Heterologous expression of peptidyl-Lys metallopeptidase of *Armillaria mellea* and mutagenic analysis of the recombinant peptidase

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A method to express, purify and modify the Peptidyl-Lys metallopeptidase (LysN) of *Armillaria mellea* in *Pichia pastoris* was developed to enable functional studies of the protease. Based on prior work, we propose a mechanism of action of LysN. Catalytic residues were investigated by site-directed mutagenesis. As anticipated, these mutations resulted in significantly reduced catalytic rates. Additionally, based on molecular modelling eleven mutants were designed to have altered substrate specificity. The S₁' binding pocket of LysN is quite narrow and lined with negative charge to specifically accommodate lysine. To allow for arginine specificity in S₁', it was proposed to extend the S₁' binding pocket by mutagenesis, however the resulting mutant did not show any activity with arginine in P₁'. Two mutants, A101D and T105D, showed increased specificity towards arginine in subsites S₂'–S₄' compared to the wild type protease. We speculate that the increased specificity to result from the additional negative charge which attract and interact with positively charged residues better than the wild type.

Keywords: enzyme kinetics/enzyme mechanism/metalloprotease/molecular modelling/site-directed mutagenesis.

Abbreviations: Am-LysN, *Armillaria mellea* peptidyl-Lys metallopeptidase; DsbA, disulfide-oxidoreductase; FRET, Förster resonance energy transfer; GAP, glyceraldehyde-3-phosphatedehydrogenase; IPTG, isopropylβ-D-1-thiogalactopyranoside; MOE; Molecular Operation Environment (CCG); MOPS, 3-(N-morpholine)propanesulfonic acid; MS, mass spectrometry; PCR, polymerase chain reaction; RMSD, root-mean-square deviation; SDS–PAGE, sodiumdodecylsulfate–polyacrylamide gel electrophoresis.

In this article, we use Schechter and Berger nomenclature (1).

Peptidyl-Lys metallopeptidase (LysN) from *Armillaria mellea* is an aspzincin metalloprotease, which cleaves in front of lysines (2). Hence, it is unusual by having its substrate specificity at the C-terminal side of the scissile bond (/K) (3). The protease is predominantly used as a tool in proteomic studies, where it serves as an alternative or complement to trypsin. The advantage of LysN is that it generates fragments which have a positive charge at the N-terminus, yielding mass spectrometry (MS) fragmentation with clear b-ion ladders (4, 5). This feature can be used to facilitate de novo peptide mapping. Recently we profiled the substrate specificity of Am-LysN and found it to be strictly lysine specific at S₁', and having decreasing specificity when moving away from the scissile bond (2, 6).

We also reported a homology model of Am-LysN based on the crystal structure of Gf-LysN (7). The two structures had a RMSD of 0.001 on backbone residues and 0.239 on the side chain residues (2). In this model Am-LysN has a narrow, and relatively short (5–7 amino acid) binding cleft. Central to the hydrolysis of peptides, is the zinc ion and the zinc binding motif 'HExxH + D'. Fushimi *et al.* showed that the aspartic acid D132 is necessary to coordinate zinc binding of the homologous protein deuterolysin (8). A 7-amino acid peptide was fitted into the binding cleft of Am-LysN using molecular dynamics (2). We found two aspartic acids and two glutamic acids forming a negatively charged cavity causing the strict lysine specificity. Binding of an arginine's guanidinium group to the E159 of the active site, distorts the peptide backbone away from the zinc, rendering the substrate unsuited for catalysis. This homology structure served as the basis for protein engineering. The specificity of LysN limits the application possibilities, and for this reason we aim to alter the specificity of LysN.

In the present study we have developed an expression system of Am-LysN in *Pichia pastoris*. We report cloning and expression of rAm-LysN in *Escherichia coli* strain BL21 (DE3) and *P. pastoris*. The homologous Gf-LysN has previously been expressed in *P. pastoris*, but in low quantities of 100 ng from an undisclosed volume (9). Our expression system in *P. pastoris* yields approx. 0.25 mg/L of active protease after one-step purification. The strict lysine specificity at P₁' was preserved in the recombinant rAm-LysN.

Nine site-directed mutants were designed to investigate catalytically important residues while eleven single substitution mutants were constructed aiming to make mutant proteases with altered substrate specificity. Activity of recombinant proteases was assayed by measuring hydrolysis of fluorogenic peptides (2, 10).

Materials and Methods

All genes and primers used for generating plasmids for *P. pastoris* were ordered from MWG Eurofins and codon optimized for expression in *P. pastoris*. Genes and subcloning for *E. coli* was ordered from Genescript and codon optimized for expression in *E. coli*.

Structural modelling of LysN and molecular dynamics in MOE

The homology model of Am-LysN was built in MOE (2, 11). Engineering of the protease was done in the presence of fitted substrates, and new molecular dynamic simulations based on the previously optimized model were run for 1 h at 350 kelvin to accommodate the changes (2).

Construction of expression plasmids

To construct pAAR1, pAAR2 and pAAR3 the genes encoding DsbA-fused LysN, Propeptide fused LysN or the mature LysN were optimized for *E. coli* and subcloned by Genescript. The *lysN* was under control of the T7 promoter and is flanked by the T7 terminator (12). pAAR4, pAAR5, pAAR6 were constructed by cloning the genes into a vector under control of the *P. pastoris* GAP promoter and flanked by a GAP terminator (13, 14).

