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Direct Evidence of Intracellular Alkalinization in *Saccharomyces cerevisiae* KNU5377 Exposed to Inorganic Sulfuric Acid

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Abstract The toxicity of inorganic sulfuric acid as a stressor was characterized in *Saccharomyces cerevisiae* KNU5377. In this work, we examined physiological responses to low extracellular pH (pH_{ex}) caused by inorganic H_2SO_4, which could not affect cell growth after pH was adjusted to an optimum with Trizma base. The major toxicity of sulfuric acid was found to be reduction of environmental pH, resulting in stimulation of plasma membrane H^+-ATPase, which in turn contributed to intracellular alkalinization. Using a pH-dependent fluorescence probe, 5-(and-6)-carboxy SNARF-1, acetoxymethyl ester, acetate (carboxy SNARF-1 AM acetate), to determine pH_{in}, we found that color was dependent on the changes of intracellular pH which coincided with calculated pH_{in} of alkalinization up to approximately pH 7.3. This alkalinization did not seem to affect survival of these cells exposed to 30 mM sulfuric acid, which lowered the pH_{ex} of the glucose containing growth media up to approximately pH 3.0; however, the cells could grow only up to 70% of the maximum growth in the same media, when 30 mM sulfuric acid was added.

Key words: Inorganic sulfuric acid, intracellular alkalinization, carboxy SNARF-1 AM acetate, p-type H^+-ATPase

One of the most crucial and fundamental tasks of a living cell is to maintain homeostasis of intracellular pH (pH_{in}) and ion concentration. Yeast cell membranes are not freely permeable to hydrogen ions, and the transmembrane proton gradient is established by active proton-pumping mechanisms. The electrochemical transmembrane proton gradient is generated by H^+-translocating ATPase enzymes, which provide the driving force for the transport of many yeast nutrients.

With regard to pH homeostasis, the H^+-ATPases encoded by the *PMA1* and *PMA2* genes are instrumental in modulating both intra- and extracellular pH. The pH_{in} remains relatively constant even when the extracellular pH (pH_{ex}) fluctuates [6]. This constancy is maintained primarily through the activities of the plasma membrane H^+-ATPase, which is inextricably linked with yeast growth and is capable of generating a 10,000-fold difference between the concentrations of protons on either side of the membrane. The plasma membrane H^+-ATPase plays a crucial role in the physiology of most fungi and plants [25, 27], and generates the transmembrane proton motive force that modulates overall cell growth, including nutrient uptake, osmotic balance, ion homeostasis, and stress tolerance [1, 18, 20]. Most *Saccharomyces cerevisiae* strains grow at pH values of 2.5–8.5, and the kinetics of growth and fermentation are not affected at pH between 3.5 and 6.0, because of tight control of the pH_{in} [16].

Intracellular acidification is important in food microbiology, because it is responsible for the effect of weak acid preservatives (e.g. benzoic, propionic, and sorbic acids) in inhibiting growth of spoilage yeasts [2, 7, 17]. The plasma membrane H^+-ATPase activity is strongly stimulated during copper-induced latency in a glucose-containing growth medium, reaching maximal levels when the cells are about to start exponential growth. In the present study, this activation of the ATPase activity by copper was accompanied by stimulating the H^+-pumping activity of the enzyme *in vivo* and was essentially due to the increase of apparent V_{max} for MgATP [9]. Minor fluctuations of pH_{in} are believed to play a regulatory role in many important cellular processes. pH_{in} in yeast can be measured by pH-dependent fluorescent probes [28]. Membrane-permeable prefluorochrome, 5-(and-6)-carboxy SNARF-1, acetoxymethyl ester, acetate (termed as carboxy SNARF-1 AM acetate) can be loaded into the cytosol where they are hydrolyzed by intracellular esterases to their polar forms whose fluorescence is pH dependent [4, 11], and this pH dependent fluorescence is the basis of methods that have been developed to determine pH_{in}.

