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# Identification of serum *N*-acetylmuramoyl-L-alanine amidase as liver peptidoglycan recognition protein 2☆

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**Abstract**

*N*-acetylmuramoyl-L-alanine amidase (NAMLAA) hydrolyzes bacterial peptidoglycan and is present in human serum. A peptidoglycan-recognition protein 2 (PGLYRP2) is expressed in human liver and has *N*-acetylmuramoyl-L-alanine amidase activity. Here, we determined the amino acid sequences of human serum NAMLAA and liver PGLYRP2 and tested the hypothesis that serum NAMLAA and PGLYRP2 are the same protein. Liver PGLYRP2 and serum NAMLAA had the same mass determined by mass spectrometry and polyacrylamide gel electrophoresis, and both proteins and recombinant PGLYRP2 reacted with polyclonal anti-NAMLAA and anti-PGLYRP2 antibodies, and with monoclonal anti-NAMLAA antibodies. Digestion of serum NAMLAA with trypsin, chymotrypsin, or trypsin plus V8 protease, or with CNBr yielded, respectively, 37, 40, and 3 overlapping peptides that matched 100% and covered 81% of the deduced amino acid sequence of mature PGLYRP2. These peptides overlapped all exon–intron junctions indicating no alternative splice forms. Digestion of liver PGLYRP2 with trypsin yielded 23 peptides that matched 100% and covered 44% of the deduced amino acid sequence of mature PGLYRP2. Serum NAMLAA had a C398–C404 disulfide, partial phosphorylation of S218, and deamidation of N253 and N301. These results indicate that serum NAMLAA and liver PGLYRP2 are the same protein encoded by the *pglyrp2* gene.


*Keywords:* *N*-acetylmuramoyl-L-alanine amidase; Bacterial peptidoglycan; Peptidoglycan-recognition protein; Innate immunity

## 1. Introduction

Peptidoglycan (PGN) is a polymer uniquely present in the cell walls of virtually all bacteria, composed of β(1–4)-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc), crosslinked by short peptides of alternating L- and D-amino acids [1–3]. Mammals have several PGN recognition molecules. They include four pattern recognition receptors which are members of the leucine-rich repeat protein family: cell-surface CD14 and Toll-like receptor 2 (TLR2), and intracellular nucleotide-binding oligomerization domain (NOD)-containing proteins, NOD1 and NOD2 [4,5]. CD14 and TLR2 recognize

*Abbreviations:* NAMLAA, *N*-acetylmuramoyl-L-alanine amidase; NOD, nucleotide-binding oligomerization domain; PGLYRP or PGRP, peptidoglycan-recognition protein; PGN, peptidoglycan; TLR2, Toll-like receptor 2

☆ The protein sequence data reported in this paper have been submitted to the SWISS-PROT and TrEMBL knowledgebase under the accession number Q96PD5.

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extracellular polymeric PGN [4–6], and NOD1 and NOD2 recognize intracellular PGN fragments [7–10]. Interaction of PGN with CD14, TLR2, and NOD has highly pro-inflammatory effects due to the induction of secretion of numerous chemokines and cytokines and other mediators of inflammation [3–5]. The combined effect of these mediators in vivo is responsible for the normal antimicrobial host defenses and can be observed as typical clinical manifestations of bacterial infections, such as inflammation, pus formation, leukocytosis, fever, acute-phase response, hypotension, sleepiness, decreased appetite, and arthritis [1,5,11]. Microbial stimulation of the innate immune system through pattern recognition receptors is also required for the initiation of the adaptive immune response [12,13], which accounts for the well-known immune adjuvant effect of microbial components, including PGN [14,15].

Mammals also have two enzymes that digest PGN. The first, lysozyme (EC 3.2.1.17), is present in the serum, body secretions (such as tears, sweat, and saliva), phagocytic granules, and intestinal Paneth cells, and hydrolyzes the glycosidic bond between MurNAc and GlcNAc of PGN [16–18]. The second, *N*-acetylmuramoyl-L-alanine amidase (NAMLAA, EC 3.5.1.28), is primarily present in the serum and hydrolyzes the amide bond between MurNAc and L-Ala and thus removes stem peptides from the PGN molecule [19–26]. Digestion of PGN with lysozyme and NAMLAA reduces or eliminates the biologic activities of polymeric PGN [18,27–30], although it may also generate NOD-activating PGN fragments [7–10,31].

