CHARACTERIZATION OF MINOR FRAGMENTS AFTER DIGESTION OF *ESCHERICHIA COLI* MUREIN WITH ENDO-*N*,*O*-DIACETYLMURAMIDASE FROM *CHALAROPSIS*, AND DETERMINATION OF GLYCAN CHAIN LENGTH

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1. Introduction

The murein (peptidoglycan) of *Escherichia coli* consists of glycan chains composed of alternating *N*-acetylated glucosamine and muramic acid with tri- or tetrapeptide side chains attached to the *O*-lactyl groups of the muramic acid residues. Using hen egg white lysozyme for murein degradation predominantly disaccharide peptide monomers, called C_5 and C_6, and peptide-crosslinked bis-disaccharide peptide dimers, called C_3 and C_4, were obtained [1,2]. However, small amounts of monomer species exhibiting higher R_F-values in chromatographic systems were also observed. In addition, C_4 was identified as a ring-shaped dimer formed by a transglycosylase reaction during murein degradation with lysozyme [1,2].

Here, we report on the characterization of minor murein monomer and dimer species obtained after enzymatic digestion of *E. coli* murein with endo-*N*,*O*-diacetylmuramidase from *Chalaropsis*. Since these fragments were found to be native building blocks of the murein sacculus rather than artifacts produced by enzymatic digestion they will be useful for further characterization of the murein network.

2. Experimental

Cells of *E. coli* K12 were grown in complex diluted medium, synchronized by sucrose density centrifugation and labelled with *N*-acetyl-D-[1-^{14}C]glucosamine for 100 min in the presence of 2 μg mecillinam/ml essentially as in [3]. Cells were harvested, mixed with 100 times the number of unlabelled cells from a batch culture, and the polymeric murein sacculi were isolated. Digestion of murein with endo-*N*,*O*-diacetylmuramidase from *Chalaropsis*, separation of monomers and dimers and the analytical procedures employed were detailed in [3–5].

3. Results and discussion

3.1. *Isolation of murein fragments*

When we followed the incorporation of radioactive *N*-acetyl-glucosamine into the murein sacculus of synchronized cells of *E. coli* we observed small amounts of new fragments after thin-layer chromatography of isolated murein monomers and dimers obtained after murein digestion with endo-*N*,*O*-diacetylmuramidase from *Chalaropsis*. Furthermore, the yield of these new fragments increased in the presence of 2 μg mecillinam/ml during the labelling period. Details of these experiments will be reported elsewhere. To facilitate detection and isolation of these minor murein fragments on a preparative scale, we mixed synchronized cells radioactively labelled in the presence of mecillinam with unlabelled cells from a batch culture. The isolated murein monomers and dimers were separated by thin-layer chromatography, and the various monomer and dimer species were rechromatographed for purification. A chromatogram of the purified fragments is shown in fig.1.

3.2. *Characterization of the fragments*

The various fragments were subjected to amino acid analysis after acid hydrolysis with and without preceeding reduction with sodium borohydride. The data are listed in table 1. In monomer A and dimer A, muramic acid residues were fully reducible since

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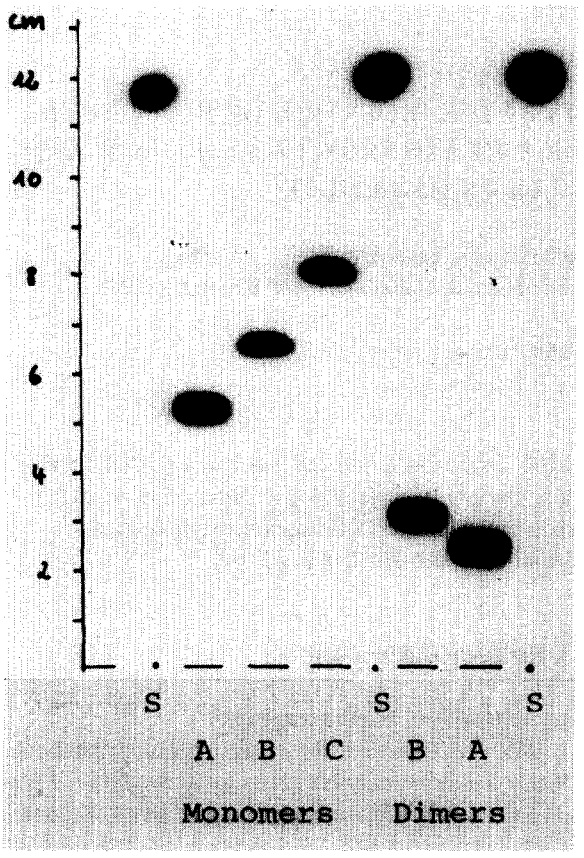

