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Bacillus subtilis CwlQ (previous YjbJ) is a bifunctional enzyme exhibiting muramidase and soluble-lytic transglycosylase activities

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ABSTRACT

CwlQ (previous YjbJ) is one of the putative cell wall hydrolases in *Bacillus subtilis.* Its domain has an amino acid sequence similar to the soluble lytic transglycosylase (SLT) of *Escherichia coli* Slt70 and also goose lysozyme (muramidase). To characterize the enzyme, the domain of CwlQ was cloned and expressed in *E. coli.* The purified CwlQ protein exhibited cell wall hydrolytic activity. Surprisingly, RP-HPLC, mass spectrometry (MS), and MS/MS analyses showed that CwlQ produces two products, 1,6-anhydro-*N*-acetylmuramic acid and *N*-acetylmuramic acid, thus indicating that CwlQ is a bifunctional enzyme. The site-directed mutagenesis revealed that glutamic acid 85 (Glu-85) is an amino acid residue essential to both activities.

Introduction

*Bacillus subtili*s contains more than 35 putative cell wall hydrolase genes of which about 70% of the gene products have been characterized. The gene products are known to be associated with functions in cell growth, cell separation, cell wall turnover, motility, cell lysis, sporulation and germination [1,2,3]. The function of the *cwlQ* (*yjbJ*) gene, expressed during the transition phase in the DSM medium (data not shown) is unknown. *cwlQ* encodes a 181-amino acid polypeptide which has high sequence similarity to the soluble lytic transglycosylase Slt70 of *Escherichia coli.* The SLT domain covers 61 % of the entire CwlQ. The SLT domain contains soluble lytic transglycosylase and muramidase (Fig. 1), though the domain name is “SLT”. SLT and muramidase cleave the β-1,4-glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in

peptidoglycan, however the digested products by SLT and muramidase are 1,6-anhydro-*N*-acetylmuramic acid and *N*-acetylmuramic acid, respectively (Fig. 1) [4,5,6,7]. At present, lytic transglycosylases and muramidases cannot be distinguished by their amino acid sequences. In *B. subtilis*, CwlT (YddH; ICE*Bs1* protein), YomI (phage SP-beta protein), YqbO, and XkdO (defective phage PBSX protein) are paralogs of CwlQ, and recently we reported that the SLT domain of CwlT is a muramidase [8]. In this study we expressed CwlQ in *E. coli* and investigated the enzymatic activities of CwlQ. We identified the reaction products of CwlQ by mass spectrometry, and determined that one amino acid replacement of CwlQ resulted in an enzyme which completely lacked both SLT and muramidase activities.

Methods

Bacterial strains. The plasmids and primers used in this study are listed in Tables 1 and 2, respectively. The strain of *Escherichia coli* JM109 (*recA1* Δ[*lac-proAB*] *endA1 gyrA96 thi-1 hsdR17 relA1 supE44* [F' *traD36 proAB+ lacI*^{q} *lacZ* ΔM15]) used in this study was grown in LB medium [9] at 30°C containing 100 μg/ml ampicillin (final concentration). If necessary, 2% glucose and 1 mM IPTG (isopropyl-beta-D-thiogalactopyranoside; final concentration of 1 mM) were added to the medium.

Construction of plasmid for overexpression of CwlQ (YjbJ). The *cwlQ* gene was amplified by PCR with BF-YjbJ and KR-YjbJ primers with the *B. subtilis* 168 DNA as a template. The PCR fragment was digested with *Bam*HI and *Kpn*I, and ligated to the

corresponding site of pQE-30, resulting in pQE-CwlQ. pQE-CwlQ was used for transformation of *E. coli*, and overexpression of CwlQ in *E. coli* was performed by the addition of IPTG as described previously [10].

Construction of plasmids to identify the active site of CwlQ. To overexpress site-specific mutations of the CwlQ proteins, new plasmids were created from pQE-CwlQ as a template with a QuikChange II site-directed mutagenesis kit (Stratagene), according to the manufacturer's instructions. The amplification of entire plasmids with site-specific mutations was performed with two complementary DNA oligomers as primers (Table 2). The created plasmids, pQECwlQ-E85A and pQECwlQ-E85Q were utilized for overexpressing the mutated CwlQ proteins (E85A and E85Q, respectively).

Purification of cell wall and peptidoglycan from B. subtilis *168.* Cell wall and peptidoglycan from *B. subtilis* 168 were prepared as described previously [11,12,13].

