Macromolecular antimicrobial glycoprotein, achacin, expressed in a methylotrophic yeast *Pichia pastoris*

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Abstract A cDNA encoding achacin, an antimicrobial glycoprotein from the body surface mucus of giant African snail *Achacina fulica* Férussac, was expressed in a methylotrophic yeast, *Pichia pastoris*, and recombinant achacin (rAch) was secreted in yeast minimal medium in a polyglycosylated form with 80 kDa. Carbohydrate analysis revealed that the glycosylated moiety of rAch was composed of 50 mol mannose and 2 mol *N*-acetylglucosamine residues. Antimicrobial activity using *Escherichia coli* and *Staphylococcus aureus* showed that the rAch had a behavior similar to its native counterpart. The rAch showed so wide an antimicrobial spectrum that 0.1 mg/ml rAch inhibited the growth of *Pseudomonas fluorescens*, *Staphylococcus epidermidis*, and *Streptococcus faecalis* in addition to *E. coli* and *S. aureus*, whereas it did not appreciably affect the growth of *Proteus mirabilis*, *Bacillus cereus* and *Micrococcus luteus*. The rAch was also effective in preventing growth of *Vibrio anguillarum* and *Vibrio parahaemolyticus*. The results suggested that the rAch had great potential of using as an antimicrobial agent.

Key words: Achacin; Antimicrobial activity; Glycosylation; Polymannosylation; Snail; *Pichia pastoris*

1. Introduction

Recently food poisoning has frequently erupted throughout the year resulting in serious consequences. The production of antimicrobial agents with a wide antimicrobial spectrum should be valuable for food safety. However, the use of antibiotics to control the bacterial infection has been a concern because it may incite a generation of drug-resistant bacteria strains. Therefore, safe antimicrobial agents which are harmless to humans have been sought in the natural environment.

The macromolecular antibacterial glycoprotein from edible snail could be useful as a harmless food additive to prevent bacterial growth. Unlike vertebrates, it is assumed that the snail has a special defense mechanism against bacterial infection. An antibacterial factor named achacin that acts on both Gram-negative and Gram-positive bacteria was found in the body surface mucus of the giant African snail, *Achacina fulica* Férussac [1]. Achacin has been characterized as a glycoprotein, which shows a bactericidal effect against *Escherichia coli* and *Staphylococcus aureus* [1–3]. Recently, a cDNA encoding achacin precursor was cloned from the tissue of the snail collar [4], and four tentative *N*-glycosylation sites, Asn-Xaa-Ser/Thr, were discovered based on the deduced amino acid sequence [4]. Morphological studies revealed that achacin attacked the cytoplasmic membranes of bacteria and then killed the bacteria [5]. Our previous study suggested that recombinant unglycosylated achacin produced by *E. coli* expression system did not exhibit any antimicrobial activity (data not shown). Thus, the *N*-glycosylation moiety of achacin might be highly related to the antibacterial activity. A lower eukaryote, e.g. yeast, may be suitable for the production of a biologically active achacin with carbohydrate chains. We were successful in obtaining active proteins with polymannosyl chains using two yeast expression systems [6,7]. The chain length of mannosyl groups of protein derived from a methylotrophic yeast *Pichia pastoris* was shorter and more homogeneous than that from *Saccharomyces cerevisiae* [7]. Therefore, we expressed the achacin gene in *P. pastoris* The objective of this study was to produce a bioactive achacin by genetic engineering.

2. Materials and methods

2.1. Materials

P. pastoris KM71 (Invitrogen, San Diego, CA) was used as a host in this study. *E. coli* JM 109 (*recA endA1 gyrA96 thi hsdR17 supE44 relA1 D (lac-proAB) mcrA F′ traD36 proA*$^+$ *proB*$^+$ *lacIq lacZDM15*) was used for routine plasmid amplification. T4 DNA ligase, alkaline phosphatase and restriction enzymes were purchased from Promega (Madison, WI). Q-Sepharose Fast Flow, Superdex 200HR, and concanavalin A-Sepharose were from Pharmacia (Baie d'Urfé, Que., Canada). All other chemicals were of analytical grade for biochemical use.

