Effect of Lysozyme-Potentiating Treatments on Escherichia coli Cell Wall Protein[1]

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While most gram-positive bacteria are sensitive to the lytic action of lysozyme, the majority of gram-negative cells are unaffected by this enzyme. According to Weidel, Frank and Martin (1960), gram-negative organisms appear to have three layers. The outer layer is a lipoprotein coat; the middle layer is a lipopolysaccharide while the innermost layer is the mucopeptide substrate of lysozyme and is thought to bestow cellular rigidity. Lysozyme cleaves the linkage between the N-acetylglucosamine and N-acetylmuramic acid of the mucopeptide.

Current research suggests that resistance of gram-negative cells to lysozyme is due to the covering of the lysozyme substrate by the external layers of lipid, protein, or lipoprotein. Grula and Hartsell (1954), Becker and Hartsell (1954), Noller and Hartsell (1961), and Repaske (1958), have reported that certain treatments of the gram-negative cell such as low pH, heat, solvents, polybasic antibiotics, and versene are capable of enhancing lysozyme action on whole cells. It is presently presumed that these treatments dissociate components of the cell wall which protect the mucopeptide. The rigid mucopeptide is then degraded by lysozyme.

The purpose of these studies is to further investigate lysozyme-potentiating treatments by observing the changes in protein and carbohydrate composition that occur in isolated gram-negative cell walls.

MATERIALS AND METHODS

Escherichia coli (ATCC 8739) was selected as test culture on the basis of lytic sensitivity of whole cells to lysozyme-potentiating systems. Cells were grown in ten-liter batches of nutrient broth + 0.2% dextrose for 18 hrs at 37 C, harvested by centrifugation, and ruptured by a Raytheon sonic oscillator. Cell walls were purified by the differential centrifugation scheme of Schocher, Bayley and Watson (1962), lyophilized, and stored in a desiccator.

Fifty-mg samples of preheated cell walls (15 min at 70 C) were exposed to lysozyme, trypsin, and lysozyme plus trypsin at 45 C for 1 hr. Fifty-mg samples of unheated cell walls were exposed to lysozyme, trypsin, and lysozyme plus trypsin in the presence and absence of n-butanol or polymyxin (phosphate buffer pH 7) or versene (Tris buffer, pH 8) at 45 C for 1 hour. Nakamura treatment of cell walls was as indicated in Fig. 1.

Following the 1-hour incubation intervals, all treatment systems were centrifuged to remove wall residues and the supernatant liquid was analyzed as indicated in Fig. 2.

RESULTS AND DISCUSSION

Any protein released from the cell wall by lysozyme-potentiating treatments was found to contain fourteen of the sixteen amino acids reported for *E. coli* by Salton (1960). Histidine and phenylalanine were

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absent. Efforts have not yet been made to quantitate individual amino acids although some differences in spot intensities have been noted following different potentiating treatments.

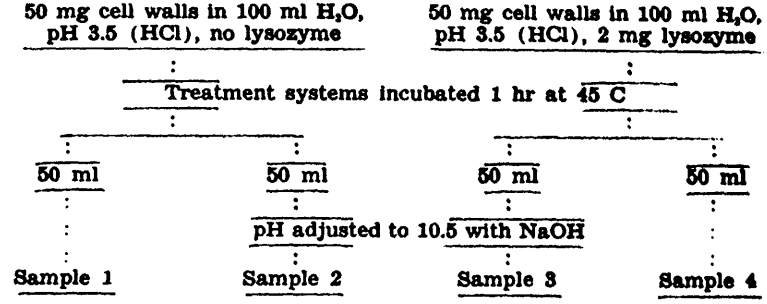

Figure 1. Procedure for the Nakamura treatment of cell walls.

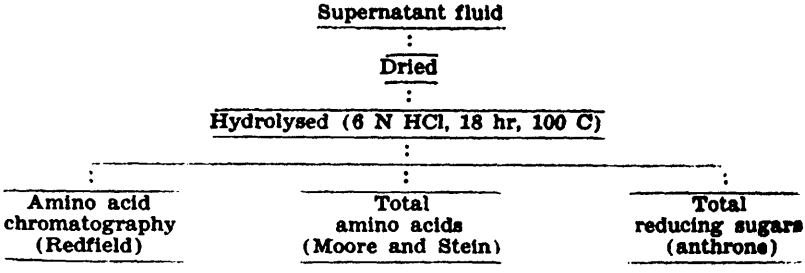

Figure 2. Analyses performed on hydrolyzed supernatant fluid from cell walls following selected pre- and co-lytic treatments.

Using heat-pretreated cell walls (Table 1), lysozyme combined with trypsin causes greater release of protein from the walls than the additive effect of the enzymes acting singly. It appears likely that with the combined enzymes, tryptic hydrolysis unmasks lysozyme substrate. Lysozyme activity then results in release of additional amino acids and/or permits further tryptic digestion. This is supported by the increased amounts of reducing sugar released when the enzymes act in combination.