SDS-PAGE and zymography. SDS-PAGE and zymography were performed as described by Sambrook et al. [9] and Leclerc and Asselin [14,15], respectively.

Overexpression and purification of CwlQ and the mutated proteins. The CwlQ and the mutated proteins were overexpressed in *E. coli* JM109. The strains were incubated at 30°C in LB medium containing 100 μg/ml ampicillin with 2% glucose. When cell growth reached an absorbance of 0.6-1.0 at 600 nm, 1 mM IPTG (final concentration) was added to the culture and further incubated for 3 hours. The proteins were extracted from the cells and purified with a HiTrap Chelating HP column (GE Healthcare), according to the manufacturer's instructions.

Determination of the optimum pH value and temperature. The pH and

89 temperature required for optimum hydrolytic activity of CwlQ were determined by
90 measurement of the cell wall density with a spectrophotometer (V-560, JASCO) as
91 described previously [16].

92 *Preparation of N-acetylated glycan strands containing GlcNAc-MurNAc*
93 *polymer.* Preparation of glycan strands (-[GlcNAc-MurNAc]n-) from *B. subtilis*
94 peptidoglycan was performed by digestion with CwlH amidase as described previously
95 [17]. The prepared glycan strands were *N*-acetylated by the modified method with
96 anhydroacetic acid as described previously [17]. The purified glycan strands (5 mg) were
97 dissolved in 1 ml of purified water and 1.25 ml of saturated $NaHCO_3$ then 1.25 ml of 5%
98 anhydroacetic acid was mixed in the solution, followed by stirring at 4°C for 30 min.
99 After a further 1.8 ml of 5% anhydroacetic acid was added to the sample, the mixture was
100 stirred at 4°C for 30 min and then incubated at 37°C for 1 hour. The *N*-acetylated glycan
101 strands were separated with a HiTrap Desalting column (GE Healthcare), according to the
102 manufacturer's instructions, and then glycan strands were freeze-dried.

103 *Digestion of N-acetylated glycan strands by CwlQ.* The *N*-acetylated glycan
104 strands (1 mg) were dissolved in 1 ml of 20 mM MES buffer (pH 6) and 0.1 mg/ml of the
105 CwlQ enzyme was added to the sample, followed by incubation at 37°C for 6 hours. The
106 non-reduced or reduced samples (by treatment with $NaBH_4$), were prepared as described
107 previously [8].

108 *Separation of digested glycan strands by CwlQ.* The digested samples
109 ("non-reduced sample" and "reduced sample") were separated by reverse phase
110 (RP)-HPLC as described previously [8, 17].

Identification of separated peaks on RP-HPLC. The separated peaks on RP-HPLC were freeze-dried, followed by the addition of 50% CH_3CN containing 0.05% TFA. The samples were analyzed using electrospray ionization (ESI)-mass spectrometry (MS) and ESI-MS/MS (Agilent 1100 series LC/MSD Trap VL).

Results

Overexpression, purification, and determination of cell wall lytic activity of CwlQ

To prepare the purified CwlQ protein, we cloned the entire gene of *cwlQ* except the predicted signal sequence in *E. coli* using a pQE30 plasmid and the CwlQ protein with a histidine tag at its N-terminal was expressed in the presence of 1 mM IPTG. After purification with a His-trap column, CwlQ was subjected to SDS-PAGE and zymography (Fig. 2A and Supplemental Fig. 1A). The single band on SDS-PAGE at 17 kDa contained cell wall lytic activity which corresponds to the calculated M_r 16,403 of CwlQ with a histidine tag. The CwlQ had an optimum pH at 6.0-6.5 although the activity was almost completely lost at pH 7.5 (Supplemental Fig. 1B). The optimum temperature was 37-40°C (Supplemental Fig. 1C) and the enzyme was most active without any addition of salt, while higher salt concentrations completely inhibited its activity (Supplemental Fig. 1D).

