jpht

Journal of Postharvest Technology
2017, **05**(2): 64-78
http://www.jpht.info

RESEARCH ARTICLE

Detection of fruit deterioration biomarkers on fresh-cut pineapple via gene expression assay based on electrochemical biosensor

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Received: 12.01.2017 Accepted: 18.03.2017

ABSTRACT

A technique for the semi-quantitative determination of target deterioration markers through gene expression in tissues of fresh cut pineapple based on electrochemical biosensor was developed. This method employed RT-PCR amplification of a specific domain corresponding to a target ATP synthase, polyphenol oxidase, superoxide dismutase gene and a common housekeeping 18S rRNA gene, and a phenomenon of DNA aggregation induced by Hoechst 33258 in conjunction with changes in anodic current peaks measured via a carbon screen printed electrode on linear sweep voltammetry. Anodic current peaks of the resulting cDNA products from pineapple tissues during storage were between 1.16-2.35 µA. Semi-quantitative analysis for the level of deterioration markers through gene expression was measured using the comparative ratio between copy numbers of target gene and housekeeping 18S rRNA genes. The expression of ATP synthase, polyphenol oxidase, superoxide dismutase gene at 25 ^{o}C was detected as early as 6 hours after storage. SOD expression level having more abundance than the rest studied might play key role in quality deterioration in pineapple. Semi-quantitative studies on gene expression revealed the increase in patterns of gene expression especially that of SOD up to 89.14% comparing with that of a housekeeping gene. These results provided gene expression configuration in responding to postharvest conditions of fresh cut pineapple. The technique has several merits on its rapidity and simplicity in quantifying gene expression. This is also a cost effective technique since no sophisticated devices and expensive reagents are needed.

Keywords: Electrochemical biosensor; pineapple; Polyphenol oxidase; ATP synthase; Superoxide dismutase.

Citation: Kaewdee, M. and Chaumpluk, P. 2017. Detection of fruit deterioration biomarkers on fresh-cut pineapple via gene expression assay based on electrochemical biosensor. *Journal of Postharvest Technology*, 5(2): 64-78.

INTRODUCTION

Pineapple is a popular fruit among consumers worldwide for its nutritive value and health promoting properties. For Thailand, it is also the most agronomical important fruit with annual gross export value more than 690 million US$. Recently, product of pineapple in term of minimal processed has become widely accept to markets as it is convenience and ready to eat. However, fresh-cut pineapple is very perishable and has shorter shelf-life than the intact fruit. This shelf-life limit to 2-3 days was mainly due to massive quality decay, including pulp browning, accumulation of liquid in the packaging, off-flavors formation and microbial growth (Iversen et al., 1989). Since pineapple is classified as a non-climacteric fruit whose processes of ripening cannot markedly be continued once removed from the plant (Kader, 1999). The deterioration is then directly involved with senescence processes rather than the ripening one. Although these physiological changes associated with senescence processes, in general, were the results of several enzymatic activities including that of ethylene synthesis,

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ISSN: 2348-4330

oxidation of phenolic substances, conversion of carbohydrates, de-methylation of pectin substances in plant tissues (Mayer and Harel, 1979), there were few studies focusing on genetic mechanisms, especially the expression of the involved genes in controlling the processes.

To assess gene expression, so far, technique based on nucleic acid hybridization of cDNA amplified with PCR, had routinely been employed (Peterhaenel et al., 1998; Martel et al., 2002). However, running the technique had several draw backs on laborious step of probe preparations, and hybridizations. Moreover, further quantitative analysis requires expensive devices, i.e. real-time-PCR, and fluorescence or radioactive isotopes, all limiting their applications in terms of cost, simplicity and rapidity.

Recently, biosensor has become a popular tool for nucleic acid detection due to its simplicity and rapidity during the assay. In principle, system requires both biomolecules, having highly specific elements for target nucleic acid recognition, and a transducer that converts molecular recognition events into quantifiable signals (Hashimoto et al., 1994a; Gau et al., 2005). So far several systems including fluorescence (Selinger et al. 2000), mass spectrometry (Isola et al., 2001), surface plasmon resonance spectroscopy (Nelson et al., 2001), quartz crystal microbalance (Liu et al., 2002) and an electrochemical assay (Park and Hahn, 2004), had been demonstrated. Electrochemical techniques showed several advantages among these both in its simplicity, its high sensitivity and its worth of economic scale. To monitor nucleic acid hybridization electrochemically, several protocols with various signal mediators have been proposed. For example, the metal coordination complexes protocol (Johnston et al., 1995; Napier and Thorp, 1997) and intercalating organic compounds protocol (Hashimoto, 1994b; Kelly et al., 1999) were commonly employed for nucleic acid hybridization as indicators. The electrochemical responses of these induce change upon nucleic acid hybridization.

