Improved antimicrobial spectrum of the N-acetylmuramoyl-L-alanine amidase from *Latilactobacillus sakei* upon LysM domain deletion

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Research Article

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Abstract

The gene encoding N-acetylmuramoyl-L-alanine amidase in *Latilactobacillus sakei* isolated from a fermented meat product was cloned in two forms: its complete sequence (AmiC) and without one of its anchoring LysM domains (AmiLysM4). Deletion of the LysM domain is believed to affect the target microorganism's affinity to the cell wall, which influences antimicrobial activity. To compare activity and inhibitory spectra, AmiC and AmiLysM4 were expressed in *Escherichia coli* BL21. Using the zymography technique, two bands with lytic activity were observed, which were confirmed by LC-MS/MS analysis, with molecular masses of 71 kDa (AmiC) and 66 kDa (AmiLysM4). The recombinant proteins were active against *Listeria innocua* and *Staphylococcus aureus* strains; however, the inhibitory spectrum of AmiLysM4 was broader because AmiLysM4 could inhibit *Leuconostoc mesenteroides* and *Weissella viridescens*, which are microorganisms associated with food deterioration. Optimal temperature and pH values were determined for both proteins using L-alanine-*p*-nitroanilide hydrochloride as a substrate for N-acetylmuramoyl-L-alanine amidase activity. Both proteins showed similar maximum activity values for pH (8) and temperature (50°C). Furthermore, *in silico* predictions did not show differences for the catalytic region, but differences were found for the region called *3dom*, which includes 3 of the 5 LysM domains. Therefore, the modification of the LysM domain offers new tools for the development of novel food biopreservatives.

Key Points

Successful cloning of N-acetylmuramoyl-L-alanine amidase from *L. sakei*

A broader inhibitory spectrum of N-acetylmuramoyl-L-alanine amidase upon LysM domain deletion

Possible applications of N-acetylmuramoyl-L-alanine amidase recombinant proteins as food biopreservatives

Introduction

Latilactobacillus sakei is a lactic acid bacterium (LAB) used as a starter culture in fermented products. Although it was initially characterized from rice wine's "sake" and is considered as a potential protective culture for meat and fish products (Zagorec et al. 2017). *L. sakei* resists high salt concentrations, low water activity, and low temperatures. Due to lactic acid production and other antagonist molecules such as bacteriocins (Anba-Mondoloni et al. 2013; Lorenzo et al. 2018), sakacins A and P, that can prevent the growth of spoilage microorganisms and even show high inhibitory activity against foodborne pathogens such as *Listeria monocytogenes* (Mapelli et al. 2018). Other proteins related to this inhibitory activity include peptidoglycan hydrolases (PGHs), which can hydrolyze covalent bonds in peptidoglycan (PG), the main component of the bacterial cell wall, that consists of alternating units of N-acetylmuramic acid and N-acetylglucosamine bound by β-1,4 glycosidic bonds. Depending on the type of bond that they

hydrolyze, PGHs are classified as N-acetylmuramidases, N-acetylglucosaminidases, N-acetylmuramoyl-L-alanine amidases, and endopeptidases (Vollmer et al. 2008).

Several studies have shown the potential of PGHs to control and treat infections caused by drug-resistant strains, such as some strains of *Staphylococcus aureus*, which has served as a model to study cell wall structure, as well as the function and the mechanism of lysis caused by PGHs in the degradation of PG (Do et al. 2020). PGHs have also been considered for the prevention of pathogenic and spoilage microorganisms in food. *L. monocytogenes* is one of the main causes of food intoxication, whereas the genera *Leuconostoc* and *Weissella* are responsible for the formation of slime and sensory defects such as the greening of products (Kamboj et al. 2015; Lorenzo et al. 2018). *Leuconostoc mesenteroides* negatively affects the flavor, texture, and color of sliced and vacuum-packed meat products (Comi et al. 2016), while *Weissella viridescens* is considered an opportunistic pathogen that causes packaging to swell or rupture due to the presence of CO_2. Both microorganisms are regarded as undesirable in food (Kim et al. 2017).

Some PGHs are modular enzymes composed of catalytic units linked to other domains, such as WxL, SH3b, CWBD, CHAP, LytM, and LysM, which are involved in the anchoring and hydrolysis of PG (Visweswaran et al. 2014). Some of the catalytic units may be attached to one or several LysM domains, which are spacing sequences consisting mainly of Ser, Thr, and Asp or Pro residues that may form a flexible region between LysM domains. These sequences have differences in length and composition and do not share significant homology. The domains contain secondary structures such as hydrogen bonds and disulfide bonds, but they have not been related to their protein functions (Buist et al. 2008). LysM is a domain containing approximately 50 residues and has been defined as a carbohydrate-binding domain with an affinity for N-acetylglucosamine polymers (Akcapinar et al. 2015; Buist et al. 2008; Mesnage et al. 2014). The principles of PG recognition and the regulatory mechanisms of the enzymes involved in its metabolism are unknown. The affinities of bacterial LysM domains for their substrates have not been described in detail (Wong et al. 2015). The activity of PGHs with LysM domains has been described for the recombinant protein AcmA. This protein has N-acetylglucosaminidase activity and contains three LysM domains. Mutants with 1.5, two, and four LysM domains have been generated, but the highest activity against *Micrococcus lysodeikticus* was observed with three LysM domains (Steen et al. 2005). In mutants of the CwlS endopeptidase of *Bacillus subtilis*, Wong and Blaise (2020) observed that as the number of LysM domains decreased, their ability to bind to PG decreased. Similar results have been reported in the PGH AtlA from *Enterococcus faecalis*, whose activity decreases drastically when all LysM domains are deleted (Eckert et al. 2006). When different proteins are modified or shortened, different effects can occur. For example, deleting the cell wall-targeting domain of lysostaphin (a PGH with endopeptidase activity) extended its inhibitory spectrum to *Staphylococcus staphylolyticus* and *S. aureus* (Sabala et al. 2012).

