ELSEVIER

Biochimica et Biophysica Acta 1289 (1996) 57–64

BBA Biochimica et Biophysica Acta

Purification and characterization of *N*-acetylmuramyl-L-Alanine amidase from human plasma using monoclonal antibodies

Maarten A. Hoijer [a,*], Marie-José Melief [a], Wolfgang Keck [b], Maarten P. Hazenberg [a]

[a] *Department of Immunology, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, the Netherlands*
[b] *Pharma Research Departement, F. Hoffmann-La Roche Ltd., CH-4002 Basel, Switzerland*

Received 19 May 1995; revised 29 August 1995; accepted 11 September 1995

Abstract

N-Acetylmuramyl-L-alanine amidase (EC 3.5.1.28) cleaves the amide bond between *N*-acetyl muramic acid and L-alanine in the peptide side chain of different peptidoglycan products. The enzyme was purified from human plasma using a three-step column chromatography procedure. Monoclonal antibodies were produced against the purified human enzyme. By coupling of a high affinity monoclonal antibody to sepharose beads an immunoadsorbent column was prepared. Using this second purification method it was possible to purify large amounts of the amidase from human plasma in a single step. SDS-PAGE showed one single band of 70 kDa and two-dimensional electrophoresis showed the presence of multiple isomeric forms of the protein with pI between 6.5 and 7.9. Two different methods were used for determination of substrate specificity, a HPLC method separating peptidoglycan monomers from the reaction products after incubation with amidase and a colorimetric method when high molecular weight peptidoglycan was used as a substrate for amidase. It is shown that the disaccharide tetra peptide, disaccharide penta peptide and the anhydro disaccharide tetrapeptide are good substrates for the amidase and that muramyl dipeptide and disaccharide dipeptide are not a substrate for the amidase. Using one of the monoclonal antibodies against the amidase it was shown in FACScan analysis that *N*-acetylmuramyl-L-alanine amidase is present in granulocytes but not in monocytes from unstimulated peripheral blood of a healthy donor. The presence of *N*-acetylmuramyl-L-alanine amidase in granulocytes is a novel finding and perhaps important for the inactivation of biologically active peptidoglycan products still present after hydrolysis by lysozyme.

Keywords: *N*-acetylmuramyl-L-alanine amidase; Monoclonal antibody; Granulocyte

1. Introduction

Peptidoglycan polymers, oligomers and monomers have potent biological effects. An important factor in the induction of the biological effects is the ability of the peptidoglycan products to persist in human tissues. Bacterial cell wall products can be degraded by 3 different human enzymes: lysozyme [1], β-*N*-acetylglucosaminidase [2] and the not well characterised *N*-acetylmuramyl-L-alanine amidase (NAMLAA). This NAMLAA hydrolyses peptidoglycan by cleaving the lactamide bond between *N*-acetyl muramic acid and L-alanine in the peptide side chain of the peptidoglycan molecule. *Bordetella pertussis* tracheal cytotoxin is an anhydro-disaccharide tetrapeptide peptidoglycan monomer which is capable of reproducing the respiratory cytopathology observed during pertussis [3,4]. NAMLAA is able to degrade this anhydro monomer very rapidly in vitro and might be the most important enzyme involved in the degradation of the tracheal cytotoxin in vivo. We hypothesize that this amidase, which we found in all human sera tested until now [5], plays an important role in the degradation and inactivation of biologically active peptidoglycan polymers and monomers in human tissue.

Amidase (NAMLAA) activity in human serum was first described by Ladesić et al. in 1981 [6]. One year later Valinger et al. from the same group partially purified NAMLAA from human and mouse serum and defined its enzymatic activity [7]. In the present study we describe two methods for the purification of NAMLAA from human plasma. The first method is based on DEAE, heparin sepharose and hydroxylapatite column chromatography. The protein obtained by this three-step method was used to

Abbreviations: NAMLAA, *N*-acetylmuramyl-L-alanine amidase; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, High performance liquid chromatography; p*I*, Isoelectric point

* Corresponding author. Fax: +31 10 4367601.

0304-4165/96/$15.00 © 1996 Elsevier Science B.V. All rights reserved
SSDI 0304-4165(95)00136-0

raise monoclonal antibodies against the enzyme. With these antibodies an immunoadsorbent affinity column was prepared with which amidase was purified in one single step with a 36% yield of the enzymatic activity.