Hashimoto et al., (1994b) proposed the use of an organic fast redox coupling dye called Hoechst 33258 as an indicator. Demonstrations of this using a DNA probe, a modified gold electrode, and Hoechst 33258 as a label had been carried out (Hashimoto et al., 1994b; Hashimoto et al., 1998). Later improve method requiring no probe and immobilization steps had also been developed (Kobayashi et al., 2004). The method employed the aggregation phenomenon of the DNA in the presence of Hoechst 33258 solution (Saito et al., 2004). Direct DNA aggregation provides a clue to quantifying DNA even at a trace limited level through the measurement of anodic current peaks. Decreasing of anodic current in proportion to the titration of dsDNA aggregation led to DNA quantification (Chaumpluk, et al. 2006). A success in the fabrication process of the electrode unit, and an integration of the system for detection of DNA in reaction solution without DNA purification, enables the detection of target DNA in a rapid, simple, and cost effective fashion. We had previously demonstrated a method for semi-quantification of DNA in samples based on the relationship between DNA titration and anodic current peak changes (Chaumpluk et al., 2006; Chaumpluk et al., 2007). Although the demonstration of DNA quantification had been extensively studied, there had been very few reports in the literature on RNA detection and quantification using Hoechst 33258 induced aggregation with electrochemical biosensor.

In this report, we have further demonstrated an application of the electrochemical principle in postharvest technology for the detection of RNA abundance of three genes involving fruit deterioration, *ATP synthase, SOD* (superoxide dismutase), *PPO* (polyphenol oxidase) through gene expression study and quantification of the level of these gene expressions in fresh cut pineapple tissues in accordance with their quality deterioration during storage.

MATERIALS AND METHODS

Plant materials

Smooth Cayenne type of pineapple fruits, *Ananus comosus* (L.) Merr., cv. Siracha., were used in the experiment. They were obtained from a commercial GAP certified orchards in Chonburi Provinces, Thailand. Fruits were selected with similar characteristics of ripening (skin color and flat eyes) and harvested 120 days after anthesis. They were soaked in 10% clorox solution for 30 min for surface sterilization, rinsed with sterile water, cut and stored at 25^0C for both gene reference and gene expression studies.

Gene expression study

Gene expression was investigated using RT-PCR. Design primers were based on aligned nucleotide sequences of ATP synthase, superoxide dismutase, and polyphenol oxidase gene of pineapple (Accession Number AY098513.1, AJ 250667, AA 016863.1) and 18S rRNA gene (Accession number AB478330.1).

Selected primers, 5'cgtacataacctactccaacat3' and 5'gtatcagaccggataactggtt3' for the ATP synthase gene, 5'tgagggtgttaaaggcacaatctacttcac3' and 5'gcgggtctcatcttcaggtgcgccatgttc3' for the superoxide dismutase gene, 5'ctgtagggcgctctttggcgtcggctttgc3' and 5'gtacgtgtggaggaaccttggcggcaagca3' for the polyphenol oxidase gene and 5'aagaaagagctctcagtctcaatc3' and aagggcaccaccaggagtggagcc3' for the 18S rRNA gene, were based on criteria of specific DNA amplification without leaving non-specific DNA products. Several RT-PCR conditions including the Mg^{2+} ion concentration and the annealing temperature were also adapted to guarantee amplification of specific cDNAs.

For DNA signal identification, first total RNA were extracted from 300 mg of thin layer surface section of fresh-cut pineapple with TRIZOL solution according to the manufacturer's protocol (Invitrogen, USA). Single-step RT-PCR of each sample was performed using one step RT-PCR reagents (Qiagen, USA) with condition of 42 ^{o}C for 1 hour at RT step and 95 ^{o}C for 10 min for reaction termination before amplification at 93 ^{o}C for 40 sec; at 60 ^{o}C for 1min for ATP synthase gene, at 58 ^{o}C for 1min for superoxide dismutase gene, at 60 ^{o}C for 1min for polyphenol oxidase gene and at 55 ^{o}C for 1min for 18S rRNA gene; and at 72 ^{o}C for 1 min, at 40 cycles. Parts of RT-PCR products were visualized after electrophoresis.

Electrochemical signal detection

Hoechst 33258 (2'-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5'-bi (1*H*-benzi midazole) was obtained from Sigma-Aldrich (Sigma, USA). Signals were induced after mixing 25 µM Hoechst 33258 with RT-PCR products. The measurement of electrochemical signals was further carried out in PBS buffer using linear sweep voltammetry (LSV) and a fabricated sensor based on disposable electrochemical printed (DEP) carbon electrode (area 1.96 mm^2) (Biodevice Technology, Japan) with Ag/AgCl as reference as described (Chaumpluk et al., 2006). Electrode was used once for each experiment and discarded after use.