Due to the lytic activity of PGHs against foodborne pathogens or spoilage bacteria, their application to agricultural production and packaging, and distribution of the final product has been suggested (Chang 2020). Their antimicrobial effects have been tested in products such as cheese and wine. Many authors

have studied their effects in lengthening the shelf-life of unpasteurized beer, when incorporated into packaging materials, in the control of pathogens in biofilms and on surfaces, and in other applications (García Cano et al. 2020; Liburdi et al. 2014).

Different PGHs have been identified from LAB isolated from food. Five strains with antimicrobial activity were isolated from salami manufactured in Mexico; among them, a protein with N-acetylmuramoyl-L-alanine amidase activity produced by *L. sakei* (GenBank accession number: MT814885) was identified, which exhibited broad antimicrobial activity, preventing the growth of pathogenic microorganisms and other LAB. This protein was identified by LC/MALDI-TOF/TOF, containing in this sequence five LysM domains were described (García-Cano and Ponce-Alquicira 2015; Lopez-Arvizu et al. 2021).

This study aimed to clone and express the full-length N-acetylmuramoyl-L-alanine amidase produced by *L. sakei* UAM-MG3 and a truncated sequence without one of the LysM domains for a comparative analysis of their activity since gene deletion from one of the five LysM domains has been reported to preserve this N-acetylmuramoyl-L-alanine amidase's activity (Najjari et al. 2016). This study contributes to knowledge regarding proteins that may have applications as antimicrobial agents in food preservation and even in the treatment of infections caused by antibiotic-resistant strains.

Methodology

Culture conditions, strains, and plasmids

L. sakei UAM-MG3 was isolated from salami and grown at 37°C under static conditions in casein glucose broth (CGB) made of 0.5% yeast extract (Bioxon, Mexico), 2% peptone-biotryptase (Bioxon, Mexico), 1% dextrose (Bioxon, Mexico), 0.005% manganese sulfate (JT Baker, Mexico), 0.2% ammonium citrate (JT Baker, Mexico), disodium phosphate 0.2% (JT Baker, Mexico), magnesium sulfate 0.01% (JT Baker, Mexico), and 0.10% Tween 80 (Hycel, Mexico) at pH 7. Brain heart infusion (BHI, BD Difco, USA) and De Man Rogosa and Sharp (MRS) broths (BD Difco, USA) were used to cultivate the strains shown in Table 1. *Escherichia coli* BL21 (DE3) was grown in Luria-Bertani (LB) broth, 1.0% Bacto Tryptone (BD Difco, Detroit, MI, USA), 0.5% extract of yeast, and 0.5% NaCl (JT Baker, Mexico) at pH 7.5. The cultures were incubated at 37°C with shaking at 200 rpm. The medium was supplemented with ampicillin (100 µg/mL) when necessary.

Table 1
Bacterial strains and plasmid used in this work

Bacterial strains	Description	Source/reference
Latilactobacillus sakei UAM-MG3	Target strain, CGB broth	Lopez-Arvizu et al. (2021)
Enterococcus faecium UAM-MG4	Target strain, CGB broth	Lopez-Arvizu et al. (2021)
Leuconostoc mesenteroides FQ	Target strain, MRS broth	Dra. Gloria Díaz (UNAM-Mexico)
Listeria innocua ATCC 33090	Target strain, BHI broth	Garcia-Cano et al. (2015)
Micrococcus lysodeikticus ATCC 4698	Target strain, BHI broth	Sigma-Aldrich
Staphylococcus aureus ATCC 6538	Target strain, BHI broth	Garcia-Cano et al. (2015)
Weissella viridescens UAM-MG3	Target strain, MRS broth	Lopez-Arvizu et al. (2021)
Escherichia coli BL21 (DE3)	F $^{-}$ mpT *hsdS* $_{B}$ (r_{B}^{-} m_{B}^{-}) *gal dcm*, LB broth	Invitrogen
Plasmid		
pET 22-b(+)	Expression vector, T7 *lac* promotor, ampicillin resistance, with an N-terminal pelB signal and C-terminal His•Tag	Novagen

DNA extraction

An 18-h culture of *L. sakei* (1.5 mL) was centrifuged at 8,000 *g* for 2 min (Eppendorf 5810R, Hamburg, Germany). The pellet was used for DNA extraction using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) following the manufacturer's specifications. The presence and integrity of DNA was determined by electrophoresis in 0.8% agarose gel/TAE buffer (10 mM Tris-acetate, 1 mM EDTA, pH 8).

Molecular identification of L. sakei **and amplification and cloning of** amiC **and** amiLysM4