2. Material and methods

2.1. Purification of NAMLAA with three-step procedure

300 ml human plasma was dialyzed 3 times against 2 l 25 mM sodium phosphate buffer pH 7.5. After dialysis it was centrifuged for 15 min at 20 000 × *g*. The supernatant was then loaded onto a 500 ml DEAE sepharose 4B (Pharmacia) column. The column was washed with 8 l 25 mM sodium phosphate buffer pH 7.5 until no more protein could be eluted. Elution of the amidase containing fraction was performed by changing the buffer to a 25 mM sodium phosphate buffer with 0.5 M NaCl. Amidase-containing fractions were pooled and dialyzed 3 times against 25 mM sodium phosphate buffer pH 7.5 and loaded onto a 50 ml heparin sepharose Cl-4B column (Pharmacia). The column was washed with 10 column volumes of the sodium phosphate buffer until the eluate did not contain any protein. Elution was performed using a linear gradient of 300 ml sodium phosphate pH 7.5 with 300 ml sodium phosphate pH 7.5 with 0.25 M NaCl. Amidase-containing fractions were pooled and dialyzed 3 times against 2 l 25 mM sodium phosphate pH 6.8 and loaded onto a 15 ml hydroxylapatite (Biorad) column. Amidase does not bind to this column. The flow through was collected and dialysed against 20 mM NH_4HCO_3 pH 8.0. All steps were performed in a cold room (4°C). The NAMLAA activity in the samples was determined using the colorimetric assay described below.

2.2. Peptidoglycan substrates

Peptidoglycan monomers from *Brevibacterium divaricatum* (ATCC 14020) GlcNAc-MurNAc-L-Ala-D-isoGln-*m*-Dpm]-(D)-amide-(L)-D-Ala-D-Ala pentapeptide, and GlcNAc-MurNAc-L-Ala-D-isoGln-*m*-Dpm-D-Ala tetrapeptide were prepared as described by Hazenberg and de Visser [5].

MurNAc-L-Ala-D-isoGlu (MDP) (Sigma), GlcNAc-MurNAc-L-Ala-D-isoGlu (GMDP) (Calbiochem corp), GlcNAc-MurNAc-anhydro-L-Ala-D-Glu-*m*-Dpm-D-Ala (anhydro disaccharide-tetrapeptide) isolated from *Escherichia coli* was a kind gift from A. Dijkstra, Hoffman La Roche.

E. coli, B. divaricatum, Eubacterium aerofaciens, Bifidobacterium adolescentis and *Streptococcus pyogenes* peptidoglycans were prepared according to Severijnen et al. [8]. *Micrococcus lysodeikticus* was obtained from Sigma. The polymeric peptidoglycans were degraded by incubating 250 μg/ml overnight with a 25 μg/ml lysozyme solution in 10 mM sodium acetate buffer, pH 5.6. Peptidoglycan concentrations vary between different experiments.

2.3. Detection of NAMLAA activity by a colorimetric method

Amidase activity was determined as described by Hazenberg and de Visser [5] with some modifications. The method is based on determination of the increase of a free lactyl group in the peptidoglycan substrate due to the removal of the peptide side chain. Peptidoglycan monomers from *B. divaricatum* (ATCC 14020) were used as a substrate. 200 μl amidase sample and 200 μl substrate diluted in 20 mM NH_4HCO_3 pH 8.0 to a final concentration of 500 μg muramic acid/ml were incubated for 15 min at 37°C. The reaction was stopped by adding 200 μl 1 M NaOH and incubating for 30 min. at 37°C. For determination of background, substrate was incubated with buffer for 15 min at 37°C. In these samples muramic acid was determined as follows. 100 μl sample + 1 ml (conc) H_2SO_4 was boiled for 3.5 min and rapidly cooled in ice water. Then 10 μl 0.16 M $CuSO_4 \cdot 5H_2O$ and 20 μl 0.09 M *p*-hydroxydiphenyl in ethanol were added. After a 30 min incubation period at 30°C the absorbance at 570 nm was measured using a Titertek Multiskan (Flow Lab., Irvine, Scotland). 0–100 μg/ml muramic acid solutions were used as standards. For determining the specific activity of NAMLAA using polymeric peptidoglycan lower concentrations of peptidoglycan were used.

2.4. Detection of NAMLAA activity by HPLC

Substrate specificity was determined using *B. divaricatum* GlucNAc-MurNAc-pentapeptide and tetrapeptide. Also MurNAc-L-Ala-D-isoGlu (MDP), GlcNAc-MurNAc-L-Ala-D-isoGlu (GMDP) (Calbiochem Corp.), GlucNAc-MurNAc[anhydro] tetrapeptide isolated from *E. coli* were used. 500 μg/ml samples were incubated with affinity-purified amidase in a final concentration of 1 μg/ml 25 mM NH_4HCO_3 buffer, pH 8.0, at 37°C. For determination of the background the substrate was incubated with buffer. The reactions were stopped after 15 min by diluting the samples 10 times with 25 mM sodium phosphate pH 3.5 (HPLC buffer A). Peak areas were used to calculate the enzymatic activity. 1 unit is defined as the amount of substrate (μmol) hydrolysed per minute at pH 8.0, 37°C.

