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Reversed-phase high-performance liquid chromatographic method for the determination of peptidoglycan monomers and structurally related peptides and adamantyltripeptides

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Abstract

The reversed-phase HPLC method using UV detection was developed for the determination of (a) immunostimulating peptidoglycan monomers represented by the basic structure GlcNAc-MurNAc-L-Ala-D-*iso*Gln-*meso*-DAP(ωNH_2)-D-Ala-D-Ala (PGM) and two more lipophilic derivatives, Boc-Tyr-PGM and (Ada-1-yl)-CH_2-CO-PGM, (b) two diastereomeric immunostimulating adamantyltripeptides L- and D-(adamant-2-yl)-Gly-L-Ala-D-*iso*Gln and (c) peptides obtained by the enzyme hydrolyses of peptidoglycans and related peptides. The enzymes used, *N*-acetylmuramyl-L-alanine amidase and an L,D-aminopeptidase are present in mammalian sera and are involved in the metabolism of peptidoglycans and related peptides. Appropriate solvent systems were chosen with regard to structure and lipophilicity of each compound. As well, different gradient systems within the same solvent system had to be applied in order to achieve satisfactory separation and retention time. HPLC separation was developed with the aim to use this method for the study of the stability of the tested compounds, the purity during preparation and isolation and for following the enzyme hydrolyses.

Keywords: Peptides; Peptidoglycan monomers; Adamantyltripeptides

1. Introduction

Peptidoglycans are common constituents of bacterial cell walls and are essential for the physical integrity of the cell. Natural peptidoglycans are polymers composed of glycan chains built of alternating β-1,4 linked *N*-acetylglucosamine and *N*-acetylmuramic acid residues and of peptide units that consist of alternating L- and D-amino acids. In a mammalian host, peptidoglycan fragments exhibit various biological activities (immunomodulating, inflammatory, antitumor and antimetastatic activity, pyrogenicity, and toxicity) depending upon the size and composition of each fragment [1,2].

Various peptidoglycan fragments were isolated from bacteria, and numerous lower molecular mass compounds have been synthesized. Of particular interest are the lower molecular mass peptidoglycans devoid of toxic properties, which affect the immune system of the host [3–5]. In our earlier studies, we have demonstrated the immunostimulating effects of

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several structurally related compounds obtained from natural sources or synthesized, as explained below. The basic compound, obtained from natural sources is the peptidoglycan monomer, the disaccharide pentapeptide GlcNAc-MurNAc-L-Ala-D-*iso*Gln-*meso*-DAP(ωNH_2)-D-Ala-D-Ala (PGM) [6,7] originating from *Brevibacterium divaricatum*. Its two more lipophilic derivatives, *tert.*-butyloxycarbonyl-L-tyrosyl-PGM (Boc-Tyr-PGM) [8] and (adamant-1-yl)-CH_2-CO-PGM ((Ada-1-yl)-CH_2-CO-PGM) [9] were obtained by semisynthetic modifications (Fig. 1). In addition to the natural PGM and its synthetic derivatives, two diastereomeric adamantyltripeptides L- and D-(Ada-2-yl)-Gly-L-Ala-D-*iso*Gln (Fig. 2) containing the dipeptide L-Ala-D-*iso*Gln characteristic for peptidoglycan structures, were synthesized [7,10]. In the present study, the purity of all prepared compounds was checked by HPLC.

We have also shown that peptidoglycan monomers are the substrates for hydrolases from mammalian sera that are involved in the metabolism of peptidoglycans in mammals.

