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[Short communication]

Prevention of biofilm formation on titanium surfaces modified with conjugated molecules comprised of antimicrobial and titanium-binding peptides

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Running title: Antimicrobial activity of antimicrobial and Ti-binding peptides

Abstract

Specific binding of antimicrobial peptides to titanium surfaces may serve to prevent biofilm formation, leading to a reduction in peri-implantitis. This study evaluated the binding behavior of conjugated molecules consisting of antimicrobial and hexapeptidic titanium-binding peptides (minTBP-1) using the quartz crystal microbalance (QCM-D) technique, and investigated the effect of modification of titanium surfaces with these peptides on the bioactivity of *P. gingivalis*. Four kinds of peptide were prepared: Histatin 5 (DSHAKRHHGYKRKFHEKHSHRGY), minTBP-1 + Histatin5 (RKLPDAPDSHAKRHHGYKRKFHEKHSHRGY), Lactoferricin (FQWQRNMRKVR), and minTBP-1 + Lactoferricin (RKLPDAPGGFQWQRNMRKVR). The QCM-D analysis demonstrated that significantly larger increases in peptide adsorption were observed in the conjugated peptides than in antimicrobial peptides alone. In addition, ATP activity in *Porphyromonas gingivalis* in peptide-modified specimens significantly decreased compared to that in the titanium control. These results indicate that surface modification with conjugated molecules consisting of antimicrobial and titanium-binding peptides is a promising method for reduction of biofilm formation on titanium surfaces.

Keywords: antimicrobial peptide; titanium-binding peptide; histatin 5; lactoferricin; *P. gingivalis*

Introduction

Accumulation of microbial plaque around dental implants may develop into peri-implantitis, with accompanying bone loss. Many types of periodontopathic bacteria have been confirmed in such cases where a titanium implant was used (Sumida et al, 2002). This indicates the importance of maintaining biofilm-free surfaces on both the supra- and sub-gingival portions of dental implants in preventing peri-implantitis.

One important candidate in the enhancement of antimicrobial activity is the loading of antimicrobial peptides onto the titanium implant. Antimicrobial peptides are a new and promising class of antimicrobial agents with a low tendency to induce resistance *in vitro* (Hancock, 1997; Zasloff, 2002; Gordon et al, 2005). This kind of peptide may offer an alternative to antibiotics as there is no resistance, such as with methicillin-resistant *Staphylococcus aureus*, and there are little known side-effects (Helmerhorst et al, 1999; Gusman et al, **2001a**; **2001b**). Clinical applications, such as saliva substitute antimicrobial peptide-loaded xanthan in the treatment of oral candidiasis, were investigated (Ruissen et al, 1999, 2002).

Histatins, a family of basic peptides secreted by the major salivary glands in human, **possess** antimicrobial activities. These antimicrobial activities are believed to regulate biofilm formation in the oral cavity, and have fungicidal and fungistatic effects on *Candida albicans* cells, in particular (Edgerton et al, 1995; Ruissen et al, 1999). In a previous study, we found that *C. albicans* colonization on poly(methyl methacrylate) was reduced significantly by adsorption of histatin 5 (Yoshinari et al, 2006). In addition, histatin 5 may play an important role in the regulation of periodontopathic bacteria such as *Porphyromonas gingivalis*, which is involved in inflammatory periodontal diseases and peri-implantitis (Imatani et al, 2000; Gusman et al, 2001; Kato et al, 2004).

Lactoferricins are highly basic peptides generated by gastric pepsin cleavage of various

lactoferrins, and **their structures and characteristics have been investigated** (Kuwata et al, 1998; Wakabayashi et al, 2003; Hunter et al, 2005). Lactoferricins displayed antimicrobial activity against several bacterial strains, including *P. gingivalis* (Shi et al, 2000; Chen et al, 2006; Haug et al, 2007). Recently, it was shown that a much smaller peptide, the 11-residue FQWQRNMRKVR peptide, homologous to just over half the loop region, had potent antibacterial activity, and was suggested that it might account for all the activity of the longer isoforms of this peptide (Odell et al, 1996; Azuma et al, 1999). Therefore, surface-loading of these peptides by either adsorption or chemical cross-linking on titanium implants might reduce periodontopathic biofilm formation, thus preventing peri-implantitis.

Over the last few years, a number of peptide aptamers (binders) capable of recognizing the surfaces of various inorganic materials have been isolated by evolutionary engineering methods, including a peptide phage system (Baneyx et al, 2007). One such aptamer, identified as a titanium-binder, is TBP-1 (Sano and Shiba, 2003). This peptide was originally isolated as a 12-mer peptide, and mutational analyses revealed that the N-terminal hexapeptide RKLPDA (named minTBP-1) was sufficient for binding (Sano and Shiba, 2003). Mutational studies also indicated that the first arginine (R1), fourth proline (P4) and fifth **aspartate** (D5) were important for binding. Because the surface of titanium is covered with an oxide film displaying both positively- and negatively-charged hydroxyl groups under physiological conditions (Jones, 2001), electrostatic interactions between $-O^-$ and R1, and $-OH_2^+$ and D5, have been proposed to underlie the interaction between minTBP-1 and titanium (Sano and Shiba, 2003). A recent study using an atomic force microscope has supported this electrostatic interaction model (Hayashi et al, 2006). Furthermore, it was also demonstrated that accretion of surfactant reduced nonspecific interactions, dramatically enhancing the selectivity and specificity of this Ti-binding peptide (Sano et al, 2005a; 2005b; 2007).

Recent studies have confirmed the usefulness of the quartz crystal microbalance-dissipation (QCM-D) technique in evaluating surface-related processes with real-time measurement in liquids, including protein adsorption, complementary activity on biomaterials, and analysis of DNA hybridization (Rodahl et al,1996; Miyachi et al, 2000). The results of this study revealed a clear difference in frequency shift among the conditions set, indicating that the QCM-D technique is effective in the evaluation of adsorption behavior of peptides, including surfactants.