Site-directed mutagenesis of LysN

Site-directed mutagenesis was performed by the method of Lei *et al.* (15). Mutations were done in a one-step manner using the expression vector pAAR4 as template. Mutagenic primers are listed in Supplementary Table S1, see Supporting Information. All mutations were verified by DNA sequencing before transformation into *P. pastoris*, using primers SeqA and SeqB.

Expression of Am-LysN in *E. coli*

Constructs pAAR1-pAAR3 were transformed into *E. coli* BL21(DE3) and the transformed cells were grown in LB medium containing 100 mg/L ampicillin at 37°C in a shaking incubator with shaking of 200 rpm. Protein expression was induced at a cell density of $A_{600} = 1.2$ with 1 mM of isopropyl- β -thiogalactopyranoside (IPTG, Sigma) and incubated for 4 h. Cells were harvested by centrifugation at 4,500 g for 15 min at 4°C. Cells were suspended in 20 mM Tris-HCl, pH 8 and disrupted by 5 min in a SonoPuls HD 3100 (Bandelin) sonicator at 40% amplitude by 10 s on-off cycles. The sonicated cell suspension was centrifuged at 10,000×g for 10 min. The supernatant was saved, and the pellet comprising of inclusion bodies was dissolved in 20 mM Tris-HCl, pH 8. SDS-PAGE analysis showed that LysN was only present in the inclusion bodies. To express LysN in the soluble fraction, cells were cultivated in LB medium containing ampicillin at 25°C. Protein expression was induced at a cell density of A_{600} 0.8 with 0.1 mM IPTG. Approx. three h after incubation the cells stopped growing and after 24 h the final A_{600} was at 2, while the uninduced control was A_{600} of 4.8. Analysis of expression was done by SDS-PAGE and Western blotting.

Transformation of *Pichia pastoris* CBS 704

Electrocompetent *P. pastoris* CBS 704 cells were prepared as described by Letchworth *et al.* (16). A total of 5 μ g of *SapI*-linearized plasmids were transformed into *P. pastoris* strain CBS 704 by electroporation using GenePulserXcell (Biorad). Immediately after the pulse, 1 mL of ice-cold YPD 1 M sorbitol was added to the cells and was subsequently incubated for 4 h at room temperature. 100 μ L of transformants were spread on YPD plates containing 100 mg/L Zeocine. The plates were incubated at 30°C until colonies appeared (3–4) days. To verify the gene integrated into the *Pichia* genome, colonies were picked up and grown in 5 mL of YPD-100 mg/L Zeocine overnight and genomic DNA was isolated as described by

Løoke *et al.* (17). PCR amplifications were carried with 2 μ L of the genomic DNA using primers SeqA and SeqB.

Expression in and purification of Am-LysN from *P. pastoris*

Expression levels of yAAR4-yAAR6 were tested by 100 mL batch cultivations in a minimal medium containing 6% glucose. Cells were cultivated for 72 h with an initial pH of 5.4, 30°C and 280 rpm. Cells were removed from the culture medium by centrifugation at 3,000×g for 30 min. The supernatant was concentrated by ultrafiltration (Vivaspin 20) approx. 5 times and washed with 50 mM Na₂PO₄ 300 mM NaCl pH 8 by 10 kDa cut-off filters (GE Healthcare). The whole procedure was done at 4°C. The concentrated medium was then loaded to a column packed with Ni-NTA agarose (QIAGEN) pre-equilibrated with 50 mM Na₂PO₄ 300 mM NaCl 10 mM imidazole pH 8. The column was washed three times with 50 mM Na₂PO₄ 300 mM NaCl 20 mM imidazole pH 8 and bound protease was eluted twice with 1 mL 50 mM Na₂PO₄ 300 mM NaCl 500 mM imidazole pH 7. The eluate was subsequently concentrated to 100 μ L and buffer exchanged with 50 mM MOPS pH 7 by 10 kDa cut-off filters (Milipore). A batch cultivation of yAAR4 of four liters was cultivated in a minimal medium containing 6% glucose. Cells were cultivated for 72 h at pH 5.4 30°C 400 rpm. Cells were removed from the culture medium by centrifugation at 3,000 g for 30 min. Approximately three liters of culture medium was diluted 4× in 50 mM sodium phosphate 300 mM NaCl 10 mM Imidazole. The diluted culture medium was applied to a 5 mL Excel Ni-sepharose, washed with 50 mM sodium phosphate 300 mM NaCl 30 mM Imidazole, and eluted with 50 mM sodium phosphate 300 mM NaCl and 500 mM Imidazole. The eluate was buffer exchanged to 50 mM MOPS pH 7. A single colony of each mutant was cultured in 100 mL of medium, and purification was done as described above.

Synthesis and purification of fluorogenic peptides

Synthesis of fluorogenic peptides was carried out in Ødum *et al.* (2). The reference peptide Pep1, has the sequence of K(Abz)-S-A-Q-K-M-V-S-K(Dnp).

Protein quantification and enzyme activity assays

The protein concentrations were determined by micro BCA assay (Thermo Scientific) following the manufacturers' instructions (18). A set of protein standards of known concentration was prepared by diluting bovine serum albumin supplied in the kit. Each mutant (10 μ L) was used to measure protein concentration against the standard. The enzymatic hydrolysis of the FRET-peptides by recombinant Am-LysN was performed in 20 mM MOPS pH 7.5. Each reaction was performed in a total volume of 200 μ L reaction mixtures, with a concentration of FRET-peptide of 2, 4, 8 and 16 μ M. The reactions were initiated by addition of protease to the reaction solution, and hydrolysis of the FRET peptides were quantified using an Enspire Multimode Plate Reader (Perkin Elmer) spectrofluorometer measuring the emission of Abz at 420 nm with excitation at 320 nm. Initial rates of hydrolysis were determined and fitted to the Michealis-Menten equation by nonlinear regression (GraphPad Prism 5).

