# Cloning and Identification of a New Antimicrobial Protein, Salvic, from Human Salivary Gland

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Abstract: From a subtracted cDNA library of human salivary gland a C77-91 gene totally unknown to date was named as Salvic that expresses 46 amino acids peptide (pI=9.45) possessing an antimicrobial activity on E. coli. Salvic is consisted of a typical hydrophobic amino acid rich domain in the N-terminus, a cluster of basic amino acids, carbohydrate attachment sites, a possible transglutaminase catalyzed crosslinking site, and multiple consensus sequences of phosphorylation site in the C-terminus. Western blot analysis using the monospecific antibody to the synthetic Salvic peptide showed strong interacting proteins in the extracts from submandibular gland and parotid saliva, and the immunohistochemical staining detected a strong positive reaction in the cytoplasmic secretory granules of interlobular ductal cells of salivary gland. The Salvic was also distributed in the human sebaceous gland and prostate. These data suggest that the identification of Salvic may add further understanding of greater role of salivary proteins providing innate immunity by protecting and stabilizing the mucosal epithelium in the maintaining homeostasis of oral mucosa.

Key words: salvic, antimicrobial protein, human, salivary gland, oral mucosa

## Introduction

The oral cavity is exposed to a variety of environmental challenges. Oral mucosal defenses range from simple mechanical rinsing by salivary flow to complex defense mechanisms of host innate and adaptive immunity[1)]. Saliva is crucial in maintaining the oral health. Salivary secretions not only have essential functions in relation to the digestive process that includes taste, mastication, bolus formation, enzymatic digestion, and swallowing, but also play a critical role in defending epithelial cells and mucosa from infection[2)].

In order to find yet undefined orphan genes specifically expressed in human salivary gland, a subtracted cDNA library of human submandibular gland was constructed and 200 clones were partially sequenced. Thirteen clones were found to be nonredundant in the databases of GenBank (National Center for Biotechnology Information, NCBI), European Molecular Biology Laboratory (EMBL), and DNA DataBank of Japan (DDBJ). Among them four clones were found predominantly expressed in the salivary epithelia. Their whole cDNA sequences were analyzed and model structures were constructed from the deduced amino acid sequences of each clone. Monospecific antibodies of each clone were generated against the synthetic peptide, and each antibody expression in various human tissues was measured by immunohistochemistry and Western blot. C77-091 orphan gene is intensely expressed in the interlobular ductal and serous acinar cells of human submandibular gland, and it is totally unknown to date was named as Salvic (Saliva Victory, GenBank; AY177672) that expresses 46 amino acids peptide (pI=9.45) possessing an antimicrobial activity on E. *coli.*

## Materials and Methods

*Subtracted cDNA library*

Human submandibular gland was obtained from the 35 years old man, who received radical neck dissection surgery due to the squamous cell carcinoma involving left mandible. A cDNA library of human submandibular gland was constructed in the Uni-ZAP XR vector (Stratagene, La Jolla, CA, USA) by use of mRNA from human submandibular gland and ZAP-cDNA$^{â}$ Gigapack$^{â}$ III Gold Cloning Kit (Stratagene).

*Sequencing and homology search*

Selected clones were sequenced by the dideoxynucleotide chain termination method[3)] using the ALF-express auto sequencer (Amersham Phamacia Biotech, Sunnyvale, CA, USA) and ALF-express autocycle sequencing kit (Amersham Phamacia Biotech).

*Antibody production*

The C-terminal 35 amino acids were synthesized (Anygen, Kwangju, Korea) and conjugated with Keyhole Limpet Hemocyanin (Calbiochem, San Diego, CA, U.S.A.) for the enhancement of antibody production. Polyclonal antibodies against the synthetic peptides were produced in New Zealand white rabbits by multi-site dermal immunization of 0.5 ml of the emulsified Freund's complete adjuvant (Pierce, Rockford, IL, U.S.A) containing 500 mg of the synthetic peptide dissolved in PBS. Monospecific antibody was obtained by antigen affinity column purification using the activated AminoLinkâ Coupling gel (Pierce).