Accordingly, we hypothesized that surface modification with conjugated molecules consisting of antimicrobial and titanium-binding peptides (minTBP-1) might provide for a reduction of biofilm. The purpose of this study was to investigate the influence of surface modification with these peptides on the bioactivity of *P. gingivalis* as well as the binding behavior of these peptides to titanium surfaces using a QCM-D technique.

Materials and Methods

Peptide synthesis

All peptides (Table 1) were synthesized using the Fmoc (9-fluorenylmethyloxycarbonyl) method (Carpino et al, 1970), and purified to >95% by high performance liquid chromatography (Anygen, Gwang-ju, Korea).

Adsorption assay of peptides

For the adsorption assay of the synthesized peptides, a QCM-D instrument (QCM-D300, Q-Sense AB, Göteborg, Sweden) with AT-cut single-crystal quartz sensors with 14 mm in diameter was used, onto which Ti was coated, using an axial flow chamber consisting of a T-loop to thermally equilibrate the sample (Figure 1). Measurements were carried out at $25^{\circ}\text{C} \pm 0.05^{\circ}\text{C}$, and data were collected at 14.8 MHz for analysis. Monitoring the resonance behavior of piezoelectric oscillation

enables measurement of mass adsorption at the surface in real time, usually as a function of the decrease in resonance frequency (f). Frequency shift (Δf) is related to adsorbed mass (Δm). The amount of adhered protein was estimated according to the Sauerbrey equation (Sauerbrey, 1959):

$$\Delta f = \Delta m (-n/C),$$

where n indicates number of overtones, and C is the constant for a given kind of sensor. According to this equation, a 1 Hz decrease in frequency at an overtone of 14.8MHz corresponds to approximately 5.98 ng/cm² sensor mass gain. It should be mentioned that this relationship is strictly applicable to sufficiently thin and rigid films.

In this experiment, surfactant (Tween 20: polyoxyethylene sorbitan monolaurate, Polysorbate 20, Wako Pure Chemical, Japan) was used as a blocking agent to block nonspecific protein binding to the sensors instead of albumin because the nature of this surfactant is known in advance (Sano *et al.*, 2003). The sequence of injections into the QCM cell for an experimental run was as follows: 0.5 mL PBS (-) for 5 minutes, 0.5 mL PBS-T (PBS plus 0.1% -Tween 20) for 5 minutes, 0.5 mL each peptide solution in PBS-T (100 $\mu\text{g mL}^{-1}$, pH = 7.4) for 20 minutes, and 0.5 mL PBS-T for 5 minutes. Lysozyme solution (100 $\mu\text{g mL}^{-1}$) in PBS-T as a negative control and lysozyme solution (100 $\mu\text{g mL}^{-1}$) in PBS were also used. Lysozyme is a globular hydrophilic protein that is positively charged under the conditions used for the artificial proteins. In our previous study, injection of lysozyme reduced the resonance frequency by only 2.5 Hz, indicating a weak association with the Ti sensor (Kokubun et al, 2008). The results were expressed as the mean \pm SD of five specimens.

X-ray photoelectron spectroscopy (XPS)

Surface characteristics of the peptide-treated titanium disks (**Furuuchi Chemical, Japan**) were analyzed using an X-ray photoelectron spectroscope (XPS, AXIS-ULTRA, KRATOS Analytical,

GBR) with X-ray source of Al K α (monochromator), 15kV and 15mA. Before analyzing, titanium plates were ultrasonically cleaned with distilled water for 10min. Depth analysis was performed to support the QCM analysis with argon-ion etching of 2kV, 20mA for 0, 10, 20, 30, 40, 50 and 60s. The etching rate under this condition was approximately 0.6nm/min on the SiO₂. It is easily expected that the etching rate on the peptides was much larger than that on SiO₂. The binding energies of each of the spectra were calibrated with C1s of 285.0 eV.

Bioactivity assay against P. gingivalis

Commercially pure wrought titanium disks (JIS, Japan industrial specification H4600, 99.9 mass% Ti, **Furuuchi Chemical, Japan**) with a diameter of 13 mm and a thickness of 1mm were polished with 1200 SiC paper in final, and then ultrasonically cleaned for 10 min, once with acetone and once with distilled water. The titanium specimens were then sterilized in an autoclave. Subsequently, to bind the peptides onto the titanium surfaces, the specimens were immersed in 5 mL solution including minTBP-1+His5 or minTBP-1+ Lfcin (100 $\mu\text{g mL}^{-1}$, pH7.4) for one hour at room temperature, and washed with distilled water.

The bioactivities of *P. gingivalis* ATCC 33277 (ATCC, American Type Culture Collection) on peptide-modified titanium were evaluated by ATP-bioluminescent assay (Takahashi et al, 2007). ***P. gingivalis* was grown on blood agar plates consisting of Tryptic soy agar (Becton Dickinson Microbiology System, Cockeysville, MD) supplemented with 10% defibrinated horse blood, hemin (5 mg/mL; Sigma Chemical Co., St. Louis, MO), and menadione (0.5 mg/mL; Wako Pure Chemical Industries, Osaka, Japan).** Culture was performed in an anaerobic chamber (N₂: 80%, H₂: 10%, CO₂: 10%) at 37°C.