The identity of the strain used in this study was confirmed by 16S rRNA gene sequencing as reported by García-Cano et al. (2015). First, 100 ng of genomic DNA, 1.25 U of *Pfu* DNA polymerase (Thermo Scientific, Pittsburgh, USA), 1 µM of primers, and 10 µM of deoxynucleotides (dNTPs; Thermo Scientific, Pittsburgh, USA) all in a reaction of 50 µL were mixed and reacted under the following conditions: one cycle at 95°C (3 min); 35 cycles at 95°C (2 min); 42°C (30 s); and 72°C (4 min); and one cycle at 72°C (10

min). The DNA extracted from *L. sakei* was used as a template to amplify the complete gene of N-acetylmuramoyl-L-alanine amidase (2004 bps; *amiC*). The primers used were AmiCF 5'- CTC **G GA TCC** GAA AGA GAC TAG AAA ACAA-3' and AmiCR 5'- ACG **C TC GAG** CTT CAA TTT CAA GAC TTG-3'. The primers used to amplify the sequence without one of the LysM domains were AmiCF 5'- CTC **G GA TCC** GAA AGA GAC TAG AAA ACAA-3' and AmiLysM4R 5'- TCG **C TC GAG** TGT TTT ATT AGG CGT TGT-3'. Both sets of primers included BamHI (forward primers) and XhoI (reverse primers) as restriction sites (bold letters). PCR reactions included 1.25 U of Dream *Taq* DNA polymerase (Thermo Scientific, Pittsburgh, USA), 100 ng of genomic DNA, primers at 0.2 µM, and dNTPs (Thermo Scientific, Pittsburgh, USA) at 0.2 mM. The PCR conditions for the *amiC* and *amiLysM4* genes were as follows: one cycle at 95°C (2 min), 35 cycles at 95°C (30 s), 58°C (30 s), and 72°C (1.30 min); and one cycle at 72°C (10 min; Eppendorf Mastercycler, Hamburg, Germany).

The amplified and purified fragments and pET-22b(+) plasmid (750 ng/µg; Novagen, Darmstadt, Germany) were digested using 5 U of each restriction enzyme (BamHI and XhoI; Thermo Scientific, USA) at 37°C for 1 h. To generate the constructs, a vector: insert ratio of 1: 3 of DNA was used with 1 U of T4 DNA Ligase (Thermo Scientific, USA), followed by incubation at 22°C for 1 h. *E. coli* BL21 (DE3) was transformed, and selection was performed on LB agar plates containing ampicillin (100 µg/mL). The constructs obtained were named pET22b-AmiC and pET22b-AmiLysM4 and were verified by sequencing (Macrogen, Seoul, Korea).

Expression of recombinant proteins

Recombinant protein expression was carried out using *E. coli* in 100 mL of LB medium supplemented with ampicillin followed by incubation at 37°C and 200 rpm until reaching an optical density (OD_{600nm}) between 0.5 and 0.8. The OD_{600nm} was measured in a microplate spectrophotometer (Epoch Biotek, Vermont, USA). Isopropyl-β-D-1-thiogalactopyranoside (IPTG; Sigma-Aldrich, Missouri, USA) was used as an inductor and added at different concentrations (0.2, 0.4, 0.8, 1, 1.5, and 2 mM), and the optical density was monitored at 1, 2, and 3 h of incubation under the conditions described above.

The cultures of recombinant clones were centrifuged at 8,000 *g* for 10 min, the cell-free supernatant was adjusted to pH 6.5 with 0.1 M NaOH, and cells were eliminated by filtering through a membrane (0.22 µm, Millipore, GV, Ireland). The resulting filtrate or crude extract (CE) was used to determine the protein profile and antimicrobial activity by agar diffusion and zymography. The CE was concentrated by ultrafiltration using a 50-kDa membrane (YM-50NMWCO 50 kDa; Millipore, USA), and the protein was purified by affinity chromatography using the PUR025 Proteus IMAC Mini Sample kit (Langford Lane, Kidlington, England) according to the supplier's specifications. The purified crude extract (PCE) was assayed for its specific activity on L-alanine-*p*-nitroanilide.

Determination of protein profiles and lytic activity by zymograms

The profile of the protein purification fractions was evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The gels were prepared at 10% according to Laemmli (1970). Zymograms were prepared by adding 0.2% of lyophilized *M. lysodeikticus* cells as a substrate embedded in the gel (Leclerc and Asselin 1989). After methylene blue staining, hydrolysis of the substrate was identified through translucent bands, which were used to calculate the apparent molecular masses, and relative protein quantification was performed to determine the optimal induction conditions with IPTG (Gel Doc version, Bio-Rad, CA, USA) since only the loading volume was the same. The purified proteins were sequenced in Campus Chemical Instrument Center, Mass Spectrometry, and Proteomics Facility at The Ohio State University (Columbus, Ohio, USA). Protein bands were subjected to in-gel trypsin digestion, and the resulting peptides were analyzed by capillary LC-MS/MS. Peptide fragments generated by tandem MS were compared against the MASCOT database to obtain the amino acid sequence. Proteins with at least two peptide fragments matching the database were considered reliable for identification.

Determination of antimicrobial activity by agar diffusion

The activity of the recombinant proteins was measured by agar diffusion assay (García-Cano et al. 2015). Plates with a lower layer of tryptic soy agar (TSA; BD Difco, USA) were used. Soft agar (TSA with 0.8% agar) was used as a second layer and was inoculated with 70 µL of the target microorganism (10^6-10^7 CFU/mL; Table 1). Wells were created on the soft layer in which 100 µL of each sample was placed, followed by incubation at 37°C for 18 h to observe the clear zones of inhibition. The activity was determined by the ratio of the inhibition halo (mm) to the amount of protein contained in each sample (mg). Protein determination was performed according to the Bradford assay (Bio-Rad, CA, USA). An analysis of variance (ANOVA) was performed to evaluate statistical differences ($P < 0.05$) in the antimicrobial activity of AmiC and AmiLysM4.

In silico **prediction of the structures of recombinant proteins**

The tools ExPASy (SIB Bioinformatics Resource Portal), ProtParam (https://web.expasy.org/protparam/), and SignalP 4.1 (http://www.cbs.dtu.dk/services/eleSignalP/) were used to analyze both recombinant proteins *in silico* (Gasteiger et al. 2005). The beta sheets and alpha helices that constitute the secondary structure were predicted with the Garnier–Osguthorpe–Robson (GOR) IV algorithm (Prabi-Gerland Rhone-Alpes Bioinformatic Pole Gerland site: https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_gor4.html). Protein modeling was performed using Swiss Model software (https://swissmodel.expasy.org) and a homology modeling server (Waterhouse et al. 2018) using RasMol 2.7.5 viewer.

