Note

Inhibition of the Bacterial Surface Protein Anchoring Transpeptidase Sortase by Medicinal Plants

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Received July 11, 2002; Accepted August 7, 2002

Inhibition by medicinal plant extracts of a recombinant sortase was evaluated for antibacterial drug discovery. The coding region of sortase, a transpeptidase that cleaves surface proteins of Gram-positive bacteria, was amplified by PCR from the chromosome of Staphylococcus aureus ATCC 6538p with the exception of an N-terminal membrane anchor sequence, expressed in Escherichia coli, and purified by metal chelate affinity chromatography. The purified sortase had maximum activity at pH 7.5 and was stable at 20–45°C for the cleavage of a synthetic fluorophore substrate. The enzyme inhibitory activity in medicinal plants was also evaluated for antibacterial drug discovery. Among 80 medicinal plants tested, Cocculus trilobus, Fritillaria verticillata, Liriope platyphylla, and Rhus verniciflua had strong inhibitory activity. The extract with the greatest activity was the ethyl acetate fraction derived from the rhizome of Cocculus trilobus (IC50 = 1.52 μg/ml).

Key words: Staphylococcus aureus; sortase expression; medicinal plants; inhibitory activity

Sortase may be universal in Gram-positive bacteria. Various surface proteins are anchored to the cell walls of Gram-positive pathogens by the transpeptidase sortase. In the case of Staphylococcus aureus protein A, immunoglobulins are captured on the microbial surface and camouflage the bacteria during the invasion of host tissues. Protein A is cleaved by sortase between the threonine and the glycine of a conserved LPXTG motif. The carboxyl group of threonine is amide-linked to the amino group of the pentaglycine cross-bridge of the peptidoglycan. This reaction, called cell-wall sorting, probably occurs in most Gram-positive bacteria. Therefore sortase is a very promising target for identifying inhibitors of general use in therapeutics against Gram-positive bacteria. Mainstream medicine is increasingly receptive to the use of antimicrobial and other drugs derived from plants, as traditional antibiotics (products of microorganisms or their synthesized derivatives) become ineffective and as new diseases remain intractable to this kind of drug. Another factor in the renewed interest in plant antimicrobials in the past 20 years has been the rapid rate of plant species extinction. From this viewpoint, medicinal plants are resources for antibacterial drug discovery. In this study, a sortase gene amplified by PCR from the genomic DNA of S. aureus ATCC 6538p was expressed in Escherichia coli and the enzymatic properties of the purified sortase were investigated. In addition, the inhibitory activity of medicinal plant extracts on the purified sortase was evaluated.

The primers 5'-AAACCCATACTTCAATT-ATC-3' and 5'-TTATTTGACTTCTGACGCTACAA-3' were used for PCR of the sortase gene (with the exception of the N-terminal membrane anchor sequence) from the genomic DNA of S. aureus ATCC 6538p. The DNA fragment was inserted into the pBAD/Thio-TOPO vector (Invitrogen, Groningen, Netherlands), used to transform E. coli TOP10 (Invitrogen), and selected on Luria-Bertani (LB) agar containing ampicillin (50 μg/ml). The recombinant plasmid was isolated from E. coli transformants and its nucleotides were sequenced with an ABI Prism 377 DNA sequencer. The positive transformant was grown in LB broth containing 50 μg/ml ampicillin at 37°C for 13 h. These cells were used to inoculate 10 ml of LB broth and the culture incubated with shaking at 37°C until the OD600 reached approximately 0.5. At this point, 0.002% arabinose was added to the medium and incubation was continued for an additional 6 h to induce fused sortase expression. Cells were harvested by centrifugation at 3,000 × g for 30 min and lysed by lysozyme treatment and sonication. The cell extract was centrifuged at 29,000 × g for 30 min, and the fused protein was purified with Xpress protein purification system, by the manufacturer’s instructions. Fractions containing N-terminal fused protein were collected and treated with enterokinase (in 50 mm Tris-HCl containing 1 mm CaCl2 and 0.1% Tween-20, pH 8.0; incubation...
Medicinal plants were obtained from drugstores in Korea as herbal medicines meant for clinical use in traditional Korean prescriptions. Extracts of each medicinal plant were prepared as described previously. The n-hexane, ethyl acetate fractions, and an aqueous layer were independently evaporated under reduced pressure to dryness. For sortase inhibition, each of the dried solvent fractions was dissolved in 100% dimethyl sulfoxide (Me$_2$SO) and diluted with sterilized distilled water in the range of 40-0.1 µg/ml before use. One hundred microliters of reaction mixture containing the prescribed concentration of test sample was added to each well of a 96-well microtiter plate and treated as stated above. The inhibitory activity was defined as the concentration (µg/ml) giving 50% inhibition (IC$_{50}$) relative to the negative control (final concentration 1% Me$_2$SO). p-Hydroxymercuribenzoic acid (pHMB), a known sortase inhibitor, was used as a positive control.