Fig.1. Thin-layer chromatography of the isolated murein fragments. About 5000 cpm of each purified fragment were applied to a silica gel thin-layer plate (Macherey and Nagel, Düren). The plate was developed twice in isobutyric acid/1 M ammonia (5:3, v/v). The bands were visualized by autoradiography. S, standard *N*-acetylglucosamine. The migration distances relative to *N*-acetylglucosamine are listed in table 1.

muramic acid disappeared completely from hydrolysates of the compounds after reduction. As seen from the alanine content, these fragments represented mixtures of species either possessing or lacking the terminal D-alanine residue. About 90–95% of the total material was found in fragments A. Therefore, monomer A comprised a mixture of compounds C_5 and C_6, and dimer A was equivalent to compound C_3. These compounds were characterized previously as main products of murein cleavage by lysozyme [1,2].

Monomer B resisted reduction with sodium borohydride although it yielded a muramic acid content similar to that of the respective fragment A. This behaviour is typical for the presence of muramic acid in its 1,6-anhydro-form. Hence, we conclude that monomer B is identical to the monomer species containing a 1,6-anhydromuramic acid residue as isolated and characterized in [6,7]. With dimer B, however, ~50% of the muramic acid could be reduced, indicating that only one of the muramic acid residues involved was reducible. Further proof of such a dimer composition was obtained by cleavage of the dimer fragments with endopeptidase (fig.2). Whereas dimer A was completely converted to monomer A as expected, dimer B yielded 2 cleavage products which were chromatographically identical with monomers A and B, respectively (fig.2).

This result showed conclusively that dimer B consisted of one disaccharide peptide containing a reducible muramic acid residue, peptide-crosslinked to another disaccharide peptide containing the muramic acid in the 1,6-anhydro-form. Therefore, dimer B was different from the lysozyme artifact C_4 characterized

Table 1
Characterization of murein fragments

Fragment		M_{GlcNAc}[a]	Molar ratio[b]						Spec. act. of GlcN[c]
			Glu	Ala	A_2pm[c]	GlcN[c]	MurN[c]	MurN[c] after reduction	
Monomer	A	0.44	1.00	1.63	0.94	0.90	0.93	–	86.3
	B	0.55	1.00	1.55	0.90	0.83	0.88	0.78	106.4
	C	0.67	1.00	1.01	0.08	0.90	0.89	–	70.1
Dimer	A	0.20	1.00	1.47	0.85	0.93	0.88	–	47.3
	B	0.26	1.00	1.67	0.92	0.83	0.83	0.39	81.2

[a] Migration relative to *N*-acetylglucosamine on thin layer plates (see fig.1)
[b] Molar ratio relative to glutamic acid
[c] *Abbreviations:* A_2pm, *meso*-diaminopimelic acid; GlcN, glucosamine; MurN, muramic acid