Effect of pH and temperature over N-acetylmuramoyl-L-alanine amidase activity

N-acetylmuramoyl-L-alanine amidase activity was measured using L-alanine-*p*-nitroanilide as a substrate (Garcia-Cano et al. 2015). In a 96-well microplate, 85 µL of deionized water, 10 µL of the substrate L-alanine-*p*-nitroaniline hydrochloride (1 mg/mL; Sigma-Aldrich, Missouri, USA), and 5 µL of the sample

were added. The reaction was incubated at 37°C for 30 min, and the absorbance was determined at 405 nm in a microplate spectrophotometer (Epoch Biotek, Vermont, USA). One unit was defined as the amount of enzyme that would hydrolyze 1.0 µmol of L-alanine-*p*-nitroaniline hydrochloride per minute. As a negative control, 1 mg/mL lysozyme was used (Garcia-Cano et al. 2015). The same test was performed to measure the effect of pH, where water (85 µL) was replaced by a mixture of sodium acetate (JT Baker), MES (Sigma-Aldrich), and HEPES (Sigma-Aldrich), each at a concentration of 100 mM, and the mixture was adjusted to different pH values (4, 5, 6, 7, 8, and 9). To measure the effect of temperature, the assay was performed without modification by incubating at 20, 30, 40, 50, 60, and 70°C (García-Cano et al. 2015).

Results

Cloning and expression conditions of the recombinant proteins

The complete sequence of the gene encoding the PGH was amplified by obtaining the DNA region of 2,004 bps (668 amino acids; Fig. 1a) and a region of 1,872 bps (624 amino acids; Fig. 1b) corresponding to the gene sequence without one of the LysM domains.

The concentrations of 0.2 and 0.4 mM of IPTG for pET22b-AmiC and 1 mM of IPTG for pET22b-AmiLysM4 were the best for expressing the recombinant proteins (Fig. 2). The protein profile obtained under these conditions is shown in Fig. 2a. In lanes 2 and 3, two bands with molecular masses of 88 and 77 kDa were observed, and these bands showed activity on zymograms at the same molecular masses (Fig. 2b, lanes 1 and 2). As shown in Fig. 2a, in lane 3, a band with a molecular mass of 77 kDa was observed, which coincided with the activity observed on the zymogram (Fig. 2b, lane 3). The LC/MALDI-TOF/TOF analysis confirmed the identity of the AmiC protein, with a coverage percentage of 62% and a molecular mass of 71.4 kDa. For AmiLysM4, the coverage percentage was 19%, and the molecular mass was 66.5 kDa. These data coincide with the *in silico* analysis performed previously, which yielded masses of 71.6 kDa for AmiC and 66.9 kDa for AmiLysM4 and theoretical isoelectric points (pI) of 9.95 and 9.90, respectively.

Antimicrobial spectrum

Table 2 shows the inhibitory spectra for both recombinant proteins. AmiC protein can inhibit 5 of the 7 microorganisms evaluated. On the other hand, AmiLysM4 showed a broad spectrum due to its ability to inhibit all 7 strains evaluated, although a reduction in activity was observed. For instance, the inhibitory activity against *S. aureus* ATCC 6538, *Enterococcus faecium* MXVK29, and *Listeria innocua* ATCC 33090 was reduced by 15, 20, and 42.15%, respectively, compared with that of the unmodified AmiC. In contrast, the modified AmiLysM4 protein could inhibit *L. mesenteroides* FQ and *W. viridescens* UAM-MG3.

Table 2
Antibacterial spectra of the recombinant proteins by agar diffusion assay

Strains	AmiC (mm halo/mg prot)	AmiLysM4 (mm halo/mg prot)
E. faecium UAM-MG4	122.20 ± 3.95 [a]	97.68 ± 12.34 [a]
L. sakei UAM-MG3	60.61 ± 1.29 [a]	67.03 ± 4.5 [a]
L. mesenteroides FQ	nd	82.65 ± 6.96
L. innocua ATCC 33090	109.81 ± 3.00 [a]	63.52 ± 0.46 [b]
M. lysodeikticus ATCC 4698	74.32 ± 19.19 [a]	42.68 ± 17.31 [a]
S. aureus ATCC 6538	50.91 ± 11.18 [a]	43.25 ± 3.11 [a]
W. viridescens UAM-MG3	nd	62.92 ± 1.69

nd = not detected. Different letters indicate a significant difference ($P < 0.05$)

Modeling of the recombinant proteins

The predicted secondary structures of the recombinant proteins showed 20.21% alpha-helices, 22.60% extended strands, and 57.19% random coils for AmiC and 19.07% alpha-helices, 22.92% extended strands, and 58.01% random coils for AmiLysM4. The instability indices of the *in silico* analysis showed values of 26.14 for AmiC and 27 for AmiLysM4. The numerical limit for the stability of proteins is less than 40 (Prabi-Gerland Rhone-Alpes Bioinformatic Pole Gerland site: https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_gor4.html).

Eleven models were obtained for each protein, and two predictions with the best homology modeling were selected to compare their structural differences. The first model encompassed the catalytic site (from amino acids 148 to 316) and was named *catsite*. The second model covered amino acids 411 to 611, included the sequences of the second (LysM2), third (LysM3), and fourth (LysM4) LysM domains, and was called region *3dom*. In Fig. 3a, the superimposed model of *catsite* from both recombinant proteins (*catsite*-AmiC and *catsite*-AmiLysM4) does not show structural differences. Figure 3b shows the predicted models of the recombinant *3dom*-AmiC and *3dom*-AmiLysM4 proteins, where the regions of the three domains present in the model are detailed, and the overlap of the two regions shows structural differences in the three domains.