Reversed phase HPLC was used for separation of the reaction products. 10 μl samples were analyzed using a Pharmacia-LKB 2248 single pump solvent delivery system and VWM 2141 UV-VIS monitor both connected to a computer working with HPLC manager software to control the pump, gradient mixer and UV-VIS detector operating at 205 mm. Integration and analysis of chromatograms was performed using the same software (Pharmacia, Sweden). The samples were separated using a Pharmacia Superfac Sephasil C18, 5 μm, 4 × 250 mm column. The flowrate

was 1 ml/min and the buffers were A: 25 mM sodium phosphate pH 3.50; B: 15% methanol in 25 mM sodium phosphate pH 4.70. At 0, 2, 10, 12.5, 13, 15 min the percentage B was 0, 0, 100, 100, 0, 0, respectively.

For determination of K_m and maximal rate of catalysis (V max), substrate concentrations were used between 2mM and 0.1 mM GlucNac-MurNAc-pentapeptide of *B. divaricatum*. The aminoacid and aminosugar composition of the separated peaks before and after amidase incubation was determined according to the method described in a previous paper [9].

2.5. Preparation of monoclonal antibodies

The purified amidase was used for preparing monoclonal antibodies. A male Balb/c mouse was injected intraperitoneally with 50 μg of NAMLAA in complete Freund's adjuvant. After 6 wk, the mouse received a booster of 10 μg NAMLAA in incomplete Freund's adjuvant. After another 6 wk, the mouse received a second booster of 10 μg amidase in incomplete Freund's adjuvant, intraperitoneally and 10 μg amidase in PBS, intravenously. Three days later, the mouse was sacrificed and cells isolated from popliteal, inguinal and axillary lymphnodes and the spleen were fused with the Sp2/0 plasmacytoma cell line in a ratio of 5:1 and 5:2 (spleen: Sp2/0) using standard procedures for the production of hybridomas [13]. Cells were seeded at a concentration of 8.10^4 cells/well in the presence of human growth factor (40 U/ml) [10,11]. The supernatants of 3000 wells were tested for the production of antibodies against amidase in an ELISA. The amidase-positive supernatants were tested in an isotype ELISA [12]. Only IgG-producing clones were used for further experiments. Monoclonal antibody AAA4 showed the highest affinity for NAMLAA and was used for preparing a FITC-labeled monoclonal antibody (α-NAMLAA-FITC) and for preparing an immunoadsorbent column.

2.6. Preparing immunoadsorbent column and purification of NAMLAA

Monoclonal antibodies were purified over a 10 ml Immunopure Immobilized Protein G column (Pierce) using the standard protocol of the supplier. 7.2 mg of monoclonal antibody AAA4 was dialyzed 3 times against 2 l 0.1 M $NaHCO_3$ + 0.5 M NaCl and coupled to 2 g CNBr-activated Sepharose 4B (Pharmacia LKB Biotechnology) according to the protocol of the supplier. Human plasma (obtained from the blood bank Rotterdam) was dialyzed against PBS and diluted 2 times in PBS before it was loaded onto the column. The column was washed with PBS until no more protein was detected in the eluate. The amidase was eluted by changing the buffer to 0.1 M Gly · HCl, pH 3.0. The peak fractions were immediately dialyzed against 20 mM NH_4HCO_3 pH 8.0.

2.7. Immunofluorescence labeling, flow-cytometric analysis

Labeling of AAA4 with fluorescent isothiocyanate (FITC) was performed according to standard procedures [13]. Human peripheral blood cells were stained for intracellular antigens according to Syrjilä [14]. In short: 100 μl citrate blood was lysed and fixed by adding 2 ml of FACS lysing solution (Becton Dickinson) and incubating for 10 min at room temperature. The cells were centrifuged and washed 2 times with PBS + 0.5% BSA. Then the mouse α-NAMLAA-FITC or rabbit α-human lysozyme (DAKO, ITK Diagnostics) were added and the mixture was incubated for 10 min at room temperature. The cells were washed 2 times with 2 ml PBS-BSA. The rabbit α-lysozyme-treated cells were incubated with the second antibody goat-α-rabbit-FITC for 10 min, washed 2 times with PBS-BSA and finally resuspended in 200 μl FACS flow (Becton Dickinson). The analyses were performed with a FACScan cytofluorimeter (Becton Dickinson, Sunnyval, CA).

2.8. SDS-polyacrylamide gel electrophoresis and immunoblot analysis

The amidase was subjected to 10% SDS-PAGE to check for purity. Affinity-purified amidase samples of 20 μl were boiled for 3 min together with 5 μl loading buffer (60 mM Tris. HCl pH 6.8; 23% glycerol 3% SDS; 0.06% bromophenol blue; 10% β-mercapto ethanol) 10 μl of these samples were analyzed on 10% SDS-PAGE (Mini Protein, BioRad). Samples were visualized by Coomassie Blue staining or transferred to nitrocellulose in 25 mM Tris, 190 mM glycine and 20% methanol transfer buffer. Nitrocellulose sheets were blocked in low-fat milk for 30 min and subsequently washed three times with 0.5% Tween-20 in PBS. Nitrocellulose sheets were then incubated for 1 h with monoclonal antibodies 500 times diluted in PBS-Tween at room temperature. Following three washes, goat anti-mouse IgG conjugated to alkaline phosphatase (TAGO) was added in a 1000 times dilution and incubated for 1 hour at room temperature. The blot was washed three times with PBS-Tween and then three times with PBS. For visualization of antibody-antigen complexes the alkaline phosphatase substrate, nitroblue tetrazolium/5-bromo-4-chloro indoxyl phosphate (NBT/BCIP), was used as described [20].