*Immunohistochemistry and in situ hybridization*

The biopsy specimens taken from normal intact submandibular gland were fixed with 10 % buffered formalin solution, embedded in paraffin and 5 mm sections were prepared. Tissue sections were deparaffinized, hydrated, and endogenous peroxidases were inactivated using 3% hydrogen peroxide for 10 min. The sections

Fig. 1

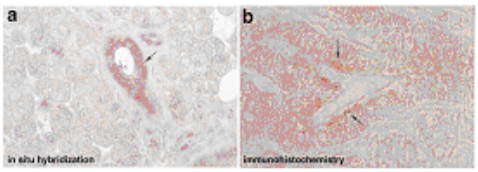


Fig.1. Salvic gene expression in RNA *in situ* hybridization (a) and immunohistochemistry (b). a: most intense in ductal cell (arrow), b: granular positive reaction in the luminal cytoplasms of ductal cells

Fig. 2

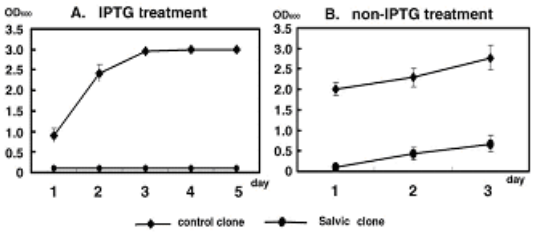


Fig. 2. E. *coli* killing effect of Salvic pBluescript clone by IPTG induction (A) was stronger than by non-IPTG treatment (B).

were treated to block any non-specific protein interaction with immunoglobulin using normal goat serum (DAKO), and three layer immuno-labeling was performed with those prepared monospecific antibodies.

The RNA probe for Salvic gene was generated from the plasmid vector (pBluescript II SK (-), C77-91 clone). Digoxigenin-UTP-labeled single strand antisense and sense RNA probe were prepared by T7 RNA polymerase and T3 RNA polymerase, respectively, using a RNA labeling kit (Boehringer Mannheim, Indianapolis, IN). The biopsy specimens taken from human submandibular gland were immediately fixed with 4% paraformaldehyde in PBS, embedded in paraffin and 5 mm sections were prepared by using RNase protection method. Hybridizations were performed at 50° C for 16 hours in a humidified chamber in the 10 mM Tris HCl, pH 7.6 buffer containing 50% formamide, 200 mg/ml tRNA, 1x Denhardt's solution, 10% dextran sulfate, 0.6 M NaCl, 0.25% SDS, and 1 mM EDTA. Detection of *in situ* hybridization was carried out using the Genius Detection system (Boehringer Mannheim, Indianapolis, IN).

*Antimicrobial assay of Vector-expressed Salvic on E. coli growth*

The diluted number ($0.5x10^3$/mL) of E. *coli* containing C77-091 clone (SOLR strain, Stratagene) and a control E. *coli* containing pBluescript SK(-) plasmid were cultured with 20 ml of ampicillin-LB broth (one liter solution containing 10 g tryptone, 5 g yeast extract, 5 g NaCl, 1 ml 1 N NaOH, and 100 mg/ml ampicillin) in shaking incubator (37°C, 150 rpm) for 3-5 days. Cell growth was monitored by optical density at 600 nm. Induction of the vector expression was carried out by addition of 20 mL isopropyl-b-D-thiogalactopyranoside (IPTG 1 mM, Sigma) into the LB broth.

*Scanning electron micrography (SEM) of microbes treated with Salvic*

*Staphylococcus aureus* were incubated with 10 μM Salvic for 10 and 20 min, and they were immediately fixed with glutaraldehyde, dried on the Millipore membrane, and coated with gold. SEM observation was performed for abnormal morphologies of each microbe where loss and shrinkage of original cell shapes as the time lapses.

## Results and Discussion

Fig. 3

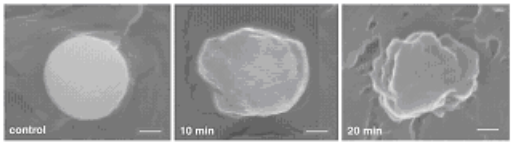


Fig. 3. SEM observation. *Staphylococcus aureus* was completely destroyed by Salvic peptide (10 mM) within 20 min.

C77-091 clone is composed of 527 bp encoding an open reading frame (+15 - +155) and expresses 46 amino acids peptide (pI=9.45, 5.252 kDa). This Salvic is composed of 8 serine, 8 valine, 6 leucine, and just one cystein residue in the central region. Besides a signal peptide in the N-terminal area the hydrophobic residues are clustered in the N-terminal domain, while hydrophilic residues are gathered in the C-terminal domain together with the consensus sequences of asparagine glycosylation site, protein kinase C phosphorylation site, and a possible transglutaminase-catalyzed crosslinking site. The immunostaining and *in situ* hybridization of Salvic gene is strongly positive in the ductal cells of human salivary gland, especially as multiple excretory granules at the luminal side of ductal cells (Fig. 1).