Peptide-modified titanium and unmodified titanium (Ti) specimens (control) were placed in 24-well cell culture plates with the modified surface of the specimen facing upward. **For ATP-bioluminescent assay, *P. gingivalis* was precultured, taken from 4 days culture on blood agar plate, for 24 h in 4 mL of Trypticase soy broth supplemented with hemin and menadione. Aliquots of 10 μ L precultured cells were inoculated into the wells, which contained 1 mL trypticase soy broth supplemented with hemin and menadione. Culture was performed in an anaerobic chamber at 37°C for 5 days.**

After incubation, media and unattached bacterial cells were removed from the wells, and specimens were transferred to new 24-well culture plates. Remaining loosely bound cells were removed by rinsing twice with 200 μ L/well distilled water. After addition of 1 mL distilled water, an adenosine triphosphate (ATP)-bioluminescence assay was performed using the Promega BacTiter-Glo™ Microbial Cell Viability Assay Kit (Madison, WI). Aliquots of 100 μ L suspended solution + 100 μ L acTiter-Glo™ reagents were added to the culture plates. Finally, bioluminescence was measured with an auto lumicounter (Lumicounter, Microtec CO., LTD, Funabashi, Japan). The results were expressed as the mean \pm SD of five specimens.

Scanning electron microscopy (SEM)

Peptide-modified titanium and unmodified titanium (Ti) specimens incubated with *P. gingivalis* were prepared for SEM observation under the following standard procedures. Specimens were fixed in 2.0% glutaraldehyde in PBS for 2 h at room temperature. They were then washed three times with PBS and dehydrated through a series of graded ethanol solutions (70, 80, 90, 95, and 100%). The specimens were subsequently freeze-dried, sputter-coated with Au-Pd, and observed using a scanning electron microscope (JSM-6340F, JEOL, Japan).

Statistical analysis

Data from the adsorption assay were analyzed for statistical significance using an analysis of variance (ANOVA) followed by the Scheffé's test for multiple comparisons. The Mann-Whitney U test was used for the bioactivity assay.

Results

Adsorption of peptides

Shift in frequency (Δf) against time for exposure of Ti sensor to various peptides obtained by QCM-D as a typical example of the raw data was demonstrated (Figure 2). Decrease in frequency shows mass adsorption on the surfaces. A comparatively large frequency change was observed in lysozyme solution without blocking agent (Lysozyme w/o blocking), indicating that a greater amount of lysozyme was adsorbed. On the other hand, little frequency was observed in lysozyme solution with blocking agent as a negative control (Lysozyme), indicating that surfactant PBS-T induced a reduction in non-specific binding. These peptides desorbed slightly when PBS-T was injected. Both minTBP-1+His5 and minTBP-1+ Lfcin showed the large decrease in frequency compared to His5 and Lfcin under using the blocking agent.

The adsorbed mass per unit area of peptides was demonstrated in Figure 3. Net changes in adsorbed mass of minTBP-1+His5 and minTBP-1+ Lfcin were approximately 3 times greater than those of His 5 or Lfcin alone (**p<0.05**).

XPS analysis

The depth analyses of N1s and O1s spectra by the XPS analysis on titanium surfaces treated with His5 and minTBP1+His5 were shown in Figure 4 and Figure 5, respectively. An N1s peak at 400.0 eV and an O1s peak at 532.2 eV were derived from the amide groups of the peptides. An O1s peak

at 530.2 eV was derived from TiO₂. The N1s peaks and O1s peaks at 532.2 eV of the His5 specimen almost disappeared after only 20 s of argon-ion etching. In contrast, on the TBP1+His5 specimen, those peaks still remained on the argon-ion etched surface after around 40 s. The intensity of the O1s peaks at 532.2 eV decreased as the argon-ion etching time increased, whereas that of the O1s peaks at 530.2 eV increased as the argon-ion etching time increased. On the Lfcin and minTBP1+ Lfcin specimens, the same results were obtained as the His5 and minTBP1+His5 specimens.

Bioactivity against P. gingivalis

The ATP activities of *P. gingivalis* were evaluated on peptide-immobilized titanium and the unmodified control (Figure 6). The ATP activity of *P. gingivalis* on peptide-modified specimens significantly decreased compared to that on the Ti control ($p < 0.05$).

Scanning electron micrographs of the minTBP1+His5, minTBP1+Lfcin and Ti specimens incubated with *P. gingivalis* ATCC33277 were shown in Figure 7. Bacterial adhesion apparently decreased on both minTBP1+His5, minTBP1+Lfcin compared that on Ti specimen.

Discussion

The purpose of this study was to determine whether the binding abilities of antimicrobial peptides to titanium surfaces were enhanced by conjugation with titanium-binding peptides, and whether the adsorption of those peptides was effective in the reduction of *P. gingivalis* biofilm formation.

We used the *P. gingivalis* ATCC33277 for bioactivity assay. It is considered that *Streptococcus* species are the primary colonizers to which *P. gingivalis* will attach (Gatewood et al, 1993; Fürst et al, 2007; Amarante et al, 2008). In addition to this mechanism, the pathogenicity around the dental implants is also assumed to be originated by direct attachment of periodontopathic bacteria such as

P. gingivalis from the point of view of peri-implantitis. Several investigators have shown adherence heterogeneity among *P. gingivalis* strains. *P. gingivalis* strains are classified as invasive or noninvasive (Watanabe et al, 1992; Naito et al, 1993). Naito et al. reported that noninvasive strains bonded more to collagen-coated hydroxyapatite than did invasive strains. The fimbriae from noninvasive strains also had high hydrophobicity and bound strongly to collagen or saliva-coated hydroxyapatite. *P. gingivalis* ATCC 33277 is type strain and is categorized as noninvasive. Thus, in the present study, we evaluated the direct attachment of *P. gingivalis* ATCC 33277 to the titanium surfaces.