2.9. Two-dimensional electrophoresis

25 μl of affinity-purified NAMLAA was mixed with 25 μl sample buffer (0.3% SDS, 200 mM dithiothreitol, 28 mM Tris · HCl and 22 mM Tris-base) and heated for 4 min at 100°C. Two-dimensional electrophoresis was performed with the Millipore Investigator system. We used ampholytes with a pH range from 3–10 for the first dimen-

Table 1
Purification of *N*-acetylmuramyl-L-alanine amidase from human plasma by three-step procedure

	Protein conc (mg/ml)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
plasma	48.7	15.6×10^3	4.6×10^{-2}	100
DEAE	13.6	2.7×10^2	0.11	41
heparin	0.2	15.4	3.2	6.9
hydroxylapatite	1.0×10^{-2}	1.0	6.7	0.9

Protein concentrations were determined according to the method of Bradford. Enzymatic activity was determined by the colorimetric assay (see Section 2.3).

sion. For the second dimension we used 10% Duracryl gels. Glyceraldehyde-3-phosphate dehydrogenase carbamalytes (Pharmacia) were used as isoelectric-focussing markers. Proteins were visualized with a silver staining technique. All steps were performed according to the manufacturer's instructions.

2.10. Gel permeation chromatography

A superdex 200 HR 10/30 column with 10 mm internal bore diameter and 30 cm length ($V_c = 24$ ml) was used (Pharmacia). The column was connected to a standard FPLC system (Pharmacia), consisting of a LCC chromatography controller, two P-500 pumps, a MV-7 injector with a 200 μl sample loop, a UV-1 monitor at 280 nM operating at 0.05 AUFS sensitivity and a FRAC 100 fraction collector. Routinely, isocratic elution with PBS was used. Column selectivity was determined by using mixtures of molecular weight marker proteins (Pharmacia) ranging from 670 kDa to 17 kDa. 200 μl purified NAMLAA was injected on the column. Enzymatic activity was determined in the peak fractions.

2.11. Immunohistochemistry

Blood smears of a healthy person were fixed for 10 min in acetone. After rinsing in PBS with 0.2% bovine serum albumin (BSA), the smears were incubated for 1 h at room temperature with monoclonal antibody AAA3 (diluted 40 μg/ml in PBS-BSA) or, for control staining, an irrelevant monoclonal antibody of the same isotype and concentration. Subsequently, the smears were rinsed in PBS-BSA and incubated for 30 min at room temperature with rabbit anti-mouse immunoglobulin (Z259, Dakopatts) diluted 1:20 in PBS-BSA with 1% normal human serum. After rinsing in PBS-BSA, a 1:40 dilution of alkaline phosphatase-mouse-anti-alkaline phosphatase complex (APAAP, D651, Dakopatts, Denmark) was applied for 30 min. To develop the stain the smears were incubated for 30 min at room temperature with new Fuchsin substrate (Chroma), which stained positive cells red. After rinsing in distilled water the nuclei of the cells were stained blue by incubation for 1 min with haematoxylin and rinsing in fresh water for 10 min. Finally, the smears were mounted in Kaiser's glycerol gelatin (Merck).

3. Results

3.1. Purification of NAMLAA from human plasma

Table 1 shows the purification scheme of the three step purification. The amidase activity in dialyzed and centrifuged plasma was 46 mU/mg protein. The final pure amidase had a specific activity of 6.7 U/mg protein. Most of the total activity was lost during this purification procedure (99%). Starting with 300 ml plasma it was possible to purify about 1 mg NAMLAA using this method.

3.2. Monoclonal antibodies and immunoaffinity purification

Six different monoclonal antibodies against NAMLAA were obtained. Monoclonal AAA4 showed the highest affinity for the human amidase in a competition ELISA (not shown) and was therefore used to prepare an immunoadsorbent column. 7.2 mg purified monoclonal antibody was coupled to CNBr-activated sepharose beads. The capacity of the column was sufficient for the 50 ml plasma used. No amidase activity could be detected in the flow-through when diluted plasma was applied to the column. After elution with 0.1 M Gly · HCl pH 3.0, pure amidase was eluted from the column. From 50 ml plasma it was possible to obtain 0.6 mg pure amidase.