N-terminal sequencing of rAm-LysN

5 μ L of the purified enzyme was injected into Waters Acquity UPLC-HRMS system. The enzyme was eluted to a Waters Synapt G2 HDMS mass spectrometer using aqueous 0.05% TFA and as B eluent: 0.04% TFA in MeCN with a linear gradient from 10% to 95% eluent B over a period of 15 min. The LC column used was a Waters Acquity BEH C4 (1×150 mm; 1.7 μ m; 300 Å) with a flow rate of 0.1 mL/min and column temperature of 60°C. MSE fragmentation was obtained in the source at and elevated cone voltage ramped from 100 to 150 V for increased sensitivity. From the nil fragmentation trace the intact mass was deduced by MaxEnt1 deconvolution with a Gaussian peak width of 1 Da. The fragmentation trace was deconvoluted with MaxEnt3 at a resolution setting of 1e4 and using the peptide algorithm. The C-terminal part until the y43-ion, Asp132, was observed confirming the sequence.

Results and Discussion

Previous studies of Am-LysN have primarily focused on its specificity and its use in proteomics (2, 4, 6, 19,

Table I. List of plasmids used in this study

Strain name	Plasmid	Host	Promoter	N-terminal fusion	C-terminal fusion
eAAR1	pAAR1	<i>E. coli</i> BL21(DE3)	T7	N/A ^a	His ₆
eAAR2	pAAR2	<i>E. coli</i> BL21(DE3)	T7	DsbA-DDDDK	His ₆
eAAR3	pAAR3	<i>E. coli</i> BL21(DE3)	T7	<i>Am-LysN</i> Propeptide-DDDDK	His ₆
yAAR4	pAAR4	<i>P. pastoris</i> (CBS704)	GAP	OST1- <i>Am-LysN</i> Propeptide-KR	His ₆
yAAR5	pAAR5	<i>P. pastoris</i> (CBS704)	GAP	OST1-Mating factor α – KR	His ₆
yAAR6	pAAR6	<i>P. pastoris</i> (CBS704)	GAP	OST1-Mating factor α – KR – <i>Am-LysN</i> propeptide-KR	His ₆

^aNot available.

20). In this study, we successfully expressed *Am-LysN* as an active recombinant enzyme in *P. pastoris*. The purified enzyme was characterized with respect to substrate specificity, pH and NaCl dependency. Heterologous expression allows sufficient amounts of recombinant enzyme to be purified and analyzed. Table I lists the plasmids used in this study. The primary sequence of *Am-LysN* was acquired from the MEROPS database, <http://merops.sanger.ac.uk/cgi-bin/aaseq?mernum=MER005595> (21).

Strategy and expression of *Am-LysN* in *E. coli*

For *E. coli*, we constructed three expression vectors, pAAR1, pAAR2, pAAR3. T7 promoter was used to control expression. In pAAR1 the open reading frame encoded just the mature *Am-LysN* with the addition of a C-terminal His₆ tag. In pAAR2, the bacterial disulfide-oxidoreductase DsbA was fused to the N-terminus of *Am-LysN* linked with an enterokinase cleavage sequence DDDDK, and a His₆ tag on the C-terminal of *Am-LysN*. DsbA is known to facilitate folding of disulfide bridges (22). In pAAR3, the DsbA was replaced with the native propeptide of *Am-LysN*.

For the related metalloprotease thermolysin, the propeptide is thought to be essential for expression and refolding in *E. coli* (23). Plasmids pAA01/pAA02/pAA03 was transformed into *E. coli* BL21 (DE3) and expression of r*Am-LysN* was induced with 1 mM IPTG. SDS–PAGE analysis of the total cellular proteins showed an extra band corresponding to the anticipated size of the recombinant protease, which was not present in uninduced total cellular proteins. However it was not possible to measure any enzymatic activity of the expressed proteins, and fractionation analysis showed the recombinant protease in all cases was insoluble and formed inclusion bodies. Refolding of inclusion bodies was not pursued in this study. In an attempt to facilitate better folding, the incubation temperature was lowered to 25°C and induction concentration of IPTG lowered to 0.1 mM. The cells harboring pARE1 now stopped growing three h after induction. We speculate that the r*Am-LysN* folded correctly thus becoming toxic. It was not possible to capture and purify the protease at this point.

Strategy, expression and characterization of r*Am-LysN* in *P. pastoris*

In the *P. pastoris* expression track, three expression vectors were constructed, pAAR4, pAAR5 and pAAR6. In all three constructs the glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter was used to drive expression (13, 14). To ease the purification and to minimize

internal proteolytic activity the constructs were designed for secretion. All three constructs contained the signal sequence of *S. cerevisiae* OST1, a signal sequence known to result in co-translational translocation into the ER (24). The function of the *Armillaria mellea* propeptide has not been definitively determined, but it was predicted by PSORT (psort.hg.jp) that it would be secreted. The pAAR4 contained the native full length coding sequence, except for the signal sequence, with addition of a C-terminal His₆ tag. For cloning reasons the C-terminal serine was removed and an N-terminal serine was introduced. The amino acid numbering in this paper is in relation to the added N-terminal amino acid. The native full length sequence includes a Kex2p site (KR) between the propeptide and the mature enzyme. pAAR5 had the native *Am-LysN* propeptide exchanged with the propeptide of mating factor alpha pheromone of *S. cerevisiae*. As with pAAR4, a C-terminal His₆ tag was introduced. The construct pAAR6 was a fusion of pAAR4 and pAAR5, by linking the native propeptide to the propeptide of mating factor alpha pheromone.