Effects of temperature and pH on the specific activity of the enzyme

The values obtained for the aliphatic index were 63.41% for AmiC and 59.92% for AmiLysM4. As the pH changed (Fig. 4b), both recombinant proteins showed similar results, with optimal values at pH 8. At

neutral pH, both proteins had approximately 70% activity. The proteins had activity at pH values lower than 6. At pH 9, AmiC had lower activity, while AmiLysM4 showed protein instability and full loss of activity.

The experimental analysis revealed that both proteins showed activity up to 50°C for 30 min. Above 60°C, both lost activity (Fig. 4a). The effect of temperature was similar in both cases, although between 40 and 50°C, AmiLysM4 had lower activity than AmiC.

Discussion

The isolation and purification of proteins with antimicrobial activity, such as PGHs, have low yields because these enzymes can hydrolyze the PG of host cells. In this study, an N-acetylmuramoyl-L-alanine amidase was successfully cloned (AmiC), as well as a trimmed sequence with one LysM domain was deleted (AmiLysM4). Similar results were observed by Najjari et al. (2016), where an *L. sakei* strain with the gene deleted from one of the five LysM domains showed reductions in molecular mass and lytic activity. However, in this work, relevant changes were observed in the inhibitory spectra presented by AmiC and AmiLysM4. The molecular masses of the recombinant proteins determined *in silico* were 71.6 kDa for AmiC and 66.9 kDa for AmiLysM4, which differed from those determined by SDS-PAGE at 88 kDa for AmiC and 77 kDa for AmiLysM4. The molecular masses showed differences of 23% for AmiC and 15% for AmiLysM4. Serrano-Maldonado et al. (2018) reported molecular masses of 71 and 75 kDa for *E. faecalis* AltD using protein electrophoresis and exclusion chromatography, respectively; however, the expected molecular mass was 52 kDa. The authors attributed the increased molecular mass to posttranslational modifications such as acetylation, palmitoylation, and O-linked glycosylation. In this study, the difference in molecular mass was approximately 10 kDa and can be attributed to variations in protein separation due to factors that can influence proteins' migration, such as the state of aggregation and the tertiary structure of the protein, among others (Shi et al. 2012). Examples of cytosolic proteins that migrate in SDS-PAGE at rates that are inconsistent with their molecular masses have been reported. In addition, higher concentrations of the denaturing agent can improve electrophoretic migration, and the substitution of amino acids or posttranslational modification can cause unpredictable changes in electrophoretic mobility (Shi et al. 2012).

The comparison of the antimicrobial activity of AmiC and AmiLysM4 against the evaluated target microorganisms showed no significant differences except for AmiLysM4, which showed lower activity against *L. innocua* than AmiC. Similar effects have been reported for other enzymes, such as *Lactococcus lactis* AcmA and *E. faecalis* AtlA, whose activity decreased after LysM domains were deleted. These domains recognize the N-acetylglucosamine of the PG via noncovalent bonds, thereby binding to the cell surface and facilitating hydrolysis by the catalytic site (Eckert et al. 2006; Steen et al. 2005). However, AmiLysM4 showed a broader antimicrobial spectrum than AmiC since it inhibited microorganisms such as *L. mesenteroides* and *W. viridescens.* Similar results were reported by Sabala et al. (2012). Deleting the cell wall-targeting domain from an endopeptidase (lysostaphin from *S. staphylolyticus*) does not affect the enzyme activity and may even facilitate recognition of

microorganisms that were previously no inhibited. In the case of AmiLysM4, the inhibition of *L. mesenteroides* and *W. viridescens* may be related to the compositions of their cell walls since both genera belong to the family *Leucostocaceae* and are often confused with *Latilactobacillus* (Comi et al. 2016; Kamboj et al. 2015). The content of N-acetylglucosamine in *L. mesenteroides* has been reported to be low compared to those in other microorganisms (Harney et al. 1967), and the LysM domains are preferentially anchored to this fraction (Akcapinar et al. 2015). However, the mechanism through which AmiLysM4 without one LysM domain can bind to the few N-acetylglucosamine fractions in the *Leuconostoc* cell wall remains unclear. The variability in the carbohydrate-binding specifications of proteins with LysM domains should also be the subject of future research. The production of exopolysaccharides reported in *Leuconostoc* and *Weissella* may be linked to the recognition of carbohydrates in the outer shells of bacterial cells since these carbohydrates and the LysM domains are both associated with the formation of biofilms (Park et al. 2013; Sun et al. 2020). In this study, the activity of the recombinant protein AmiLysM4 (without one LysM domain) showed antibacterial activity against microorganisms that are often responsible for decomposition in meat products.