Table 2
Purification of *N*-acetylmuramyl-L-alanine amidase from human plasma by immunoaffinity chromatography

	Protein conc (mg/ml)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
plasma	48.7	2435	3.1×10^{-2}	100
Immunoaffinity	0.1	0.6	46	34

Protein concentrations were determined according to the method of Bradford. Enzymatic activity was determined by the HPLC method (see Section 2.4).

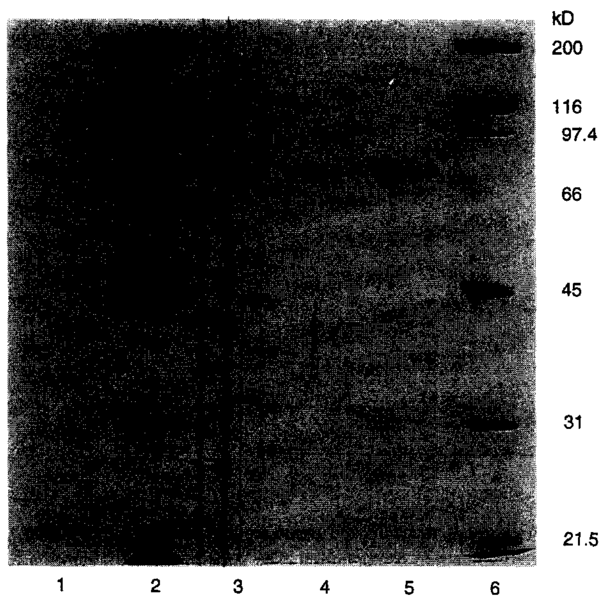

Fig. 1. Polyacrylamide gel electrophoreses of purified *N*-acetylmuramyl-L-alanine-amidase. 10-μl samples of three different concentrations were analyzed on 10% PAGE-SDS. Lane 1 is amidase purified using the three-step procedure. Lane 2 and 6 are molecular weight markers and lane 3, 4 and 5 are 20, 40 and 100 μg/ml dilutions (resp.) of immunoaffinity-purified amidase. NAMLAA is clearly shown as a single band of 70 kDa. The gels were stained with Coomassie Blue.

Table 2 shows the purification scheme. The amidase activity in this dialyzed and centrifugated plasma was 31 mU/mg protein, determined by the HPLC method. The purified amidase solution obtained by this method contained a specific activity of 46 U/mg protein and 36% of the enzymatic activity was recovered. Therefore, by using the immunoadsorbent column it was possible to increase the yield 38-fold and the final product had a specific activity 6.9-fold higher compared with the three-step procedure.

3.3. SDS-polyacrylamide gel electrophoresis and immunoblot analysis

Fig. 1 shows a 10% SDS-PAGE. Purified amidase is electrophoresed as a single band with a molecular mass of 70 kDa. After deglycosylation by *N*-glycosidase-F digestion, the molecular mass decreased to 60 kDa (not shown).

Western blot analysis of purified NAMLAA before and after deglycosylation showed that four of the six monoclonal antibodies including AAA4, the antibody used for purification, were able to recognize NAMLAA in both forms.

3.4. Two-dimensional electrophoresis

Fig. 2 shows affinity-purified NAMLAA subjected to iso-electrofocussing in the first dimension and 10% polyacrylamide gel electrophoresis in the second dimension. At least 8 different isomeric forms are visible of this glycoprotein. The p*I* values vary between 7.0 and 7.9 although