TABLE 1. Release of amino acids and reducing sugars from heat pretreated cells walls by lysozyme and/or trypsin.

50 mg pre-heated cell walls 0.0067M PO_4, pH 7	Total amount released*	
	Amino acids (as μmole leucine)	Reducing sugars (as mg glucose)
Buffer only	[12] —	[2.2] —
Lysozyme (20 μg/ml)	4	0.1
Trypsin (10μg/ml)	28	0.0
Lysozyme + trypsin	74 (32)**	0.6

*Corrected for buffer values shown in brackets.
**Values in parentheses are sums of amounts released by individual components of system.

There is some trypsin and lysozyme action on unheated walls (Table 2), probably due to partial denaturation of the walls during their preparation. However, *n*-butanol markedly increases trypsin activity but does not increase lysozyme action. Since these results were obtained after 60 min incubation and trypsin action is very rapid in the presence of butanol, lysozyme effects are not apparent in the lysozyme-trypsin-butanol system. A similar situation was reported by Noller and Hartsell (1961) when lysis of whole cells by the three-fold system was observed after 60 min. Synergism between lysozyme and trypsin would be expected to occur only during the initial 10 min of reaction.

As also indicated in Table 2, polymyxin is rather ineffective in potentiating lysozyme or trypsin action on their substrates unless the enzymes are in combination. Then a situation similar to preheated cell walls appears to occur. Tryptic action permits lysozyme to reach its substrate with subsequent increase in trypsin activity.

TABLE 2. Release of amino acids and reducing sugars from unheated cell walls by lysozyme and/or trypsin in the presence of butanol or polymyxin.

50 mg unheated cell walls 0.0067M PO_4, pH 7	Total amount released*	
	Amino acids (as μmole leucine)	Reducing sugars (as mg glucose)
Buffer only	[22] —	[1.2] —
Lysozyme (20 μg/ml)	34	1.0
Trypsin (10 μg/ml)	64	0.8
Lysozyme + trypsin	82 (98)**	1.8
Butanol (5% v/v)	2	0.0
Butanol + lysozyme	34 (36)	0.9
Butanol + trypsin	128 (66)	0.8
Butanol + lysozyme + trypsin	122 (100)	1.2
Polymyxin (70 U/ml)	6	0.4
Polymyxin + lysozyme	40 (40)	0.8
Polymyxin + trypsin	64 (70)	1.2
Polymyxin + lysozyme + trypsin	110 (104)	1.3

*Corrected for buffer values shown in brackets.

**Values in parentheses are sums of amounts released by individual components of system.

Versene has no potentiating effect on the release of protein or carbohydrate residues from cell walls by lysozyme and trypsin alone or in combination (Table 3). These observations place doubt on the protein-dissociating action of versene suggested by Noller and Hartsell (1961) and opens the cation-chelation mechanism proposed by Repaske (1958) to re-study.

As shown in Table 4, exposure of cell walls to acid in the absence of lysozyme causes release of some protein. Additional protein is released upon addition of alkali. When lysozyme is included in the system, further release of protein occurs under either acid or akaline conditions. However, greater release of sugar residues from lysozyme action occurs under alkaline conditions.

TABLE 3. Release of amino acids and reducing sugars from unheated cell walls by lysozyme and/or trypsin in the presence of versene.

50 mg unheated cell walls 0.033M Tris, pH 8	Total amount released*	
	Amino acids (as μmole leucine)	Reducing sugars (as mg glucose)
Buffer only	[28] —	[3.0] —
Lysozyme (20 μg/ml)	12	0.0
Trypsin (10 μg/ml)	58	0.2
Lysozyme + trypsin	76 (70)**	0.5
Versene (133 μg/ml)	8	0.0
Versene + lysozyme	16 (28)	0.0
Versene + trypsin	46 (66)	0.2
Versene + lysozyme + trypsin	76 (78)	0.3

*Corrected for buffer values shown in brackets.

**Values in parentheses are sums of amounts released by individual components of system.

TABLE 4. Release of amino acids and reducing sugars following Nakamura treatment of unheated cell walls.

Sample number (Fig. 1)		Total amount released per 50 mg cell walls*	
		Amino acids (as μmole leucine)	Reducing sugars (as mg glucose)
	w/o Lysozyme		
1	pH 3.5	4.5	1.0
2	pH 3.5, then pH 10.5	10.0	0.7
	w/Lysozyme (20 μg/ml)		
4	pH 3.5	17.0	0.9
3	pH 3.5, then pH 10.5	17.0	1.5

*25 mg cell walls per sample (data are 8× actual values/sample).

SUMMARY

With the exception of versene, the treatments which potentiate lysis of whole cells in gram-negative bacteria by lysozyme and trypsin appear to have similar effects on the degradation of isloated cell walls by these same enzymes. The suggestion that heat, acidity, butanol or polybasic antibiotics have the common effect of dissociating the protein or lipoprotein portion of the wall exposing the mucopeptide to lysozyme action is supported by these experiments with isolated cell walls of *Escherichia coli*.

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