Significantly larger peptide adsorptions were observed in the conjugated peptides which included minTBP-1 than in histatin 5 or lactoferricin alone. Furthermore, QCM analysis revealed little frequency change in lysozyme with blocking agent, despite a comparatively large frequency change in lysozyme solution without blocking agent, suggesting that PBS-T surfactant reduced non-specific binding. These results indicated that conjugation with minTBP-1 effectively increased **the binding ability of part of a complex community in the peptide.**

Reversible specific binding on metal surfaces, where irreversible nonspecific binding can be avoided, may play an important role in achieving efficacious bio-fictionalization in metal surface modification. MinTBP-1 is considered to fulfill this function. The specific binding of minTBP-1 is due to double electrostatic bonds between the charged residues and the surface groups of the substrate. It has also been demonstrated that the accretion of surfactant reduced nonspecific interactions, dramatically enhancing the selectivity and specificity of Ti-binding peptides (Sano and Shiba, 2003, Sano et al, 2005; Hayashi et al, 2006; **Yamashita et al, 2006**). The binding of biomolecules, e.g., antimicrobial peptides, to the surface of implants is critical for their proper function. The results in the present study demonstrated that conjugated molecules adsorbed on the titanium surfaces with specific binding. Therefore, surface modification with conjugated

molecules consisting of antimicrobial and minTBP-1 peptides on titanium surfaces may induce antimicrobial activity.

The bioactivity test revealed that both ATP activity **and** SEM observation in *P. gingivalis* on the peptide-modified specimens significantly decreased in comparison with that in the control. These results indicate specific binding of antimicrobial peptides to titanium surfaces and antimicrobial activity.

There are two possible explanations for the mechanism of reducing biofilm formation on the titanium surfaces modified with conjugated peptides. One **the** on hand, the action of the released antimicrobial peptides could be responsible for this mechanism; on the other hand, direct antimicrobial activity of the adsorbed molecules **may be** responsible for this mechanism. **We think that** the latter mechanism, caused by the adsorbed molecules, **may be primarily responsible** for the antimicrobial activity. **This is supported by the specific binding of the conjugated molecules.** Thus surface modification with conjugated molecules consisting of antimicrobial and minTBP-1 peptides on titanium surfaces may induce antimicrobial activity even though these peptides are not released. **However, more experiments are needed to demonstrate this directly.**

Histatin 5 molecules possess cationic ions ($pI > 9$, positively charged at $pH = 7.4$), and are amphipathic, having both hydrophilic and hydrophobic domains (Amerongen et al, 2002) . It was predicted that histatin 5 would be absorbed on a negatively-charged Ti surface. However, only small amounts of histatin 5 were absorbed compared to minTBP1-His 5. Therefore, we believe that this titanium binding peptide may play an important role in increasing specific binding to titanium. Further investigation should be performed to clarify its bonding mechanism. Many studies have reported that histatin 5 exhibited antimicrobial activity in the treatment of oral fungal infections such as *C. albicans* (Edgerton et al, 1995 ; Kavanagh et al, 2001; Yoshinari et al, 2006). In this study, minTBP1-His 5 exhibited antimicrobial activity against *P. gingivalis*. This result supports those of

earlier studies which found that histatin 5 inhibited inflammatory cytokine induction in human gingival fibroblasts by *P. gingivalis* (Imatani et al, 2000; Kato et al, 2004).

Lactoferricins (Lfcins) are also positively-charged peptides **similarly to histatin 5**. Lactoferricin B, a 25-amino acid-long peptide located at the N-lobe of bovine lactoferrin, also exhibited antimicrobial activity against *P. gingivalis* cells (Shi et al, 2000). The 11-residue peptide FQWQRNMRKVR used in this study exhibited potent antibacterial activity, and may account for all the activity of the larger peptides; in particular, the multiple-antigen peptides (MAP) of this 11-residue peptide exerted significant antibacterial effects against a broad spectrum of bacteria, including MRSA (Odell et al, 1996; Azuma et al, 1999).

In summary, the results of the present study indicate that surface modification with conjugated molecules consisting of antimicrobial and titanium-binding peptides is a promising approach to the reduction of biofilm formation on titanium surfaces. **These findings indicate that the antimicrobial peptides may not only have activity against *P. gingivalis* but also may inhibit biofilm development against other bacterial pathogens** if the conjugated molecules consisting of the specific antimicrobial and material-binding peptides were developed. It should be noted that controlled binding and release of biomolecules to implant surfaces is critical in the development of intelligent implant materials. Further study is necessary to clarify the underlying mechanisms of antimicrobial activity in such conjugated peptides, and determine the stability of adsorbed peptides by exposure to simulated saliva.

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Figures captions

Figure 1 Schematics of axial flow chamber in QCM-D instrument for adsorption assay. In a flow configuration, the sample flows radially from the center of the cell to the exit at the circumference of the cell.

Figure 2 Shift in frequency (Δf) against time for exposure of Ti sensor to various peptides obtained by QCM-D as a typical example. At black arrowhead, 100 $\mu\text{g mL}^{-1}$ peptide in PBS-T was injected, followed by successive injection of PBS-T (white arrowhead). Decrease in frequency shows mass adsorption on the surfaces. All experiments except “Lysozyme w/o blocking” were carried out with the surfactant, i.e. with blocking.

Figure 3 The adsorbed mass per unit area of peptides derived by subtracting increased ΔF at injection of PBS-T from decreased ΔF at injection of peptides in PBS-T, at 14.8 MHz by QCM-D. Identical letters indicate no significant difference ($p > 0.05$).

Figure 4 Depth analysis of N1s spectra by the XPS evaluation on titanium surfaces treated with His5 (**left**) and minTBP1+His5 (**right**).

Figure 5 Depth analysis of O1s spectra by the XPS evaluation on titanium surfaces treated with His5 (**left**) and minTBP1+His5 (**right**).

Figure 6 ATP activities of *P. gingivalis* ATCC33277 on peptide-modified and unmodified titanium (mean \pm standard deviation). RLU: Relative Light Unit, Identical letters indicate no significant difference ($p > 0.05$). The ATP activity of *P. gingivalis* on peptide-modified specimens significantly decreased compared to that on the Ti control ($p < 0.05$).

Figure 7 Scanning electron micrographs of a: minTBP1+His5, b: minTBP1+Lfcin and c: Ti specimens incubated with *P. gingivalis* ATCC33277.

