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Molecular weight-determination of biosynthetically modified monomeric and oligomeric muropeptides from *Escherichia coli* by plasma desorption-mass spectrometry

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The presence of certain D-amino acids in the growth media of *Escherichia coli* results in the accumulation of 2 major and 3–5 minor new muropeptides in the murein sacculus. Preliminary data suggested that the major muropeptides correspond to a monomer and a cross-linked dimer with one residue of D-amino acid per molecule. We have analyzed several D-amino acid-modified muropeptides by plasma desorption-mass spectrometry. Our results confirmed that the general structures of the major modified muropeptides are: GlucNAc-MurNAc-L-Ala-D-Glu-*m*-A2pm-D-X, and GlucNAc-MurNAc-L-Ala-D-Glu-*m*-A2pm-D-Ala; GlucNAc-MurNAc-L-Ala-D-Glu-*m*-A2pm-D-X, being X a residue of the D-amino acid. These results corroborate the usefulness of this technique for the structural analysis of muropeptides.

High performance liquid chromatography; Plasma desorption-mass spectrometry; Molecular weight determination; D-Amino acid; Peptidoglycan

1. INTRODUCTION

The murein sacculus of the eubacterial cell wall is a giant macromolecule that completely surrounds the cell. This structure plays an essential morphogenetic role in the life cycle of the bacteria. The sacculus has a net-like structure in which long polysaccharide chains made up of (GlucNAc-MurNAc)-oligopeptide units are cross-linked by means of peptide bridges. The nature of the oligopeptide and peptide bridge are both variable among bacterial groups [1]. In Gram-negative, and some Gram-positive, bacterial species the basic monomeric units (GlucNAc-MurNAc)-L-Ala-D-Glu-*meso*-A2pm-D-Ala, are cross-linked by means of D-Ala-*meso*-A2pm and *meso*-A2pm-*meso*-A2pm peptide bridges of DD and LD conformation, respectively [2].

In *Escherichia coli* murein the proportions of the different kinds of constituent muropeptides show a certain degree of variability in response to genetic and physiological changes [3,4]. However, the chemical composition of murein remains apparently constant during the life cycle of the bacteria. Addition of certain D-amino acids as D-Met, D-Trp, D-Phe, etc., to *E. coli* cultures results in the incorporation of these compounds into murein therefore modifying the chemical composition of the sacculus [5–8]. HPLC analysis of murein from D-amino acid-treated cells revealed the accumulation of 2 major and 3–5 minor new muropeptides. The major components typically account for more than 90% of total new muropeptides. Depending upon time of incubation and concentration of the D-amino acid the proportion of new muropeptides can be as high as 45–50% of total muropeptides. Chemical analyses of the major modified muropeptides accumulated in the presence of D-Met and D-Phe suggested that these compounds correspond to monomeric (shorter retention time-component) and cross-linked dimeric (longer retention time-component) muropeptides with the following general structures: GlucNAc-MurNAc-L-Ala-D-Glu-*m*-A2pm-D-X, and GlucNAc-MurNAc-L-Ala-D-Glu-*m*-A2pm-D-Ala; GlucNAc-MurNAc-L-Ala-D-Glu-*m*-A2pm-D-X, D-X being a residue of the added D-amino acid [7,8]. The C-terminal position of the D-amino acid residue in modified dimers was deduced from biochemical evidence. Formation of modified dimers is penicillin-insensitive and no D-amino acid-modified soluble precursors (UDP-muramyl-pentapeptide analogues) have been detected. Therefore synthesis of modified dimers could only be due to modification of a pre-existing normal dimer, and in the absence of β-lactams, to a DD-transpeptidation reaction in which the acceptor muropeptide was a D-amino acid modified one. Both reactions would lead to accumulation of a C-terminal modified dimer.

A detailed investigation of this phenomenon is interesting for a number of reasons: (i) it might lead to the identification of new enzymes involved in murein metabolism; (ii) it might help to study the cellular response

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against alterations in murein structure; (iii) it could provide a practical way for large scale production of 'tailored' muropeptides with potential pharmacological interest [9]. However, an obvious prerequisite to proceed forward is the unambiguous determination of the structure of D-amino acid-modified muropeptides. As an attempt to solve this problem modified muropeptides accumulated upon growth of *E. coli* in the presence of several D-amino acids have been purified and analyzed by means of plasma desorption-mass spectrometry (PD-MS). Study of these compounds had also a considerable interest from the analytical point of view. The series of equivalent modified muropeptides constitute families of related glycopeptides differing only in the nature of one amino acid residue. Until recently mass spectrometric

