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LambdaSa1 and LambdaSa2 Prophage Lysins of *Streptococcus agalactiae*

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Running title: LambdaSa1 and LambdaSa2 Lysins

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Putative N-acetylmuramoyl-L-alanine amidase genes from LambdaSa1 and LambdaSa2 prophages of *Streptococcus agalactiae* were cloned and expressed in *Escherichia coli*. The purified enzymes lysed the cell walls of *Streptococcus agalactiae*, *Streptococcus pneumoniae*, and *Staphylococcus aureus*. The peptidoglycan digestion products in the cell wall lysates were not consistent with amidase activity. Instead, the structure of the muropeptide digestion fragments indicated that LambdaSa1 and LambdaSa2 lysins both exhibited γ-D-glutaminyl-L-lysine endopeptidase activity. The endopeptidase cleavage specificity of the lysins was confirmed using a synthetic peptide substrate corresponding to a portion of the stem peptide and cross-bridge of *Streptococcus agalactiae* peptidoglycan. The LambdaSa2 lysin also displayed β-D-N-acetylglucosaminidase activity.

Bacteriophages often lyse their bacterial hosts at the end of an infective cycle and release their progeny into the surrounding medium. Phages of gram-positive bacteria usually accomplish this by employing a two-component lytic system consisting of a holin and a lysin (11). The holin makes the bacterial cell membrane porous, thereby exposing the peptidoglycan layer of the cell wall to the degradative action of the lysin. Phage lysins and the related endogenous bacterial cell wall hydrolases have been found that cleave many different bonds in peptidoglycan (11, 12). They include β-D-N-acetylmuramidases, β-D-N-acetylglucosaminidases, lytic transglycosylases, N-acetylmuramyl-L-alanine amidases, and various endopeptidases that cleave either the stem peptide or cross-bridge peptide of peptidoglycan (11, 12).

The observation that many phage lysins specifically lyse susceptible gram-positive bacteria, which have been exposed to the enzyme externally, has led to recent intense interest in using them as antimicrobial agents. For example, Nelson and coworkers showed that nasopharyngeal colonization of mice with *Streptococcus pyogenes* could be cleared using the C1 phage lysin from a group C streptococcus (14). Schuch *et al.* reported that the phage enzyme, PlyG, killed vegetative cells of *B. anthracis* and protected mice from an otherwise fatal intraperitoneal infection with *Bacillus cereus* (16). Similarly, Jado and coworkers showed that a single intraperitoneal injection of either of the pneumococcal phage lysins Pal, an amidase, or Dpl-1, a lysozyme, was sufficient to protect mice in a murine model of pneumococcal sepsis (7). These workers also demonstrated a synergistic effect on pneumococcal killing when the two enzymes were administered together. Similar synergism among different peptidoglycan cleavage activities may be why many phage lysins evolved with more than one type of lytic activity. The different peptidoglycan cleavage activities are usually located on distinct

enzymatic domains, often along with one or more cell wall binding domains (11, 12). We recently described the cloning and elucidation of cleavage specificities of the B30 lysin from a bacteriophage of *Streptococcus agalactiae* (1, 15). It contains an endopeptidase domain, a glycosidase domain, and an SH3b putative cell wall binding domain (1, 15). The endopeptidase activity was shown to cleave between the D-alanine of the stem peptide and L-alanine in the cross-bridge, and the glycosidase had the specificity of an N-acetylmuramidase (1). We initially cloned the genes for the two lysins described in this report expecting them to exhibit N-acetylmuramyl-L-alanine amidase activity, however, as described below, characterization of their cleavage specificities showed that they were not amidases.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Streptococcus agalactiae* and *Staphylococcus aureus* strains from our collections were routinely plated on Trypticase soy agar (BBL) containing 5% sheep blood or cultured in Todd-Hewitt broth (Difco) at 37° C without shaking. *S. pneumoniae* were cultured in Todd-Hewitt broth containing 0.5% (w/v) yeast extract. Serotype V strain 2603 V/R *Streptococcus agalactiae* was obtained from the ATCC (No. BAA-611). *Escherichia coli* strains INVαF′ and BL21/DE3 were routinely grown at 37°C with shaking in Luria-Bertani (LB) broth. However, for protein expression, cultures were grown at room temperature.