The enzyme *N*-acetylmuramyl-L-alanine amidase hydrolyses the peptidoglycan monomers to the di-

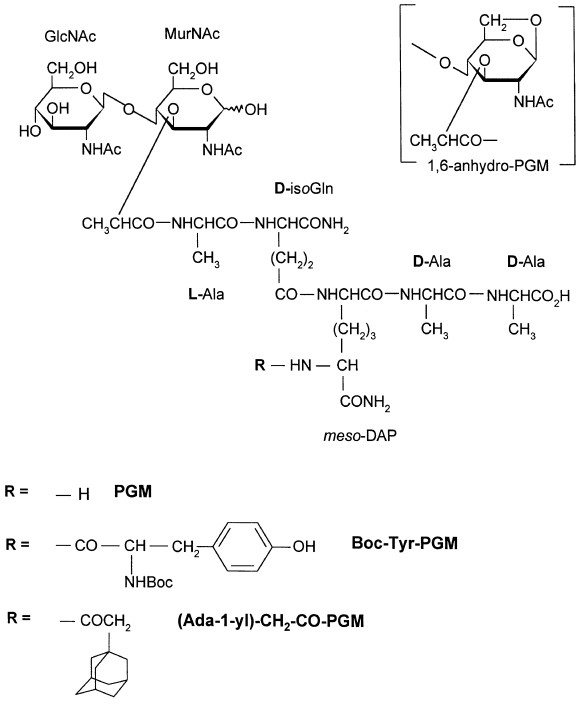

Fig. 1. Chemical structures of peptidoglycan monomer (PGM) and its derivatives, Boc-Tyr-PGM and (Ada-1-yl)-CH_2-CO-PGM.

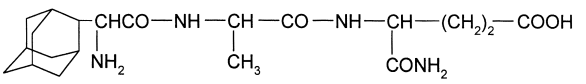

Fig. 2. Chemical structures of adamantyltripeptides: D-(adamant-2-yl)-Gly-L-Ala-D-*iso*Gln, AdTP1; L-(adamant-2-yl)-Gly-L-Ala-D-*iso*Gln, AdTP2.

saccharide and the respective peptide portions [9,11] (Fig. 3). These peptides are further substrates for an L,D-aminopeptidase, which cleaves L-alanine from the amino end of the peptide [12]. In the present study we have used RP-HPLC to follow the course of hydrolyses with the amidase or with the aminopeptidase. Subsequently, the purity of products isolated from the incubation mixtures with respective enzymes was checked by RP-HPLC.

Several reports on HPLC of peptidoglycans and related compounds have been published so far. Some examples listed here deal with higher molecular mass fragments and the analyses of the mixtures obtained by degradation of peptidoglycan polymer [13,14] or synthetic lower molecular mass muramylpeptides [15–17]. Peptidoglycan monomer was also studied and analyses were done by reversed-phase HPLC in two different solvent systems, using a gradient of acetonitrile in water (containing TFA) [18] or methanol in a phosphate buffer [19].

In the present study we have developed reversed-phase HPLC assays with UV detection for several structurally related compounds that differ in structure and lipophilicity. Except for PGM, no data at all have been available on HPLC of compounds studied in this work. Most of the compounds tested were analyzed using a gradient of acetonitrile in water (containing TFA), but appropriate adjustments of the gradient composition were required for successful separations and satisfactory retention time. Peptides obtained by enzyme hydrolyses were chromatographed in the isocratic systems.

2. Experimental

2.1. Reagents and chemicals

Acetonitrile and trifluoroacetic acid (TFA) were of HPLC grade (Merck, Darmstadt, Germany). A daily supply of water was obtained from a Symplicity–