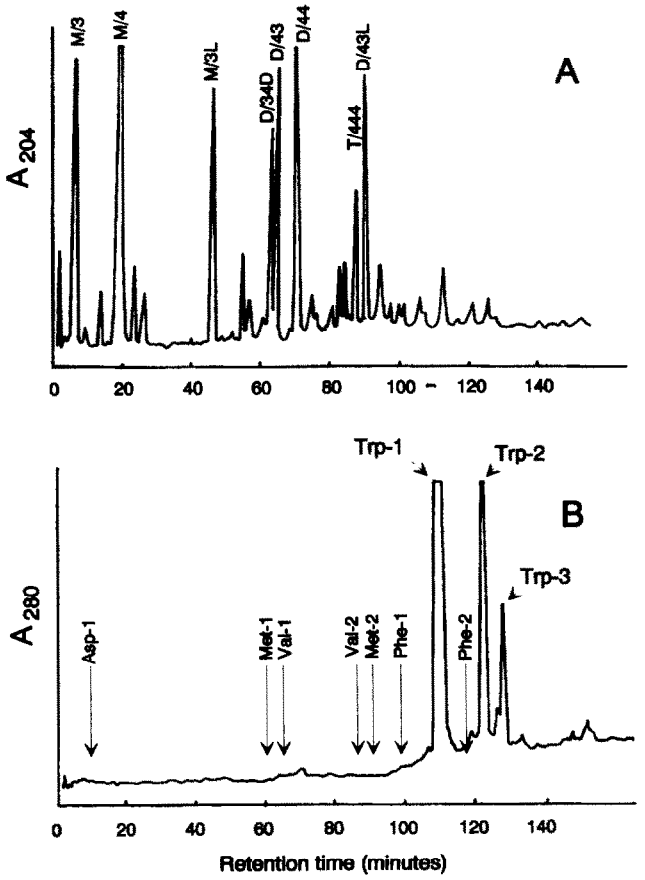

Fig. 1. Muropeptide composition of *E. coli* murein grown in the presence of D-amino acids. (A) HPLC elution pattern of unmodified murein recorded at 204 nm. The position of the more abundant muropeptides is indicated: M/3, disaccharide tripeptide; M/4, disaccharide tetrapeptide/ M/3L, disaccharide tripeptide-Lys-Arg; D/34D, *bis*-disaccharide tri-tetrapeptide dimer crosslinked by a A2pm-A2pm bridge; D/43, *bis*-disaccharide tetra-tripeptide dimer; D/44, *bis*-disaccharide tetrapeptide dimer; T/444, *tri*-disaccharide tetrapeptide trimer; D/43L, *bis*-disaccharide tetratripeptide-Lys-Arg dimer. (B) HPLC elution pattern of D-Trp-modified murein recorded at 280 nm. Trp-1, putative D-Trp-modified disaccharide tetrapeptide; Trp-2, putative D-Trp-modified *bis*-disaccharide tetrapeptide dimer; Trp-3, D-Trp modified minor muropeptide. The *Rts* for the muropeptides modified with the other D-amino acids used in this work are indicated using the standard three letter abbreviation plus a number. Number 1 indicates the position of the putative D-amino acid-modified disaccharide tetrapeptides, and number 2 that of putative D-amino acid-modified *bis*-disaccharide tetrapeptide dimers.