2.2. Construction of expression plasmid, transformation, and culture condition

The construction of *P. pastoris* expression vector was performed based on the method described in the manual of the *Pichia* expression kit (Invitrogen). To express mature achacin with a native N-terminus, the signal sequence with the *kex*2 cleavage (E-K-R) was introduced at the upstream of the N-terminal amino acid (E) of achacin by PCR using 5′-AGG CTC GAG AAA AGA GAA GCT CCC CAG TGC-3′ as a primer. Plasmid pDR-A2 carrying the complementary DNA of achacin [4] was used as a template for the PCR procedure. A 1.6 kbp achacin cDNA with the signal sequence was ligated into the *P. pastoris* expression vector pPICZα-A. The resulting vector was linearized with a restriction enzyme *Dra*I, and the linearized DNA was introduced into *P. pastoris* cells according to the EasyComp transformation protocol in the manual. Integration of achacin in the recombinant genomes of *P. pastoris* was confirmed by genomic PCR using 5′AOX1 primer paired with 3′AOX1 primer, 5′-GAC TGG TTC CAA TTG ACA AGC-3′ and 5′-GCA AAT GGC ATT CTG ACA TCC-3′. Transformants were grown in 5 ml of the yeast minimal medium (YMM) containing 0.4 mg/l biotin at 30°C for 24 h, then subcultured in fresh one liter YMM containing 0.5% methanol as the sole carbon source.

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2.3. Purification

Culture of the recombinant yeast was centrifuged at 10 000×*g* for 10 min, and the supernatant was concentrated using an ultrafilter system with 3000 mol wt cut-off (Pellicon cassette filter, PLBC 000 05, Millipore, Bedford, MA). The concentrated culture was applied to a Q-Sepharose Fast Flow column (1.3×10 cm) pre-equilibrated with 20 mM Tris-HCl buffer (pH 7.5). The column was washed with the same buffer until a protein-free fraction was obtained, and then the protein was eluted with a linear gradient to 500 mM NaCl in the buffer. Fractions including achacin were combined after Western blotting as described later. The combined fractions were then concentrated by pressure ultrafiltration using a Diaflo YM30 membrane (Amicon, Danvers, MA). The sample was applied to a Pharmacia FPLC Superdex 200HR column (1.0×30 cm) pre-equilibrated with 20 mM Tris-HCl buffer (pH 7.5). Protein content in each fraction eluted was determined by measuring absorbance at 280 nm. Carbohydrate content was measured from absorbance at 490 nm after color development using the phenol-sulfuric acid reaction [8]. All fractions containing glycoproteins were combined and applied to a concanavalin A-Sepharose column (1.3×5 cm) previously washed with 10 bed volumes of 20 mM Tris-HCl buffer (pH 7.5) containing 500 mM NaCl, followed by washing again with the same buffer until the washed solution became protein free. The adsorbed glycoprotein was then eluted with 100 mM methyl α-D-mannopyranoside in the 20 mM Tris-HCl buffer (pH 7.5) containing 500 mM NaCl, and served for further experiments after dialysis against deionized water.

2.4. SDS-polyacrylamide gel electrophoresis and Western blot analysis

SDS-polyacrylamide gel electrophoresis was carried out according to the method of Laemmli [9] using 10% acrylamide separating gel and 3% stacking gel containing 1% SDS. Samples were heated at 100°C for 5 min in Tris-glycine buffer (pH 8.8) containing 1% SDS and 1% β-mercaptoethanol. Electrophoresis was run at a constant current of 10 mA for 5 h in an electrophoretic buffer of Tris-glycine containing 0.1% SDS. The gel sheets, thereafter, were stained with 0.025% Coomassie brilliant blue R-250. For Western blot analysis, the gel sheet was transferred to a PVDF membrane after electrophoresis as described [10,11]. The membrane was incubated for 30 min at room temperature in 10 mM Tris-HCl buffer (pH 8.0) containing 5% skim milk, 150 mM NaCl and 0.05% Tween 20. Then the membrane was incubated with the rabbit antiserum raised against the wild-type achacin [4] for 60 min at room temperature. Finally, the antigen, reacted with goat anti-rabbit IgG conjugated with alkaline phosphatase on the membrane, was detected by the enzyme-linked coloration reaction [12,13].