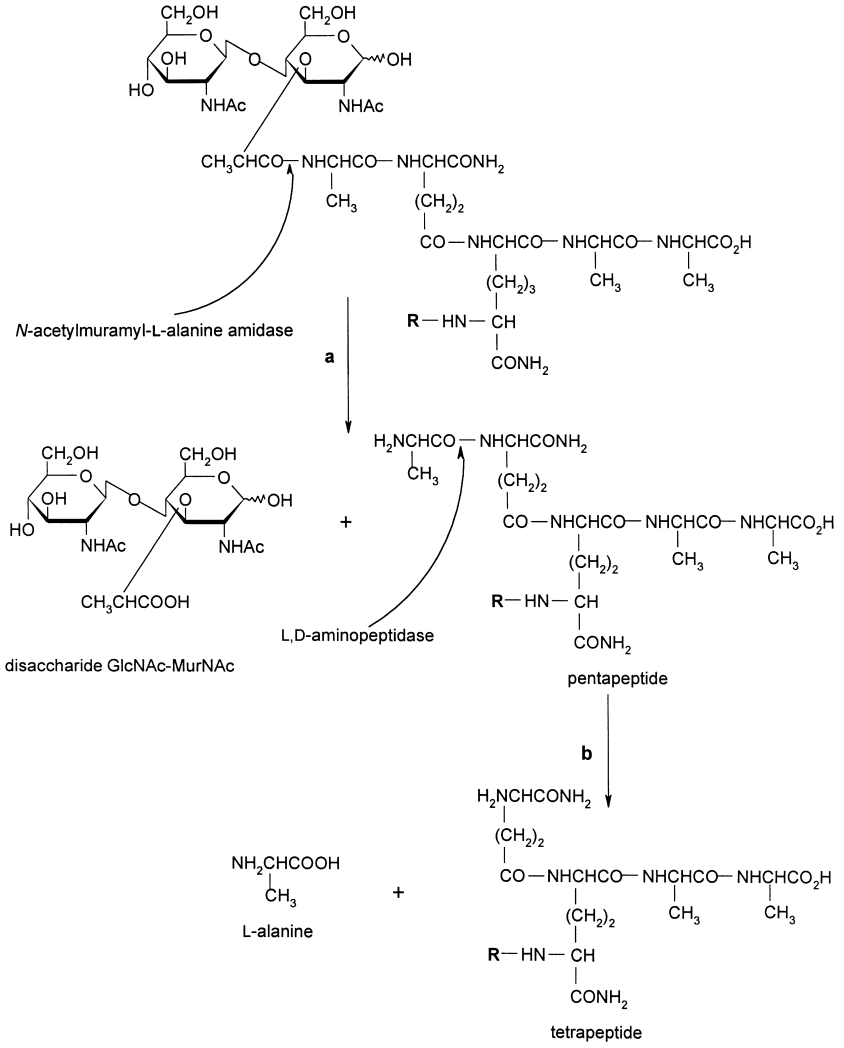

Fig. 3. Enzymatic hydrolysis of (a) PGM and derivatives with *N*-acetylmuramyl-L-alanine amidase and (b) the pentapeptides with L,D-aminopeptidase. **R** as in Fig. 1.

Personal ultrapure water system (Millipore, Bedford, MA, USA). All other chemicals used for buffers and enzyme hydrolyses were of analytical grade and purchased from Sigma (St. Louis, MO, USA).

2.2. Peptidoglycans, peptides and adamantyltripeptides

Peptidoglycan monomer was prepared in PLIVA, Chemical and Pharmaceutical Works (Zagreb, Croatia), according to the previously described procedure [6]. Boc-Tyr-PGM was prepared as described in Ref. [8] and (Ada-1-yl)-CH_2-CO-PGM in Ref. [9]. The pentapeptides were obtained by enzyme hydrolyses of peptidoglycans as described in Refs. [9,11] and tetrapeptides as in Ref. [12]. The diastereoisomers L- and D-(Ada-2-yl)-Gly-L-Ala-D-*iso*Gln were prepared as described in Ref. [10].

2.3. Preparation of samples

Peptidoglycans, adamantyltripeptides and peptides isolated from reaction mixtures after enzyme hydrolyses were dissolved in water in a concentration of 2

mg/ml unless otherwise stated, and 5–10 μl of the solution were analyzed (10–20 μg).

For the study of the course of enzyme hydrolyses, the reaction mixtures were treated in the following manner: 1 ml of 96% EtOH was added to 0.4 ml of the reaction mixture and the resulting mixture was left for at least 60 min at +4 °C. The precipitated proteins were removed by centrifugation; the supernatant was decanted and evaporated to dryness under reduced pressure. The dry residue was dissolved in 300 μl of water and filtered through a 0.45 μm filter; 5–10 μl each were injected to the column.