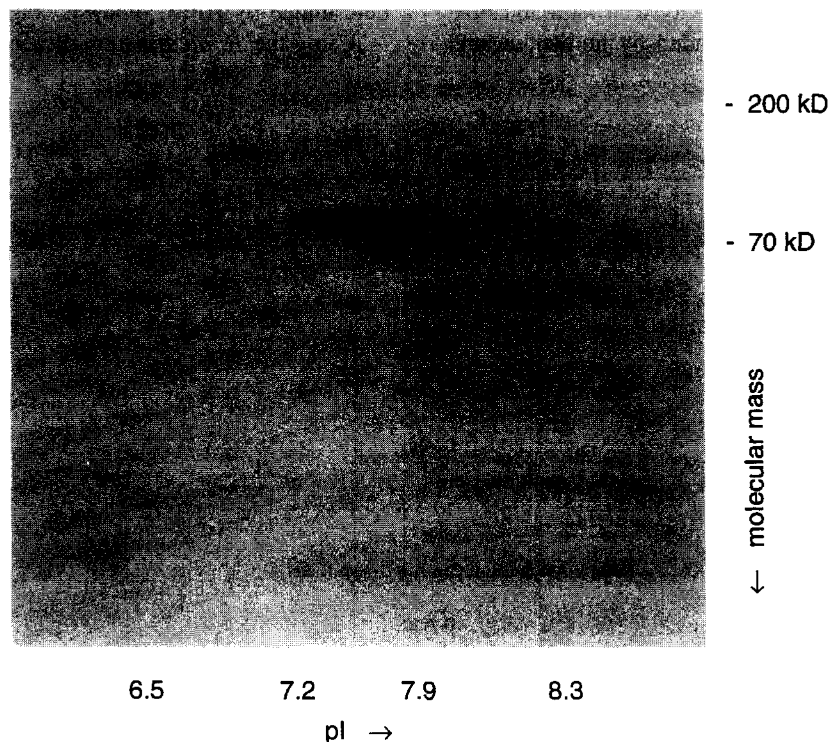

Fig. 2. Two-dimensional electrophoresis of affinity-purified NAMLAA. First dimension isoelectro-focussing in a pH range of 3–10 and second dimension 10% Duracryl electrophoresis separation on molecular weight. Thirteen different isomeric forms of the glycoprotein are visible. The silverstained gel does not show any impurities.

Fig. 3. Reversed phase high performance liquid chromatography chromatogram of *B. divaricatum* disaccharide pentapeptide before (A) and after (B) incubation with NAMLAA. The large peak with retention time 12.8 min in **A** represents the disaccharide pentapeptide. The small peak with retention time 11.8 min is the disaccharide tetrapeptide. The large peak in **B** with retention time 2.8 min (void volume) contains the peptide product released from the sugar moiety. The small peak at 5 min contains the disaccharide. The difference in area of the peaks in A with the corresponding peaks in B is a direct measure for the amount of substrate degraded by NAMLAA.

the main spots are in the 7.5–7.8 range. No impurities are visible after silver staining.

3.5. Detection of NAMLAA activity by HPLC

NAMLAA activity was tested with different peptidoglycan products. Muramyl dipeptide (MDP) was tested because this product is the smallest component of peptidoglycan known to posses biological activity. Harrison and Fox [15] described that MDP was degraded by human serum. We did not find this activity for the purified amidase. OON-acetylglucosamine-*N*-acetylmuramic acid dipeptide (GMDP) was not a substrate for NAMLAA either. Disaccharide tetrapeptide and the anhydro-disaccharide tetrapeptide were good substrates for NAMLAA. The anhydro form was degraded four times slower than the non-anhydro disaccharide-tetrapeptide. The disaccharide-pentapeptide was degraded at the same velocity as the tetrapeptide, as determined by HPLC analysis (Fig. 3).

Using the *B. divaricatum* disaccharide pentapeptide as a

Table 3
NAMLAA activity related to differences in peptide bridges in peptidoglycan from 6 bacterial species

Peptidoglycan Source	Aminoacids in peptide bridge	Specific activity (U/mg)
E. coli	D-Ala NAM-L-Ala-D-Glu-M-Dpm–	57
B. divaricatum	D-Ala NAM-L-Ala-D-isoGln-M-Dpm–	111
E. aerofaciens	D-Ala NAM-L-Ala-D-Glu-L-Lys–	12.5
B. adolescentis	D-Ala NAM-L-Ala-D-Glu-L-Orn-Ala-D-Asp-D-Ser–	2
S. pyogenes	D-Ala NAM-L-Ala-D-Glu-L-Lys-D-Ala-L-Thr-L-Ala–	0
M. lysodeikticus	NAM-L-Ala-D-Glu-L-Lys-D-Ala-L-Lys-D-Glu-L-Ala– Gly Gly	0

Enzymatic activity was determined with the colorimetric method (see Section 2.3).