Cloning the genes for LambdaSa1 and LambdaSa2 lysins. Chromosomal DNA was prepared from GBS strain 2603 V/R by a procedure described previously (9). Briefly, this involved digesting washed cells with mutanolysin at 60° C for 30 min, adding sodium dodecyl

sulfate (2% final concentration) to lyse the cells, cooling to 37° C and treating with ribonuclease, purifying the DNA by phenol-chloroform extraction, and finally precipitating the DNA with isopropanol. The two lysin genes were PCR amplified with chromosomal DNA as template. The LambdaSa1 gene was amplified using the primers SAG0604F, 5′ GTA CAG TCC ATA TGG TAA TTA ATA TTG AGC AAG CTA TC 3′ and SAG0604R, 5′ CGT ACA TGC TCG AGC ATA TCT GTT GCA TCA ATT AAG TAA G 3′. The primers for the LambdaSa2 gene were SAG1837F, 5′ GTA CAG TCC ATA TGG AAA TCA ACA CTG AAA TAG CCA TTG 3′ and SAG1837R, 5′CGT ACA TGC TCG AGA ACT GGC TTT TTA GTC AGT TCA T 3′. The PCR products were cloned into the pGEM T-vector (Promega, Madison, WI) and transformed into *E. coli* strain INVαF′. Purified plasmid DNA was cleaved with the restriction endonucleases NdeI and XhoI and the inserts cloned into similarly digested expression vector pET21a (Novagen). This construct was then transformed into *E. coli* strain BL21/DE3 for enzyme expression. The sequence of the resulting plasmid was confirmed by DNA sequencing.

Expression and purification of cloned lysins. Starter cultures of E. *coli* BL21/DE3 containing the recombinant plasmids were grown overnight in LB medium containing 50 μg/ml ampicillin at 37°C with shaking at 250 rpm. This culture was diluted with twenty volumes of fresh medium (3 liters) and grown at 37°C until it reached mid log phase ($OD_{550\ nm}$ of 0.5). Lysin expression was induced by adding isopropyl-β-D-thiogalactopyranoside to give a final concentration of 1 mM. After growing for an additional 4.5 h at room temperature, the cells were harvested by centrifugation and washed three times with extraction buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). The pellet was resuspended in 100 ml of extraction buffer and sonicated in an ice bath for a total of 5 min at 60% of maximum power using a model 300 Fisher

Sonic Dismembrator. Cell debris was removed by centrifugation and the resulting supernate passed over a 10 ml Ni-NTA affinity column (Qiagen), which was then washed and eluted according to the manufacturer's instructions. Imidazole was removed from the sample by passing it through a 2.5 x 14 cm column of Bio-Gel P4 run in 50 mM ammonium acetate, pH 6.8. Fractions with enzyme activity were pooled and concentrated by ultrafiltration to give final approximate concentrations of 10 mg/ml for LambdaSa1 lysin and 1 mg/ml for LambdaSa2 lysin.

Turbidity reduction assays were carried out on washed cells or bacterial cell walls essentially as we described previously (15). However, the buffer used was 50 mM ammonium acetate, pH 6.8. Bacterial cell walls were also prepared as described previously (15). Plate assays for lytic activity were performed in 1% agarose in the above buffer containing a suspension of cells or cell walls sufficient to make the layers turbid. Holes (5 mm) were punched in the layers and 10 µl samples were added. After incubation at 37° C for several hours, or overnight, distinctive clear zones form around sample wells containing lysin activity.

Lysin endopeptidase activity was estimated by measuring the free amino groups released during digestions. Cell walls were enzymatically digested as described above, but with 50 mM sodium acetate substituting for ammonium acetate. After centrifugation of the digests, aliquots of the supernatants were assayed for free amino groups by a spectrophotometric method employing picryl sulphonic acid (13). In order to determine the site of endopeptidase cleavage, a synthetic peptide corresponding to a portion of the stem peptide and cross-bridge of GBS peptidoglycan (Fig.1) was lysin digested. The hexapeptide Ala-D-γ-Glu-Lys-D-Ala-Ala-Ala was custom synthesized by Genscript Corp. (Piscataway, NJ) and the two carboxyl groups of the

peptide were amidated (8, 17) to yield Ala-D-γ-Gln-Lys-D-Ala-Ala-Ala-amide. Digestion products were characterized by mass spectrometry.

Sample preparation for mass spectrometry. Washed cell walls of different bacterial species were treated with purified lysins overnight. In some cases double digests using both mutanolysin and a phage lysin were carried out. In all cases the digestion mixtures were centrifuged and the supernates were passed through a 5 K cutoff centrifugal ultrafilter (Ultrafree-MC Centrifugal filter, Millipore Corp., Billerica, MA) prior to analysis in order to remove proteins and capsular polysaccharides. Alkaline de-O-acetylation of glycopeptides was accomplished by treating samples with 5% triethylamine at room temperature for 30 min followed by evaporation to dryness in a vacuum centrifuge. Acetate released from glycopeptides was measured enzymatically using a kit supplied by R-Biopharm Inc. (Marshall, MI 49068).