NAMLAA activity was reported in human [19–26] and mouse [19,20] serum and tissues [21,26]. NAMLAA protein was purified from human serum by anion exchange chromatography [22,24,25]. Monoclonal antibodies (mAbs) to human serum NAMLAA were then obtained [25] and used to purify NAMLAA protein from human serum by affinity chromatography [25]. The sequence of 15 N-terminal amino acids of purified human serum NAMLAA was also obtained [24], but the NAMLAA cDNA was never cloned, the NAMLAA gene has not been identified, and the entire sequence of the protein is unknown.

Mammals and insects have a family of PGN Recognition Proteins (PGRP). When first insect and mammalian PGRPs were cloned in 1998, it was noticed that they all contained an amidase-homology domain [32]. In 2000, a family of 13 PGRP genes in *Drosophila* [33] and a year later a family of 4 PGRPs in humans [34] were identified. They were named PGRP-S (for "short" transcript), PGRP-L (for "long" transcript), and PGRP-Iα and PGRP-Iβ (for "intermediate" transcript). Recently, the Human Genome Organization Gene Nomenclature Committee approved the names Peptidoglycan Recognition Protein 1, 2, 3, and 4, and the symbols PGLYRP1, PGLYRP2, PGLYRP3, and PGLYRP4 for human PGRP-S, PGRP-L, PGRP-Iα, and PGRP-Iβ, respectively, and this new nomenclature is used in this article.

Mammalian PGLYRP1 is present in granulocyte granules and has antibacterial properties [35–37]. Mammalian PGLYRP2 is primarily expressed in the liver [34] and has *N*-acetylmuramoyl-L-alanine amidase activity [38,39]. The sequence of the 15 N-terminal amino acids of the human serum NAMLAA [24] is identical to the N-terminal sequence of the mature PGLYRP2 protein, deduced from the cDNA sequence [34], thus suggesting that the serum amidase and PGLYRP2 may be the products of the same gene and may be the same protein. The objective of the current study was to determine the amino acid sequences of human serum NAMLAA and liver PGLYRP2 and to test the hypothesis that human serum NAMLAA and liver PGLYRP2 are the same protein, or, alternatively, that they are different proteins or different splice forms coded by the same gene. The possibility of alternative splicing of human PGLYRP2 was investigated, because alternative splicing was reported for mouse PGLYRP2 [40], and because two alternative cDNA sequences for human PGLYRP2 have been previously submitted to GenBank.

## 2. Materials and methods

### 2.1. Reagents

Acetonitrile (HPLC grade), urea, formic acid (sequencing grade), ammonium bicarbonate, and dithiothreitol (DTT) were from Fisher Scientific (Fair Lawn, NJ). Iodoacetamide was from Bio-Rad (Hercules, CA). Modified trypsin (sequencing grade) was from Promega (Madison, WI). Modified chymotrypsin (sequencing grade) was from Princeton Separations (Adelphia, NJ). All other reagents, unless otherwise indicated, were from Sigma (St. Louis, MO).

### 2.2. Plasmids and recombinant PGLYRPs

Human PGLYRP1, PGLYRP2, PGLYRP3, and PGLYRP4 in pcDNA3.1 vector [34] were used for transient expression in Cos-7 or HEK293T cells [34,39]. Enzymatic de-glycosylation was performed with N-glycosidase (glycopeptidase F from *Flavobacterium meningosepticum*) as previously described [41]. PGLYRP2 deletion mutants Δ1–322 (N-terminal deletion), Δ323–555 (C-terminal deletion of the entire amidase/PGRP domain), and Δ474–555 (deletion of a C-terminal portion of the amidase/PGRP domain) were the same as described previously [39], but note that the amino acid numbering used here is based on the mature protein sequence and does not include the 21 amino acid signal peptide that was included in the numbering reported previously [39]. All the PGLYRP plasmids were also subcloned into the inducible pET-32 bacterial expression vector with N-terminal thioredoxin, 6×His, and S tags (Novagen, Madison, WI) and analyzed by restriction digests and by sequencing as described previously [34]. Recombinant PGLYRP proteins were expressed in *Escherichia coli* as inclusion bodies, which were isolated using BugBuster kit (Novagen) and differential centrifugation as recommen-