The bacterial growth rate in LB broth was compared between the E. *coli* transfected with pBluscript vector containing Salvic gene and the E. *coli* transfected with pBluscript vector only. The E. *coli* containing Salvic gene showed retarded growth during 3

Table 1. Amino acid sequence homology of Salvic in comparison with other antimicrobial proteins registered in Antimicrobial Peptide Database (APD; University of Nebraska Medical Center, USA, http://aps.unmc.edu/AP/main.html).

| types | amino acid sequences |
|---|---|
| Salvic | +MHDFWVLWVL+LEYIYN+S+ACSVLSATSS+VSSRVL+NR++SL+QVK++V+VKITN |
| Sapecin | ATCD+++L++LSTGI+NHS+AC++++A+AH+CLLR+G+NRGGYC+NGK+AV |
| Phormicin | ATCD+++L++LSGTGI+NHS+AC++++A+AH+CLLR+G+NRGGYC+NGK+GV |
| Opistoporin 2 | ++++GKV+W++++DWI+K+STA+KKL+WNSEPV++KELKNT++ALNAAKNFV |
| Opistoporin 1 | ++++GKV+W++++DWI+K+STA+KKL+WNSEPV++KELKNT++ALNAAKNLV |
| Caerin 1.4 | +++++++++GL+L++++++S+++S+L+++SS+VAKHVL++++++P+HV+++VPV+IAE |

Salvic (GenBank; AY177672): Human saliva, Sapecin (AP00227, PDB; 1L4V): Flesh fly, Phormicin (AP00216, PDB; 1L4V): Black blowfly, Opistoporin 2 (AP00375, Swissprot; P83314): African yellow leg scorpion, Opistoporin 1 (AP00374, Swissprot; P83313): African yellow leg scorpion, Caerin (AP00243, Swissprot; P56229): Green tree frog.
* Underlined letter: homologous residue, bold letter: hydrophobic residue (repeated pattern for a-helix)

days incubation, displaying the cell density measured as optical density at 600nm ($OD_{600}$) showed 0.0 after one day, 0.4 at two days, and 0.7 at three days. While the control group cells showed rapid growth reaching a plateau stage (2.3 $OD_{600}$) in two days. Furthermore, when the E. *coli* culture was supplemented with a promoter, IPTG (1 mM), the E. *coli* transfected with pBluescript vector containing the Salvic gene showed severe growth arrest, its cell density ($OD_{600}$) was not detectable until 5$^{th}$ day, while E. *coli* transfected with pBluescript vector only overgrew within 2 days (Fig. 2). And more, 10 mM Salvic peptide destroyed the cell membrane of *Staphylococcus aureus* within 20 min (Fig. 3).

In the oral cavity a lot of normal flora and pathogenic microbes are inoculated in warm body temperature. The salivary mucin and proline rich proteins initially function for the aggregation of microorganisms, and antimicrobial proteins including defensins and histatins may play a role for the bactericidal effect on the oral microorganisms. Salvic is one of candidate genes for the secretory antimicrobial protein in the saliva, because both of recombinant protein (data not shown) and synthetic peptide of Salvic showed the bactericidal effect on E. *coli*. The amino acid composition and simulated protein structure of Salvic (Fig. A-1,2) showed strong hydrophobic residues in N-terminal and a-helical hydrophilic residues with several consensus sequences of asparagine glycosylation site, protein kinase C phosphorylation site, and a possible transglutaminase-catalyzed crosslinking site in C-terminal, mimicking the adhering and leakage mechanism of typical antimicrobial peptide[4]. Homology search for the amino acid sequence of Salvic performed on the antimicrobial peptide database[5] revealed that Salvic showed about 30% amino acid sequence homology to several secreted antimicrobial peptides having helix structures, such as sapecin from flesh fly, phormicin secreted from the larvae of black blowfly, opistoporin 1 and 2 secreted from the venom gland of African yellow leg scorpion, and caerin secreted from the skin parotid and/or rostral glands of green tree frog (Table 1). Salvic has about 60% sequence homology of hydrophobic residues to these antimicrobial peptides. Antimicrobial peptide prediction program indicated that Salvic may form alpha helices and it may have at least 16 residues on the same hydrophobic surface. Thus it may directly interact with membranes and have a chance to be an antimicrobial peptide. Therefore, it is supposed that the Salvic is a novel antimicrobial peptide relevant to the evolutionary defensive secretions, and that it may be crosslinked on the oral mucosa or skin for the prolonged antimicrobial effect.

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