2.4. Chromatography

Chromatographic separations were carried out using the Waters HPLC System: Waters 600E System Controller, Waters 486 Tunable Absorbance Detector and Waters 746 Data Module-integrator. A Merck Lichrosorb RP-18 column, 244 mm×4 mm, 5 μm, and a Merck guard column LiChrospher 100 RP-18 (5 μm) were used. The samples were applied using a Rheodyne 4598 loop injector with an effective volume of 20 μl. Analyses were run at a flow-rate of 1.0 ml/min at room temperature and the eluate was monitored at 214 nm. The percentage of each peak in the respective chromatograms was calculated by the integration of the UV response (peak area). All analyses were performed at ambient temperature.

The gradient solvent systems used were made of acetonitrile containing 0.035% TFA and water containing 0.05% TFA. Three gradient systems were used for the tested compounds. Each system contained a different percentage of acetonitrile and the amount of acetonitrile was changed at the indicated running times. For system A with a running time 25 min, the percentage of acetonitrile at 0, 20 and 25 min was 3, 17 and 3, respectively. For system B with a running time 30 min, the percentage of acetonitrile at 0, 25 and 30 min was 10, 30 and 10, respectively. For system C with a running time 20 min, the percentage of acetonitrile at 0, 15 and 20 min was 10, 30 and 10, respectively.

The isocratic system D was water containing 0.05% TFA. System E was a combination of water containing 0.05% TFA (75%) and acetonitrile containing 0.035% TFA (25%). The running time was 15 min.

3. Results

3.1. Peptidoglycans

Peptidoglycan monomer was analyzed in solvent system A. Three distinct peaks were obtained (Fig. 4a), as expected for such a compound [18,19]. Retention times were 12.20 min (32.6%) and 14.57 min (62.7%) for β- and α-anomers and 18.23 min (4.6%) for anhydro-PGM, respectively.

The analyses of the more lipophilic Boc-Tyr-PGM and (Ada-1-yl)-CH_2-CO-PGM required longer time of analyses and different gradient compositions. Chromatographic separation was achieved in system B (Fig. 4b and c, respectively). Due to the increased lipophilicity, (Ada-1-yl)-CH_2-CO-PGM exhibited higher retention times than Boc-Tyr-PGM. Three distinct peaks denoting the α- and β-anomer and anhydro-component were detected in chromatograms of both derivatives as in original PGM, but the resolution was not so distinct as for PGM.

The reaction mixture following hydrolysis of PGM with the *N*-acetylmuramyl-L-alanine amidase could also be resolved satisfactorily in system A (Fig. 5). All eight peaks of the reaction mixture could be assigned to relevant components of the mixture, based on retention times determined with purified compounds (the disaccharide GlcNAc-MurNAc and respective pentapeptide).

3.2. Peptides

The pentapeptides were obtained from PGM, Boc-Tyr-PGM and (Ada-1-yl)-CH_2-CO-PGM, by the use of *N*-acetylmuramyl-L-alanine amidase. As noted in Section 3.1 these peptides could not only be chromatographed in system A, but also in system D, which is more suitable for simultaneous chromatography together with respective tetrapeptides. For example, in system A, the pentapeptide originating from PGM has a retention time 5.25 min and in system D 12.64 min.

Upon hydrolysis with L,D-aminopeptidase, L-alanine is cleaved off the amino end of the penta-