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In the present work, we investigated a major cellular response against sulfuric acid as a stressor and detoxification system, focusing on modulation of intracellular pH against low environmental pH. Under inhibitory concentrations of the chemical, physiological responses of the cells were studied by restoring intracellular pH perturbation and concomitant visualization of cytoplasmic pH. Moreover, the expression patterns of major intracellular pH modulator genes, *PMA1* and *PMA2*, revealed their strong implication in cytoplasmic pH regulation under sulfuric acid stress.

MATERIALS AND METHODS

Yeast Strain and Culture Condition

A yeast strain used in this study was isolated from Korean soil [13, 14, 19] and identified as *Saccharomyces cerevisiae* KNU5377, which could produce ethanol at high temperature as well as at 30°C. Precultures of *S. cerevisiae* KNU5377 were inoculated and aerobically grown in YEPD (yeast extract 1%, peptone 2% and dextrose 2%) medium at 30°C and pH 6.3. Additionally, if necessary, various concentrations of inorganic sulfuric acid were added to this medium after autoclaving. To rectify the very low extracellular pH due to the sulfuric acid, YEPD with inorganic sulfuric acid was adjusted to pH 6.3 with 1 M Trizma base.

Sensitivity Test Against Sulfuric Acid

Mid-log cultured cells were challenged with an increased concentration of sulfuric acid for up to 120 min, and the cells were diluted 10- and 100-fold, and then plated in triplicate onto YEPD agar media to monitor cell viability. Percent survival was expressed as relative to the untreated control culture (100%). Additionally, the diluted cells were spot-tested onto YEPD media. Exponentially growing cells were exposed to 30 mM sulfuric acid for time increases of 10 min up to 130 min and diluted to OD_{600}=3 and 0.3, and 5 μl each were then spotted onto YEPD plates.

Intracellular pH Calculation Curve

A pH-sensitive fluorescence probe, 5-(and-6)-carboxy SNARF-1, acetoxymethyl ester, acetate (carboxy SNARF-1 AM acetate) (Molecular Probes, Leiden, Netherlands) was selected to study the changes of intracellular pH in *Saccharomyces cerevisiae*. Ten μl of stock solution (10 μM/μl in DMSO) was added to 4 ml of BSA (50 μg/ml) containing 100 mM phosphate buffer at pH values between 5.8 and 8.0 by increments of 0.2 of a pH unit.

Fluorescence determinations were made on a Shimadzu RF-5301PC fluorospectrophotometer with a 4 ml optically clear quartz cuvette (Helma). Using carboxy SNARF-1 AM acetate, all readings were followed at an excitation wavelength of 534 nm with an emission scan between 550 nm and 650 nm (bandwidths 10 nm). Calibration curves for this fluorescence probe were constructed in a phosphate buffer by plotting the ratio of fluorescence intensities (excitation wavelength 525 nm) at emission wavelengths of 580 nm (pH-dependent point) and 610 nm (pH-independent point) as a function of pH. Intracellular pH was calculated from this calibration curve.

Measurement of Intracellular pH

Exponentially growing cells were exposed to 30 mM sulfuric acid, corresponding to about extracellular pH 3.0, at a time interval of 10 min. After washing the cells three times with ice-cold PBS, cells were diluted to an OD_{600} of approximately 0.3. Carboxy SNARF-1 AM acetate was added at a concentration of 5 μM, and the cells were then incubated at 30°C for 1 h with shaking. After incubation, the cells were washed three times with PBS and then resuspended in 4 ml of PBS. Following the treatment with sulfuric acid, the level of fluorescence from the loaded cells was determined fluorospectrophotometrically as described above, and intracellular pH was obtained from the calibration curve.

