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Identification of active site serine and histidine residues in *Escherichia coli* outer membrane protease OmpT

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Abstract ***Escherichia coli*** **outer membrane protease OmpT has been characterised as a serine protease based on its inhibitor profile, but serine protease consensus sequences are absent. By site-directed mutagenesis we substituted all conserved serines and histidines. Substitution of His^{101} and His^{212} by Ala, Asn or Gln resulted in variant enzymes with 0.01 and 9–20% residual enzymatic activity towards a fluorogenic pentapeptide substrate, respectively. The mutations S140A and S201A did not decrease activity, while variants S40A and S99A yielded 0.5 and 0.2% residual activities, respectively. When measured with a dipeptide substrate the variant S40A demonstrated full activity, whereas variant S99A displayed at least 500-fold reduced activity. We conclude that Ser^{99} and His^{212} are essential active site residues. We propose that OmpT is a novel serine protease with Ser^{99} as the active site nucleophile and His^{212} as general base.**

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Key words: Active site; Serine protease; OmpT; Outer membrane protein; *Escherichia coli*

1. Introduction

OmpT (EC 3.4.21.87) is a 33.5 kDa protease of 297 amino acid residues present in the outer membrane of *Escherichia coli* [1,2]. The enzyme cleaves peptides and proteins preferentially between two consecutive basic amino acids [1]. OmpT has been suggested to be involved in urinary tract disease [3], in DNA excision repair [4] and in the breakdown of antimicrobial peptides [5], but the exact biological function remains to be elucidated.

Proteases are divided into four classes based on their catalytic mechanism: cysteine proteases, aspartic proteases, metalloproteases and serine proteases. OmpT lacks cysteines and consequently is not a cysteine protease. In all aspartic proteases known so far the amino acid sequence D(T/S)G is conserved [6] and all metalloproteases characterised to date contain the signature sequence HExxH or HxxEH [7]. OmpT does not contain either of these motifs. The fourth class comprises the serine proteases, which are subdivided into more than twenty families [8]. Consensus sequences surrounding the catalytic serine and histidine residues in the well-characterised chymotrypsin family (peptidase family S1; including trypsin, elastase and others) are GDSGg and tAaHc (showing absolutely conserved amino acids in capitals) [8]. For the large subtilisin family (S8) the conserved sequences are GTSma and nxHGT [8]. OmpT does not contain any of these classical serine protease consensus sequences, but several serine proteases are known which also lack these motifs, e.g. human cytomegalovirus [9]. Obviously, it is not possible to classify OmpT on basis of conserved sequences in the known classes. An alternative approach to protease classification is to study the susceptibility of the enzyme to various protease inhibitors. The proteolytic activity of OmpT is not or hardly inhibited by many common protease inhibitors such as the trypsin inhibitor tosyl-L-lysine chloromethyl ketone, the metalloprotease inhibitor EDTA and peptides like antipain and leupeptin, which are inhibitors of several aspartic and cysteine proteases [1,10,11]. Only at high concentrations did the serine protease inhibitors diisopropylfluorophosphate (DFP) and phenylmethylsulfonyl fluoride (PMSF) decrease OmpT activity significantly [1]. Based on this inhibitor profile, OmpT was classified as a member of a novel serine peptidase family called omptin (S18) [8]. Four proteins homologous to OmpT have been identified so far: PgtE of *Salmonella typhimurium* (mature part 49% identical to OmpT) [12], Pla of *Yersinia pestis* (50% identical) [13], OmpP of *E. coli* (72% identical) [14] and SopA of *Shigella flexneri* (60% identical) [15]. Alignment of OmpT with these homologues (not shown) demonstrated that Ser^{40}, Ser^{99}, Ser^{140}, Ser^{201}, His^{101} and His^{212} are conserved.

In this study we aimed at identifying the active site serine and histidine to provide experimental support for the classification of OmpT as a serine protease. Using site-directed mutagenesis, the four conserved Ser residues of OmpT are replaced by Ala and the two His residues are substituted by Ala, Asn and Gln. Membrane fractions of cells expressing the resulting variants are isolated and residual activities are measured using two different spectroscopic assays.