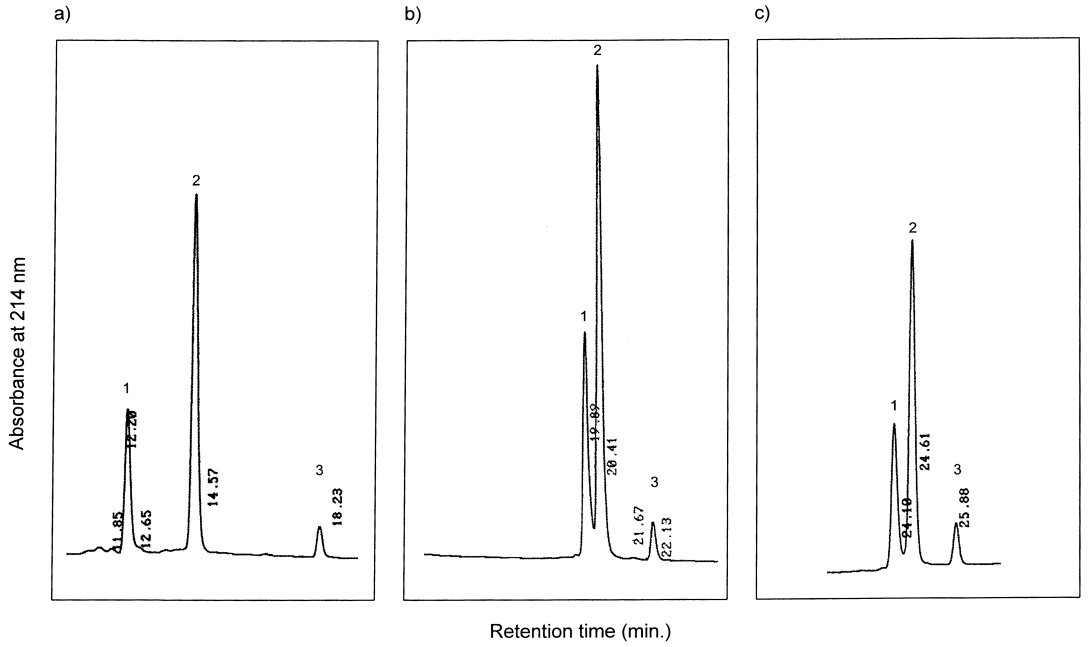

Fig. 4. Chromatogram of: (a) PGM; peak 1, β-anomer; peak 2, α-anomer; peak 3, anhydro-PGM; gradient system A; (b) Boc-Tyr-PGM; peak 1, β-anomer; peak 2, α-anomer; peak 3, anhydro-component; gradient system B; (c) (Ada-1-yl)-CH_2-CO-PGM; peak 1, β-anomer; peak 2, α-anomer; peak 3, anhydro-component; gradient system B.

peptides. For analyses of the reaction mixtures following hydrolyses with aminopeptidase the convenient system was D (Fig. 6) where the alanine has a retention time 2.79 min, the tetrapeptide 7.49 min and the pentapeptide 12.64 min. Tris from the Tris buffer elutes first at 2.38 min.

For the analyses of the reaction mixtures after hydrolysis of Boc-Tyr-pentapeptide and (Ada-1-yl)-CH_2-CO-pentapeptide, the isocratic system D was modified to system E with the addition of acetonitrile (Fig. 7a and b) due to the increased lipophilicity of these peptides. Peptides containing the adamantyl residue exhibited higher retention times. With these peptides better resolution was also achieved between pentapeptide and tetrapeptide than for the Boc-Tyr-substituted peptides.

3.3. Adamantyltripeptides

Synthetic diastereomeric adamantyltripeptides L- and D-(Ada-2-yl)-Gly-L-Ala-D-*iso*Gln were analyzed in system C (Fig. 8) and required shorter time of analysis than the peptidoglycans and peptides tested. The two diastereomers exhibit different retention times: L-(Ada-2-yl)-Gly-L-Ala-D-*iso*Gln 13.19 min and D-(Ada-2-yl)-Gly-L-Ala-D-*iso*Gln 17.70 min. A mixture of two diastereomers is formed in the course of the synthesis as described before [10]. The reaction mixture can be analyzed using RP-HPLC, and also the purity of isolated diastereoisomers can be tested.