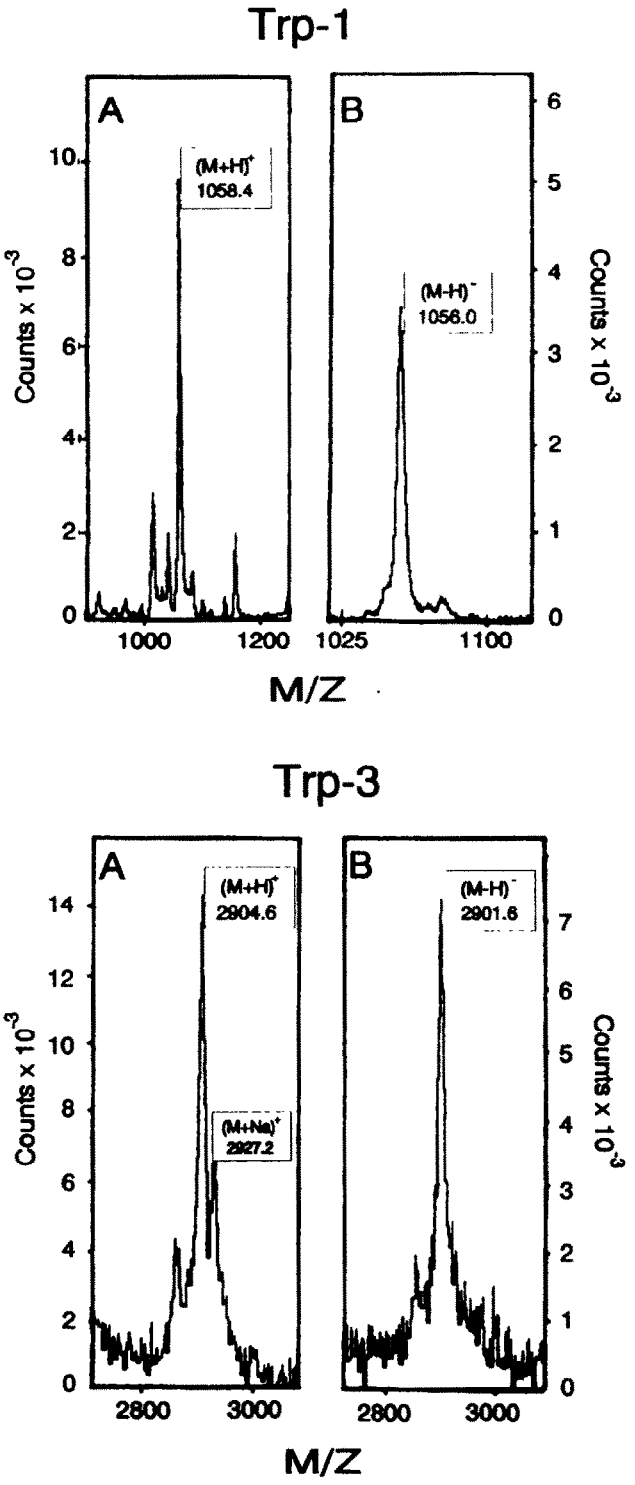

Fig. 2. Positive (A) and negative (B)-ion PD-M spectra of the D-Trp modified-disaccharide tetrapeptide monomer (Trp-1) and *tri*-disaccharide tetrapeptide trimer (Trp-3).

analysis of muropeptides by means of so-called soft ionization techniques as fast atom bombardment and plasma desorption, was essentially restricted to monomeric muropeptides [10–12] with a few exceptions [13–15]. Here we describe the application of this technique to the analysis of oligomeric muropeptides, and to the fast determination of the molecular weights of a series of D-amino acid-modified muropeptides.

2. MATERIALS AND METHODS

2.1. *Bacterial strains and growth conditions*

Escherichia coli MC6 RP3 (K12, F⁻, *proA leuA thr dra drm lysA A2pmA thi*) [7] was used throughout this work. Cultures were grown in LB [16] media at 37°C under vigorous aeration. Media were systematically supplemented with 8 mg/ml 2,6-*meso*-A2pm, 40 mg/ml L-Lys and one of the following D-amino acids at 20 mM final concentration: D-Asp, D-Val, D-Met, D-Trp and D-Phe. Cell growth was followed by monitoring the OD_{550} of the cultures.

2.2. *Analysis of murein, isolation of modified muropeptides and desalting of muropeptides by HPLC*

Murein was purified from late-exponential phase (OD_{550} = 2) cultures and was analyzed by HPLC using an Hypersil RP18 column (3 μm particle size, 250 × 4 mm), as described by Glauner et al. [17]. Muropeptides were identified by their retention times after periodical calibration of the HPLC equipment with a mixture of known, purified muropeptides. Detection was performed by monitoring the A_{204} of the eluate. The muropeptides of interest were collected, lyophilized and desalted by HPLC on a μBondapak RP18 column (10 μm particle size, 300 × 4.6 mm). The column was eluted at a flow rate of 1 ml/min, starting with 0.05% trifluoroacetic acid in water (5 min) and then with

Table I

Calculated and measured *m/z* values for the protonated and deprotonated quasimolecular ions of natural and modified muropeptides[a]