The kinetic parameters, k_{cat} , K_M and k_{cat}/K_M were determined to be 26.54 s⁻¹, 4.3 μ M and 6.17 μ M⁻¹ s⁻¹ respectively by plotting reaction velocity V_0 as a function of substrate concentration [S] for the reference substrate Pep1, visualized in Fig. 1a. These values are similar to those of the native *Am-LysN* (2). The specificity of the recombinant r*Am-LysN* was assessed by measuring relative activity against 19 FRET-peptides with substitutions of lysine on position P₁' of the reference peptide, Pep1 K(Abz)-S-A-Q-K-M-V-S-K(Dnp), summarized in Fig. 1b. The recombinant *Am-LysN* exhibits strict lysine specificity, exactly like the native enzyme (2). The pH profile and NaCl profile of r*Am-LysN* was determined with Pep1, and was very similar to that of the native *Am-LysN*, with optimal activity at pH 7.5 and 0 mM of NaCl, see supporting information.

Plasmids pAAR4, pAAR5 and pAAR6 were transformed into *P. pastoris*. The protease was purified directly from the culture medium on Ni-NTA column with a yield of 0.25 mg/L. The purified proteases had an apparent molecular mass of 20 kDa on SDS–PAGE similar to the native protease, visualized in lane 2 of Fig. 2. A screening of 48 clones of both yAAR4 and yAAR6 showed that the expression levels of yAAR4 were in general higher than yAAR6. In light of this the native propeptide of *Armillaria mellea*, yAAR4, was used for further studies. The N-terminus of r*Am-LysN* was analyzed by tandem mass spectrometry and

Fig. 1 (A) Plot of the reaction velocity as a function of substrate concentration of Pep 1. This plot was used to calculate kinetic constant of K_M , k_{cat} and k_{cat}/K_M of the recombinant wildtype *Am*-LysN. (B) Substrate specificity of *rAm*-LysN, determined by hydrolysis of 19 FRET peptides. The x-axis indicates the 19 substituted amino acids in position P₁' of the substrate, given in the header. *rAm*-LysN exhibits strict lysine specificity.

Fig. 2 SDS-PAGE analysis of *rAm*-LysN after Ni⁺ purification stained with Instant Bluecoomassie stain. Lane 1: Seeblue 2 plus ladder. Lane 2: Native *Am*-LysN purified from the fruiting bodies of *Armillaria mellea*. Lane 3: Seeblue 2 plus ladder. Lane 4: Wild type *rAm*-LysN. Lane 5: M2. Lane 6: M3. Mutations are listed in Table II (2).

was found to be Ser-Ile-Ser-Tyr-Gln. The mass spectrometry also confirmed the full molecular size to be as expected. This is in accordance with the expected Kex2p site of the propeptide. Genome sequencing of *Armillaria mellea* reveals a homologous gene to that of *KEX2* of *S. cerevisiae*, further supporting that this is the activation pathway (25).

In order to investigate whether *rAm*-LysN expressed in *P. pastoris* has autocatalytic activity, we designed a double and triple mutant, M2 and M3. The double mutant had the native Kex2p site substituted from KR to QN and an additional Kex2p site seven amino acids upstream substitution from RR to RN. The triple mutant had additionally substitution of the catalytic glutamic acid E120, in the zinc binding motif to an asparagine. Mutants are listed in Table II. Mutants were purified in a similar manner with yields spanning from 0.20 mg/L to 0.63 mg/L, enough to study the activity. The triple mutant M3 had an apparent molecular mass

Table II. Mutations in the propeptide to investigate the processing of *Am*-LysN

Mutant name	Mutations	Apparent processed size
Wildtype	None	20 kDa
M2	KR→QN, RR→RN	15 kDa, 20 kDa, 28 kDa, 31 kDa, 36 kDa
M3	KR→QN, RR→RN, E120Q	36 kDa

of 36 kDa as visualized in lane 4 in Fig. 2, which corresponds to the full length proenzyme. The double mutant M2 however, had a band corresponding to the wild type protease at 19 kDa, a band at 15 kDa corresponding to free propeptide, and weak bands at 28 kDa and 31 kDa, which coincide with lysines in the propeptide, as visualized in lane 3 in Fig. 2. Thus, the M2 mutant appears to slowly auto activate after purification. These findings suggest the possibility of autocatalytic activity of LysN, but the main activation pathway is still likely to be the Kex2p in both yeast and *Armillaria mellea*.

Characterization of site-directed mutants of *rAm*-LysN

Am-LysN is part of the M35 family of the MEROPS classification. Sequence alignment of the two homologs *Am*-LysN and *Gf*-LysN show a similarity of 60.4%. When aligned with two other family members, deuterolysin and penicillolysin, the overall similarity falls to 17.7%. The alignment is presented in Fig. 3 and indicates residues which were targeted by site-directed mutation, where the red circles indicate residues involved in the active site, and the blue circles indicate residues which were mutated to change the specificity of *rAm*-LysN.

Mechanism of hydrolysis and catalytic site mutations.