4. Discussion

The peptidoglycan monomer (PGM) is a complex molecule containing a disaccharide and a pentapeptide portion. The RP-HPLC of the PGM that we analyzed resulted in three peaks. The muramic acid residue in the disaccharide is a reducing sugar with free OH-group at C-1. In solutions, a free OH-group of a half acetal exists in both the α- and the β-configuration and therefore, two peaks can always be expected with HPLC using appropriate columns and systems [15,18,20]. On the basis of these previous results we have ascribed β- and α-configuration to

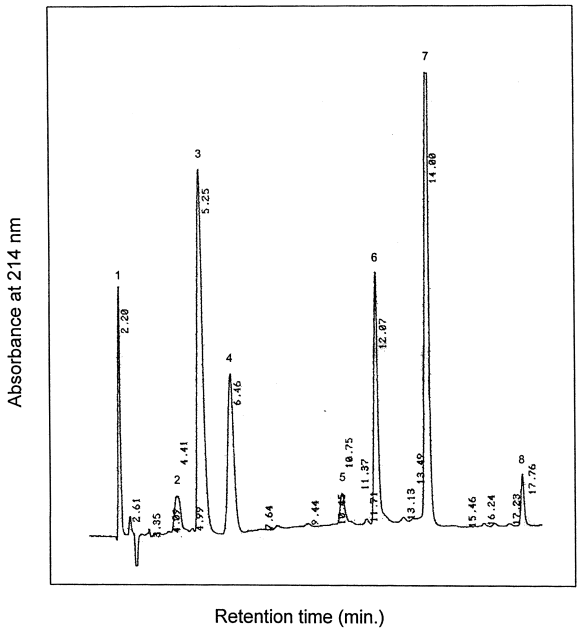

Fig. 5. Chromatogram of reaction mixture following hydrolysis of PGM with *N*-acetylmuramyl-L-alanine amidase: peak 1, TRIS (buffer); peak 2, disaccharide β-anomer; peak 3, pentapeptide; peak 4, disaccharide α-anomer; peak 5, disaccharide anhydro-component; peak 6, PGM β-anomer; peak 7, PGM α-anomer; peak 8, anhydro-PGM; gradient system A.

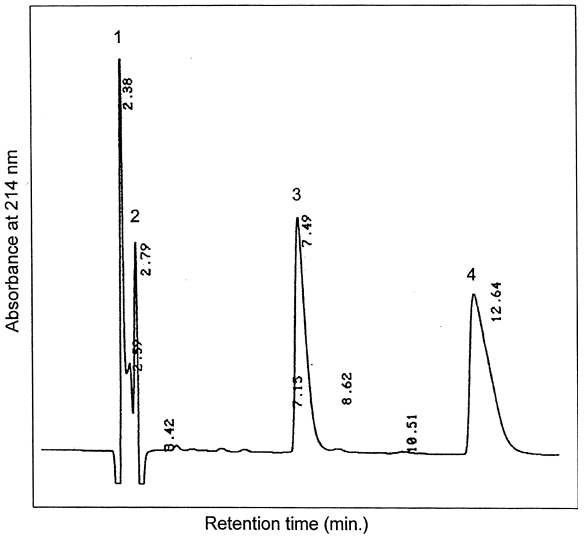

Fig. 6. Chromatogram of reaction mixture following hydrolysis of pentapeptide with L,D-aminopeptidase: peak 1, TRIS (buffer); peak 2, alanine; peak 3, tetrapeptide; peak 4, pentapeptide; isocratic system D.