Fig.1

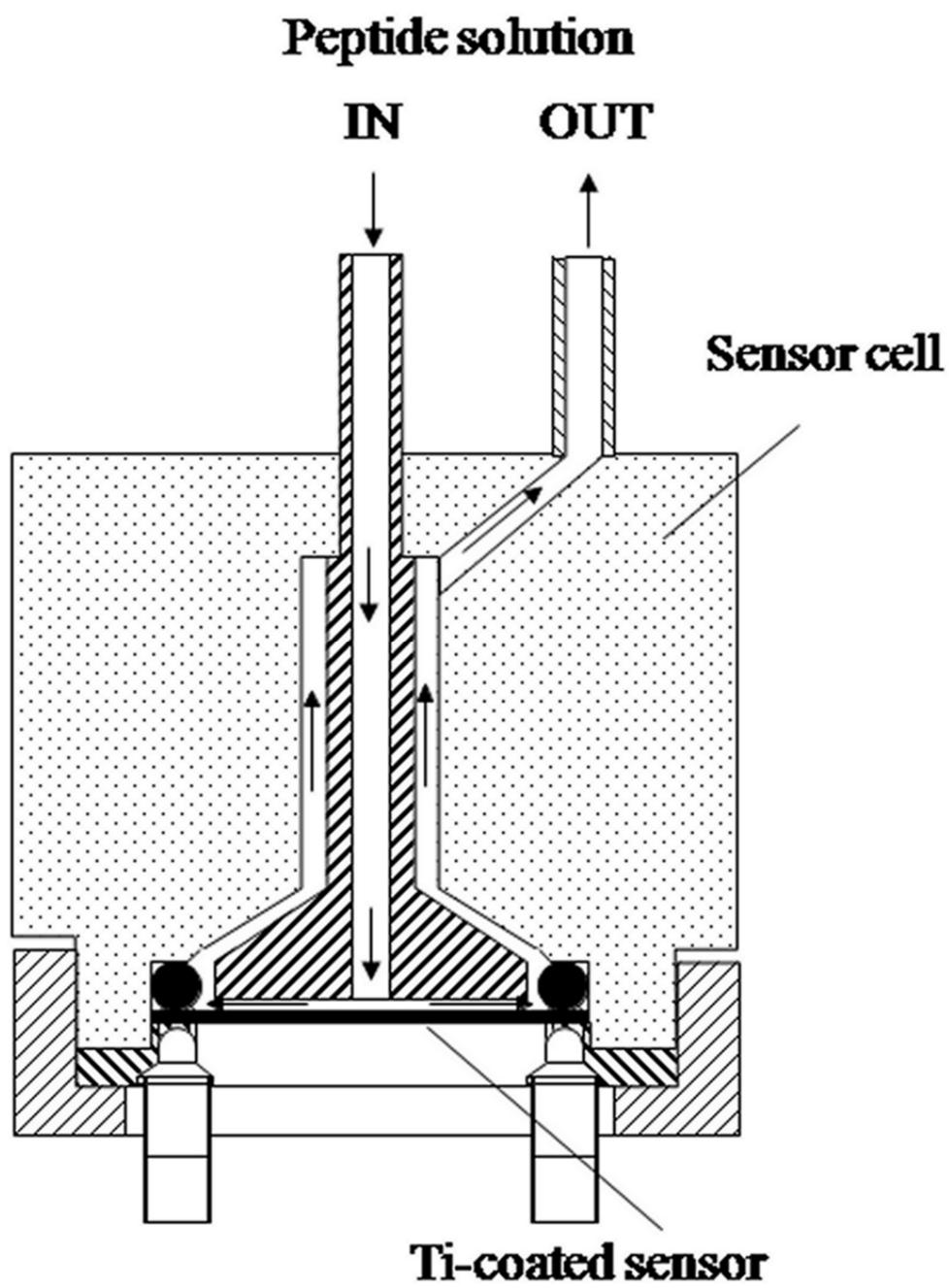


Fig.2

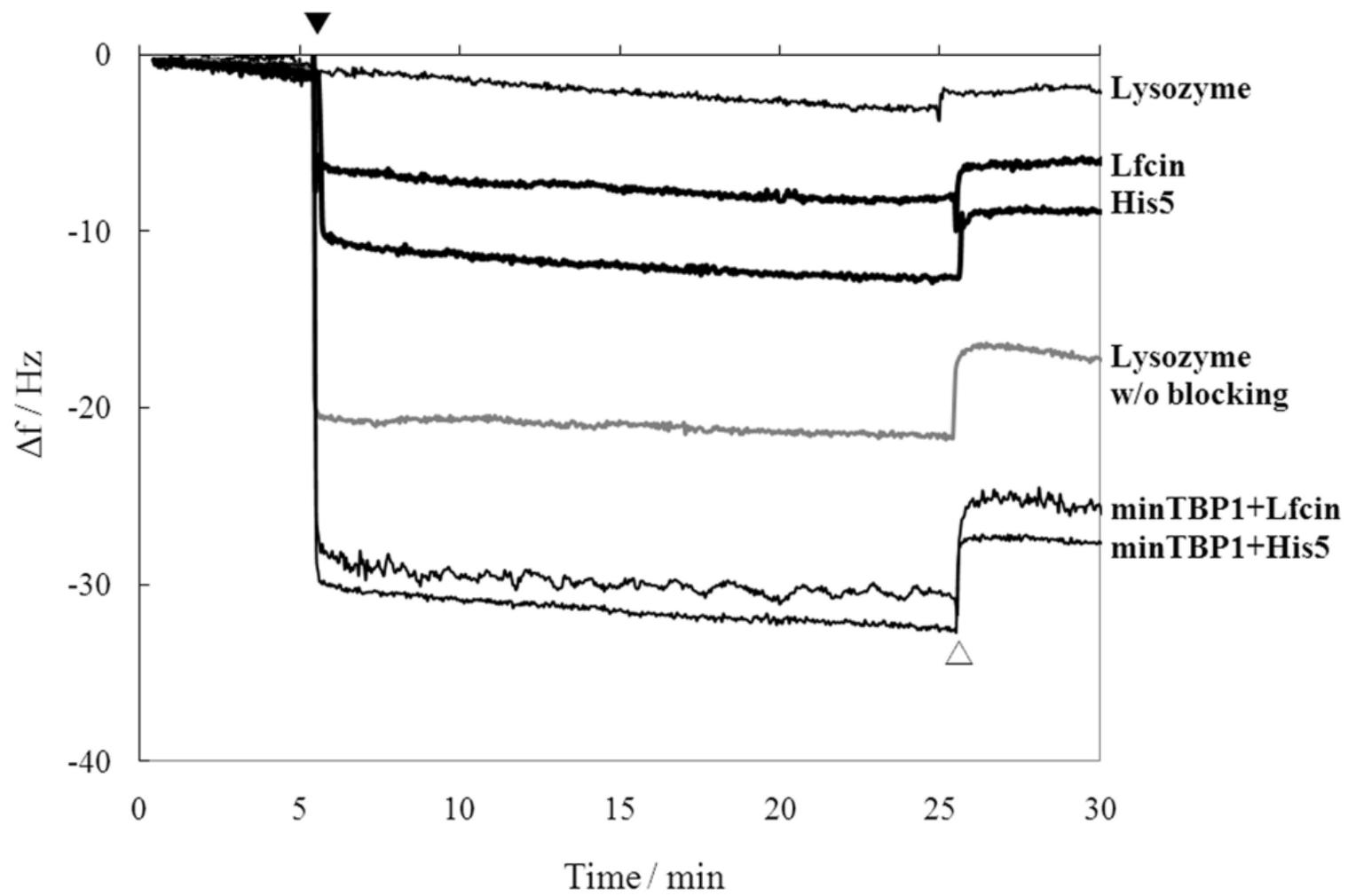