2. Materials and methods

2.1. Materials

DNA restriction enzymes were purchased from New England Biolabs. Oligonucleotides were bought from Amersham Pharmacia Biotech. Polyethyleneglycol *tert*-octylphenyl ether (Triton X-100) was obtained from Serva and polyoxyethylene sorbitanmonolaurate (Tween 20) was obtained from Bio-Rad. The chromogenic substrate IAA-Arg-Arg-pNA (IAA = indole-3-acetic acid, pNA = *p*-nitroaniline) was a generous gift of Dr. A.J. Slotboom (Utrecht University). The fluorogenic substrate Abz-Ala-Arg-Arg-Ala-Tyr(NO_2)-NH_2 (Abz = *o*-ami-

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Abbreviations: Abz, *o*-aminobenzoyl; DFP, diisopropylfluorophosphate; GAMAP, goat anti-mouse IgG alkaline phosphatase conjugate; IAA, indole-3-acetic acid; PMSF, phenylmethylsulfonyl fluoride; pNA, *p*-nitroaniline; Triton X-100, polyethyleneglycol *tert*-octylphenyl ether; Tween 20, polyoxyethylene sorbitanmonolaurate; Tyr(NO_2), 3-nitrotyrosine

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nobenzoyl, Tyr(NO_2) = 3-nitrotyrosine) was purchased from Research Genetics (Huntsville, USA). Anti-OmpT monoclonal antibodies were obtained by immunisation of a mouse with native wild-type OmpT, purified from *E. coli* as described by Sugimura and Nishihara [1]. Goat anti-mouse IgG alkaline phosphatase conjugate (GAMAP) was bought from Promega. All other chemicals were of analytical grade.

2.2. Bacterial strains and plasmids

The *E. coli* K-12 strain DH5α [16] was used in the cloning procedures. The *E. coli* strain BL21(DE3) [17] lacks the *ompT* gene and was used for expression. Plasmids pND9 and pND10 [18] are derivatives of pUC19 [19], containing the *ompT* gene including its own promoter sequence oriented clockwise and counterclockwise with the *lac* promoter, respectively.

Mutations were introduced into plasmid pND10 using PCR according to the manufacturers protocol provided with the QuikChange Site-Directed Mutagenesis Kit (Stratagene). To facilitate the screening for the mutations, silent mutations resulting in the introduction or deletion of restriction sites were made concomitantly with the amino acid mutations. All mutations except S40A, S140A and S201A were subcloned from the pND10 derivative into plasmid pND9 as follows. For mutations at position 99 or 101, the pND10-derived plasmids were digested with *Hin*cII and *Xcm*I, followed by cloning of the 257 bp fragment containing the desired mutation into *Hin*cII/*Xcm*I digested pND9. For mutations at position 212, the pND10-derived plasmids were digested with *Acc*I and *Afl*II, followed by cloning of the 580 bp fragment containing the desired mutation into *Acc*I/*Afl*II digested pND9. The sequences of the relevant parts of the constructed plasmids were checked by restriction enzyme analysis and DNA sequencing.

2.3. Expression of OmpT and isolation of membrane fractions

After transformation by the various plasmids, BL21(DE3) cells were grown overnight in 10 ml LB medium [20] with 50 μg/ml ampicillin at 37°C. The optical density at 600 nm was measured and cells corresponding to 6 ml culture with A_{600} of 1.0 were harvested by centrifugation. The pellet was washed with 0.15 M NaCl and resuspended in 1 ml 2 mM EDTA, 50 mM Tris, pH 8. Cells were disrupted by sonication at 0°C and intact cells were removed by short centrifugation (15 000 × *g* for 30 s). The supernatant was centrifuged at 15 000 × *g* for 15 min at 4°C and the resulting membrane pellet was resuspended in 200 μl buffer A (0.1% Triton X-100, 50 mM Tris, pH 7.5).