Am-LysN is a zinc metallopeptidase, where the zinc acts as a catalyst and stabilizer of intermediates in the hydrolysis of peptide bonds. *Am*-LysN has three zinc coordinating residues His119, His123 and Asp132 while two water molecules act as fourth and fifth zinc

Deuterolysin	-TEVTDCKGDAESSLTALSNAAKLANQAAEAESGDES--KFEEYFKTTDQQTTRTTVAE 57
Penicillolysin	RTKETCSNASRKSALEKALSNTVKLANAAATAARSGSAS--KFSEYFKTTSSSTRSVVAA 58
<i>Am</i> -LysN	SISYNGCTSSRQTTLVSAAAAAQTYAQSSYNLYSSHTASTTRYVTWFGPYTSARHSTVLS 60
<i>Gf</i> -LysN	--TYNGCSSEQSALAAAASAAQSYVAESLSYLQHTAATPRYTTWFGSYISSRHSTVLQ 58
	 : : * * : : . . : : : : : * . . : : *
Deuterolysin	RLRAVAKEAGSTSGGSTTYHCNDPYGYCEPNVLAYTLPSKNEIANCDIYSELPLLAQKC 117
Penicillolysin	RLEAVAKEAQSSSGSTTYCSDTLGYCETNVLAYTLPARNIANCDIYSYLPALAGTC 118
<i>Am</i> -LysN	CFSNMLAYPYANYEYDCTCTESDVYAYVYPSQFG-----TIYLCGAFWQTT-----TT 108
<i>Gf</i> -LysN	HYTDMNSNDFSSYSFDCTCTAAGTFAYVYPNRFG-----TVYLCGAFWKAP-----TT 106
	: : . * . . * . . : : * . : : .
Deuterolysin	HAQDQATTTLHEFTHAPGVYQPGTEDLGYGYDAATQLSAQD---ALNNADSYALYANGKH 174
Penicillolysin	HQQDQATTTLHEFTHAPGVYSPGTDLLGYGYSAATSLSSSQ---AVLNADSYALYANAIN 175
<i>Am</i> -LysN	GTDSRGGTLIHSSHFT--IICGTQDYAYGQSAAKSLASSNPSEAIKNADNYEYFAENNP 166
<i>Gf</i> -LysN	GTDSQAGTLVHSSHFT--RNGGTDYAYGQAAAKSLATMDPKAVMNADNHEYFSENNP 164
	: : . * : * : * . * . * . * . * . * . : : * : * . : : :
Deuterolysin	V-- 175
Penicillolysin	LGC 178
<i>Am</i> -LysN	AQ- 168
<i>Gf</i> -LysN	AQS 167

Fig. 3 Sequence alignment of deuterolysin, penicillolysin, *Gf*-LysN and *rAm*-LysN. All proteases are members of the aspzincin family M35. *Gf*-LysN and *Am*-LysN are homologous sharing approximately 60% of sequence identity. '.' A period indicates weakly conserved amino acids, while ':' colon indicates strongly similar amino acids and '*' asterisk indicates fully conserved residues. The residues M65, D83, V88, A101, T105, Q131, Q137, E159 in white on black background are residues which are mutated in relation to the substrate specificity. The residues Y85, E120, H123, Thr130, D132, Y135, G136 and D156 in black on gray background are mutated in relation to the catalytic site.

ligand. The fourth ligand, the water molecule, makes a hydrogen bond to the catalytic Glu120, while the fifth ligand, also a water molecule forms a hydrogen bond with the phenolic group of Tyr135. In *Gf*-LysN the zinc is bound in a pyramidal structure, in contrast to many other metalloendopeptidases which coordinate in a tetrahedral structure (7). The exact catalytic mechanism of *Am*-LysN is unclear, but is likely to be reminiscent of other metallopeptidases. We expect, based on the mechanisms of astacin and thermolysin, that when a substrate binds into the S_1' pocket, it displaces the zinc-bound water molecule towards the Glu120 which acts as a base removing a proton from the water molecule. This allows the metal-bound hydroxide to make a nucleophilic attack on the peptide carbonyl, while the oxygen of the carbonyl interacts with the zinc (26). In this transition state, Tyr135 occupies the proper position to act as a second proton donor to stabilize the formed oxyanion(7). Glu120 donates a proton to the leaving amine, while the metal bound intermediate collapses. The two products leave the binding site and a water returns to the zinc (26). In the crystal structure of *Gf*-LysN Asp156 and Thr130 are thought to be critical for the chelating architecture of His119 and H123 respectively. In turn Gly136 is thought to stabilize the Asp156 by weak interaction. Besides the important role in the transition state Tyr135 is also thought to provide a hydrophobic environment to accommodate the alkyl part of the lysine side chain of lysine in coordination with Tyr86. A

model of the *Am*-LysN models' active site is depicted in Fig. 4, showing the involvement of the mentioned residues.

To evaluate the residues of the active site, nine site-directed mutants were constructed and expressed in *P. pastoris*. Plasmid pAAR4 served for mutations of Y85, E120, H123, T130, D132, Y135, G136 and D156. These mutants were characterized for their activity using the reference peptide, Pep1, relative to the activity of the recombinant wild type protease *rAm*-LysN. Relative activities of the mutants are shown in Table III.

The two mutations in the zinc binding motif, H123R and D132N both showed a significant decrease in activity as expected. As stated earlier both are part of the coordination of zinc, and mutation of these would decrease the zinc binding ability (8). Similar to the findings of Fushimi *et al.*, the D132N mutation had the highest relative activity of the active site mutants, with a relative activity of 16% to the wild type protease. Mutation of the catalytic E120Q, which plays an important catalytic role, has a surprisingly high activity of 3% relative to the wild type protease. We assume that the E120Q mutation is able to bind zinc, just as the case is with deuterolysin. The decrease in activity is likely to be due to the poor polarization of zinc by Gln.