The nucleotide sequence analysis showed that the sortase gene was composed of 546 bp encoding 182 amino acid residues, with the exception of an N-terminal signal peptide-membrane anchor sequence, and contained a single active site cysteine residue (Cys$^{159}$) that catalyzes (via nucleophilic attack) a highly specific cleavage of the scissile threonine-glycine peptide bond in LPXTG. Database searches found that the sequence of S. aureus ATCC 6538p sortase clone showed 99%, 78%, 24%, and 21% identity at the amino acid level with the published S. aureus, Staphylococcus epidermis, Enterococcus faecalis, and Streptococcus pneumoniae sortases, respectively (www.sciencemag.org/feature/data/1041555.shl). The concentration of expressed fusion protein was analyzed by SDS-PAGE (Fig. 1). The concentration of fusion protein content was about 20% of total protein produced in E. coli. The molecular mass of the expressed protein was calculated to be 36 kDa. Considering the fusion protein, this molecular weight was reasonable. After purification, fusion protein was treated with enterokinase and the mature form of the recombinant sortase was purified.

The enzyme activity of recombinant sortase could be assayed by monitoring the increment of fluorescence intensity with the synthetic peptide substrate Dabcyl-QALPETGEE-Edans. An elegant experiment has been reported by Ton-That et al. When the pHMB was added to the reaction mixture, the increment of fluorescent intensity was diminished (IC$_{50}$ = 40.55 µg/ml), so our assay system can be used in the search for sortase inhibitors. The recombinant sortase was stable at pH 6.0 to 8.0, with 97% activity at pH 6.0 and 80% activity remaining at pH 10.0. There was optimal activity at pH 7.5, with 40% and 60% activities at pH 6.0 and 10.0, respectively. The enzyme in 50 mM Tris-HCl buffer (pH 7.5; 160 µg/ml) was stable at temperatures up to 45°C and had maximum activity at 35°C. The sortase

Fig. 1. SDS-PAGE of the Sortase Expressed in E. coli.

The sortase was purified by Ni-chelate affinity column chromatography. The eluted protein was treated with enterokinase for cleavage between amino-terminal residues for affinity and recombinant sortase. The samples were put on a 15% polyacrylamide gel. M, protein molecular mass standard; lane 1, total proteins of host with pBAD/Thio-TOPO-sortase (6-h induction); lane 2, unbound fraction of Ni-NTA affinity column; lane 3, eluted fraction containing the fused sortase (36 kDa); lane 4, enterokinase-treated sortase separated from amino-terminal residues for affinity (20.8 kDa).

for 16 h at 37°C) to eliminate amino-terminal residues. After cleavage, the reaction mixture was put through an AKTA purifier (Amersham Pharmacia Biotech, Uppsala, Sweden) and the sortase was purified. The expressed recombinant protein was analyzed by SDS-PAGE. To measure the enzyme activity, reactions were done in 1 ml volume of 50 mM Tris·HCl buffer (pH 7.5) containing 150 mM NaCl, 2.5 µg of the synthetic peptide substrate Dabcyl-QALPETGEE-Edans, and 5 mM CaCl$_2$ with different amounts of sortase. After incubation for 1 h at 37°C, the increase in fluorescence intensity was recorded by a fluorescence spectrophotometer (SpectraMAX Gemini XS, Molecular Devices Co., Sunnyvale, CA), with 350 nm for excitation and 495 nm for recordings. To measure the pH stability of the enzyme, the purified sortase was incubated at different pHs at 37°C for 1 h. After adjustment of the pH to 7.5, the residual activity was measured. To measure the heat stability, the purified enzyme was incubated in 50 mM Tris·HCl buffer (pH 7.5) for 1 h at various temperatures (20 to 70°C), and the residual activity was assayed as described above. The effects of various metal ions and chemical reagents on the sortase activity were measured by addition of test metal ions or chemical reagents to the final concentration of 1 or 5 mM to the reaction mixture, which was then incubated at 37°C for 1 h.

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activity was increased by the addition of Ca\(^{2+}\) and Mn\(^{2+}\) to the reaction mixture (increased 25–29% at 5 mM), although the addition of several other divalent cations (Ag\(^{+}\), Zn\(^{2+}\), and Fe\(^{3+}\)) failed to stimulate peptide cleavage. Ton-That and Schneewind reported that neither sulfhydryl reducing agents nor treatment with EDTA interfered with the cell-wall sorting reaction in the pulse-labeling experiment using a whole-cell system.\(^{10}\) However, in our in vitro assay system, the sulfhydryl reducing agents dithiothreitol and mercaptoethanol and the divalent cation chelator EDTA did affect the sorting reaction. We cannot fully explain this result.

A total of 80 medicinal plant extracts were sequentially fractionated with n-hexane, ethyl acetate, and water, and the inhibitory activity of each fraction on the sortase activity was estimated. Among the 240 medicinal plant fractions tested, the ethyl acetate fractions of Coccusculus trilobus, Fritillaria verticillata, Liriopoe platiphylla, and Rhus verniciflua had potent inhibitory activity (Table 1). The extract with the greatest activity was the ethyl acetate fraction derived from the rhizome of C. trilobus (IC\(_{50}\) = 1.52 \(\mu\)g/ml). This is the first report of a sortase inhibitor from a plant. It was reported that penicillin G, a transpeptidation inhibitor, has no effect on surface protein anchoring, although vancomycin and moenomycin, inhibitors of cell-wall polymerization into peptidoglycan strands, slow the sorting reaction.\(^{10}\) As surface proteins are believed to be essential for the pathogenesis of bacterial disease, a sortase inhibitor may be useful for the therapy of human infections caused by Gram-positive bacteria that have acquired resistance to antibiotics.\(^{12}\)

### References

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