2.4. Analysis of OmpT in isolated membrane fractions

The total amount of protein in the isolated membrane fractions was determined using the Bio-Rad protein assay with bovine serum albumin as a reference. Samples of membrane fractions corresponding to equal amounts of total protein were mixed with loading buffer [20] and loaded on a 11% SDS–PAGE gel [21] either directly or after 5 min incubation at 100°C. After electrophoresis, proteins were transferred to a nitrocellulose membrane by Western blotting [22]. OmpT was detected using anti-OmpT monoclonal antibodies and GAMAP as described elsewhere [20].

2.5. Enzymatic activity assays

Samples of membrane fractions were diluted in buffer A to appropriate concentrations and OmpT activity was measured using two different spectroscopic assays.

The chromogenic substrate IAA-Arg-Arg-pNA was used in a colorimetric assay described elsewhere [18]. Assay conditions were 0.5 mM IAA-Arg-Arg-pNA, 1 mM Tween 20, 20 mM Mes, pH 7.0 and 0.1 U/ml aminopeptidase M. OmpT specific cleavage between the two arginines results in the liberation of Arg-pNA. This product is subsequently cleaved by aminopeptidase M resulting in the release of pNA, which is detected spectrophotometrically at 405 nm. The increase in absorbance was recorded and activities were determined from the linear part of the curves. Using $\varepsilon_{405} = 10\,200\ M^{-1}\ cm^{-1}$ for pNA [23], activities were calculated in enzymatic units U (= μmol substrate/min).

The internally quenched fluorogenic substrate Abz-Ala-Arg-Arg-Ala-Tyr(NO_2)-NH_2 was used in a fluorimetric assay as described previously [18]. Assay conditions were 6 μM substrate, 1 mM Tween 20, 5 mM EDTA, 10 mM Bistris, pH 6.5. Activity was measured in a fluorimeter using excitation and emission wavelengths of 325 and 430 nm, respectively. After recording the initial linear increase in fluorescence, 20 μg of trypsin was added to determine the fluorescence of completely hydrolysed substrate, which enabled calculation of the activity in enzymatic units.

3. Results

3.1. Expression of OmpT

BL21(DE3) cells expressing the active site variants of OmpT, wild-type OmpT or no OmpT were grown in triplicate and membrane fractions were isolated. Concentrations of total protein were determined and used as a measure for the amount of OmpT. To validate the assumption that expression levels of the OmpT variants were similar, Western blot analysis was performed with all samples containing equal amounts of total protein. No OmpT was detected in samples of BL21(DE3) transformed with plasmid pUC19 as expected (data not shown). In all other samples the major protein band corresponded to a protein with an apparent molecular mass of about 35 kDa, which corresponds to OmpT with a calculated molecular mass of 33,478 Da (Fig. 1). The expression level of the variants encoded by plasmid pND9 was on average approximately three times higher than that of variants encoded by pND10. This effect can be explained by the counterclockwise and clockwise orientation of the *lac* promoter with the *ompT* gene in pND9 and pND10, respectively. Wild-type and all variants were well expressed and the expression levels of variants encoded by the same plasmid were comparable within a factor of three. Although these differences were significant, they were acceptable for our purpose to identify active site mutations, since for such mutations a decrease in activity of at least 100 times was expected. A second band with low intensity was observed at approximately 31 kDa. This band has previously been shown to be a degradation product of OmpT, resulting from autoproteolytic cleavage between residues Lys^{217} and Arg^{218} [18]. When the samples were not boiled before loading, the apparent molecular mass of OmpT was about 29 kDa (data not shown). This so-called heat-modifiability is generally observed for outer membrane proteins [24] and occurs because the putative β-barrel fold of the protein is not denatured by the presence of SDS alone, resulting in a relatively fast migrating protein. The observation of this effect is a good measure for proper