Reverse phase-HPLC profile of glycan fragments digested with CwlQ

Peptidoglycan of *B. subtilis* was digested with an amidase, CwlH, and then

133 *N*-acetylated to avoid a mixture of *N*-acetylated and non-acetylated fragments. The
134 *N*-acetylated glycan was completely digested with CwlQ and then digested fragments
135 were non-reduced or reduced with $NaBH_4$, and separated by RP-HPLC (Fig. 2C and D).
136 The elution times of peaks B and E for non-reduced fragments corresponded to those of
137 peaks 2 and 4 for reduced ones, respectively. Peaks A and D for non-reduced fragments
138 did not correspond to any peaks for reduced fragments (Fig. 2C and D).
139
140 *Identification of the structures of digested glycan fragments*
141 Peaks A to E and 1 to 4 in Fig. 2C and D were analyzed by mass spectrometry.
142 The peak A material (fragment peak, *m/z* 519.3) is the $[M+Na]^+$ form of non-reduced
143 disaccharide (*M*r, 496.2) (Supplemental Fig. 2A). MS/MS analysis of the peak A material
144 indicated that it was GlcNAc-MurNAc (Supplemental Fig. 3A). Since GlcNAc-MurNAc
145 has a reducing end, CwlQ was believed to contain muramidase activity. We also
146 identified the structure of peak D material by MS/MS analysis (Supplemental Fig. 3D).
147 The Peak D material (fragment peak, *m/z* 997.5) is the $[M+Na]^+$ form of non-reduced
148 tetrasaccharide (*M*r, 974.4) (Supplemental Fig. 2D). MS/MS analysis of the peak D
149 material indicated that it was GlcNAc-MurNAc-GlcNAc-MurNAc with a reducing end
150 (Supplemental Fig. 3D). This result also supported that CwlQ contains muramidase
151 activity. After reduction with $NaBH_4$, the reduced end of MurNAc should be changed to
152 *N*-acetylmuramitol (MurNAcr). The disappearance of peaks A and D by the reducing
153 treatment supported the above result (Fig. 2C and D).
154 Peak B material (fragment peak, *m/z* 477.2) is the $[M-H]^-$ form of

anhydro-disaccharide (*M*r 478.2) (Supplemental Fig. 2B). MS/MS analysis of the peak B material clearly indicated it to be GlcNAc-anhMurNAc (1,6-anhydro-*N*-acetylmuramic acid) (Supplemental Fig. 3B). Therefore, CwlQ was capable of digesting a MurNAc-GlcNAc linkage and the activity was a transglycosylase instead of a muramidase (Fig. 1). The structure of the peak E material was also determined to be GlcNAc-MurNAc-GlcNAc-anhMurNAc (Supplemental Figs. 2E and 3E). The structure of the small peak C material (Fig. 2C and D) was determined to be a non-reduced tetrasaccharide, GlcNAc-MurNAc-GlcNAc-MurNAc (Supplemental Fig. 3C). This is the same compound as the peak D material. The reason for the different elution times (peaks C and D) of GlcNAc-MurNAc-GlcNAc-MurNAc is unknown, but it may reflect the difference of α and β anomers at the reducing end. These results indicate CwlQ has bifunctional activities (muramidase and lytic transglycosidase activities) in one domain.

Further experiments were performed for the samples reduced by $NaBH_4$. Peak 1 was not identified in non-reducing samples and found only after $NaBH_4$ reduction. We identified the structure of peaks 1 and 3 materials by MS and MS/MS analyses (Supplemental Fig. 4 and Fig. 3). The peak 1 material (fragment peak, *m/z* 497.5) is the $[M-H]^-$ form of reduced disaccharide (*M*r, 498.2) (Supplemental Fig. 4A) and MS/MS analysis of the peak 1 material indicated it to be GlcNAc-MurNAcr (Fig. 3A). When MurNAc-GlcNAc was reduced to MurNAc-GlcNAcr, peak A disappeared and peak 1 was newly produced (Fig. 2C and D). The peak 3 material (fragment peak, *m/z* 999.5) is the $[M+Na]^+$ form of a reduced tetrasaccharide (*M*r, 976.4) (Supplemental Fig. 4C) with a structure of GlcNAc-MurNAc-GlcNAc-MurNAcr (Fig. 3C). The data indicated that peak

D was converted to peak 3 by $NaBH_4$ reduction (Fig. 2C and D). These results supported that CwlQ contains muramidase activity.