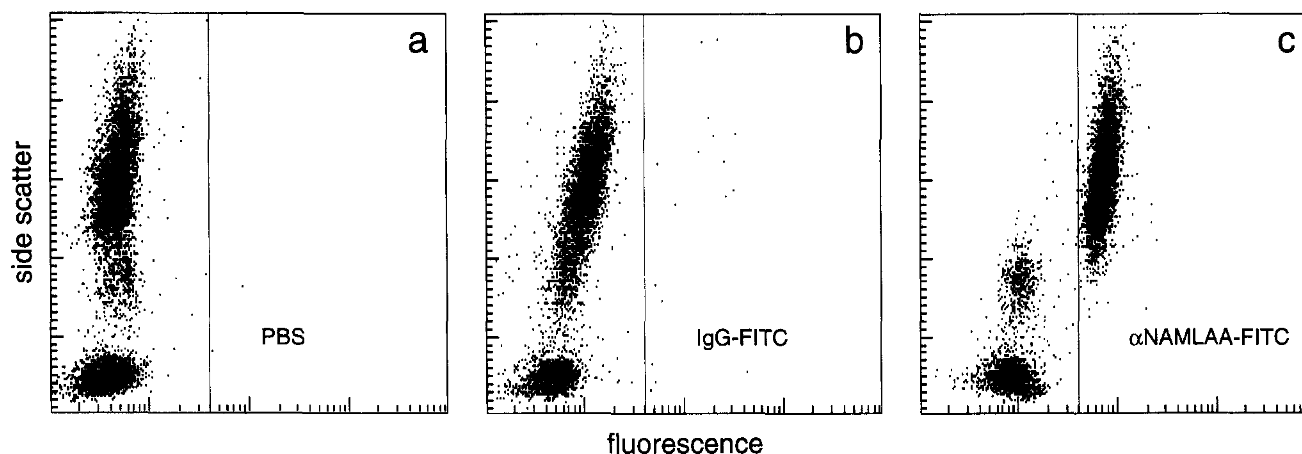

Fig. 4. FACScan analysis of nucleated fraction from blood of a healthy donor. Based on sideward scatter human peripheral blood cell suspensions can be devided in three different populations. The lower population consists of lymphocytes, the middle population consists of monocytes and de upper population are the granulocytes. Granulocytes are positively stained by α-NAMLAA-FITC (C), monocytes and lymphocytes are not stained with this monoclonal antibody. PBS (A) and IgG-FITC (B) were used as negative controls.

substrate the maximal rate of catalysis is 0.10 mmol/min and the K_m value is 2.5 mM.

3.6. Detection of NAMLAA activity by colorimetric assay

Six different lysozyme-solubilized peptidoglycan samples were incubated with NAMLAA. In Table 3 the differences in the peptide bridges of the peptidoglycans are shown with the corresponding specific activity of NAMLAA. *B. adolescentis, S. pyogenes* and *M. lysodeikticus* peptidoglycans are not degraded by NAMLAA. The substrate concentration was 350 μg/ml based on the concentration muramic acid in the reaction mixture.

3.7. Flow-cytometric analysis

FACScan analysis of the nucleated cells from blood of a healthy donor were performed to determine if NAMLAA is produced by white blood cells. In Fig. 4 it is shown that granulocytes contain NAMLAA but monocytes do not. Lysozyme could be detected in both monocytes and granulocytes (not shown). PBS and IgG-FITC were used as a negative control (Fig. 3A and 3B).

3.8. Native molecular mass determination

The selectivity curve used was: $K_{AV} = 2.309 - 0.378 \log M_r$

$$K_{AV} = \frac{V_e - V_o}{V_t - V_o} = \frac{V_{e-8}}{12.8}$$

where V_e is the elution volume, V_0 is void volume (8.0 ml) and V_t is total volume (20.7 ml). The elution volume of the NAMLAA activity containing peak was 14.0 ml. Therefore the calculated molecular mass is 69 kDa ± 10%. Which proves that the amidase is composed of one subunit.

3.9. Immunohistochemistry

The immunohistochemical staining of the blood smears of a healthy person with monoclonal antibody AAA3 showed a positive staining of the granulocytes. The mononuclear cells including monocytes were negative. No positive cells were found by using an irrelevant monoclonal antibody with the same isotype and concentration.

4. Discussion

In 1990 Vanderwinkel et al published the purification of NAMLAA from human serum [16]. They found an enzyme of Mr 120.000–130.000 in native PAGE and two bands of 57 kDa and 70 kDa under denaturating conditions and considered amidase to be a dimeric protein with pI of 4.5–5.5. They also found the *E. coli*-derived MurNAc-tripeptide to be a good substrate for the enzyme as well as some polymeric peptidoglycans. Because it was only possible to purify very small amounts of amidase with their method and the properties of the enzyme did not always correspond with the protein we were purifying we started with the development of a large scale purification to make a good characterization of the enzyme possible.

This study describes two methods for the purification of NAMLAA from human plasma. The purification with a NAMLAA specific immunoabsorbent column yielded a higher specific activity than the three-step column purification method. Apparently some of the amidase purified by the three-step method loses some of its activity during the different steps, because the specific activity is much lower

compared to the immunoaffinity-purified NAMLAA. Purification of human NAMLAA can therefore best be carried out using the specific immunoabsorbent column. It is unknown if all the immunoaffinity-purified NAMLAA is still active. It is therefore not possible to determine exactly the concentration of NAMLAA in plasma, but it must be at least 10 μg/ml plasma. This is similar to normal serum concentrations of lysozyme which varies from 7 to 20 μg/ml [1].

The pattern of dots obtained after two-dimensional electrophoresis indicates that the NAMLAA contains variable amounts of charged groups. It was shown that NAMLAA is a glycoprotein because the molecular mass decreased after incubating the enzyme with *N*-glycosidase F. This was confirmed by experiments where to some extent NAMLAA activity bound to Sambuccus Nigra Agglutinin (SNA) coupled to sepharose beads (results not shown). SNA has a specific affinity for α-NeuNAc [2–6] GalNAc [17] which is a charged group.

Enzymatic activity of the enzyme is not restricted to peptidoglycan monomers. After lysozyme degradation, polymers with molecular mass greater than 10^6 Da from *E. coli* and *E. aerofaciens* were degraded for more than 80% depending on NAMLAA concentration and reaction time. The composition of the peptide side chains of these substrates differ widely starting from the third aminoacid, counted from MurNAc (Table 3). Therefore it seems obvious that the first three aminoacids together with *N*-acetylmuramic acid are the most important in determining substrate specificity. After alkali hydrolysis of the *N*-acetylgroup of *N*-acetylmuramic acid, the remaining glycopeptide is no longer degradable by NAMLAA.