The two tyrosines, Y85 and Y135, make up major parts of the wall of the binding cleft. Y135 was proposed by Hori *et al.* to be critical in a two ways. In the monoclinic crystal structure they observed that the

Fig. 4 A model of the catalytic site of *Am-LysN*. The mutated residues are displayed as stick models. Histidine 119 is part of the zinc binding domain, and was not mutated in this study. The Zn(II) is displayed as CPK space filling model.

Table III. Proteolytic activity for hydrolysis of Pep1, K(Abz)-SAQKMVS-K(Dnp) at pH 7.5 in 20 mM MOPS of site-directed mutants of rAm-LysN relative to wild type rAm-LysN

Mutant	Proposed roles of amino acid	Activity in percent relative to the wild type
Y85L	Aromatic ring of Tyr is thought to stabilize lysine side chain of substrate ¹	3 ^b
E120Q	Glu in HEXXH + D, zinc binding motif	3 ^b
H123R	His in HEXXH + D, zinc binding motif	1 ^b
T130A	Thr, thought to interact with H123 ²	4 ^a
D132N	Asp in the HEXXH + D, zinc binding motif	16 ^b
Y135F	Aromatic ring of Tyr is thought to stabilize lysine side chain of substrate ²	5 ^a
Y135W	Aromatic ring of Tyr is thought to stabilize lysine side chain of substrate ²	5 ^a
G136L	Gly is thought to interact with D156	4 ^a
D156A	Located in the S ₁ ' , but involved in zinc coordination by stabilization of H123.	1 ^b

¹ Proposed from homology structure.

² Proposed from Hori *et al.* (7).

^a Standard deviation is 0–10%.

^b Standard deviation is 10–20%.

tyrosine Y135 interacted with the zinc ion via a water molecule. In a hexagonal crystal, the tyrosine had rotated 300° and the phenolic hydroxyl group acted as a proton donor (7). The aromatic ring of Tyr85 is thought to assist the binding of the alkyl chain of lysine in coordination with Tyr135. Mutant Y85L had its activity reduced to 3% of the wild type, which supports the hypothesis that the aromatic ring stabilizes the alkyl chain. Both Y135F and Y135W had activities lower than 5%, while the Y135F indicates that the phenolic hydroxyl group acts a secondary proton donor.

While being integral part of the S₁' binding site contributing to the negatively charged pocket, the carbonyl group of D156 is also thought to interact with the nitrogen of G136 and the delta nitrogen of H119.

These non-covalent interactions were proposed by Hori *et al.*, to be important for the zinc coordination (7). To investigate this hypothesis, we constructed a mutant G136L, where the leucine would likely interrupt the interaction with D156. Similarly the hydroxyl group of T130 is thought to help stabilize H123 in the coordination of the zinc ion. Substitution of threonine to alanine would disrupt this interaction and should lower the zinc binding ability, with decreased activity similar to that of the H123R mutation. Both T130 and G136 are conserved throughout all the known aspzincins. The G136L mutant had decreased activity, just 4% of the wild type, similar to that of D156A. While it was not possible to differentiate between the two roles of the D156A mutant, we must conclude that both G136 and D156 are important residues for maintaining

the activity of *Am*-LysN. We have with these nine mutations shown the complex and delicate nature of the catalytic site of *Am*-LysN.

Screening of site-directed mutants for altered specificity in the binding cleft. The binding of lysine in the S_1' pocket is thought to come from a tetrad of residues, two aspartic acids (D111, D156) and two glutamic acids (E159, E163). The primary residue is the E159 which interacts with the amino group of lysine. Subsites S_3 – S_1 and S_2' – S_4' are not clearly defined, but from the homology model it is speculated how they may interact with the substrates. From a protein engineering perspective it would be interesting to alter the substrate specificity, either allowing other residues in S_1' or making the enzyme more stringent in what is accepted in S_3 – S_1 or S_2' – S_4' . Altered specificity could open up for new applications of LysN.

Eleven site-directed mutants, targeting M65, D83, V88, A101, T105, Q131 and Q137, all located around the binding cleft were constructed based on plasmid pAAR4 and expressed in *P. pastoris*. These mutations were designed by modelling to have altered affinity for specific side chains using molecular dynamics. In Fig. 5, the mutants are highlighted in green, where the protease is depicted in ribbon format and the peptide Pep1 is docked into the active site. Each sub-figure can be found at high resolution individually in the Supporting Information. Mutants were characterized and their altered specificity determined by measuring relative activities of substrates compared to the wild type protease. The results are summarized in Table IV.

Methionine 65 is located in alpha-helix C, see Fig. 5A, and should not play a role in the substrate binding or activity of LysN (7). Substitution of methionine to leucine was a control substitution to evaluate residues far from the active site. As expected there was

very little change in the relative activities for the three substrates tested.

Most of the residues which are part of the binding cleft are conserved between *Gf*-LysN and *Am*-LysN. One exception is D83, which in *Gf*-LysN is a glycine. Based on the molecular modelling, we previously speculated that D83 could help direct positively charged substrates and secondly maybe assist in binding of lysine and arginine containing substrates (2). To investigate this hypothesis we constructed a D83N mutation, which we thought would have decreased affinity for arginine and lysine in subsites S_2' , S_3' and S_4' . However, this mutation surprisingly did not result in a decrease in arginine affinity as anticipated.