Confocal Scanning Laser Microscopy

To confirm intracellular pH, the loaded cells were observed under confocal scanning laser microscopy (CSLM), which was performed using a Bio-Rad MRC 1000 (Hercules, CA, U.S.A.) attached to a Nikon microscope (Eclipse E600 Nikon, Tokyo, Japan). Carboxy SNARF-1 AM acetate was excited by 488 nm lines of a krypton-argon laser (American Laser Corporation, Salt Lake City, U.S.A.), and an emission was collected at 580 nm. Standard filter sets used on the photomultipliers were 580DF32 and Blue reflection. Intracellular pH of *Saccharomyces cerevisiae* KNU5377 cells was determined by imaging of the fluorescence from the pH-sensitive dye. Each image was averaged over at least three frames to reduce background noise.

RNA Isolation and Northern Blot Analysis

After treatment with sulfuric acid, yeast cells were collected, washed 3 times with saline (0.86% NaCl), and stored at −70°C until use. Total RNA was extracted from frozen cell pellets by a hot phenol method [30]. Cells were thawed on ice and then mixed with 5 ml of hot phenol, 4.5 ml of Na-acetate buffer, and 0.5 ml of 10% SDS. The mixture was incubated at 65°C for 5 min with frequent vortex mixing, and the aqueous phase was separated from the organic phase by centrifugation. The aqueous layer was extracted once more with an equal volume of phenol/chloroform (1:1) and then with chloroform/isoamyl alcohol (24:1) before ethanol precipitation. The mRNA was purified from the total RNA with an oligo (dT) selection step (Oligotex; QIAGEN, Chatsworth, CA, U.S.A.), fractionated on 0.8% agarose gels, and then transferred to Hybond-N^+ membrane (Amersham Pharmacia Biotech, Bucks, UK) overnight in 10 × SSC. cDNA probes were amplified by

PCR, and the PCR products were radiolabeled with a Megaprime DNA labeling system kit (Amersham Biosciences, Uppsala, Sweden).

Plasma Membrane H^+-ATPase Activity Assay

Plasma membrane fractions were purified from yeast cells after treatment with 30 mM sulfuric acid at various time intervals, as described by Fernandes *et al.* [10]. Protein concentration in the plasma membrane suspension was determined to range from 3 mg/ml to 5 mg/ml by commercial protein assay kit (Bio-Rad, Mercules, CA, U.S.A.), based on the method of Bradford. Plasma membrane H^+-ATPase activity was determined essentially by a modification of the method of Serrano [15, 25]; briefly, 1.0 ml routine assay mixtures contained 50 mM MES-Tris, pH 5.6, 3 mM $MgCl_2$, 3 mM Na_2ATP, total membrane suspension, and inhibitors to specifically assay plasma membrane H^+-ATPase. ATPase activity was calculated by linear regression from the slope of released Pi vs. time (up to 10 min) and expressed in μmoles of Pi released per min (U) per mg of total membrane protein.

RESULTS AND DISCUSSION

Effects of Sulfuric Acid as Stressor

As a strong inorganic acid, sulfuric acid is very corrosive and chars sugar and wood, and easily dissociates to H^+ and SO_4^{2-} in water, resulting in reduction of environmental pH. These properties are applied to acid hydrolysis of cellulosic biomass whose hydrolysates are used by fuel fermenting yeast, *Saccharomyces cerevisiae*, which produces an alternative energy resource, fuel alcohol [22, 29]. However, it is hard for yeast to produce alcohol without neutralization of the due to low pH acid hydrolysate [24].