2.5. Carbohydrate analysis

The total sugar content of the glycosylated achacin was estimated using the phenol-sulfuric acid reaction employing mannose as a standard. HPLC analysis was used for identifying the hexose liberated from the glycoprotein by hydrolysis with 2 N HCl at 100°C for 3 h in a sealed glass ampoule. The hydrolysates were dried, dissolved in water, and chromatographed on an Asahipak NH2P-50 column (Asahi Chemical, Tokyo) in 75% acetonitrile using a Hitachi HPLC system equipped with an RI detector.

2.6. Antimicrobial assays

The test bacteria used in this study were eight Gram-negative bacteria namely *E. coli* ATCC 25922, *Proteus mirabilis* IFO 13300, *Pseudomonas fluorescens* IFO 14160, *Aeromonas hydrophila* subsp. *hydrophila* IFO 13286, *A. hydrophila* KAH 8501, *Aeromonas salmonicida* IFO 12718, *Vibrio anguillarum* IFO 13266 and *Vibrio parahaemolyticus* IFO 12711. As Gram-positive bacteria, *Staphylococcus aureus* ATCC 25923, *S. epidermidis* IFO 13889, *Bacillus cereus* IFO 13690, *Streptococcus faecalis* IFO 12970 and *Micrococcus luteus* IFO 13867 were also used.

Antibacterial activity of achacin was estimated from the growth curve of *E. coli* and *S. aureus*. These strains were subcultured and then grown in L-broth (10 g of bacto tryptone, 5 g of bacto yeast extract, 5 g of NaCl, 1 g of glucose, and water to 1 liter) at 37°C overnight. The fresh cells were diluted with L-broth and microscopically adjusted to give a concentration of 10^3 cells/ml by using a hematometer. A 100 μl aliquot of the cell suspension was then added to the tested sample with and without achacin in 5 ml saline solution. After vortexing, the inoculated samples were incubated at 37°C while rotating at 250 rpm. Bacterial number was counted on MacConkey agar (Difco, Detroit, MI) plate for *E. coli* and Mannitol salt agar (Difco) plate for *S. aureus*.

The antimicrobial spectrum was assessed using a stainless-steel cylinder cup with 8.0 mm outside diameter, 6.0 mm inside diameter 10 mm high. Overnight culture of the test bacteria was spread onto a Standard agar (Difco) plate, which was left for 1 h in a germ-free cabinet to dry the surface of the agar plate. For *V. parahaemolyticus*, the assay was carried out in 2.5% NaCl of the final concentration. 10 μl of the achacin solution was injected into the stainless-steel cup on the agar plate, and incubated for 24 h at 37°C. The diameter of an antibacterial zone which formed surrounding the stainless-steel cup was measured using slide calipers.

3. Results and discussion

3.1. Expression of achacin in P. pastoris

Mature achacin was successfully secreted from the transformants in a form of a molecular mass of 80 kDa, as shown in Fig. 1. Western blot analysis revealed that the single band in Fig. 1 cross-reacted with rabbit antiserum raised against the wild-type achacin (data not shown), thereby suggesting it to be the recombinant achacin (rAch). The rAch was analyzed for the amino acid sequence from the N-terminus using an

Table 1
Antimicrobial activity of the recombinant achacin

Bacterial strains	Recombinant achacin	
	1 mg/ml	0.1 mg/ml
Gram-negative bacteria		
Escherichia coli ATCC 25922	+++	+++
Proteus mirabilis IFO 13300	+	±
Pseudomonas fluoresces IFO 14160	+++	+++
Aeromonas hydrophila subsp. *hydrophila* IFO 13286	++	+
Aeromonas hydrophila KAH 8501	++	+
Aeromonas salmonicida IFO 12718	++	+
Vibrio anguillarum IFO 13266	+++	+
Vibrio parahaemolyticus IFO 12711	++	+
Gram-positive bacteria		
Staphylococcus aureus ATCC 25923	+++	+++
Staphylococcus epidermidis IFO 13889	+++	+++
Bacillus cereus IFO 13690	+	±
Streptococcus faecalis IFO 12970	+++	+++
Micrococcus luteus IFO 13867	+	±

+++, over 12 mm in diameter (strong antimicrobial activity); ++, 12–10 mm (moderate activity); +, 10–8 mm (weak activity); ±, 8 mm (not detected). The data are the averages of triplicate determinations.