Electrospray ionization mass spectrometry. This was performed on a Micromass Q-TOF2 mass spectrometer (Micromass Ltd., Manchester, U.K.). Samples were dissolved in either water or dilute ammonium acetate buffer. Sodium salts were avoided to minimize the formation of sodium adducts. Online injections were made into a stream of 50/50 acetonitrile/water containing 0.1% formic acid pumped at a rate of 1 μL/min. Negative ion mass spectra were obtained using a needle voltage of 2.8 kV. The mass spectral data was processed using the Max-Ent3 module of MassLynx 3.5.

Determining the mode of glycosidic cleavage. This was accomplished by identifying the amino sugar at the reducing terminus of glycopeptides obtained upon digestion with a lysin.

Procedures for the borohydride reduction, propanolysis, re-N-acetylation, and trimethylsilylation of samples have been described previously (1). Derivatized samples were analyzed on a GLC/MS (Varian 4000, Varian Inc. Palo Alto, CA) fitted with a 30-m (0.25 mm in diameter) VF-5 capillary column. Column temperature was maintained at 100°C for 5 min, then increased to 275°C at 20°C/min, and finally held at 275°C for 5 min. In an improved version of the original procedure used in this work, the column effluent was analyzed by chemical ionization mass spectrometry using acetonitrile as the reagent gas. This relatively gentle means of sample ionization results in little fragmentation and often yields ions that are highly characteristic of a particular sugar or alditol.

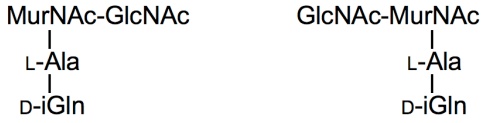
RESULTS AND DISCUSSION

The increasing incidence of antibiotic resistance in pathogenic bacteria has contributed to the recent interest in bacteriophage lysins as potential antimicrobial agents. Encouraging results from several animal experiments are likely to prompt further work directed towards using phage lysins to prevent or treat human bacterial diseases (1, 3, 6, 7, 10, 14, 16).

The sequencing of bacterial genomes often reveals the presence of prophage lysin genes. Such genes are usually identified by their sequence similarities to known lysin genes and on this basis they are generally annotated as coding for amidases, lysozymes, endopeptidases, or other types of lysins. Occasionally, lysin genes are annotated incorrectly. The sequenced genome of strain 2603V/R *S. agalactiae* contains several lysin genes, including two putative amidases from LambdaSa1 and LambdaSa2 prophages (19). These two lysin genes were cloned and expressed as described above. Crude cell lysates obtained by sonic disruption of the *E. coli* cells were found to rapidly lyse *S. agalactiae* cells and cell walls in a turbidity reduction assay and also

gave distinct zones of clearing in the lysin plate assay (data not shown). Interestingly, although both LambdaSa1 and LambdaSa2 lysins gave slightly smaller clear zones in the agar plate than did the B30 lysin we described previously, the zones showed complete clearing with no residual turbidity, unlike the B30 lysin. Presumably this is because some of the products of B30 digestion are large enough to remain insoluble. Since digestion of *S. agalactiae* cell walls with affinity-purified LambdaSa1 or LambdaSa2 lysins led to a significant increase in free amino groups, it was evident that the lysins possessed either N-acetylmuramyl-L-alanine amidase or endopeptidase activities. Further characterization of the lysin digestion products was necessary to identify the type of enzymatic activity present.

Lysin digestion products of *S. agalactiae* cell walls that had been passed though a 5 K-cutoff ultrafilter were examined by electrospray mass spectrometry in the negative ion mode. No significant peaks above background were found in LambdaSa1 lysin digests. So although LambdaSa1 lysin rapidly decreases the turbidity of a suspension of *S. agalactiae* cell walls, it does not yield fragments small enough to pass through a 5 K-cutoff ultrafilter. However, LambdaSa2 lysin digests contained low-molecular weight products that gave remarkably simple mass spectra (Fig 2A). Two prominent peaks, 694 m/z and 736 m/z were observed. The first peak corresponds to the M-1 of a disaccharide containing N-acetylglucosamine and N-acetylmuramic acid linked to Ala-Gln and will have the structure of I or II illustrated below.



I II

The production of this glycopeptide indicates that both endopeptidase and glycosidase activity are present. The second peak at 736 m/z is 42 mass units higher than the first peak and

appeared to be the same glycopeptide with an additional acetyl group. Brief alkaline treatment of the sample with aqueous triethylamine resulted in the loss of the 736 m/z peak (Fig 2B), consistent with the presence of a labile O-linked acetyl group. That acetate was released was confirmed by enzymatic measurement of released acetate (data not shown).