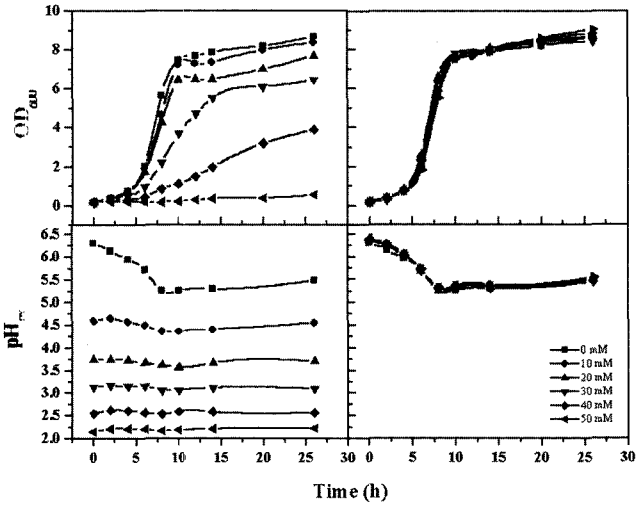

Fig. 1. Growth curve and extracellular pH (pH_{ex}) under various concentrations of sulfuric acid with and without pH_{ex} adjustment.
Exponentially growing *S. cerevisiae* KNU5377 cells were cultivated in the YEPD media containing 0, 10, 20, 30, 40, and 50 mM sulfuric acid without (left panels) and with (right panels) buffering with Trizma base to adjust the pH to pH_{ex} 6.3, and concomitantly monitoring pH_{ex} under the same condition (lower panels). Data shown are representative of two independent trials.

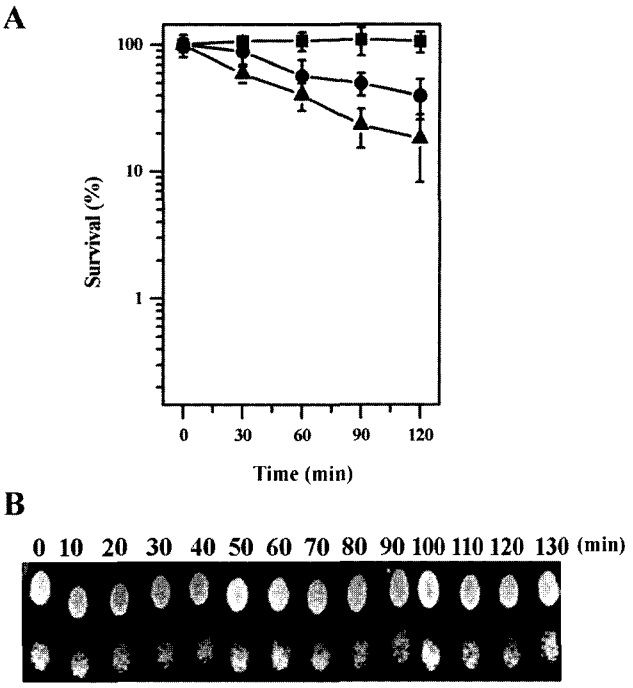

Fig. 2. Sensitivity test of *Saccharomyces cerevisiae* KNU5377 against sulfuric acid.
A: Mid-log phase cultured cells were exposed to sulfuric acid buffered with 30 mM (-■-), 90 mM (-●-), and 120 mM (-▲-) of this acid at given times. Survival was measured as the ability to form colonies on YEPD plates at 30°C for 48 h. Percentage survival was expressed relative to the untreated control culture (100%). Data are means±SD of three independent experiments. B: Exponentially growing cells were exposed to 30 mM sulfuric acid for indicated times (min) and diluted to OD_{600}=3 (upper) and 0.3 (lower), and 5 μl of each were then spotted onto YEPD plates.

To examine the toxicity of this chemical, yeast cells were treated with various doses of sulfuric acid: Different concentrations of sulfuric acid were added to the YEPD media and overnight cultured cells were inoculated into these YEPD media. Concentrations greater than 20 mM and pH_{ex} below 3.7 interfered with cell growth, and 30 mM sulfuric acid reduced the growth by 70% with no effect on survival (Fig. 1 left panel and Fig. 2). In order to distinguish toxicity of sulfuric acid from low extracellular pH, cells were cultivated in YEPD media containing various doses of sulfuric acid, which were subsequently adjusted to pH 6.3 with Trizma base. As seen in the right panel of Fig. 1, a buffered sulfuric acid regime did not affect the growth of the cells. These results indicate that the essence of the sulfuric acid stress is the reduction of extracellular pH by dissociated protons. In contrast, weak-

acid preservatives, such as sorbic and benzoic acids, are freely permeable across the plasma membrane and are dissociated in the cell, resulting in the release of charged anions and protons, and inhibitory effect of these preservatives has mainly been attributed to disturbance of intracellular pH homeostasis [3, 5, 23]. Piper *et al.* [20] reported that the ATP-binding cassette (ABC) transporter Pdr12 in the plasma membrane was induced during growth of *S. cerevisiae* in the presence of these weak-acid preservatives: Pdr12 confers resistance to weak acids by mediating extrusion of the anions [12].