Structures of the peaks 2 and 4 materials were also identified by MS and MS/MS analyses (Supplemental Fig. 4 and Fig. 3). The peak 2 material (fragment peak, *m/z* 477.1) was the $[M-H]^-$ form of an anhydro-disaccharide (*M*r, 478.2) and the structure was GlcNAc-anhMurNAc (Supplemental Fig. 4B and Fig. 3B). The peak 4 material (fragment peak, *m/z* 955.4) was the $[M-H]^-$ form of an anhydro-tetrasaccharide (*M*r, 956.4) and the structure was GlcNAc-MurNAc-GlcNAc-anhMurNAc (Fig. 3D). Therefore, upon attempted reduction, these anhydro-compounds were not converted and show similar elution times (peaks B and 2, and peaks E and 4 [Fig. 2C and D]). These results indicated CwlQ contains lytic transglycosylase activity. Since one SLT domain of CwlQ contained both muramidase and lytic transglycosylase activities, CwlQ is clearly a bifunctional enzyme.

Identification of an active site of CwlQ

The SLT domains of the characterized proteins were aligned and are shown in Supplemental Fig. 5. SLT60 of *E. coli*, goose lysozyme, and a probably conjugation-related muramidase YomI have one common essential amino acid residue, glutamic acid. CwlQ (CwlQ-BACSU) also conserves a glutamic acid residue at the corresponding position. We prepared two mutants (E85A and E85Q proteins) by site-directed mutagenesis. Supplemental Fig. 1A shows the SDS-PAGE and zymography of these mutated proteins. E85A and E85Q proteins completely lost both activities. The

lack of enzymatic activities is also confirmed by incubation of the proteins in cell wall suspensions (Fig. 2B). These results indicate that the glutamic acid residue is an essential amino acid residue for both activities.

Discussion

Many orthologs of CwlQ are found in various bacteria, and as indicated in Supplemental Fig. 5, there are four groups with amino acid sequences similar to the sequence of CwlQ. The first group contains CwlQ, and the conserved amino acid residues are highlighted in grey. The second group contains the soluble lytic transglycosylase of *E. coli* (SLT_ECOLI) whose structure was extensively analyzed by X-ray crystallography and with protein-inhibitor interactions [18]. SLT_ECOLI has an essential glutamic acid residue (E505) for enzymatic activity, and S514 and E610 play a role in stabilization of the oxocarbonium ion intermediate [18]. Q523 is associated with GlcNAc recognition [19]. E505, S514, E610, and Q523 of SLT_ECOLI are completely conserved in all members of the first group along with CwlQ (E85, S94, E167, and Q103, respectively; Supplemental Fig. 5). The third group contains the CwlT muramidase (CWLT_BACSU). CwlT contains glutamic acid (E87) and aspartic acid (D94) residues which are essential to its activity. The fourth group contains goose lysozyme (LYG_ANSAN). It has also glutamic acid residue (E73) which is essential to its activity, and aspartic acid residues (D86 and D97) which are candidates for participating in the catalytic reaction [7, 20]. Comparisons of the active sites show CwlT to be similar to goose lysozyme. Previous

reports suggested that the catalytic residue, aspartic acid, in egg white lysozyme is associated with substrate specificity rather than the critical muramidase activity on the basis of the results of crystallography and mutagenesis [4, 7]. The amino acid sequence of CwlQ is more similar to the soluble lytic transglycosylase than muramidase (lysozyme) (Supplemental Fig. 5). YomI_BACSU exhibits only muramidase activity (data to be published elsewhere). E85 of CwlQ is an essential amino acid residue (Fig. 2B and Supplemental Fig. 1A) which is common in CwlQ, YomI, SLT, CwlT, and goose lysozyme. CwlQ and YomI share more than 74% amino acid similarity. We generated K83Q, V93R, and Q166K mutants of CwlQ because these amino acid residues are different from those of YomI and they are located near the functionally important residues. The native CwlQ produced more GlcNAc-anhMurNAc than GlcNAc-MurNAc (Fig. 2C peaks B and E vs. peaks A and D), indicating that the SLT activity is stronger than the muramidase one. In the case of the mutants, the activity ratios (SLT vs. muramidase) were not significantly changed from the wild type (data not shown).

Acknowledgments

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Figure legends

Fig. 1. Peptidoglycan structure in *B. subtilis* and the cleavage site by muramidase and lytic transglycosylase. The peptidoglycan consists of glycan strands covalently bound to peptide side chains. The thick arrow indicates the cleavage site by muramidase and lytic transglycosylase. The products by muramidase and lytic transglycosylase contain *N*-acetyl muramic acid (MurNAc) and 1,6-anhydro-MurNAc, respectively [4,5,6,7]. GlcNAc, *N*-acetyl glucosamine; A_2pm, diaminopimelic acid.