The change in the structure of AmiLysM4 observed *in silico* may be related to the anchoring of the cell walls of these microorganisms. Additionally, the prediction of the structures showed differences between AmiC and AmiLysM4 in the *3dom* region where LysM domains are found, but no differences were noted for the catalytic region. The template used in the model (Swiss-Model Template Library, SMTL 4b8v.1) was LysM Ecp6, which has a region of three LysM domains that generate a composite binding site with a high affinity for chitin, a polysaccharide composed of N-acetylglucosamine units (Sánchez-Vallet et al. 2013). In contrast to reports from other works indicating that LysM domain deletion decreases or even inhibits enzymatic activity (Eckert et al. 2006; Wong and Blaise 2020), this work suggests that deletion of the fifth LysM domain may cause the remaining domains of the protein, specifically, the *3dom* region, to assume a conformation with a high affinity for the cell walls of microorganisms such as *Leuconostoc*; however, to validate this hypothesis, different experiments must be performed, including molecular coupling. On the other hand, the prediction of secondary structures showed that the randomly oriented monomer subunits constituted more than 50% of the secondary structures (random coils), which together with tertiary interactions determine the overall protein folding (Khoury et al. 2014). These regions include the sequences that interconnect with the LysM domains, and according to reports of proteins that have been crystallized, such as the endopeptidase NlpC/P60 TTHA0266, these regions are very flexible and influence the conformation of the LysM domains such that small alterations in structure may cause changes at the level of biological activity; in this case, these changes can be used mainly in biotechnological applications (Wong and Blaise 2013; Wong et al. 2015). Although the autolytic systems of several Gram-positive bacteria such as *S. aureus, B. subtilis, Bacillus thuringiensis, Pediococcus spp., L. lactis, E. faecalis*, and *Lactiplantibacillus pentosus* have been studied, the specific autolytic mechanisms and associated enzymes involved in *L. sakei* are still unclear (Najjari et al. 2016).

Paradoxically, *W. viridescens* is within the antimicrobial spectrum of the native N-acetylmuramoyl-L-alanine amidase of *L. sakei* isolated from salami; however, after being cloned and expressed as AmiC, it lost the ability to inhibit this strain. Among the disadvantages of the expression of recombinant proteins

is coupled transcription and translation. The variation in posttranslational modifications or the lack of these modifications that may be related to the loss of activity warrants thorough exploration by different strategies to explain this function (Hemamalini et al. 2020).

Another factor modulating protein binding to cell walls is pH. Maximum activity values were observed at pH 8 for both recombinant proteins, which coincides with the optimal value reported for the native protein (García-Cano and Ponce-Alquicira 2015). This pH is close to the theoretical pI values of 9.95 for AmiC and 9.90 for AmiLysM4. At pH 8, the proteins are positively charged; in contrast, the Gram-positive bacterial cell wall has a negative charge, thus favoring the binding of the protein to the cell wall (Low et al. 2011). These results diverge from those of different PGHs produced by other *Lactobacillus* strains, where the optimal activity occurs around pH 5. Most PGHs reportedly have optimal activity at neutral or slightly acidic pH values (Xu et al. 2015). However, recent studies reveal that PGHs from the genus *Bacillus* reach their maximum activity at pH values between 7 and 8 (Etobayeva et al. 2018).

Another advantage of these recombinant proteins is their high temperature tolerance. The proteins retained maximum activity at 50°C for 30 min. These results differ from those for the native protein, whose optimal temperature for activity was 37°C, which decreased by 23% when incubated at 50°C (García-Cano and Ponce-Alquicira 2015). Another factor that may influence the high temperature tolerance is the aliphatic index defined as the relative volume of a protein occupied by aliphatic side chains (alanine, valine, isoleucine, and leucine residues), which was determined to be greater than 50% *in silico*. These temperature-tolerant proteins have potential applications in industrial processes.

In this study, the antimicrobial activity of the protein without one of the LysM domains was not drastically affected, even showed the ability to inhibit microorganisms that did not inhibit the protein with the entire sequence. Future studies can focus on eliminating the other LysM domains and evaluate the importance of each domain to enzymatic activity.

In summary, both recombinant proteins represent promising alternatives in different fields, as has been mentioned in other studies. The potential applications of this new class of antimicrobial agents include medicine, biotechnology, agriculture, and food safety since they can be useful to control bacterial infection or other unwanted contamination. As mentioned by Schmelcher and Loessner (2016), they can be used to control bacterial contamination at different points within the food production chain, and high-affinity cell wall binding domains, such as the *3dom* region evaluated in this study, can be applied for rapid detection of pathogenic bacteria. Identifying and characterizing proteins that can inhibit microorganisms that cause high economic losses in the food industry, such as *L. mesenteroides* and *W. viridescens*, are important. The inhibitory spectrum of PGHs may be limited. For example, LytA from *Streptococcus pneumoniae*, which exhibits N-acetylmuramoyl-L-alanine amidase activity, is used to treat infections of the same producing strain (Afshar et al. 2020), and lysostaphin from *S. aureus* is also used to eradicate staphylococcal infections (Gonzalez-Delgado et al. 2020). To effectively inhibit bacteria that represent a health risk or cause food spoilage, leading to economic losses, PGHs used as antimicrobial agents must have a broad inhibitory spectrum.

The applications and possible benefits that PGHs can provide are increasing, and new approaches are emerging every day towards methods that can be used alone or together to increase the efficiency of bioconservation processes.

Declarations

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Ethics approval. This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest. The authors state no conflict of interest.