Sensitivity Against Sulfuric Acid

To determine the kinetics of loss of cell viability, cells were treated with different concentrations of sulfuric acid, and samples were taken from the culture at 30-min intervals over 2 h. Unlike the growth condition, survival of the cells was not affected by treatment with 30 mM, sulfuric acid, however, there was an initial rapid loss of cell viability at 120 mM sulfuric acid (Fig. 2). Furthermore, cells were treated with 30 mM sulfuric acid for 130 min at a time increase of 10 min under the same conditions and diluted YEPD to OD_{600}=3 and 0.3. Also, 5 µl each of the dilutions were spotted onto YEPD plates, and the plates were incubated for 2 to 3 days at 30°C. The result obtained confirmed that 30 mM could not affect the survival of the cells, although it could reduce the growth rate (Fig. 1). In subsequent experiments, treatment of yeast with 30 mM sulfuric acid at various time intervals was chosen as a stress condition.

Determination of Intracellular pH

The strain KNU5377 cells were grown in the YEPD medium to the mid-exponential phase, treated with 30 mM sulfuric acid at time intervals of 10 min for up to 90 min, and then loaded with carboxy SNARF-1 AM acetate. Using a fluorospectrophotometer, the ratio of fluorescence intensity at 580 and 610 nm was measured and plotted by the pH vs. fluorescence ratio calculation curve (Fig. 3). At the beginning of the stress condition by 30 mM, the intracellular pH of the strain was about 6.7, and the concomitant extracellular pH was approximately 3.0. During the stress treatment for 90 min, the value of pH_{in} was increased to about pH_{in} 7.3 for 40 min due to alkalinization, and pH_{in} subsequently retained pH homeostasis by increasing the level of protons in the cell. However, the pH value did not return to the initial level of pH_{in} 6.7 (Fig. 4). This result indicated that a decline of extracellular pH during prolonged exposure to inorganic acid induced intracellular alkalinization without significantly affecting cell viability. Consequently, pH_{in} was able to maintain its homeostasis, but it could not return to the normal level of pH_{in}.

Notably, rapid reduction of the pH_{in} should be expected after withdrawal of this chemical. As mentioned earlier, intracellular pH was gradually reduced to approximately pH_{in} 7.1 after 40 min in the presence of this chemical. If the pH homeostasis was maintained by reducing intracellular pH, pH_{in} became alkalinized under 30 mM sulfuric acid for up to 40 min, and thereafter, gradually kept pH homeostasis. Therefore, it was expected that the removal of sulfuric acid would make cells maintain pH homeostasis to promptly recover the normal state of cells. Namely, it is quite natural to keep pH homeostasis more effectively, when this stressor was removed, since cells were oriented into maintenance

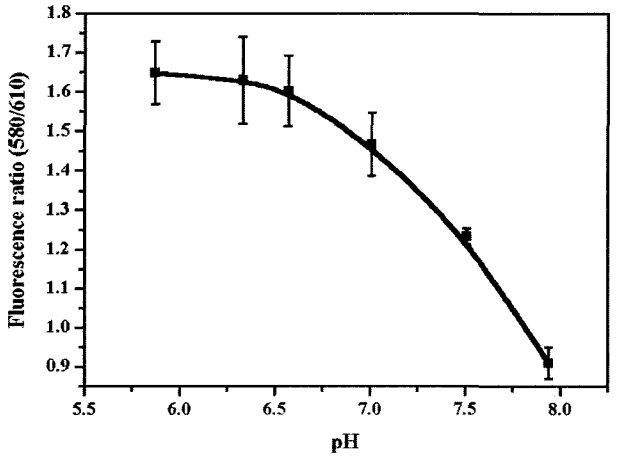

Fig. 3. Fluorescence intensity ratio (580 nm/610 nm) vs. pH calibration curve of carboxy SNARF-1 AM acetate.
The pH-adjusted 50 mM phosphate buffer was calculated in the presence of 50 µg/ml BSA *in vitro*. Data are means±SD of three independent determinations.