Fig. 2. Cell wall hydrolytic activity of CwlQ. (A) 14% polyacrylamide gel electrophoresis and zymography were performed with cell lysates or purified CwlQ from *E. coli* (pQE-CwlQ). CwlQ was overexpressed in *E. coli* JM109 (pQE-CwlQ) with or without 1 mM IPTG. Lane M, protein standards (Bio-Rad); lane 1, non-induction of CwlQ; lane 2, induction of CwlQ with IPTG; lane 3, purified CwlQ by affinity chromatography; lane 4, zymography with the purified CwlQ protein. Renaturation for zymography after SDS-PAGE was performed with 20 mM MES buffer (pH 6.0) at 37°C

for 3 h. (B) Assay of the cell wall hydrolytic activity by measuring cell wall turbidity. 15 μg/ml (final concentration) of purified CwlQ or its mutated CwlQ (E85A and E85Q) was mixed with 0.3 mg/ml (final concentration) of cell wall, and the hydrolytic reaction was performed at 37°C in 20 mM MES buffer (pH 6.0). The activity of native CwlQ, CwlQ (E85A), and CwlQ (E85Q) was shown in closed triangles, closed circles, and open squares, respectively. Arrow indicates the position of CwlQ. The 14% polyacrylamide gel electrophoresis and zymography of the mutated CwlQ proteins are shown in Supplemental Fig. 1A. (C and D) RP-HPLC of digested glycan strands (GlcNAc-MurNAc polymer) by CwlQ. After the purified glycan strands had been digested with CwlQ, and then the reduced ends of amino sugars had been non-reduced (panel C) and reduced (panel D), the samples were separated by RP-HPLC as described in Materials and Methods. The peak A to E materials corresponds to disaccharide, anhydro-disaccharide, tetrasaccharide, tetrasaccharide, and anhydro-tetrasaccharide, respectively (Supplemental Figs. 2 and 3). The peak 1 to 4 materials are identified as reduced disaccharide, anhydro-disaccharide, reduced tetrasaccharide, and anhydro-tetrasaccharide, respectively (Fig. 3 and Supplemental Fig. 4).

Fig. 3. ESI-MS-MS analysis of the peak 1 to 4 materials in Fig. 2D. (A to D) The MS-MS analysis shows that the peak 1 to 4 materials are reduced disaccharide, 1,6-anhydro-disaccharide, reduced tetrasaccharide, and 1,6-anhydro-tetrasaccharide, respectively. The ESI-MS data are shown in Supplemental Fig. 4. The ion series b and y correspond to the fragment peaks of each identified saccharide structure.

353

354

Table 1

Plasmids used in this study

Plasmids	Genotype	Source
pQE30	*bla*	Qiagen
pQE-CwlQ	*bla* Δ*lacZ* $(His)_6$-Δ*cwlQ*	This study
pQECwlQ-E85Q	*bla* $(His)_6$-mutatedΔ*cwlQ* (E85Q)	This study
pQECwlQ-E85A	*bla* $(His)_6$-mutatedΔ*cwlQ* (E85A)	This study

Table 2

Primers used in this study

Primer name	Sequence (5'→3')
BF-YJBJ	*gcgc*ggatccGCAGAAAAGCACTGAAACAG
KR-YJBJ	*gcgc*ggtaccGCGTAAGATTTCACTTCGC
E85Q-1	GCGCCGTCATCAAACAGcAATCAGGCTTTAATGC
E85Q-2	GCATTAAAGCCTGATTgCTGTTTGATGACGGCGC
E85A-1	GCGCCGTCATCAAACAGGcATCAGGCTTTAATGC
E85A-2	GCATTAAAGCCTGATgCCTGTTTGATGACGGCGC

The italic small letters and double-underlining are the tag sequences and restriction sites, respectively.