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Figures

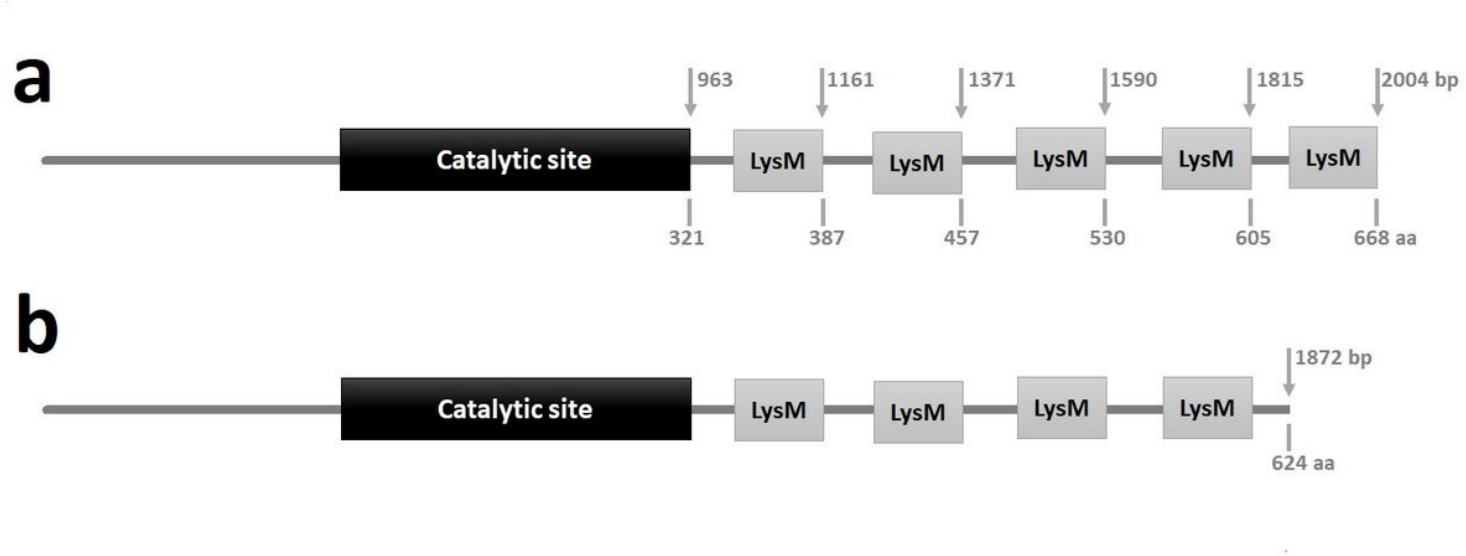

Figure 1

Graphic representation of the domains of AmiC (a) and AmiLysM4 (b) proteins.

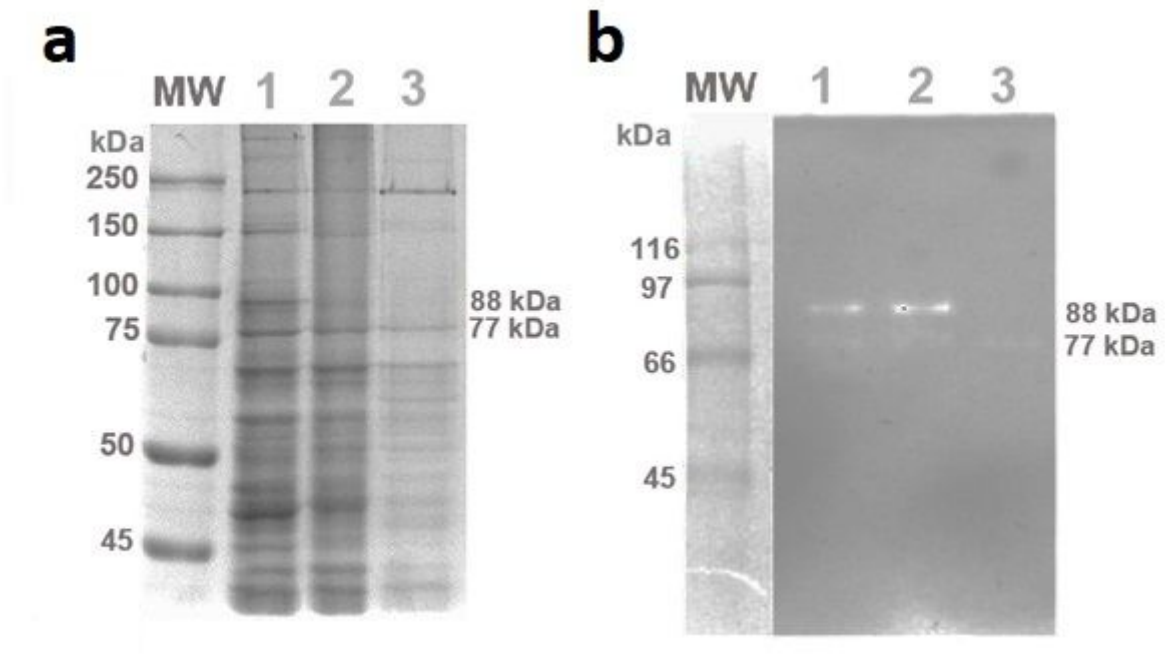

Figure 2

Protein profiles and zymograms for AmiC and AmiLysM4 under the best induction conditions with IPTG; a Protein profile SDS-PAGE (10%), b Zymogram against M. lysodeikticus. MW: Molecular weight ladder. Induction line 1: CE of pET22b-AmiC with 0.2 mM IPTG; line 2: CE of pET22b-AmiC with 0.4 mM IPTG; line 3: CE of pET22b-AmiLysM4 with 1 mM IPTG.