Applied Biosystems 476A sequencer (Foster City, CA). The N-terminal sequence was NH_2-EAPQCSRSVDVA, which was identical to that of authentic achacin [4] except for the second amino acid of A as was to be expected. The second amino acid G of authentic achacin was substituted to the amino acid A in order to increase an efficiency of recognition by signal peptidases. This result indicates that the mature protein (502 amino acids and 55.8 kDa molecular mass [4]) is correctly processed during the expression in the host cells. The yield of the rAch was calculated as 0.2 mg/l. The carbohydrate liberated from the rAch was identified as hexose. The HPLC analysis revealed that the glycosylation moiety of the rAch is composed of mannose and *N*-acetylglucosamine. The carbohydrate composition ratio was determined by employing mannose and *N*-acetylglucosamine as a standard. 50 mol of mannose and 2 mol of *N*-acetylglucosamine appeared to link per mol achacin according to the weight ratio. These results indicate that the rAch carries Man50GlcNAc2-linked form.

3.2. Antimicrobial activity of the recombinant achacin

The antimicrobial activity of achacin was determined using a typical Gram-negative bacterium *E. coli* and Gram-positive bacterium *S. aureus*. Fig. 2 illustrates the destruction of *E. coli* by 12 μg/ml of wild-type achacin (wAch) isolated from snail mucus after 3 h incubation at 37°C. At concentrations lower than 1.2 μg/ml, there was a dose-dependent delay in the onset of detectable growth. On the other hand, *S. aureus* was more sensitive against wAch as described earlier [3], the growth was totally inhibited at achacin concentrations in excess of 1.2 μg/ml. At lower concentrations, the onset of exponential growth curve was retarded. For both bacteria tested, the concentration of achacin below that necessary for total inhibition resulted in a marked delay in reaching adequate viable colony-forming units. The rAch showed a bacteriostatic effect similar to the wAch at low concentrations. The rAch prevented the growth of *E. coli* and *S. aureus* resulting in no increase in bacterial number for 9 h and 12 h, respectively (Fig. 2). The antibacterial activity of rAch at a concentration of 8.5 μg/ml was equivalent to that of wAch at 0.12 μg/ml (1/70 activity), for both *E. coli* and *S. aureus*. The carbohydrate moiety of the wAch was shown to contain not only mannose but also sialic acids, glucose, fucose and xylose [14], which may play an important role of the biological activity of the novel antimicrobial glycoprotein. Achacin makes up a relatively new class of antimicrobial agents which possess a unique mechanism, unlike vancomycin and relevant glycopeptide antibiotics, which exert their antimicrobial effect by binding to carboxy-terminal peptide targets in the bacterial cell wall preventing the biosynthesis of peptidoglycan [15]. It is worth noting that the antibacterial activity of the rAch is effective in the richer media, in which the same phenomenon was observed on the wAch in the heart infusion bouillon medium [3].

The rAch showed a broad antimicrobial spectrum (Table 1). 1 mg/ml rAch showed an antibacterial activity against all tested bacteria of eight Gram-negative and five Gram-positive bacteria. The growth of *E. coli*, *P. fluorescens*, *S. aureus*, *S. epidermidis*, and *S. faecalis* was strongly suppressed even at the achacin concentration of 0.1 mg/ml. The rAch was effec-