Figure

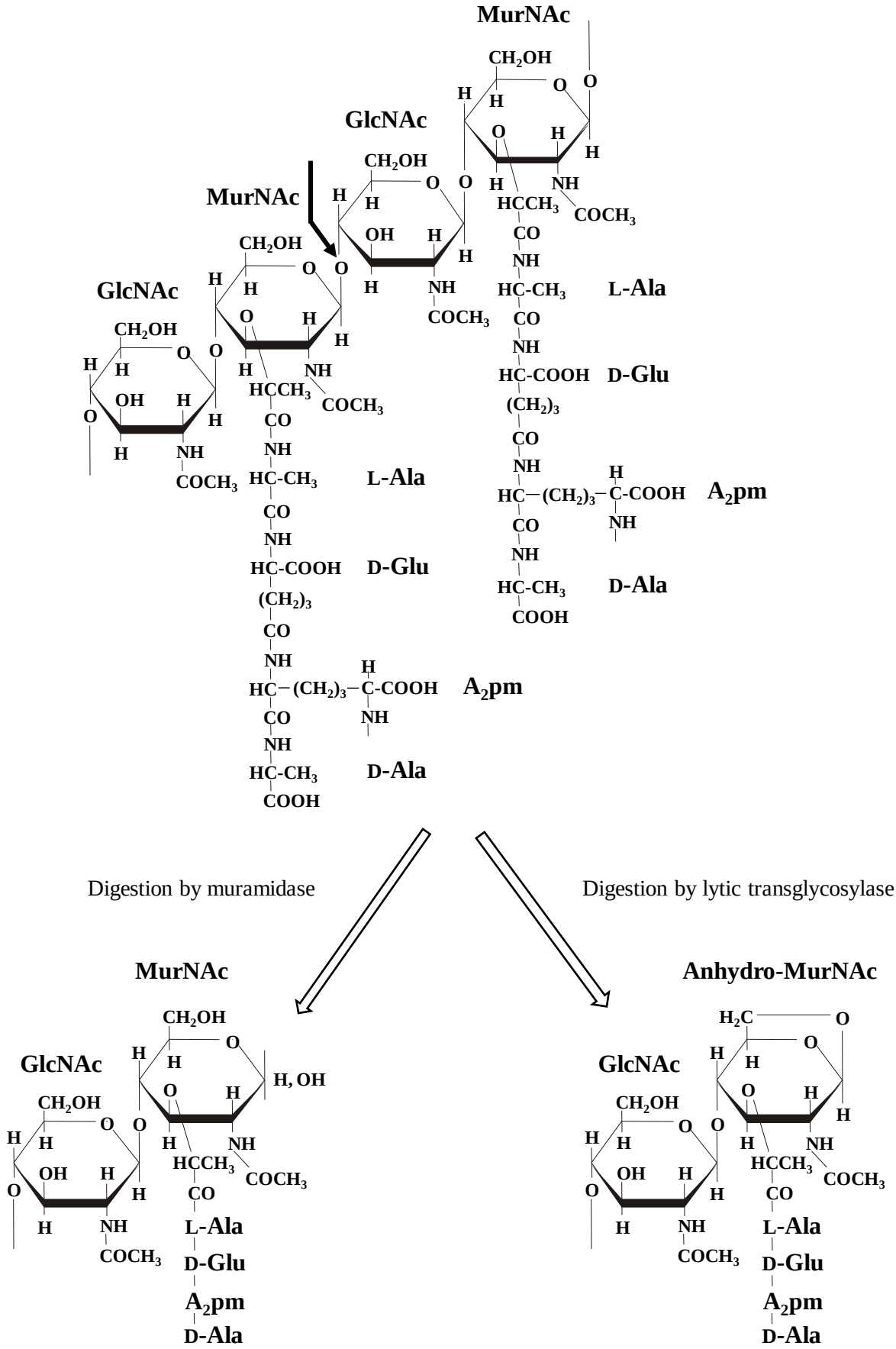
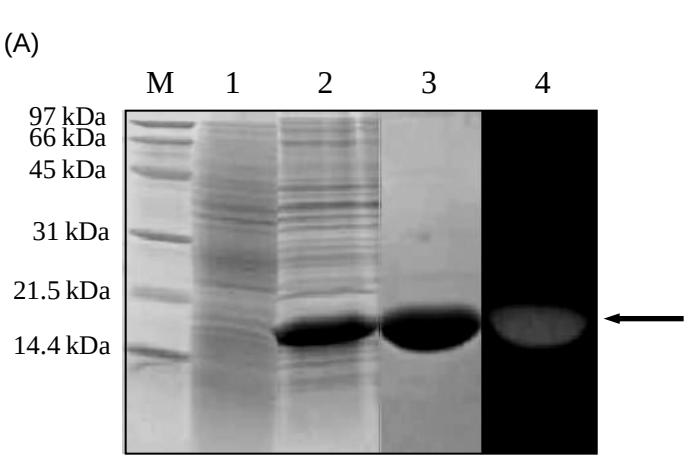
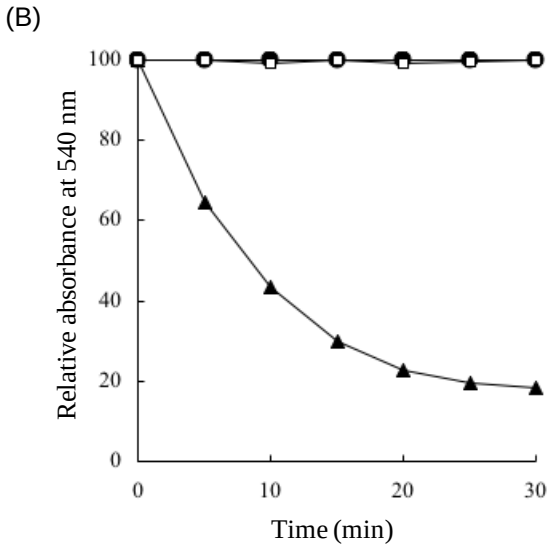


