hen egg-white	*c*	++	+++	++	I, II, III
Human leukaemic	*c*	++	+++	++	I, II, (III)
Goose egg-white	*g*	+++	++	±	I, II, (III)
Papaya		±	±	++	III
T4 phage		+++	—	—	I

A summary of the specificities of the five enzymes, together with their presumed natural substrates (see below), are given in Table III.

In the past, the search for an evolutionary connection between proteins and enzymes of various species and phyla has been attempted through the comparison of primary structures [18,19], immunological cross-reactivity [20,21], and most importantly through the comparison of three-dimensional crystallographic structure [22]. In all of these comparisons, it has been tacitly assumed that the substrate of the enzyme, or ligand of the binding protein, has not undergone any major evolution of its own. In cases where the substrates (or ligands) are peptide bonds (serine proteases), H^+ (dehydrogenases), e^- (cytochrome *c*) and O_2 (hemoglobin), the evolutionary pressure on the protein to change has certainly not come about because of some evolutionary change in the peptide bond, proton, electron or in oxygen, respectively.

The variation in composition and structure of the bacterial cell wall from a highly complex multilayered peptidoglycan as in Gram-positive bacteria (e.g. *M. luteus*) to the mono- or bi-layered peptidoglycan of Gram-negative bacteria (e.g. *E. coli*) and further to the relatively simple external cell wall of fungi and insects containing chitin, may have had a direct bearing on the differences found in the specificity of lysozymes from different sources. These changes in cell wall structure could have provided the impetus for a type of "substrate-induced evolution" of lysozymes, perhaps by the independent evolution of these enzymes from non-lysozyme protein progenitors.

Although the role of lysozymes in nature is far from being established [23], the literature abounds with evidence pertaining to the functional significance of lysozyme in various organisms as a defense mechanism against bacteria and chitin-covered pathogens. Lysozyme in invertebrates [5] may perhaps serve as a rudimentary protective system since these organisms do not produce immunoglobulins. The specificity of the lysozymes in this case is against the bacterial cell wall. The papaya and fig proliferate in climates that are favorable to fungal growth. A large number of phytopathogenic fungi possess chitin in their cell walls. It seems reasonable that the papaya enzyme would act mainly as a chitinase and in conjunction with the proteolytic enzymes of these fruits offer protection from invasion. It is of interest that another plant lysozyme, that from turnip, also acts more as a chitinase and thus resembles papaya lysozyme [24]. The phage lysozyme, which requires a peptide-substitued substrate, would also appear to be uniquely adapted to its host, *E. coli*, in which all the *N*-acetylmuramyl residues are substituted with peptides.

Lysozyme exists in high concentrations in the egg-white of many birds, and two classes of the enzyme, *c* and *g* types, have been characterized [2]. Although these vertebrates all contain immunological systems, the egg and developing embryo do not produce immunoglobulins until about 7 days before hatching [25]. It is therefore possible that the egg has a high lysozyme content to maintain protective vigilance until the embryo has the capability to produce immunoglobulins. Further, it is conceivable that the *c* type or *g* type lysozymes are distributed in the egg-white of various species of birds according to the differing potential substrates the eggs would encounter whether hatched near water, in the soil, or on land. Goose lysozyme, a *g* type enzyme, has been demonstrated to be more effective against Gram-negative *E. coli* than hen egg-

white lysozyme, a *c* type enzyme. The latter enzyme is, however, more effective against chitin and *M. luteus*. Since the eggs of birds hatched near water are more commonly exposed to Gram-negative bacteria [26], this type of environmental relationship and lysozyme selection can be envisaged.

In higher vertebrates, lysozyme has been recognized as an essential antibacterial agent. The antibody-complement system attacks to kill the bacteria, while the lysozyme breaks up the cell walls into smaller units for effective clearing [23,27,28], and possibly to reduce their ability to act as antigens capable of triggering immune reactions.

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