Muropeptide	Ion	*m/z*			
		Calculated	Measured	Δm[b]	Error[c] (%)
M/4	$(M+H)^+$	941.94	942.9	0.96	0.10
	$(M-H)^-$	939.92	940.5	0.58	0.06
D/44	$(M+H)^+$	1866.87	1867.7	0.83	0.04
	$(M-H)^-$	1864.85	1865.3	0.45	0.02
Asp-1	$(M+H)^+$	986.94	987.2	0.26	0.02
	$(M-H)^-$	984.92	985.1	0.18	0.01
Met-1	$(M+H)^+$	1003.05	1003.7	0.65	0.06
	$(M-H)^-$	1001.03	1000.9	−0.13	−0.01
Phe-1	$(M+H)^+$	1019.03	1019.0	−0.03	−0.002
	$(M-H)^-$	1017.01	1017.2	0.19	0.01
Trp-1	$(M+H)^+$	1058.07	1058.4	0.33	0.03
	$(M-H)^-$	1056.08	1056.0	−0.08	−0.007
Val-1	$(M+H)^+$	970.99	972.1	1.11	0.11
	$(M-H)^-$	969.97	968.9	−1.07	−0.10
Phe-2	$(M+H)^+$	1941.94	1942	0.06	0.003
	$(M-H)^-$	1939.92	1939.2	−0.72	−0.03
Trp-2	$(M+H)^+$	1980.98	1981.8	0.82	0.04
	$(M-H)^-$	1978.96	1978.2	−0.76	−0.03
Val-2	$(M+H)^+$	1893.90	1895.0	1.1	0.05
	$(M-H)^-$	1891.88	1892.3	0.42	0.02
Trp-3	$(M+H)^+$	2903.89	2904.6	0.71	0.02
	$(M-H)^-$	2901.87	2901.66	−0.21	−0.007

[a] The nomenclature of muropeptides was as in the legend to Fig. 1.
[b] Mass difference between calculated and measured quasimolecular ion values.
[c] [(measured mass −calculated mass)/calculated mass] × 100.

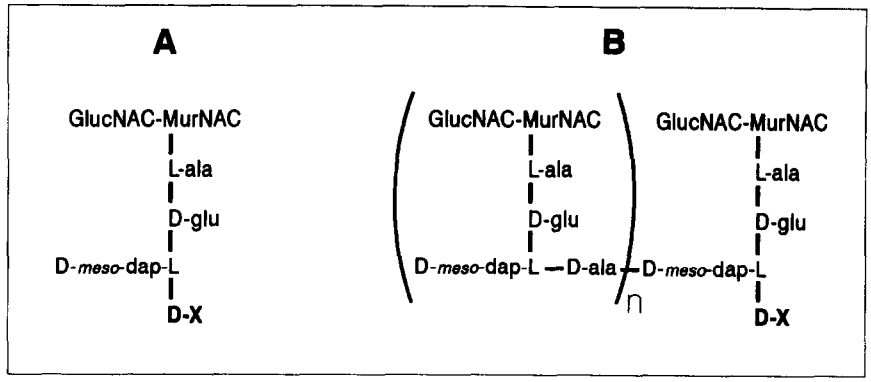

Fig. 3. Structure of the D-amino acid-modified monomeric (A) and oligomeric muropeptides (B). *n*, number of disaccharide units; $n = 1$ for a dimer, $n = 2$ for a trimer, etc.

a gradient (40 min) from 0% to 50% of 0.035% trifluoroacetic acid in acetonitrile.

2.3. *Analysis of D-amino acid modified-muropeptides by PD-MS*

Aliquots (5–10 μg) of the lyophilized, desalted muropeptides were used for PD-MS analysis. Measurements were performed on a ^{252}Cf plasma desorption time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA, USA) with a flight tube length of 15 cm. Acceleration voltages were 17 and 15 kV in the positive- and negative-ion modes, respectively. Spectra measured in the positive and negative ionization modes were accumulated for five and ten million fission events, respectively. Samples were prepared by the so-called 'nitrocellulose-sandwich technique' as described by Allmaier et al. [12]. Approximately 200 μl of a solution of nitrocellulose in acetone (2 μg/μl) were electrosprayed onto aluminized Mylar backings. After sample deposition from an aqueous solution containing 0.1% trifluoroacetic acid onto the nitrocellulose support, initial positive- and negative-ion mass spectra were recorded to assess sample purity. Afterwards, approximately 20 μl nitrocellulose-solution in acetone was sprayed over the previously deposited analyte. Finally the target was rinsed with 5 μl of 0.1% aqueous trifluoroacetic acid, and resubmitted to PD-MS analysis.