Fig. 1. Sudiarta *et al.*





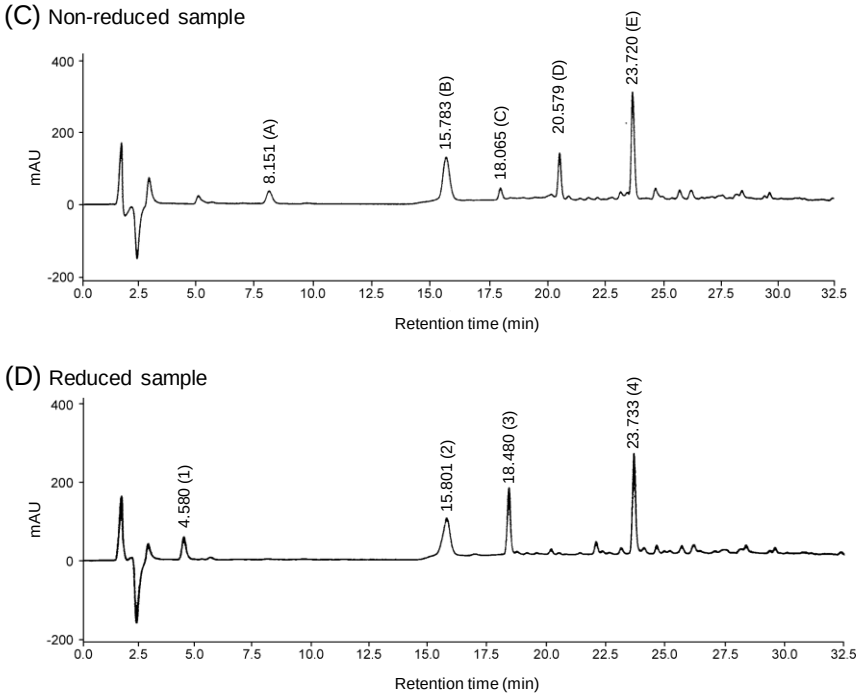
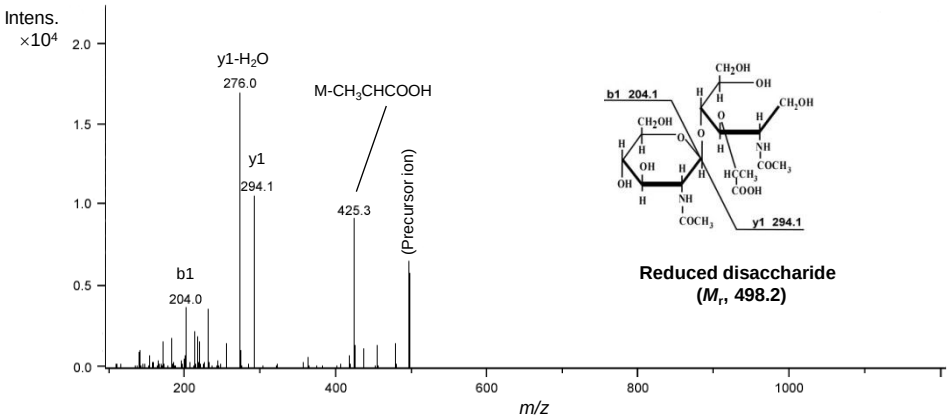
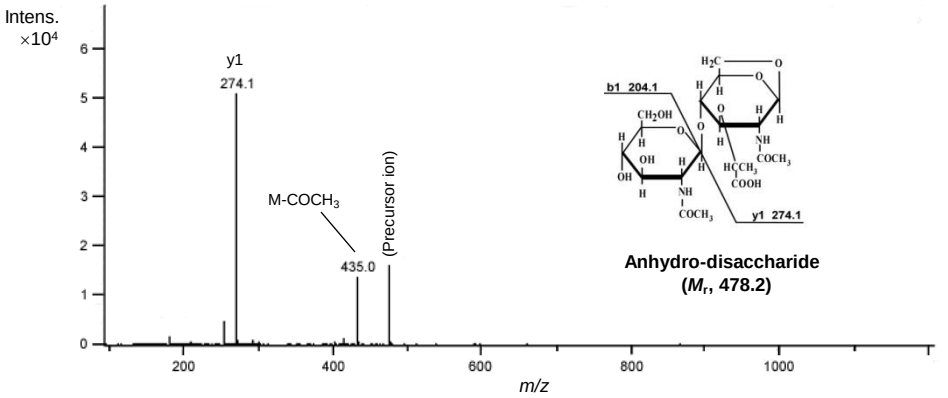


