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The *Enterococcus hirae* Mur-2 enzyme displays

N-acetylglucosaminidase activity

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Abstract

Enterococcus hirae produces two autolytic enzymes named Mur-1 and Mur-2, both previously described as *N*-acetylmuramidases. We used tandem mass spectrometry to show that Mur-2 in fact displays *N*-acetylglucosaminidase activity. This result reveals that Mur-2 and its counterparts studied to date, which are members of glycosyl hydrolase family 73 from the CAZy (Carbohydrate-Active enZyme) database, display the same catalytic activity.

1. Introduction

Peptidoglycan is the component of the bacterial cell wall which protects the cell against internal osmotic pressure [1]. Peptidoglycan consists of glycan strands of alternating β -1,4-linked *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc) residues cross-linked to each other by short peptides made of L- and D-amino acids [2]. Throughout growth, the limited cleavage of the peptidoglycan molecule ensures the insertion of new precursors and separation of daughter cells after division [3]. The enzymes responsible for this process are potentially lethal and are referred to as autolysins. Autolysins display diverse enzymatic activities: *N*-acetylmuramoyl L-alanine amidases, endopeptidases (D,L- or L,D-endopeptidases), lytic transglycosylases and glycosyl hydrolases (GHs) [4]. The GHs include two types of enzymes: *N*-acetylglucosaminidases and *N*-acetylmuramidases (lysozymes) which cleave between MurNAc and GlcNAc, generating muropeptides carrying GlcNAc or MurNAc at the reducing end of the disaccharide, respectively. Based on amino acid sequences and structural analyses, the bacterial autolysins with GH activity have been listed in the CAZY (Carbohydrate-Active enZyme) database proposed by Henrissat (<http://afmb.cnrs-mrs.fr/CAZY/index.html>; [5]). Most of the bacterial *N*-acetylmuramidases which have been biochemically characterized belong to family 25 of the CAZY database (eg. LytC from *Streptococcus pneumoniae* [6], the mutanolysin from *Streptomyces globisporus* [7] or SleM from *Clostridium perfringens* [8]). As for the autolysins with *N*-acetylglucosaminidases activity, most of them belong to family 73 of the CAZY database (eg. AtlA from *Enterococcus faecalis* [9], AcmA from *Lactococcus lactis* [10], and LytG from *Bacillus subtilis* [11]). It is interesting to note that although family 73 contains more than 400 enzymes, very few of them have an experimentally determined activity.

In *Enterococcus hirae* ATCC 9790, two autolysins (Mur-1 and Mur-2) have been described. Whereas the gene encoding Mur-1 has not been identified yet, the *Mur-2* gene has

been cloned . It encodes a 666 residue-long autolysin consisting of a catalytic domain fused to a C-terminal cell wall targeting domain composed of 6 LysM modules [12, 13]. Mur-2 has been assigned to GH family 73 in the CAZy classification. It displays a common modular organization and a strong sequence similarity to three recently characterized *N*-acetylglucosaminidases (AcmA, LytG and AtlA; *e.g.* 77% identity between Mur-2 and AtlA). Surprisingly, the *E. hirae* Mur-2 autolysin has been reported to display *N*-acetylmuramidase [14]. We recently used a tandem mass spectrometry approach to demonstrate the *N*-acetylglucosaminidase activity of *E. faecalis* AtlA [9]. Using the same method, we reinvestigated the enzymatic activity of Mur-2.

2. Material and methods

2.1. Plasmid construction

The DNA fragment encoding residues 50 to 249 of Mur-2 was PCR amplified from *E. hirae* ATCC9790 chromosomal DNA using Vent DNA polymerase (Biolabs) and oligonucleotides Mur-2_up (5'-AAACCATGGATCAAACCCCCACACAATTCGGTGCC-3') and Mur-2_down (5'-TTTGGATCCGCCTGAGTTCCCCTAGTACTTC-3'). The PCR product was digested by NcoI and BamHI and cloned into pET2818 [9], giving pMur-2, encoding the Mur-2 catalytic domain fused to a C-terminal hexa-histidine tag.

2.2. Overexpression and purification of Histidine-tagged polypeptides

Plasmid pMur-2 was transformed into *Escherichia coli* BL21 DE3 (pREP4GroESL) [15]. Expression of the recombinant protein was induced in exponentially growing cells at 37°C by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside. The protein was purified under native conditions by immobilized metal affinity chromatography using Ni²⁺-nitrilotriacetate

agarose resin (Qiagen GmbH), followed by size exclusion chromatography on a Superdex 200 HR 10/30 column (Amersham biosciences).

2.3. Analysis of Mur-2 activity

The enzymatic activity of the Mur-2 catalytic domain was tested on purified peptidoglycan extracted from *Micrococcus lysodeikticus* ATCC4698 as previously described [9]. Various amounts of Mur-2 catalytic domain were tested to identify conditions in which the velocity of hydrolysis was proportional to enzyme concentration. One enzymatic unit corresponds to the decrease of 0.001 A_{450} units per minute per μ mole of protein.