V88 is located within vicinity of both subsite S_3 and S_2 , see Fig. 5C, and a promising target to alter specificity in S_2 . *Am*-LysN prefers mostly hydrophobic residues in P_2 (2). We envisaged that a mutation of V88F would increase the hydrophobic environment of S_3 and S_2 , and that this mutant would have increased affinity towards hydrophobic residues. Phenylalanine was selected as it is bulky and the most hydrophobic residue on the hydrophobicity index at pH 7 (27). However, the protease behaved opposite to what was expected. It displayed lower relative activity with substrates with leucine, phenylalanine and valine in P_2 than the reference peptide with alanine, probably due to steric effects. As anticipated the mutant had lower relative activity with a substrate with asparagine in P_2 . Interestingly we observed no change in activity with either serine or methionine in P_2 compared to the wild type.

It was proposed that increased arginine affinity in P_3 could be achieved by placing a negative charge at Q131 see Fig. 5F. The glutamine is not conserved throughout the aspzincin family, and using molecular modelling an interaction between P_3 arginine and Q131E

Fig. 5 The proposed residues for altered substrate specificity are colored in green (Online version only). (A) M65 – which due to its position far away from the active site is anticipated to have very little effect on the substrate specificity. (B) D83 – Located on the side of the cleft, it was proposed that this aspartate is involved in attracting positively charged substrates. (C) V88 – Located close to the S_2 , so it was proposed to influence specificity of P_2 . (D) A101 – Located within vicinity of P_2' and P_3' . (E) T105 – located within distance of P_2' and P_3' . (F) Q131 – Located in the S_3 . (G) Q137- Located close to the S_3' . (H) E159 – The residue interacting with the P_1' lysine.

Table IV. Relative specificity of site-directed mutants in the substrate binding sites of *rAm-LysN* compared to the wildtype enzyme with the reference peptide SAQKMVS

Mutant	Proposed subsite interaction and effect	Substrate	Relative specificity to the wild type enzyme in percent*
Wildtype	Control	Any substrate	100
M65L	Control	SAQKRVS	108
		SAQKMRS	112
		SAQKMVR	108
D83N	S ₂ '/S ₃ '/S ₄ ' – Decreased Arg specificity compared to Wt	SAQKRVS	100
		SAQKMRS	95
		SAQKMVR	100
V88F	S ₂ – Increased specificity for hydrophobic residues	SFQKMVS	57
		SLQKMVS	40
		SMQKMVS	101
		SNQKMVS	76
		SSQKMVS	104
		SVQKMVS	67
A101D	S ₂ '/S ₃ ' – Increased specificity for positive charged residues	SAQKRVS	207
		SAQKMRS	128
		SAQKMVR	154
A101I	S ₂ '/S ₃ ' – Increased specificity for hydrophobic residues	SAQKFVS	89
		SAQKIVS	53
		SAQKLVS	57
		SAQKVVS	92
		SAQKNVS	104
		SAQKSVS	111
		SAQKMFS	127
		SAQKMLS	76
		SAQKMNS	110
		SAQKMSS	82
T105D	S ₃ '/S ₄ ' – Increased specificity for positive charged residues	SAQKRVS	292
		SAQKMRS	131
		SAQKMVR	209
T105L	S ₃ '/S ₄ ' – Increased specificity for hydrophobic residues	SAQKMFS	72
		SAQKMIS	98
		SAQKMLS	49
		SAQKMNS	113
		SAQKMSS	139
		SAQKMFV	54
		SAQKMVL	57
		SAQKMVV	213
Q131E	S ₃ – Increased specificity for positive charged residues	RAQKMVS	80
Q137D	S ₄ ' – Increased specificity for positive charged residues	SAQKMVR	110
Q137L	S ₄ ' – Increased specificity for hydrophobic residues	SAQKMFV	61
		SAQKMVL	21
		SAQKMVV	89
E159D	S ₁ ' – Change specificity to R	SAQRMVS	N/A ¹

* Relative specificity of given mutant with substrate compared to reference substrate compared to wild type enzyme. Specificity is calculated by the following formula of relative activity = $100 \cdot \frac{MT:SAQKXXX/MT:SAQKMVS}{WT:SAQKXXX/WT:SAQKMVS}$, where MT is the corresponding mutant protease and WT is the wildtype *rAm-LysN*, and SAQKXXX is the corresponding substrate while SAQKMVS is the reference substrate.

¹ Activity could not be measured.

formed almost immediately. The mutation showed opposite results than anticipated by having a decreased specificity towards arginine than the wild type protease.

Increased affinity for arginine P₄' was suggested to be achieved by mutation of Q137E, see Fig. 5G. Glutamine 137 is conserved in the lysine specific aspzincins but not in the other peptidases. Using molecular modelling, the Q137E interaction to P₄' arginine was stretched but seemed to be viable. The Q137E mutant had a minor increase in specificity for substrates with arginine in P₄' compared to the wild type. Following that, we anticipated that a Q137L mutation would have increased affinity for hydrophobic residues in P₄'. However this mutation did not have the expected effects, again probably due to steric requirements for substrate alignment.

In the previous section it was discussed that E159 interacts with the amino group of lysine in the S₁' pocket, see Fig. 5H. Based on homology modelling, it was hypothesized that an E159D mutant would make the S₁' pocket deeper, possibly enabling activity with arginine in P₁'. However, the E159D mutant did not have a significant increase in activity with arginine in P₁'. The mutant was still able to hydrolyze substrates with lysine in P₁'.