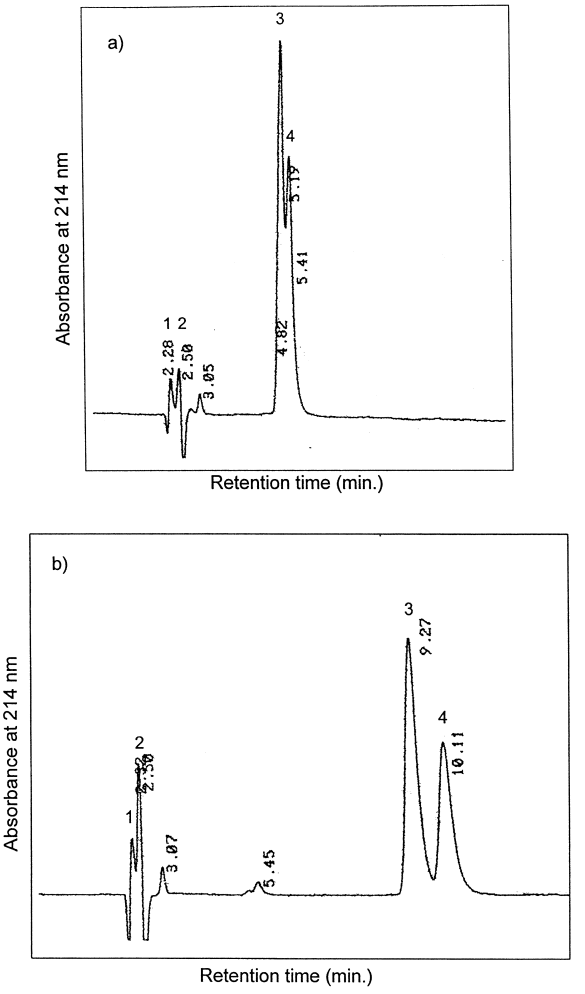

Fig. 7. Chromatograms of reaction mixtures following hydrolysis with L,D-aminopeptidase of (a) Boc-Tyr-pentapeptide: peak 1, TRIS (buffer); peak 2, alanine; peak 3, Boc-Tyr-pentapeptide; peak 4, Boc-Tyr-tetrapeptide; isocratic system E; (b) (Ada-1-yl)-CH_2-CO-pentapeptide: peak 1, TRIS (buffer); peak 2, alanine; peak 3, (Ada-1-yl)-CH_2-CO-pentapeptide; peak 4, (Ada-1-yl)-CH_2-CO-tetrapeptide; isocratic system E.

the two major peaks. The third peak was identified earlier as the anhydro-component (Fig. 1) [18]. The presence of an anhydro-component in our PGM preparations could be explained as follows. In the course of the peptidoglycan chain biosynthesis, a 1,6-anhydromuramyl residue is formed at the end of each chain via transglycosylase action [21,22]. The peptidoglycan monomer used in this study was obtained by lysozyme hydrolysis of uncrosslinked peptidoglycan chains apparently containing an

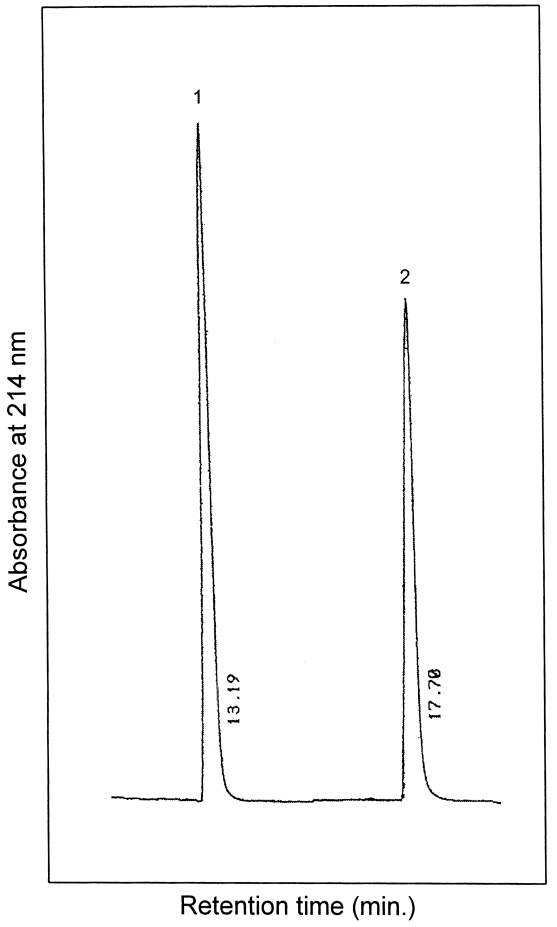

Fig. 8. Chromatogram of the diastereoisomeric mixture of adamantyltripeptides: peak 1, L-(adamant-2-yl)-Gly-L-Ala-D-*iso*-Gln, AdTP2; peak 2, D-(adamant-2-yl)-Gly-L-Ala-D-*iso*Gln, AdTP1; gradient system C.