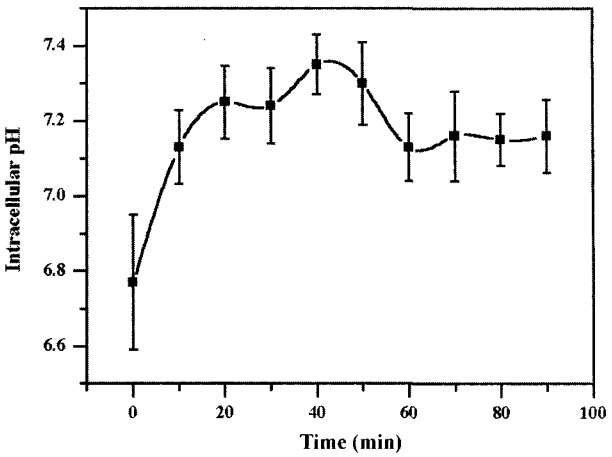

Fig. 4. Intracellular pH of *Saccharomyces cerevisiae* KNU5377 after sulfuric acid challenge.
Intracellular pH (pH_{in}) corresponding to extracellular pH value of approximately 3.0, lowered by 30 mM sulfuric acid, was calculated to plot the pH calibration curve in carboxy SNARF-1 AM acetate by measuring the ratio of fluorescence intensities (580 nm of pH-sensitive wavelength and 610 nm of pH-insensitive wavelength) at the excitation wavelengths of 534 nm. pH_{in} data are average values±SD from three independent experiments.

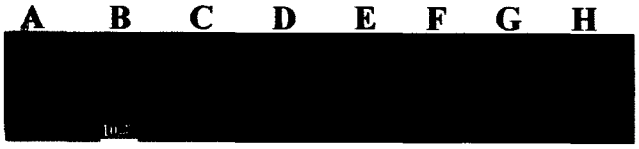

Fig. 5. Direct evidence of intracellular pH changes after exposure to sulfuric acid.
Images of *S. cerevisiae* KNU5377 cells after loading with fluorescence probes, carboxy SNARF-1 AM acetate, were taken using confocal scanning laser microscopy (CSLM) following treatment with 30 mM sulfuric acid for 0, 10, 20, 30, 40, 50, 60, and 90 min, indicated as A, B, C, D, E, F, G, and H, respectively. Images were selected from three independent trials, processed by Confocal Assistant™ version 4.02 (Bio-Rad), and then compiled by Adobe Photoshop® version 6.0.

of pH homeostasis in 40 to 50 min of the presence of sulfuric acid.

On the other hand, because of the physiological response to a highly inhibitory concentration of copper, cells cope with copper-induced plasma membrane lipid perturbation and the increase in the nonspecific proton flux. Therefore, the toxic effects of copper were shown by decreasing intracellular pH, and the plasma membrane H^+-ATPase activity was concomitantly strongly stimulated in the glucose-containing growth medium [8, 9].

Visualization of Intracellular pH by Using CSLM

Visual analysis of a change of intracellular pH was performed by CSLM at low environmental pH. A fluorescence dye, carboxy SNARF-1 AM acetate pH indicator loaded into the cell, was color-changed depending on intracellular pH, which tends to respond against extracellular pH fluctuation [30]. Color change in the cell population depends on intracellular pH. Cells with approximately 6.7 of normal pH_{in} value were red. Corresponding to environmental low pH of about 3.0, cells had a high cytoplasmic pH which was indicated by green (Fig. 5). These results coincided well with the fluorospectrophotometrically measured intracellular pH (Fig. 4).