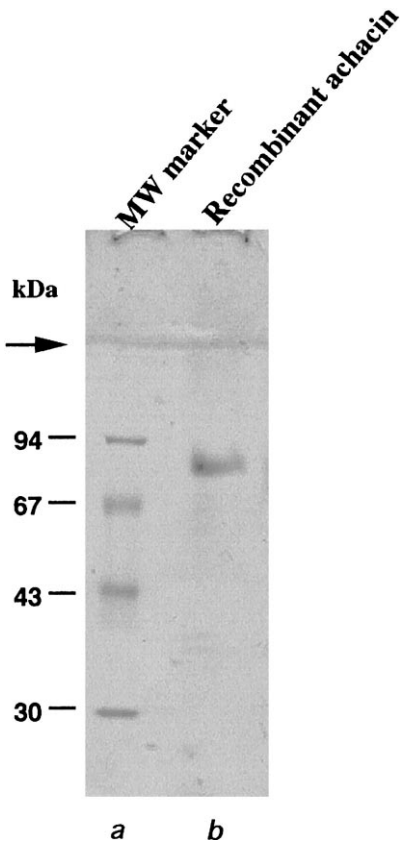

Fig. 1. SDS-polyacrylamide gel electrophoresis of the recombinant achacin secreted from *P. pastoris*. a: MW marker (94 kDa, phosphorylase; 67 kDa, bovine serum albumin; 43 kDa, ovalbumin; 30 kDa, carbonic anhydrase); b: recombinant achacin.

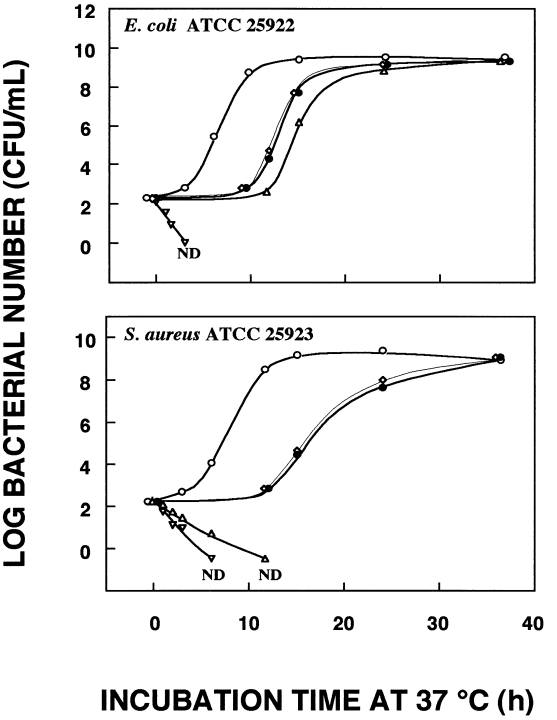

Fig. 2. Growth curve of *E. coli* and *S. aureus* with and without achacin in L-broth. Data are from a representative experiment repeated five times with similar results. ○, Control (without achacin); ◇, 8.5 μg/ml recombinant achacin, ●, 0.12 μg/ml native achacin; △, 1.2 μg/ml native achacin; ▽, 12 μg/ml native achacin. ND, not detected.

tive on *V. anguillarum* and *V. parahaemolyticus*, as well. On the other hand, the rAch did not appreciably affect the growth of *P. mirabilis*, *B. cereus* and *M. luteus*.

The achacin produced by *E. coli* expression system did not show antimicrobial activity. The present study suggested that the carbohydrate moiety could play an important role in exhibiting the biological activity of the protein, despite the fact that the role of the carbohydrate moiety recognized in the rAch from *P. pastoris* is unclear. Although it has been shown that the yeast expression system can be used to increase the stability to heating or proteolysis due to protein glycosylation, an excessive polymannosyl chain may not be suitable for producing active protein [6]. The hyperglycosylated lysozyme expressed in *Saccharomyces cerevisiae* decreased the lytic activity to only 11% when the insoluble cell wall of *Micrococcus lysodeikticus* was used as substrate [6]. Polyglycosylation in the rAch may bring about the antimicrobial activity lower than that of the wAch. It may be caused by a reduction of the harmful effects of achacin on yeast growth. Compared to a number of reports documenting high-level expression of foreign genes in *P. pastoris* [16], the amount of the rAch secreted in YMM in this study was quite low (0.2 mg/l). Achacin may be harmful for the growth of host yeast.

The present study reports that biologically active achacin with polymannosyl chains has been successfully produced using the *Pichia* expression system The effects of extracellular pH and incubation temperature during cultivation on the glycosylation patterns of heterologous proteins in yeast await for further study.

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