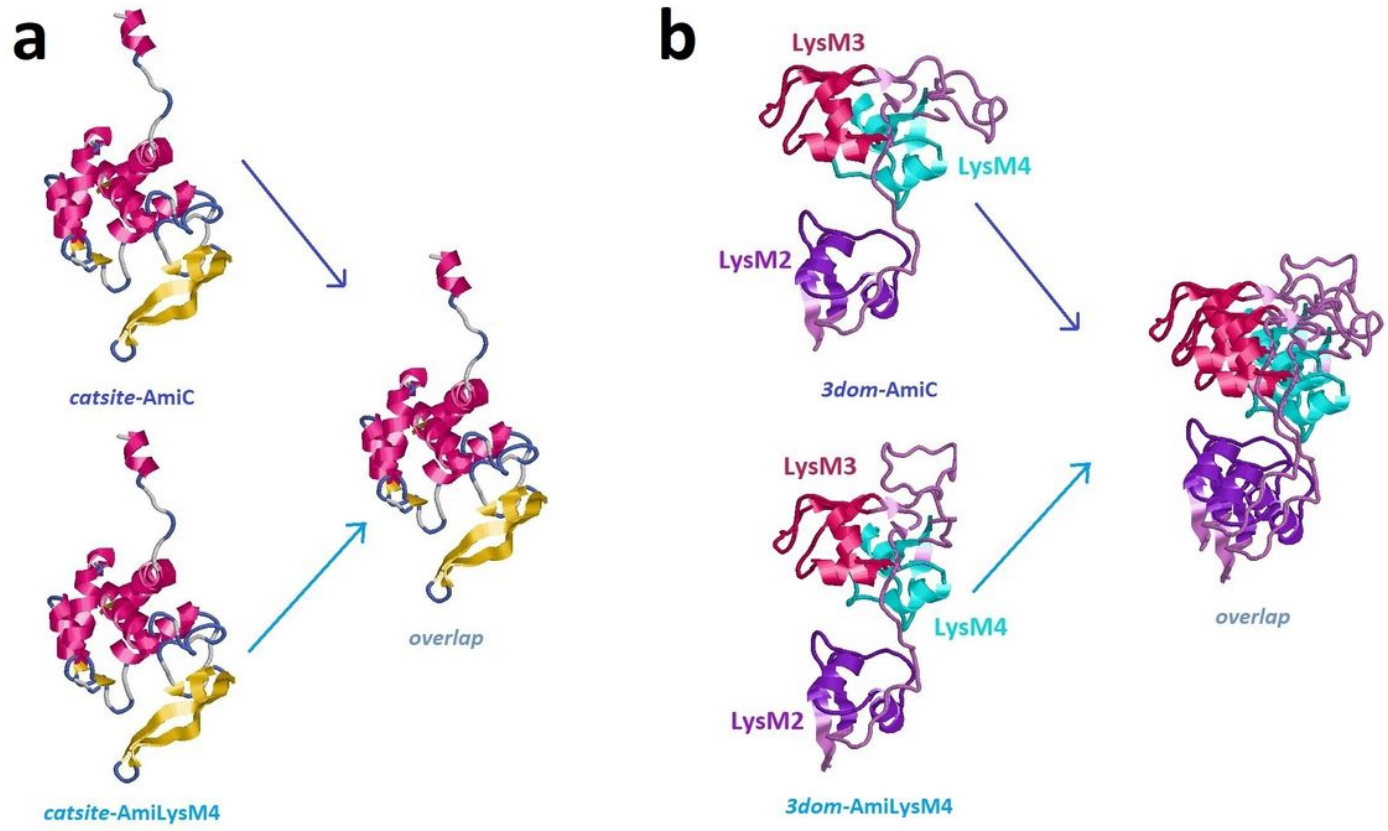

Figure 3

3D structural model for the catsite region and 3dom of AmiC and AmiLysM4; a Superposed model for catsite-AmiC and catsite-AmiLysM4; b Superposed model for 3dom-AmiC and 3dom-AmiLysM4.

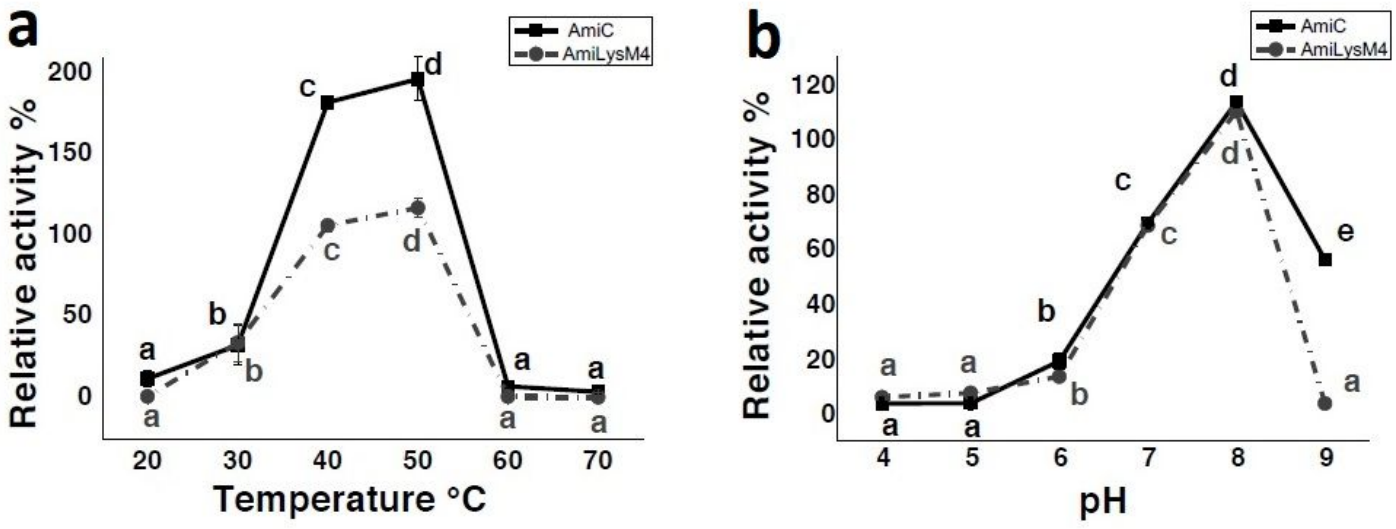

Figure 4

Effects of temperature and pH on the relative activity of recombinant proteins: a Temperature b pH.

Supplementary Files

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