The Effect of Sulfuric Acid on Expression and Activity of p-Type ATPase

To determine whether the plasma membrane H^+-transporting p-type ATPases, *PMA1* and *PMA2* genes, were induced by sulfuric acid stress and to see if regulation of these genes occurred differentially or in a coordinated manner, total RNA was isolated from KNU5377 cells which were previously treated with 30 mM sulfuric acid at time increments and examined by Northern blot analysis. The transcripts of plasma membrane H^+-transporting p-type ATPase major isoform (*PMA1*) and minor isoform (*PMA2*) were found to be dramatically overexpressed within 10 min. After that point, the expression level of both genes was downregulated to approximately normal levels, whose inductions were not proportional to the alkalinization of pH_{in} (Fig. 6A). However, cellular alkalinization appears to have stimulated the plasma membrane H^+-transporting p-type ATPase activity, since it was responsible for modulating intracellular pH by pumping out proton ion (Fig. 6B). Therefore, the visualization assay of intracellular pH revealed that plasma membrane proton ATPase was inextricably stimulated and led to cellular alkalinization under low extracellular pH sulfuric acid.

In conclusion, the present study was important to determine whether this ethanol-fermenting yeast strain is suitable to be used for this purpose: The stress response investigated in this study was to ascertain the most important prerequisite factor of a yeast strain necessary to produce ethanol from cellulolytic biomass hydrolysates that are generally prepared with sulfuric acid. As shown earlier, this strain was good enough to be used in ethanol fermentation from biomass hydrolysates containing a considerable amount of sulfuric acid.

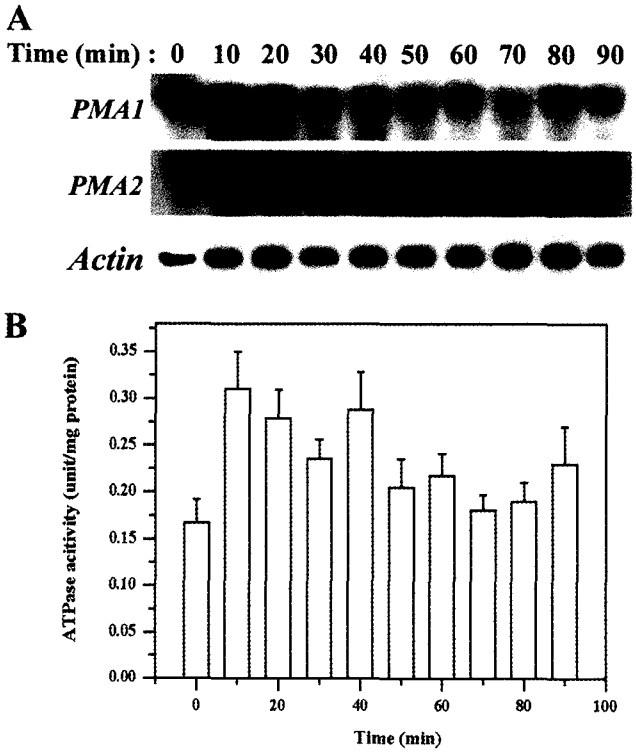

Fig. 6. The effect of sulfuric acid on expression of p-type H^+-ATPase and its activity.
A: Northern blot analysis of *S. cerevisiae* KNU5377 *PMA1* and *PMA2* expression. Total RNA was isolated from KNU5377 cells grown on YEPD media, which were then exposed to 30 mM sulfuric acid for 90 min at an increase of 10 min. The RNA was blotted onto filters, which were probed with the *PMA1* ORF (upper panel) and *PMA2* ORF (middle panel). B: P-type H^+-ATPase activity after exposure to sulfuric acid. Plasma membrane fractions prepared at each time interval were used in measurement of plasma membrane H^+-ATPase activity. The activity was expressed in unit, which was defined as μmoles of Pi released per min per mg of total membrane protein. Data are average values±SD with standard deviations from triplicate experiments.

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