The presence of alanine and glutamine in the muropeptides is evidence that the lysin possesses an endopeptidase activity capable of cleaving between D-γ-glutamine and lysine in the stem peptide of the peptidoglycan. In order to support this finding, the peptide Ala-D-γ-Gln-Lys-D-Ala-Ala-Ala-amide was synthesized from Ala-D-γ-Glu-Lys-D-Ala-Ala-Ala as described in the Materials and Methods section. Digesting this peptide with LambdaSa2 lysin resulted in the near complete loss of substrate peptide (M+1 = 558) and the appearance of a peak at 339 m/z corresponding to the M+1 ion of Lys-D-Ala-Ala-Ala-amide, confirming that it is the γ-linkage between D-Gln and L-Lys that is cleaved by this lysin.

The amino acid sequence of LambdaSa2 lysin contains two domains, annotated as amidase-5 and amidase-4 domains (see Fig. 1). In contrast, the smaller LambdaSa1 lysin lacks the amidase-4 domain and its amidase-5 domain has 71% amino acid identity to that of the LambdaSa2 lysin. Since digestion of cell walls with either lysin results in the exposure of free amino groups it was predicted that the amidase-5 domain is responsible for endopeptidase activity in both cases. The observed glycosidase activity of the LambdaSa2 lysin would then be expected to be associated with the amidase-4 domain. Since this domain is absent in the LambdaSa1 lysin we predicted that double digestion of *S. agalactiae* cell walls with both LambdaSa1 lysin and the muramidase mutanolysin would yield glycopeptide II. As shown in Fig. 2C the double digest gave the same two muropeptide fragments observed in LambdaSa2

digests. Therefore, the amidase-5 domain of the LambdaSa1 lysin is also responsible for peptidoglycan cleavage at its D-γ-Gln-Lys linkage.

LambdaSa1 and LambdaSa2 lysin also degrade the cell walls of *Staphylococcus aureus* and *S. pneumoniae,* but the relative proportions of the glycopeptides in LambdaSa2 digests varies. LambdaSa2 digests of *S. agalactiae* cell walls (Fig. 2A) contain approximately equal amounts of a glycopeptide composed of alanine, glutamine, N-acetylglucosamine and N-acetylmuramic acid (m/z 694) and a similar glycopeptide containing an additional acetyl group (m/z 736). However, as shown in Fig. 2D, LambdaSa2 digests of *Staphylococcus aureus* cell walls have a much higher proportion of O-acetylated muropeptide (736 m/z). This O-acetylated glycopeptide was a minor component in digests of *S. pneumoniae* cell walls (Fig 2E). These digests contained about equal amounts of the core disaccharide-dipeptide and an N-deacetylated form of the compound that gives a peak at 652 m/z. Acetylation of *S. aureus* cell walls occurs at the C6-OH of the N-acetylmuramic acid residues and has been reported to render the cell walls resistant to lysozyme (2). Similarly, N-deacetylation of N-acetylglucosamine residues in *S. pneumoniae* peptidoglycan has also been shown to make the cell walls resistant to lysozyme (20). Clearly, the presence of O-acetylated and N-deacetylated glycopeptides in LambdaSa2 lysin digests show that LambdaSa2 lysin, unlike lysozyme, is capable of cleaving peptidoglycan with these two types of modifications.

In order to determine whether the glycosidase activity of LambdaSa2 lysin cleaves after an N-acetylmuramic acid or an N-acetylglucosamine residue, it was necessary to establish their sequence in the glycopeptide digestion products, i.e., whether the dual action of LambdaSa2's glycosidase and endopeptidase activities release glycopeptides I or II, or both. We were unable to confidently determine this by electrospray mass spectrometry alone. We therefore reduced the

glycopeptides with sodium borohydride and identified the resulting amino sugar alditol by a GC/MS procedure. As described previously, depolymerization of samples using propanolysis greatly improves the gas chromatographic separation of the amino sugars and alditols (1). In addition, the use of chemical ionization results in very limited fragmentation and yields high-molecular mass ions that can be selectively monitored to identify the amino sugar or alditol. Unexpectedly, the mass spectra of the derivatized four sugars and alditols all possessed molecular ions indicating that not only the hydroxyl groups, but also the nitrogen of the acetamido groups, had been trimethylsilylated (data not shown). Panel 3A shows the total ion chromatogram obtained for a mixture of GlcNAc, GlcNAc-ol (reduced GlcNAc), MurNAc, and MurNAc-ol (reduced MurNAc). Selected ion monitoring of the indicated fragment ions clearly distinguishes each sugar or alditol (Figs. 3B-3E). Fig. 3F shows the results obtained for LambdaSa2 lysin digestion products when the indicated four ions were monitored. Two main peaks are present corresponding to N-acetylglucosaminitol and N-acetylmuramic acid, showing that glycopeptide I was present and was the product of N-acetylglucosaminidase action. That is to say, the LambdaSa2 lysin posseses N-acetylglucosaminidase and not N-acetylmuramidase activity. A much smaller peak due to MurNAc-ol was also present in the chromatogram showing that some MurNAc residues were at the reducing termini of digestion fragments. However, as we pointed out previously, this is most likely because peptidoglycan chains are biosynthesized from lipid-linked glycopeptide precursor units (lipid II) that have MurNAc residues at their reducing termini, and the polysaccharide chains of GBS peptidoglycan are short (1). Analysis of the double digest with LambdaSa1 lysin and mutanolysin gave peaks due to N-acetylglucosamine and N-acetylmuramitol indicating that glycopeptide II was present in this digest. This is consistent with the known N-acetylmuramidase activity of mutanolysin. Thus, it