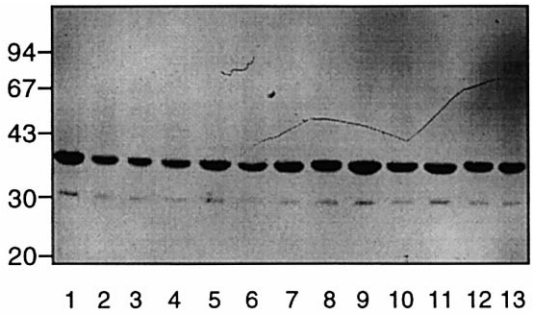

Fig. 1. Western blot analysis of OmpT in isolated outer membrane fractions. Samples corresponding to 0.5 μg of total protein were incubated for 5 min in loading buffer at 100°C. Lane 1, wild-type encoded by pND9. Lane 2, wild-type encoded by pND10. Lanes 3–6, variants S40A, S99A, S140A and S201A encoded by pND10. Lanes 7–13, variants S99A, H101A, H212A, H101N, H212N, H101Q and H212Q encoded by pND9. Numbers on the left refer to molecular masses of marker proteins.

folding and assembly in the outer membrane and all active site variants fulfilled this criterion.

3.2. Activity measurements of serine variants

The enzymatic activity of OmpT in the isolated membrane fractions was measured with a colorimetric assay. For all variants, membrane fractions of three independent cell cultures were measured. The average activities are shown in Table 1. When only buffer was added, a background increase in absorbance could be recorded. This apparent activity was equal to that of membrane fractions from BL21(DE3)(pUC19) cells (no OmpT expression) and corresponded to a value of 5.3 ± 0.4 U/g total protein. The activity of wild-type OmpT encoded by plasmid pND10 was 2.3-fold lower than that of wild-type encoded by pND9, which is in agreement with the difference in expression levels (Fig. 1). We therefore used the activity of wild-type OmpT encoded by plasmid pND10 as a reference for variants encoded by pND10 derivatives (and correspondingly for plasmid pND9). No activity was detectable for OmpT variant S99A (in plasmid pND10), whereas activity comparable to that of wild-type was measured for mutant proteins S40A, S140A and S201A. Since activity was maintained for the latter three variants, Ser^{40}, Ser^{140} and Ser^{201} were concluded not to be active site residues. A sequenced fragment of the gene carrying the S99A mutation was subcloned into plasmid pND9 and no activity was detectable for the S99A variant encoded by this plasmid as well (Table 1), confirming that Ser^{99} is the active site serine. In order to determine the activity of the serine variants more accurately, a sensitive fluorimetric assay was used. The measured activities of the S140A and S201A variants in this assay were consistent with those in the colorimetric assay (Table 1). The residual activity of the S99A variant was 0.2%, in agreement with the identification of Ser^{99} as an active site residue. Interestingly, the residual activity of the S40A variant in this assay was only 0.5%.

3.3. Activity measurements of histidine variants

All histidine mutations were sequenced and subcloned into plasmid pND9 to rule out the presence of additional mutations affecting enzymatic activity. In the colorimetric assay, no activity was detectable when His^{212} was replaced by Ala, Asn or Gln (Table 1). Although activities were low for the His^{101} variants, they were significantly above the background with highest activity for H101Q (1.0%). Since replacement of His^{101} and His^{212} both resulted in a large decrease in activity, measurement of the variants in the more sensitive fluorimetric assay was necessary to identify the active site histidine. As shown in Table 1, substitution of His^{101} resulted in activities that were only five- to ten-fold lower than wild-type activity. In contrast, the activities of variants with Ala, Asn or Gln at the position of His^{212} were at least 10 000-fold lower than that