2.4. Determination of Mur-2 specificity

To identify the peptidoglycan bond cleaved by Mur-2, we digested *M. lysodeikticus* peptidoglycan with the recombinant Mur-2 catalytic domain. The soluble muropeptides were reduced with sodium borohydride and separated by reverse-phase HPLC on a C18 column [16]. The muropeptide fractions were collected and analyzed by mass spectrometry (MS) and tandem mass spectrometry (MS/MS) with an electrospray time-of-flight mass spectrometer operating in the positive mode (Qstar Pulsar I; Applied Biosystems) as previously described [17]. The data were acquired with a capillary voltage of 5,200 V and a declustering potential of 20 V. The mass scan range was from m/z 400 to 2,500, and the scan cycle was 1 s.

3. Results and discussion

The catalytic domain of Mur-2 was overexpressed in *E. coli* and purified in a two step procedure including immobilized metal affinity chromatography and size exclusion chromatography. The final purification product eluted as a globular protein of 18.3 kDa, in agreement with the theoretical value of 22.2 kDa for a monomer (Fig. 1A). Purified Mur-2 was estimated to be more than 95% pure as estimated by SDS-PAGE (Fig. 1B). The yield of

the purification was 30mg/L. Previous work by Kawamura and Shockman addressed the substrate specificity of Mur-2 [18]. They showed that Mur-2 activity is very similar when assayed on peptidoglycan with different peptidoglycan cross-bridge structures, and that acetylation of the glycan strands had a limited impact on enzyme activity. As they showed that *M. lysodeikticus* was the most susceptible peptidoglycan tested, we tested the enzymatic activity of the Mur-2 catalytic domain on this purified peptidoglycan. In the conditions previously described, the purified catalytic domain of Mur-2 displayed peptidoglycan hydrolysis activity in the same range of order as that of the catalytic domain of *E. faecalis* AtlA (0.9 ± 0.1 U vs 4.85 ± 0.4 U, respectively).

The products of *M. lysodeikticus* peptidoglycan cleaved by the Mur-2 catalytic domain were reduced and separated on HPLC (data not shown). One of the most abundant muuropeptides with a monoisotopic mass of 1,025.51 matched the expected mass of a reduced disaccharide-hexapeptide (GlcNAc-MurNAc-L-alanyl- γ -D-glutamylglycine-L-lysyl-D-alanyl-D-alanine; DS-Penta[Gly]). To distinguish between *N*-acetylglucosaminidase and *N*-acetylmuramidase activity, we identified the sugar moiety carrying the reducing group on the $[M+H]^+$ ion at m/z 1,026.51 using tandem mass spectrometry (MS/MS) (Fig. 2). The fragmentation pattern revealed two abundant ions at m/z 803.40 and m/z 618.29, consistent with the loss of reduced GlcNAc (-223.1 mass units) and the loss of reduced GlcNAc plus the sugar moiety of the non-reduced MurNAc (-185.1 mass units). In contrast, when *M. lysodeikticus* peptidoglycan was digested with a commercially available muramidase (mutanolysin), the DS-Penta[Gly] revealed a distinct typical MS/MS fragmentation pattern in which the most abundant ion corresponded to the loss of a non-reduced GlcNAc residue (-203.1 mass units; [9]). These results demonstrate that Mur-2 displays *N*-acetylglucosaminidase, rather than *N*-acetylmuramidase activity. Further fragmentation of the DS-Penta[Gly] generated either by Mur-2 or mutanolysin (Fig. 2A and data not shown)

confirmed the structure of the *M. lysodeikticus* monomer suggested by Ghuysen *et al.* [19] and depicted in Fig. 2B.

Concluding remarks

Our characterization of the enzymatic activity of Mur-2 shows that this enzyme is an *N*-acetylglucosaminidase. Our study reveals that all the enzymatic activities of the members of GH family 73 of the CAZy database experimentally determined to date are *N*-acetylglucosaminidases. This study also suggests that *E. hirae* produces two distinct autolysins with glycosylhydrolase activity. The respective role of the corresponding enzymes remains to be investigated.

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FIGURE LEGENDS

Figure 1. Purification of Mur-2 catalytic domain. (A) Gel filtration of affinity-purified Mur-2 catalytic domain. The gel filtration experiment was carried out on a Pharmacia Superdex 200 column. The Standard proteins used for the calibration curve (inset) were thyroglobulin, ferritin, aldolase, BSA, carbonic anhydrase, cytochrome *c* and aprotinin. AU, absorbance units; V_e , elution volume; V_o , void volume. (B) SDS-PAGE analysis of purified Mur-2 catalytic domain. Lane 1, crude extract of BL21(DE3)(pREP4GroESL) transformed with pMur-2; lane 2, fractions eluted from the gel filtration column that contained purified Mur-2.

Figure 2. Determination of Mur-2 cleavage specificity by MS/MS. (A) The $[M+H]^+$ ion at m/z 1,026.51, corresponding to a *M. lysodeikticus* monomer generated by Mur-2, was analyzed by MS/MS. The m/z values of the most informative ions in the fragmentation pattern are boxed. (B) Structure of the muropeptide fragmented in (A). The one letter code is used for amino acids, lowercases referring to C-terminal residues. A, L-Ala or D-Ala; a, D-Ala; K, L-Lys; E, γ -D-Glu; g, Gly; MurNAC, *N*-acetylmuramic acid; GlcNAC^{red}, reduced *N*-acetylglucosamine. The fragmentation events generating the ions boxed in (A) are indicated by dotted arrows.