237 is concluded that the GBS prophage LambdaSa2 lysin possesses both N-acetylglucosaminidase
238 and γ-D-glutaminyl-L-lysine endopeptidase activities. The smaller LambdaSa1 lysin possesses
239 only the endopeptidase activity.

240 We previously described the cleavage specificities of the B30 phage lysin of *S.*
241 *agalactiae* (1). We found that it possessed two enzymatic domains, an endopeptidase domain
242 and an N-acetylmuramidase domain. Using synthetic peptide substrates mimicking the stem
243 peptide and cross-bridge peptide of GBS peptidoglycan we showed that the endopeptidase
244 specifically cleaves between the D-Ala of the stem peptide and an L-Ala of the cross bridge.
245 However, the endopeptidase activities of the two lysins described in this paper cleave the stem
246 peptide between D-γ-glutamine and L-lysine. It is of interest, and possible significance, that the
247 B30 lysin and both lysins described in the current study cleave after D-amino acid residues, i.e. at
248 D-Ala-L-Ala or at D-γ-Gln-L-Lys. Cleavage of linkages involving a D-amino acid may help
249 ensure that the endopeptidases target cell wall peptidoglycan and not protein components of the
250 cell wall.

251 Lopez and Garcia pointed out that lysins typically possess both substrate binding and
252 bond cleavage specificities (12). Cell wall binding domains of lysins are responsible for
253 conferring substrate specificity whereas the enzymatic domains determine which bonds of the
254 peptidoglycan are cleaved. The putative cell wall binding domain of the LambdaSa1 lysin is an
255 SH3b domain, while the two putative cell wall binding domains of the LambdaSa2 lysin are of
256 the Cpl-7 type (Fig. 1A). We focused on the enzymatic domains, however, in the current study.

257 Chimeric lysins composed of enzymatic and cell wall binding domains from different
258 sources have been constructed (4-6, 18). Some of these combinations have unique, potentially
259 useful properties. For example, Donovan *et al.* recently fused the gene for lysostaphin to either

the entire B30 lysin gene or just the endopeptidase domain of the B30 lysin. Expression of these constructs yielded chimeric enzymes that displayed lytic activity against all three major mastitis-causing pathogens of dairy cattle, *Streptococcus agalactiae* (GBS), *Streptococcus uberis*, and *Staphylococcus aureus* (6). Knowledge of the precise cleavage specificities of phage lysins therefore is important for the rational design of such engineered chimeric enzymes.