Fig. 2. Sudiarta *et al.*

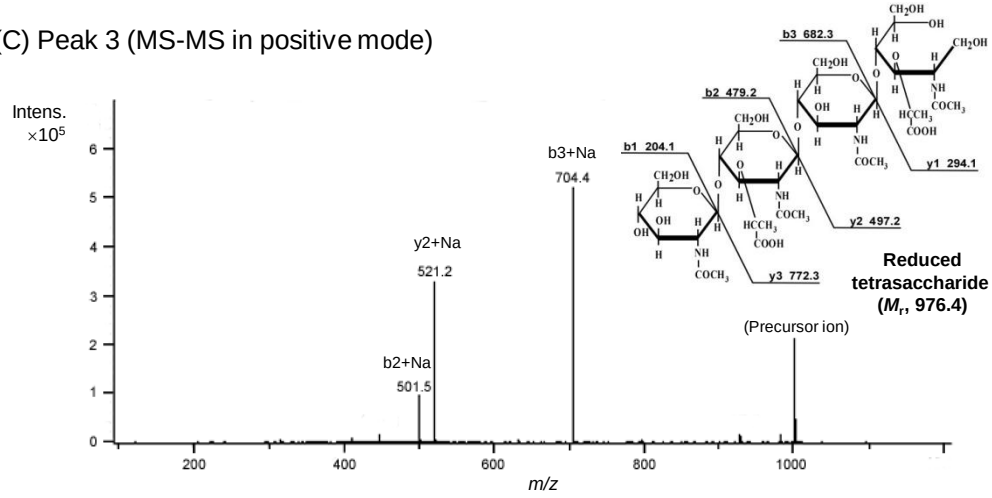
(A) Peak 1 (MS-MS in negative mode)



(B) Peak 2 (MS-MS in negative mode)



(C) Peak 3 (MS-MS in positive mode)



(D) Peak 4 (MS-MS in negative mode)

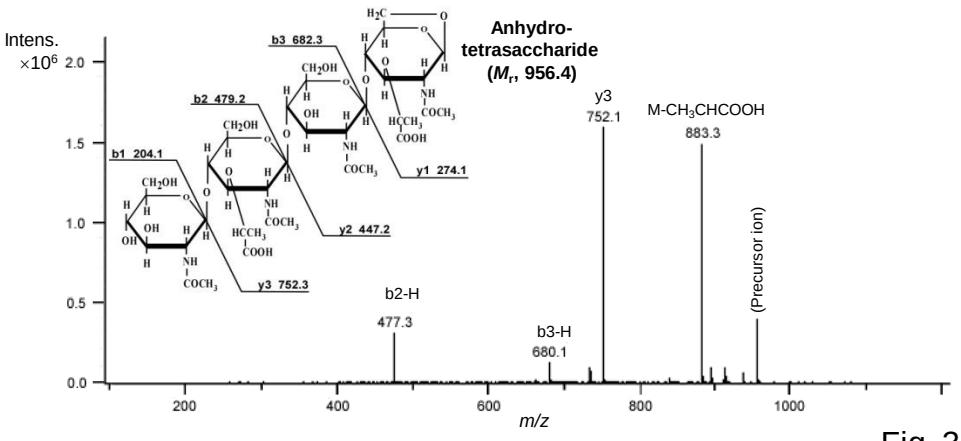


Fig. 3. Sudiarta *et al.*