The six purified monoclonal antibodies against NAMLAA all have different affinities for amidase. 3 out of 6 recognize NAMLAA in Western blot (data not shown). Flow-cytometric analysis of blood using a FITC-labeled monoclonal antibody against NAMLAA showed that granulocytes contain NAMLAA. This to our knowledge novel finding makes it very likely that the enzyme is involved in degrading peptidoglycan products in blood and tissues, which could be liberated during infections or absorbed from the gut. The monoclonal antibodies are currently being used to screen a human cDNA expression library in order to identify the gene coding for the enzyme.

Peptidoglycan is a polymer found in all bacterial cell walls. Biological activities of peptidoglycan are multifold. It has many biological effects in common with LPS but it is also able to cause chronic inflammation [18]. Peptidoglycan monomer anhydromuramyl dipeptide possesses sleep-inducing capacity [19]. All these biological activities of peptidoglycan monomers/polymers are likely to be susceptible to degradation by amidase. Further research will be done to study the influence of amidase on the biological activities of peptidoglycan. The relatively simple method described in this paper for obtaining large amounts of pure NAMLAA will make it possible to study this human enzyme in greater detail.

Acknowledgements

We gratefully thank Tar van Os and Marjo van Everdingen-Quartel for excelent assistance in preparation of the manuscript and Theo M. Luider for his indispensable help with the two-dimensional electrophoresis.

References

[1] Jollès, P. and Jollès, J. (1984) Mol. Cell. Biochem. 63, 165–189.
[2] Striker, R., Kline, M.E., Haak, R.A., Rest, R.F. and Rosenthal, R.S. (1987) Infect. Immunol. 55, 2579–2584.
[3] Cookson, B.T., Cho, H.-L., Herwaldt, L.A. and Goldman, W.E. (1989) Infect. Immunol. 57, 2223–2229.
[4] Luker, K.E., Collier, J.L., Kolodziej, E.W., Marshall, G.R. and Goldman, W.E. (1993) Proc. Natl. Acad. Sci USA 90, 2365–2369.
[5] Hazenberg, M.P. and de Visser, H. (1992) Eur. J. Clin. Chem. Clin. Biochem. 30, 141–144.
[6] Ladesic, B., Tomasic, J., Kveder, S. and Hrsac, I. (1981) Biochem. Biophys Acta 678, 12–17.
[7] Valinger, Z, Ladesic, B. and Tomasic, J. (1982) Biochem. Biophys Acta 701, 63–71.
[8] Severijnen, A.J., van Kleef, R., Hazenberg, M.P. and van de Merwe, J.P. (1989) J. Rheumatol. 16, 1061–1068.
[9] Hoijer, M.A., Melief, M.J., van Helden-Meeuwsen, C.G., Eulderink, F. and Hazenberg, M.P.(1995) Infect. Immunol. 63, No 5, 1652–1657.
[10] Shulman, M., Wilde, C.D. and Köhler, G.A. (1978) Nature 276, 269–270.
[11] Aarden, C., Lansdorp, P. and de Groot, E. (1985) Lymphok 10, 175–185.
[12] Bos, N.A., Kimura, H, Meeuwsen, C.G., et al. (1989) Eur. J. Immunol. 19, 2335–2339.
[13] Coligan, J.E., Kruisbeek, A.M., Margulies, D.H., Shevach, E.M. and Stober, W. (1990) Current protocols in immunology, Green Publ. Assoc./John Wiley & Sons, New York, 3.5–3.6.
[14] Syrjilä, M.T., Tiirikainen, M, Sansson, S.E. and Krusius, T. (1993) Am. J. Clin Pathol. 99, 298–303.
[15] Harrison, J. and Fox, A. (1985) Infect. Immunol. 50, 320–321.
[16] Vanderwinkel, E., De Vlieghere, M., De Pauw, P., Cattalini, N., Ledoux, V., Gigot, G. and Ten Have, J.-P. (1990) Biochem. Biophys. Acta 1039, 331–338.
[17] Shibuya, N., Goldstein, I.J., Van Damme, E.J.M. and Peumans, W.J. (1988) J. Biol. Chem. 262, 1596–1601.
[18] Cromartie, W.J., Craddock, J.G., Schwab, S.K. and Yang, C.H. (1977) J. Exp. Med. 146, 1585–1602.
[19] Krueger, J.M., Pappenheimer, J.R. and Karnowsky, M.L. (1982) J. Biol. Chem. 257, 1664–1669.
[20] Blake, M.S., Johnston, K.H., Russell-Jones, G.J., Gotshlich, E.C. (1984) Anal. Biochem. 136, 175–179.