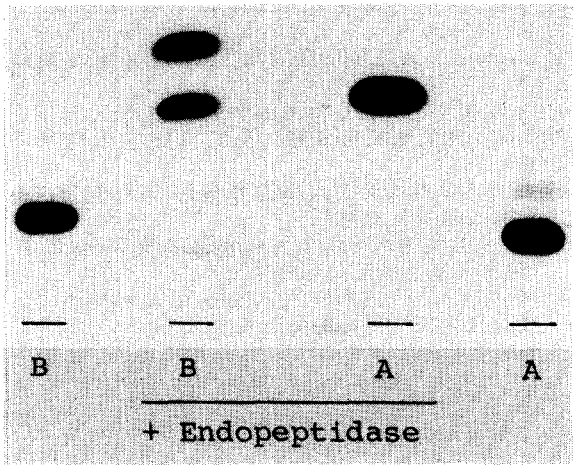

Fig.2. Thin-layer chromatography of dimer fragments after endopeptidase cleavage. About 20 nmol dimer were incubated with 2 mU DD-carboxypeptidase/transpeptidase H from *Proteus mirabilis* (which was shown to also possess endopeptidase activity and was kindly provided by Dr Schilf) in 15 µl 0.5 M Tris–HCl buffer (pH 9) containing 0.1% Genapol X-100 and 0.025 M $MgCl_2$. The mixture was incubated overnight at 30°C and then applied to a thin-layer plate as in fig.1.

as a ring-shaped dimer with cross-linked peptide side chains and the amino sugars linked to form a linear tetrasaccharide. Accordingly, C_4 would still retain its dimer form after endopeptidase action. Such a compound was not detected in our murein digests.

Monomer C, although fully reducible, lacked the terminal D-alanine residue as well as *meso*-diaminopimelic acid. This monomer species had not been described in *E. coli*, but was found also in *Proteus mirabilis* [5]. The significance of such a monomer has been discussed [5].

3.3. *Significance of the fragments containing 1,6-anhydromuramic acid residues*

Non-reducible murein monomers were first described in lysozyme digests of *E. coli* murein [1]. Monomers containing 1,6-anhydro-*N*-acetylmuramic acid were later characterized as main fragments in phage endolysin digests of *E. coli* murein [6], and as main products of murein digestion with an isolated transglycosylase from *E. coli* [7]. With the latter enzyme preparation, dimers migrating faster than C_3 were also observed but were not characterized further. Clearly, in both cases these fragments containing anhydromuramic acid were obtained through the peculiar transglycosylase/hydrolase activity of the phage endolysin or the *E. coli* enzyme. Therefore, we had to ensure that the *Chalaropsis* endo-*N,O*-diacetylmuramidase action does not lead to anhydro-formation. This possibility, however, seemed to be highly unlikely: We had observed about twice the amount of anhydromuramic acid containing fragments from murein synthesized in the presence of mecillinam (in preparation). Therefore, we analyzed a murein mixture obtained from unlabelled cells, and from cells radioactively labelled in the presence of mecillinam (section 2). After isolation and purification of the various murein fragments we determined the specific radioactivity of the glucosamine residue after acid hydrolysis and amino acid analysis (table 1, last column). Since this specific activity was much higher in the fragments B containing anhydromuramic acid than in the corresponding reducible fragments A, we conclude that the amounts of anhydro-fragments obtained from sacculi of cells labelled in the presence of mecillinam differed from the amounts present in sacculi of the unlabelled cells used in the same experiment. If the anhydro-fragments were created by action of the *Chalaropsis* enzyme, the specific radioactivity of glucosamine would have been similar in fragments A and B.

Hence, the anhydro-fragments obtained from the polymeric murein sacculus after digestion with *Chalaropsis* enzyme indicate glycan chain terminations in the native murein network. Accordingly, they can serve to calculate the average glycan chain length, and we arrived at an average chain length of ~30 disaccharide units for *E. coli* murein. Murein biosynthesis in the presence of mecillinam gave only ~15 disaccharide units/glycan chain (in preparation). An average chain length of ~64 disaccharide units was calculated in [8] based on the amount of anhydro-fragments in lysozyme digests of *E. coli* murein. However, from [8] it is not entirely clear whether dimers containing anhydromuramic acid were included in the calculation.

The characterization of dimer fragment B containing only one anhydromuramic acid residue clearly showed the overlapping of neighbouring glycan chains within the murein network. The end of one chain is peptide-crosslinked to an interior unit of the next chain. Dimer fragments with 2 anhydromuramic acid residues should migrate even faster in chromatographic systems and have not been detected in our murein digests.

Acknowledgements

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