Fig.3

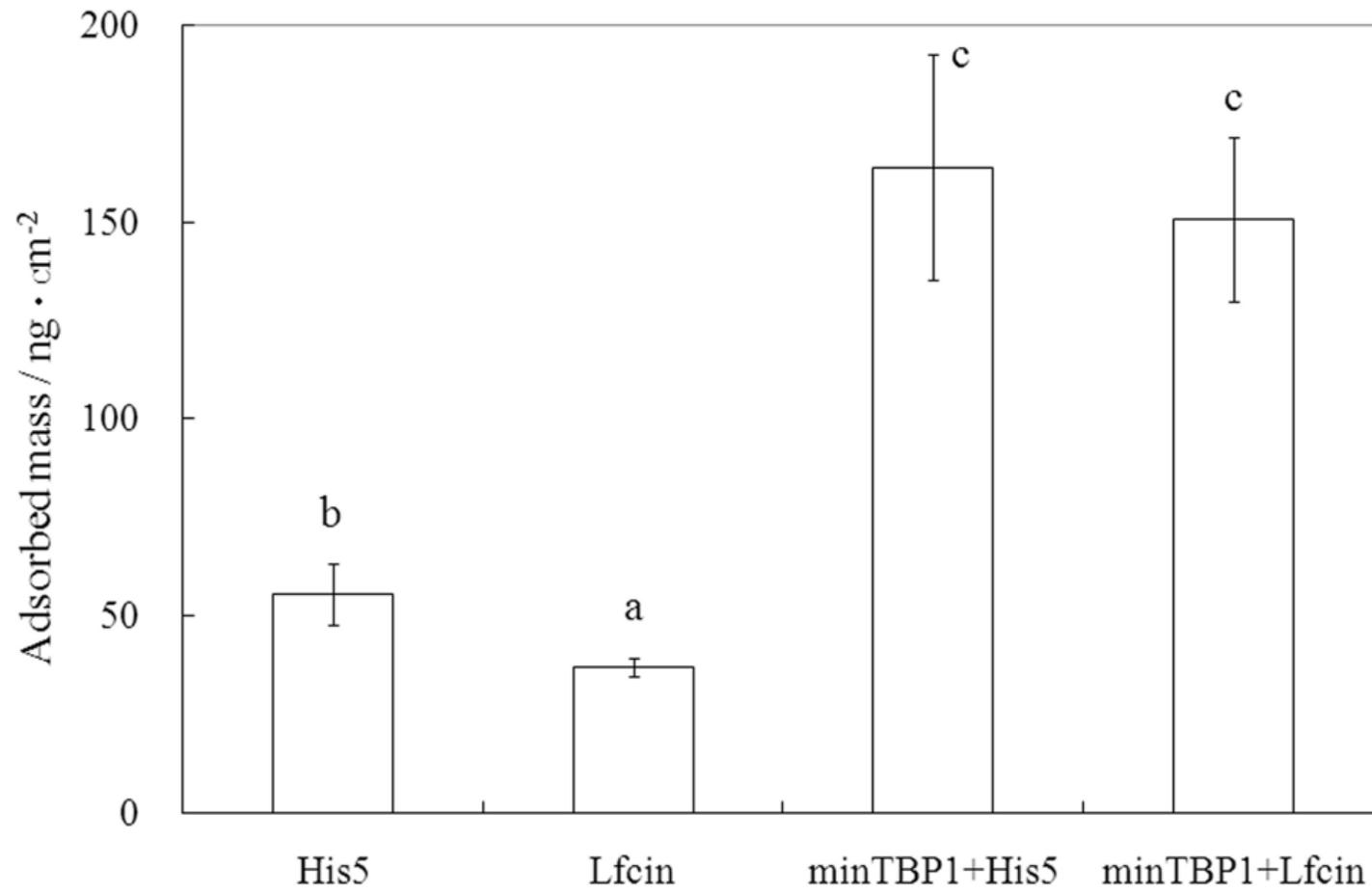


Fig.4