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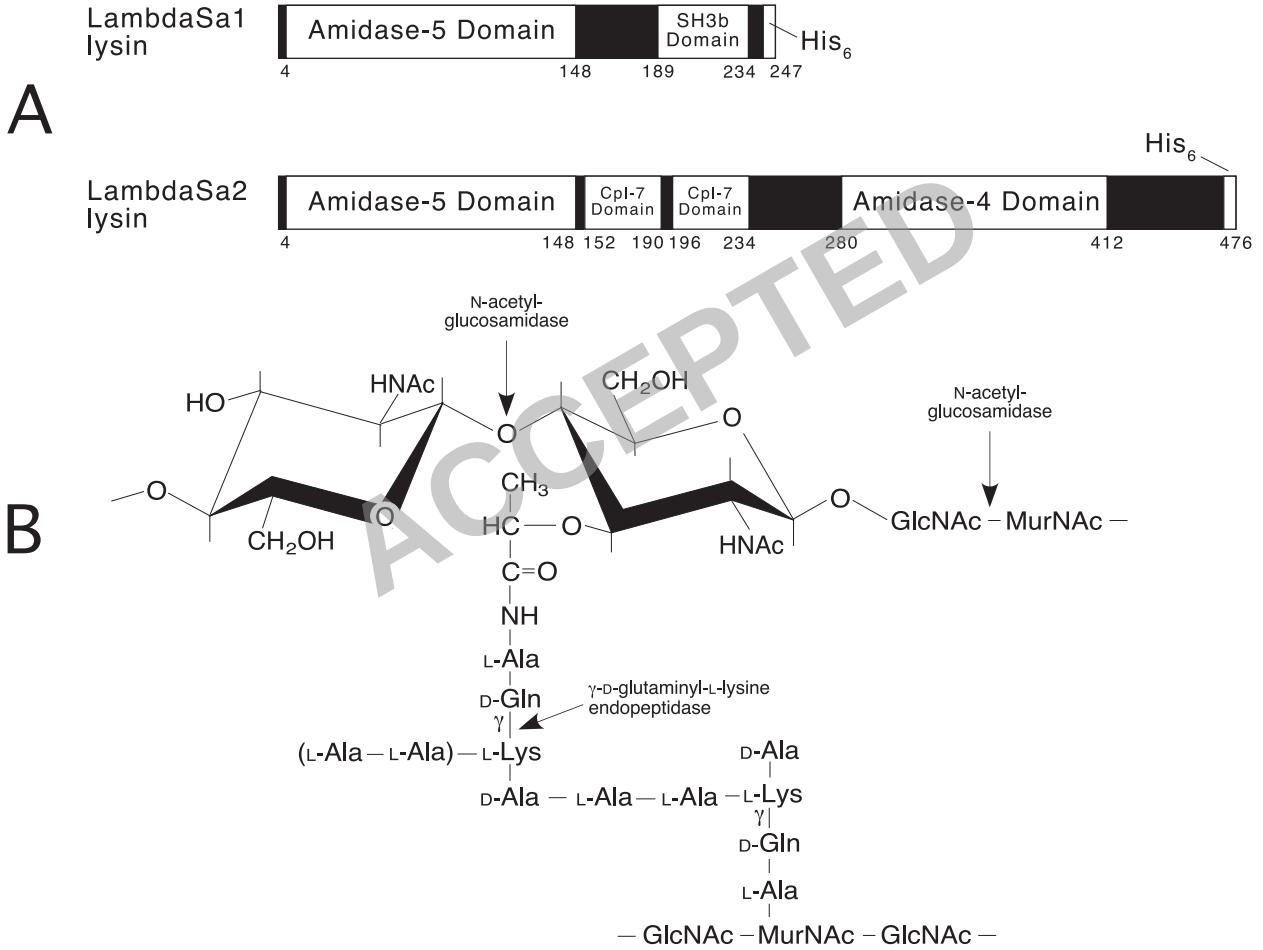
FIGURE LEGENDS

FIG. 1. Domain structure of the cloned LambdaSa1 and LambdaSa2 lysins (A) and structure of GBS peptidoglycan showing the glycosidic bond cleaved by LambdaSa2 lysin and the endopeptidase cleavage site for both lysins (B).

FIG. 2. Negative ion mass spectra of the low-molecular weight products of lysin-digested bacterial cell walls. (A) LambdaSa2-digested GBS cell walls, (B) LambdaSa2-digested GBS cell walls treated with 5% (v/v) aqueous triethylamine, (C) LambdaSa1 plus mutanolysin-digested GBS cell walls, (D) LambdaSa2-digested *Staphylococcus aureus* cell walls, and (E) LambdaSa2-digested *Streptococcus pneumoniae* cell walls. The 694.4 m/z fragment is due to a disaccharide dipeptide composed of N-acetylglucosamine, N-acetylmuramic acid, alanine and glutamine. The 652.4 m/z fragment has one less acetyl group and the 736.4 m/z fragment has one more acetyl group than the core glycopeptide (694.4).

FIG. 3. Identification of the site of glycosidic bond cleavage by GC/MS. (A) Total ion chromatogram (TIC) of a mixture of derivatized N-acetylglucosamine, N-acetylglucosaminitol, N-acetylmuramic acid, and N-acetylmuramitol. (B) Selected ion chromatograms of the same sample for m/z 584 (GlcNAC-ol), (C) m/z 552 (GlcNAc), (D) m/z 626 (MurNAc-ol), and (E) m/z 462 (MurNAc). (F) Selected ion chromatogram for the four ions of m/z 462, 552, 584, and 626 for a LambdaSa2 digest of GBS cell walls. (G) Selected ion chromatogram for the same four masses for a double digest of GBS cell walls by LambdaSa1 and mutanolysin.