anhydro-component at the end, and therefore, the anhydro-PGM was obtained as a result of the hydrolysis along with PGM molecules. The two structurally very similar molecules cannot be separated by conventional chromatography. As confirmed by the use of RP-HPLC described in this paper, the content of the anhydro-component in different PGM preparations ranged from 4 to 12%.

In some previous analyses [13,14], the problem of anomers was avoided by reduction of the starting material with sodium borohydride. For our purposes, i.e. for analyses of PGM preparations the reduction was not necessary since all three peaks (α- and β-anomer and anhydro-PGM) in the purified material can be identified (Fig. 4a) and their ratio is an indication of batch consistency. Since we use PGM preparations for testing of biological activity it is of utmost importance to carry out experiments with material of consistent composition and the use of RP-HPLC successfully measures consistency.

We have also used PGM as a starting material for the preparation of more lipophilic derivatives, Boc-Tyr-PGM and (Ada-1-yl)-CH_2-CO-PGM. The substituents were coupled to PGM via a free amino group in diaminopimelic acid in the peptide portion. We expected that the substituents would be coupled to the anhydro-PGM present in the PGM preparation and that the anhydro-PGM derivatives would be also formed in the course of the reaction. As confirmed by HPLC, both Boc-Tyr-PGM and (Ada-1-yl)-CH_2-CO-PGM contain low amount of a respective anhydro-component. Since muramic acid was not changed in the reaction, the two major peaks were considered to be β- and α-anomers of the derivatives formed.

PGM and its derivatives were shown to be the substrates for the enzyme *N*-acetylmuramyl-L-alanine amidase [9,11,19]. The peptides obtained by the hydrolysis of Boc-Tyr-PGM and (Ada-1-yl)-CH_2-CO-PGM with the amidase are novel compounds [9] and their analysis by HPLC was necessary to prove the purity and homogeneity of isolated material. As expected, the purified peptides appeared as single peaks upon HPLC in our systems. The disaccharide formed in these reactions showed three peaks (α-, β- and anhydro-disaccharide) as expected based on earlier results [18,19].

Pentapeptides are further substrates for an L,D-aminopeptidase [12]. The reaction mixture could be analyzed by HPLC in order to estimate the ratios of tetrapeptide and pentapeptide and to follow the course of hydrolysis. In the solvent systems tested in this work, tetrapeptides and pentapeptides differ sufficiently in their retention times to allow for distinct differentiation between them.

It should be noted that the pentapeptides obtained by hydrolysis with the amidase and the tetrapeptides obtained by the hydrolysis with aminopeptidase, are in fact the metabolites of peptidoglycan monomers tested, since both enzymes are present in mammalian sera. HPLC will be necessary for any further preclinical study of these biologically active compounds and their fate in mammals.

The synthetic adamantyltripeptides, L- and D-(Ada-2-yl)-Gly-L-Ala-D-*iso*Gln were shown to possess biological activity, but the activity of two diastereoisomers differed [7,10]. It is therefore important to separately test only highly purified diastereoisomers and the use of RP-HPLC can provide such data.

In conclusion, the peptidoglycan monomer, several novel derivatives of it and structurally related adamantyltripeptides have been analyzed in different systems, by reversed-phase HPLC using UV detection, without derivatization. Some of the analyzed compounds exhibit biological activity and several are the substrates for enzymes from mammalian sera. Except for the original PGM, no data on HPLC have so far been available for the compounds studied and our data represent an original contribution to the study of these compounds.

Acknowledgements

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