Alanine 101 is located close to subsites S₂' and S₃', see Fig. 5D. It is conserved between *Gf-LysN* and *Am-LysN*. From molecular dynamic modelling, we anticipated that substitution of alanine to aspartic acid would have increased activity with arginine in P₂' and P₃'. Similarly A101I was anticipated to have increased activity with hydrophobic residues in P₂' and P₃'. While the A101D did show increased

specificity for P₂' and P₃' arginine, the A101D did not show any increased specificity towards hydrophobic residues in either P₂' and P₃'.

Threonine 105 is located within vicinity of both S₃' and S₄' and T105 is predicted to be involved in binding substrates in these subsites, see Fig. 5E, T105 is one of the few amino acids not conserved in the family, with the *Gf*-LysN having an alanine. Two mutants, T105D and T105L were expressed and expected to have increased affinity for arginine or hydrophobic residues, respectively. Surprisingly the T105D mutant showed a 2.9-fold increase for P₂' arginine and around 1.3-fold increase and 2-fold increase for P₃' and P₄' arginine respectively. It was surprising that T105D had a higher relative activity with P₂' arginine, but molecular dynamics does show great flexibility of the P₂' of the substrate. It was expected that the T105L mutant would have an increased affinity for hydrophobic residues in S₃' and S₄'. We measured a decrease in activity compared to the wild type for all the tested substrates except with valine in P₄', which had a 2-fold increase. Engineering of hydrophobic specificity therefore appears to be feasible in P₄'.

Characterization of A101D and T105D

In the screening, mutants A101D and T105D both exhibited increased affinity for arginine in P₂', P₃' and P₄'. Kinetic constants of four substrates were determined for both A101D and T105D and compared to wild type recombinant protease, as summarized in Table V. Due to low concentrations of mutant A101D and T105D it was not possible to quantify exact concentrations. Relative $V_{\max}$ were calculated compared to the reference substrate for the wildtype and for the mutants. Mutants A101D and T105D both have much higher relative $V_{\max}$ with substrate SAQKRVS than the wild type, while having a similar K_M ratio to the wild type. These findings confirm the screening of both mutations to have higher specificity with arginine.

Table V. Kinetic constants for hydrolysis of FRET-substrates with the reference substrate SAQKMVS and substitutions to P₂', P₃' and P₄'. $V_{\max}^*$ is the relative $V_{\max}$ to the reference substrate

Enzyme	Substrate	$V_{\max}^*$ (s ⁻¹)	K_M (μ M)	$V_{\max}^*/K_M$ (μ M s ⁻¹)
WT	SAQKMVS	1.00 ^b	2.50 ^b	0.40 ^c
	SAQKRVS	0.26 ^a	0.37 ^b	0.70 ^d
	SAQKMRS	0.64 ^c	0.76 ^c	0.84 ^d
	SAQKMVR	1.51 ^b	3.93 ^b	0.38 ^c
A101D	SAQKMVS	1.00 ^b	6.63 ^c	0.15 ^b
	SAQKRVS	0.72 ^b	1.31 ^d	0.55 ^c
	SAQKMRS	0.66 ^a	1.76 ^b	0.37 ^a
	SAQKMVR	0.56 ^a	2.49 ^b	0.23 ^b
T105D	SAQKMVS	1.00 ^c	6.54 ^d	0.15 ^d
	SAQKRVS	2.48 ^b	3.17 ^b	0.78 ^b
	SAQKMRS	0.99 ^b	2.57 ^d	0.39 ^d
	SAQKMVR	0.38 ^c	1.22 ^c	0.31 ^c

^a Standard deviation is ± 0 –5%.

^b Standard deviation is ± 5 –10%.

^c Standard deviation is ± 10 –15%.

^d Standard deviation is ± 15 –20%.

Concluding Remarks

We have developed an expression system for *Am*-LysN. Yields in the range of 0.25 mg/L with *Pichia pastoris* were sufficient to allow full characterization of the recombinant protease. We showed that the most likely route of activation of LysN is by processing of the Kex2p site (KR). However in a mutant lacking Kex2p sites we observed fragmented propeptide after purification, demonstrating that the enzyme has autocatalytic activity. This was confirmed by an inactivate mutant, which also lacks Kex2p sites appeared to be totally unprocessed. Furthermore, we analyzed the active site by multiple site-directed mutations of residues anticipated to be involved in the hydrolysis, and characterized the effects on enzymatic activity. We previously built a homology model of LysN, and used this model to predict mutations which could have altered substrate specificities. Only two of the proposed mutations gave rise to altered substrate specificity. This indicates that substrate processing by *Am*-LysN is a complex event where sidechain interaction and steric requirements for substrate alignment has to work in close concert to achieve the productive arrangement of the scissile bond, zinc and zinc binding residues of the active site. Obtaining productive binding rather than unproductive binding is down to angstrom of residues interacting. Results show that even small changes in the enzyme structure can upset the activity a great deal. Productive binding is delicate and substrate-enzyme interactions must stabilize the transition-state in order catalyze hydrolysis. The molecular model seems to be sensible, but it appears that altering the substrate specificity of LysN is difficult compared to other enzymes such as trypsin and papain (28, 29). One explanation could be the open nature of the binding cleft, where elaborate engineering schemes might be necessary to alter the specificity. The mutant E157D was designed to make the binding pocket of S₁' deeper in order to accommodate arginine, however the resulting enzyme failed to bind a substrate with arginine in P₁' in a productive manner. Two mutants, A101D and T105D, are especially interesting as they exhibit increased affinity for arginine in subsites S₂'–S₄'.

Supplementary Data

Supplementary Data are available at *JB* Online.

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Conflict of Interest

None declared.

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