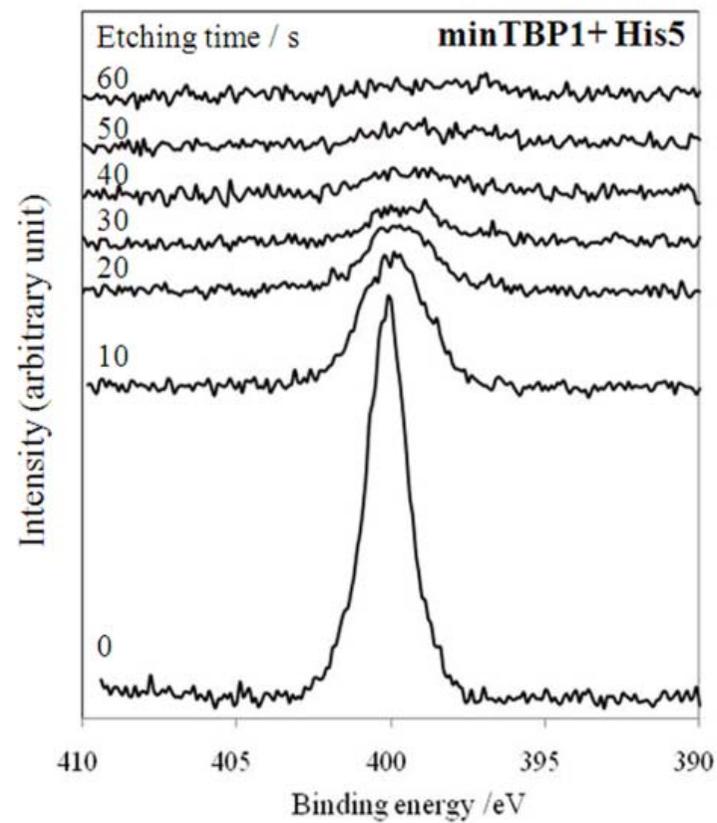
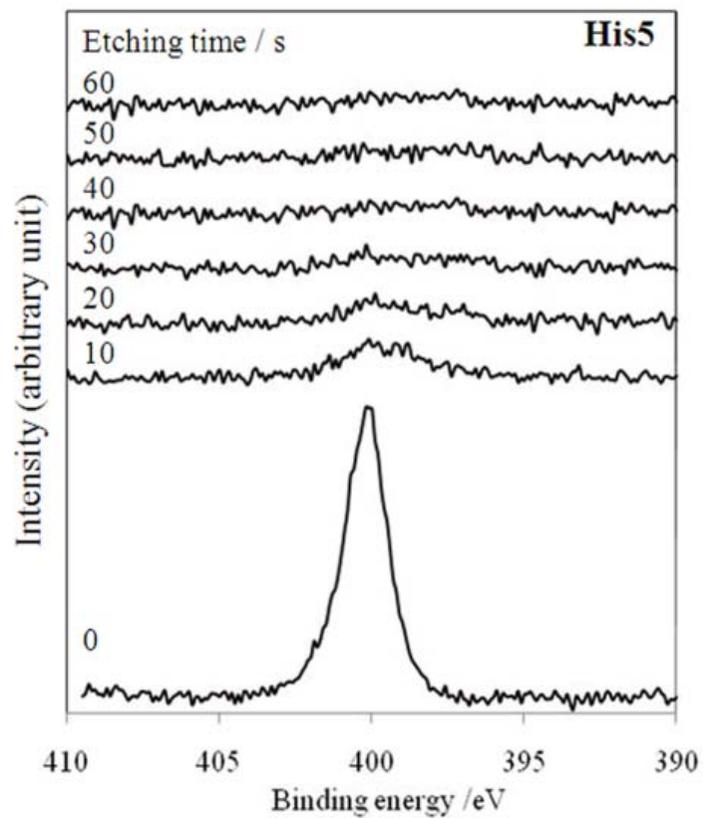