A
LambdaSa1 lysin
Amidase-5 Domain
SH3b Domain
His6
4
148
189
234
247
LambdaSa2 lysin
His6
Amidase-5 Domain
CpI-7 Domain
CpI-7 Domain
Amidase-4 Domain
4
148
152
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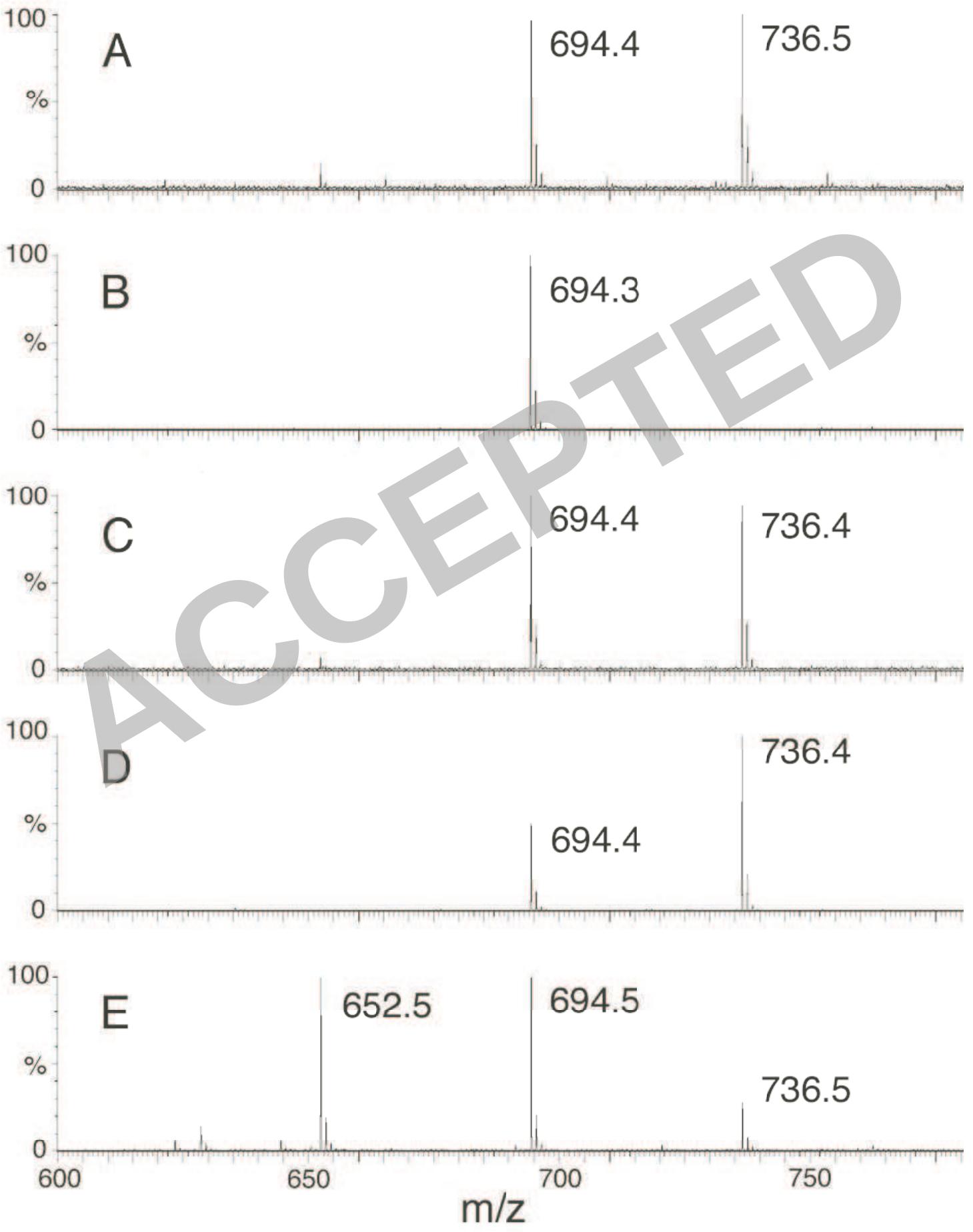
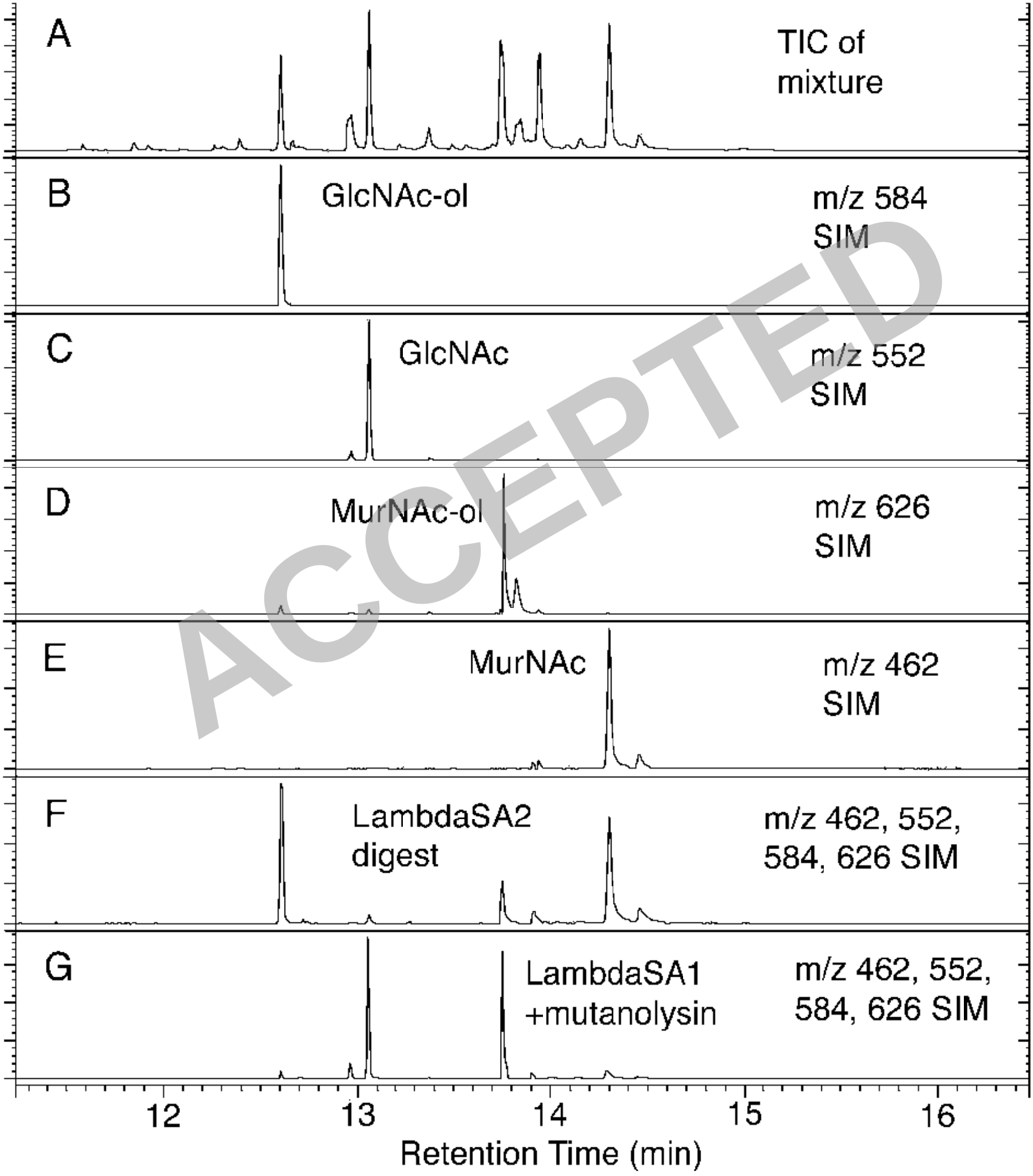
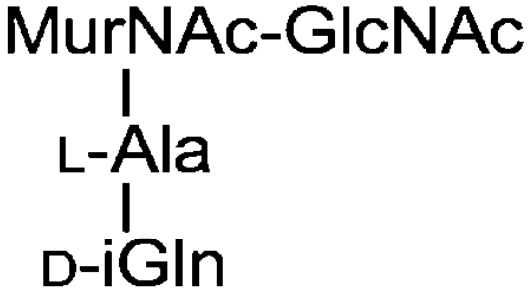
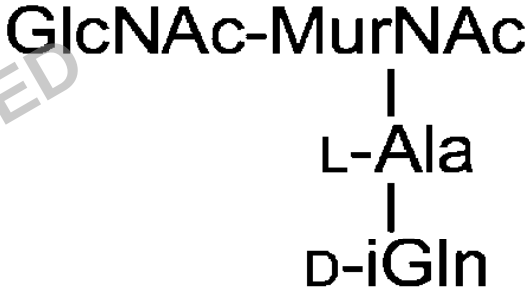
196
234
280
412
476
B
N-acetyl-glucosamidase
HO
HNAc
CH2OH
O
CH3
HC
C=O
NH
L-Ala
D-Gln
γ
γ-D-glutaminyl-L-lysine endopeptidase
(L-Ala — L-Ala) — L-Lys
D-Ala — L-Ala — L-Ala
D-Ala
L-Lys
γ
D-Gln
L-Ala
— GlcNAc — MurNAc — GlcNAc —
N-acetyl-glucosamidase
GlcNAc — MurNAc —
ACCEPTED


A
694.4
736.5
B
694.3
C
694.4
736.4
D
736.4
694.4
E
652.5
694.5
736.5
100
%
0
600
650
700
750
m/z


A
TIC of mixture
B
GlcNAc-ol
m/z 584 SIM
C
GlcNAc
m/z 552 SIM
D
MurNAc-ol
m/z 626 SIM
E
MurNAc
m/z 462 SIM
F
LambdaSA2 digest
m/z 462, 552, 584, 626 SIM
G
LambdaSA1 +mutanolysin
m/z 462, 552, 584, 626 SIM
12
13
14
15
16
Retention Time (min)



I



II