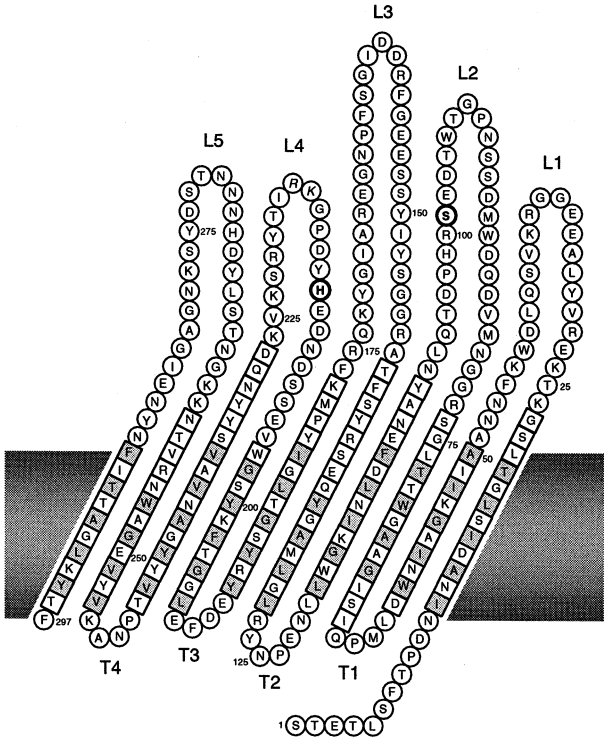

Fig. 2. Topology model for *E. coli* OmpT (adapted from [18]). Numbers refer to the amino acid positions in mature OmpT. Residues in β-strands are shown in squares; other residues are in circles. Hydrophobic β-strand residues pointing to the lipid phase are shaded. The grey area represents the approximate position of the membrane relative to the protein. The N-terminus and the short turns (T1–T4) are localised in the periplasm and the large loops (L1–L5) face the extracellular side of the membrane. Active site residues Ser^{99} and His^{212} are printed bold. The autoproteolytic cleavage site Lys^{217}-Arg^{218} is given in italics.

Table 1
Activities of OmpT and its variants measured in two different spectroscopic assays

OmpT	Plasmid	Activity in colorimetric assay (U/g total protein)	Activity in fluorimetric assay (U/g total protein)
None	pUC19	5.3 ± 0.4	0[a]
Wild-type	pND10	120.0 ± 6	12000 ± 4000
S40A	pND10	243.0 ± 34	60 ± 17
S99A	pND10	5.3 ± 0.2	26 ± 6
S140A	pND10	33.0 ± 6	12000 ± 7000
S201A	pND10	142.0 ± 25	18000 ± 11000
Wild-type	pND9	275.0 ± 73	48000 ± 24000
S99A	pND9	5.3 ± 0.3	41 ± 17
H101A	pND9	6.3 ± 0.3	4500 ± 1500
H101N	pND9	6.0 ± 0.6	6700 ± 1400
H101Q	pND9	8.0 ± 0.2	9700 ± 2900
H212A	pND9	5.3 ± 0.4	5 ± 2
H212N	pND9	5.5 ± 0.3	3 ± 2
H212Q	pND9	5.3 ± 0.1	4 ± 2

[a]No detectable increase in fluorescence.

of wild-type. These results show that His^{212} is an active site residue.

4. Discussion

The inhibitor profile of OmpT [1,10,11] suggested that the enzyme is a serine protease and in this study we tested that hypothesis by site-directed mutagenesis of all conserved serine and histidine residues. Single mutant proteins of OmpT were made with conserved serine residues replaced by alanine or with conserved histidine residues substituted by alanine, asparagine or glutamine. Due to variations in expression levels for the different mutant proteins there was a considerable spread in the measured activities (Table 1). However, for the aim of active site residue identification this was not a major drawback. By SDS–PAGE and Western blotting it was shown that all variants were folded correctly.