3. RESULTS AND DISCUSSION

Peptidoglycan from *E. coli* cells grown in the presence of D-Asp, D-Met, D-Phe, D-Trp, and D-Val for 8 generations was subjected to enzymatic digestion with muramidase (Cellosyl, Hoechst, Germany) and solubilized muropeptides were resolved by HPLC. The muropeptides of interest were collected, lyophilized, and desalted by HPLC as indicated in section 2. Fig. 1 shows the elution profiles corresponding to D-Trp-modified and control mureins. Control sample was monitored at 204 nm to visualize all muropeptides, whilst the record for D-Trp-containing murein was monitored at 280 nm, a wavelength at which normal muropeptides do not absorb, to make D-Trp-modified muropeptides more conspicuous. In the particular case of the sample analyzed in Fig. 1, modified muropeptides (D-Trp-1 + D-Trp-2 + D-Trp-3) accounted for 32% of total muropeptides. The very long retention times (*Rts*) of D-Trp-modified muropeptides permitted purification of one of the minor modified muropeptides (Trp-3) in addition to the major ones (Trp-1 and Trp-2). Purification of minor muropeptides modified with the other D-amino acids tested was not possible because they co-eluted with normal, more abundant, muropeptides, as was the case for D-Met-2. The *Rts* of the major muropeptides modified by D-Asp, D-Phe, D-Met and D-Val are also indicated in Fig. 1. For reference we used as compounds of known structure the two more abundant natural muropeptides in *E. coli*: disaccharide tetrapeptide (GlucNAc-MurNAc-L-Ala-D-Glu-*m*-A2pm-D-Ala)(M/4) and *bis*-disaccharide tetrapeptide (GlucNAc-MurNAc-L-Ala-D-Glu-*m*-A2pm-D-Ala; GlucNAc-MurNAc-L-Ala-D-Glu-*m*-A2pm-D-Ala)(D/44).

A major problem for the MS characterization of muropeptides is their high affinity to alkali cations which remain bound even after extensive HPLC-purification. Multiple attachment of these cations to the analyte molecules causes signal suppression and obscures exact molecular weight determination. A promising alternative to overcome this problem could be the application of the so-called 'nitrocellulose sandwich' technique for sample deposition. This technique has been successfully applied to the analysis of monomeric muropeptides [10]. The main advantage is that it permits washing of the samples without large losses of sample material due to the low adsorption of muropeptides to the nitrocellulose.

The values measured for each muropeptide in both the positive and the negative ionization modes are listed in Table I. The theoretical values calculated for each muropeptide are also listed in Table I for comparison. Theoretical values were calculated assuming that early (Asp-1, Met-1, Phe-1, Trp-1, and Val-1) and late (Phe-2, Trp-2, and Val-2) eluting muropeptides would correspond to disaccharide tetrapeptides and *bis*-disaccharide tetrapeptides with a residue of the D-amino acid at the C-terminal position, respectively. Measured and calculated values matched well within ±0.1% in all instances. These results strongly support the structures

for the D-amino acid modified muropeptides predicted by chemical analyses [7].

The measured *m/z* values of Trp-3, the minor muropeptide purified from D-Trp grown cells, indicate that this muropeptide is a cross-linked trimer with a molecule of D-Trp as the C-terminal amino acid (GlucNAc-MurNAc-L-Ala-D-Glu-*m*-A2pm-D-Ala; GlucNAc-MurNAc-L-Ala-D-Glu-*m*-A2pm-D-Ala, GlucNAc-MurNAc-L-Ala-D-Glu-*m*-A2pm-**D-Trp**). As shown in Table I the predicted molecular weight for such a muropeptide fits very closely with the experimental value. Interestingly, quality of the PD-MS spectra for the monomeric (Trp-1) and trimeric (Trp-3) muropeptide was comparable (Fig. 2). This result further confirms the suitability of PD-MS to the analysis of oligomeric muropeptides.

The identification of a modified trimer is also of interest in the context of the enzymology of D-amino acid incorporation. Existence of this compound suggests that the murein D-amino acid L,D-transpeptidase which catalyzes the reaction might accept trimeric muropeptides as substrate, in addition to monomers and dimers [7].

The results presented above confirm the hypothesis that whenever a D-amino acid is incorporated into the murein of *E. coli*, equivalent modified muropeptides are accumulated. These muropeptides correspond to monomers and cross-linked oligomers with the general structure shown in Fig. 3. The determination of the structures of these muropeptides confirms our hypothesis about the enzymatic reaction responsible for the post-insertional incorporation of D-amino acids into the murein of *E. coli* [7].

In conclusion, PD-MS in combination with the 'nitrocellulose-sandwich technique' proved to be a very useful tool for the rapid and accurate identification of modified and oligomeric muropeptides.

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