Fig.5

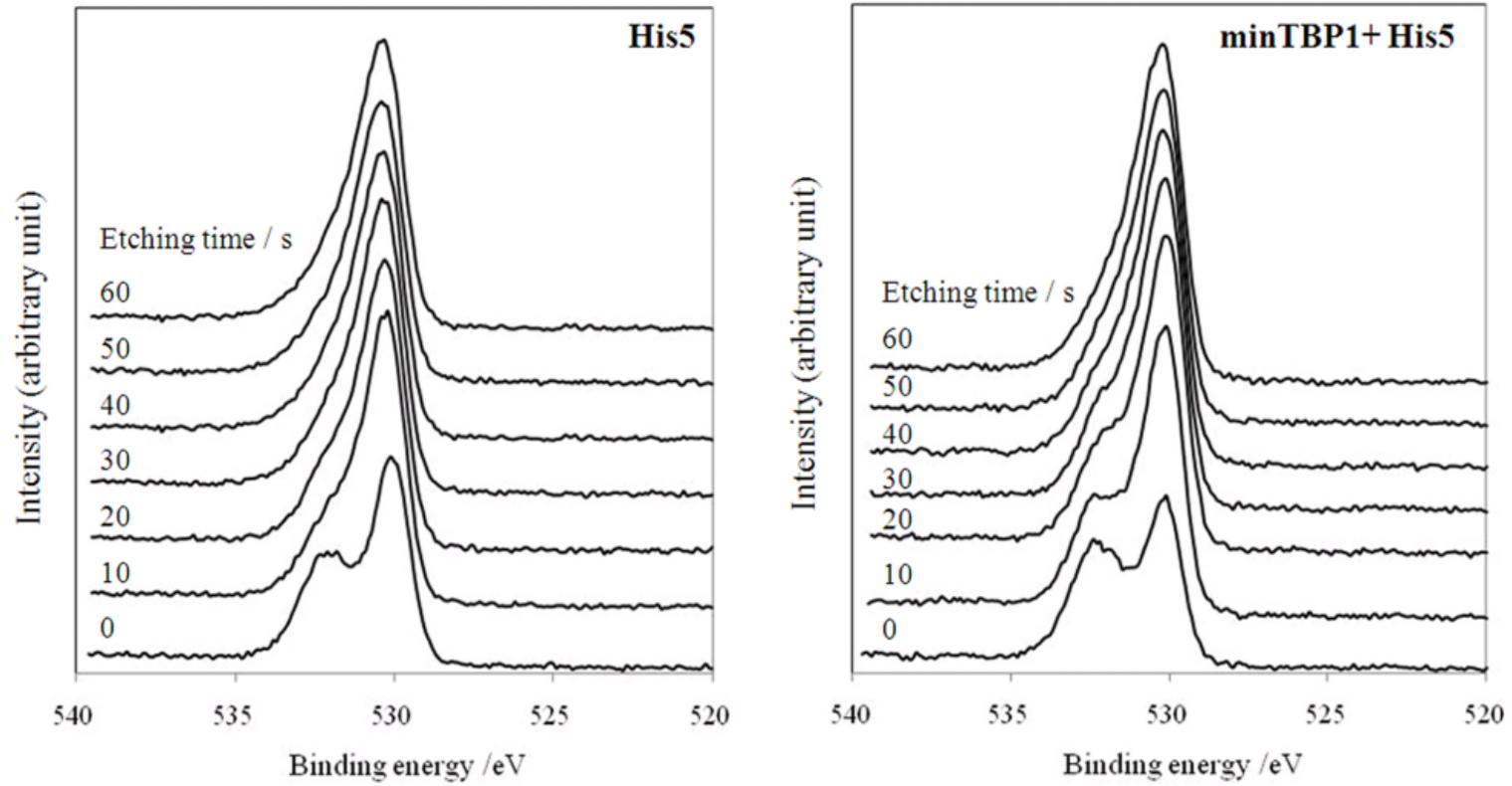


Fig.6

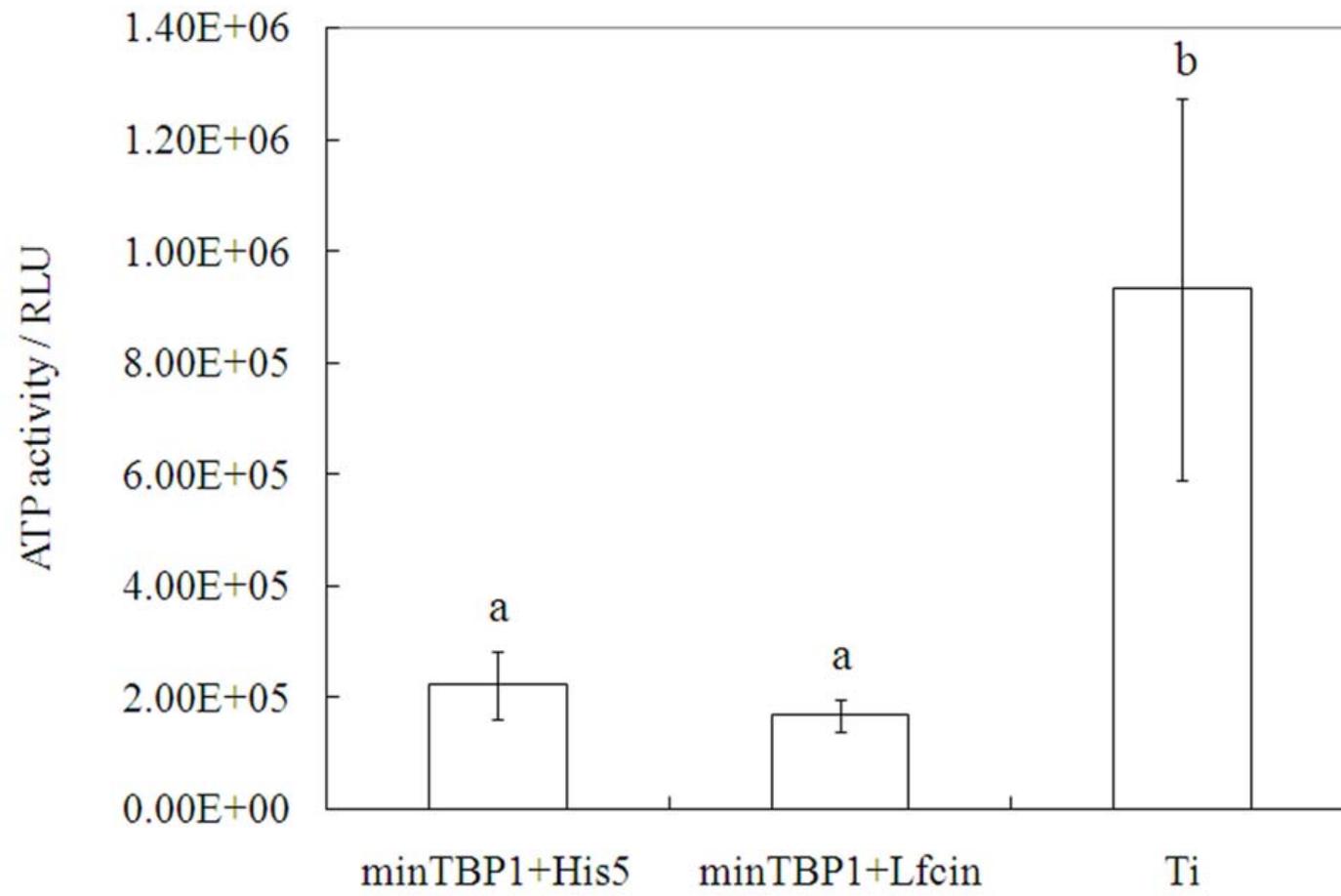


Fig.7