Two different spectroscopic assays were used to measure the residual enzymatic activities of the OmpT variants. For variants in which Ser^{40} or His^{101} was replaced the outcome of the two assays showed high variability. Variant S40A displayed 207 and 0.5% activity in the colorimetric and the fluorimetric assay, respectively. In contrast, variants with His^{101} substituted by Ala, Asn or Gln demonstrated on average about 30-fold higher relative residual activity in the fluorimetric assay. When the assays are compared, two important differences can be noted. Firstly, it is clear from Table 1 that the absolute activities for equal amounts of wild-type OmpT are two orders of magnitude higher in the fluorimetric assay. Secondly, the fluorogenic substrate is larger than the chromogenic substrate, with six and three backbone carbonyl moieties, respectively. These differences are illustrated by our recent findings [18] that the kinetics of wild-type OmpT in the colorimetric assay were characterised by low affinity and activity (K_M = 0.5 mM, $k_{cat} = 0.4\ s^{-1}$), whereas these parameters were substantially better in the fluorimetric assay ($K_M = 0.3\ \mu M$, $k_{cat} = 38\ s^{-1}$). Substitution of an amino acid in an enzyme can lead to a loss of activity because the original residue participates directly in enzymatic catalysis. Alternatively, the substituted residue may be involved indirectly via interactions with the substrate and we propose that this is the case for residues Ser^{40} and His^{101} of OmpT. We hypothesise that the side chain of Ser^{40} interacts with the N- or C-terminus of the large fluorogenic substrate, which can explain the large loss of activity for the S40A variant in the fluorimetric assay. In the colorimetric assay Ser^{40} would not be able to interact with the shorter substrate, which explains why the activity is not affected in this assay. Furthermore, we believe that His^{101}, being only two residues away from Ser^{99}, may be required for correct positioning of the substrate into the active site to make the Arg-Arg peptide bond optimally accessible for the nucleophilic attack by Ser^{99}. This productive binding may be achieved more easily for the larger fluorogenic substrate because of the potential additional interactions. In this respect it is interesting to note that the residual activity of variant H101Q was substantially higher than that of variant H101A. This suggests that glutamine can take over the hydrogen-bonding capacity of the histidine residue, indicating that His^{101} may be involved in substrate interaction indeed. Additional studies are needed to confirm our hypotheses, preferably by solving the crystal structure of OmpT in complex with a substrate analogue.

In contrast to the conflicting results of the two assays for replacement of Ser^{40} and His^{101}, the results of both assays were consistent for all other mutations (Table 1). We conclude that Ser^{99} and His^{212} are active site residues of OmpT. We propose that Ser^{99} performs the nucleophilic attack on the scissile peptide bond of the substrate, catalysed by residue His^{212} that acts as a general base by abstracting a proton from the hydroxyl group of Ser^{99}. However, other possibilities for the exact function of the active site serine in OmpT should not be ruled out, since the residual activity of OmpT variant S99A was relatively high (0.2%) when compared to variants of other serine proteases in which the active site serine is substituted. Removal of the catalytic serine in subtilisin for example reduced the k_{cat} value of the protease 10^6 times [25]. Most serine proteases have an active site composed of a Ser-His-Asp/Glu catalytic triad [8], in which the carboxylate group of the third member stabilises the positive charge of the protonated histidine. Within the omptin family eleven acidic amino acids are conserved which are candidates for being the third member besides Ser^{99} and His^{212}. Identification of this residue was beyond the scope of the present study, but is currently pursued.

Active site residue His^{212} of OmpT is located close to residues Lys^{217} and Arg^{218}, which form an autoproteolytic cleavage site in OmpT. Mutation of these residues to stabilise the protein led to significant losses in enzymatic activity [18]. We now understand these observations by reasoning that the mutations could have disturbed the active site conformation and/or substrate binding.

We previously proposed a β-barrel topology model for OmpT [18]. The recently published crystal structure of the homologous *E. coli* OmpX protein [26] (with 31 of the 148 amino acids of mature OmpX identical to OmpT) enabled us to refine this model (Fig. 2). The active site residues Ser^{99} and His^{212} are positioned in two different extracellular loops, restricting the folding of both loops because of the required close proximity between these two residues. The extracellular location of the active site of OmpT is experimentally supported by the observation that T7 RNA polymerase and initiation factor IF2α were degraded by OmpT when added to intact *E. coli* cells [27,28]. Furthermore, OmpT activity was also observed when intact *E. coli* cells were measured in our colorimetric assay (unpublished results). At present, the only outer membrane enzyme for which the active site and the crystal structure are known is *E. coli* outer membrane phospholipase A [29–31]. Interestingly, the active site serine (Ser^{144}) and histidine (His^{142}) of this enzyme are located on one of the β-strands, separated by only one amino acid. This indicates that at least two possibilities exist for the active site architecture of serine hydrolases that reside in the outer membrane of Gram-negative bacteria.

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