Quantification of the transcription profile of each target gene was based on calibration of the amount of cDNAs, comparing between cDNA of each target gene (ATP synthase, superoxide dismutase, polyphenol oxidase), with that of 18S rRNA house keeping. The known copies number references were obtained from cloned portions of these target genes, and the 18S rRNA gene in pSC A^{TM} plasmid (Statagene, USA). The DNA was purified using Micro Spin S200-Column (GE, USA). Measurements were taken after 30 cycles of amplification in PCR step. Amplification was based on reference target gene and

18S rRNA gene with conditions as described earlier, at 1, 0.1, 0.01, 0.001, 0.0001 0.00001 0.000001 and 0.0000001 ng of DNA template compared with those of selected samples described above (Anonymous, 2003). Calibration of level of cDNA was based on the relationships between copy numbers of each target gene and the 18S rRNA gene to each anodic current peak drawn against copy number via standard curve method as described (Kuribara et al., 2002; Chaumpluk et al., 2006). Ratio of gene expression was concluded in terms of target gene expression per housekeeping gene (the 18S rRNA) expression.

Fruit physiological studies

Effect of storage time on qualities and physiological changes were also studied using thin layer specimen from the same pool set of samples. Respiration rate and ethylene production were measured according to Baur and Workman (1964). Change of color in terms of a, b, L and hue angle was carried out (McGruire, 1992) using Minolta Colorimeter model CR-10, Japan. Texture evaluation in each postharvest condition was detected by TA-TX2 Texture Analyzer (Stable Micro Systems, Ltd. Surrey, England) using 50 g of sectioned slices. The results were reported as resistance to shear in N per g fresh weight (Bartolome and Paull, 1986). Total soluble solid was measured in the exudation from the Kramer Shear cell with a hand refractometer (PAL 1, Atago . Japan). Measurement of ethylene production and respiration were measured by a gas chromatograph equipped with a GDX-502 pack column and flame ionization detector. The temperature of the carrier gas, detector and column was 120, 200 and 80^{o}C respectively. The rate of ethylene production was expressed as nmol ethylene per hour per gram. The respiration was measured as CO_2 production using Infrared Gas Analyzer. The results were expressed as mg CO_2 per hour (Rojas-Grau *et al*., 2008). Measurements of titration acidity was measured by titrated with 0.1 N NaOH until color changes to pink color as described by Horwitz, 2000. The results were expressed as percentage of citric acid (g citric acid per 100 g fresh weight). Measurement of vitamin C in the juice (50 ml) obtained from titration of pineapple was detected by the dye-titration method (AOAC,1985).

RESULTS AND DISCUSSION

The losing quality of fruit was depending on relationships among genes involving in shelf-life determination. These genes include *ATP synthase, superoxide dismutase (SOD),* and *polyphenol oxidase (PPO),* (Leon *et al*., 2002). Here, the representative *ATP synthase*, *superoxide dismutase (SOD)*, *polyphenol oxidase (PPO)* of pineapple were used as biomarkers for genetic analysis.

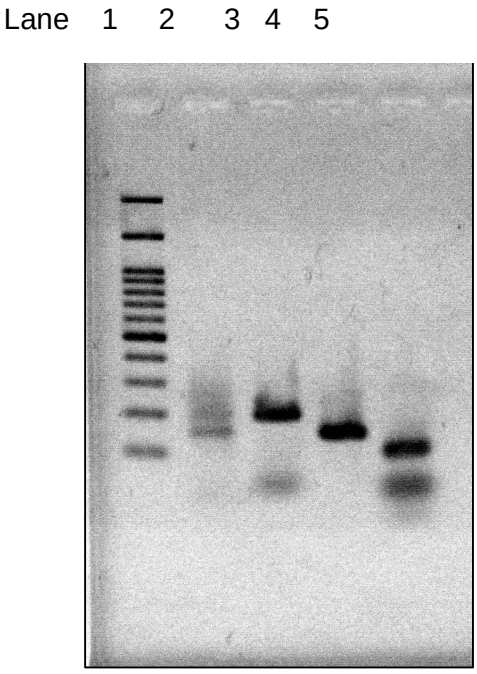
In all living cells, ATP synthase was an indispensable enzyme in ATP production. Any cells deprived of it did not survive (Klingenberg, 2008). Thus the expression of this gene might be used as an indicator for freshness determination. SOD was to catalyze the dismutation of superoxide ion to hydrogen peroxide and molecular oxygen during oxidative energy processes to protect cells from oxidative damage (Pilon et al., 2011).

It was considered to be an important enzyme in neutralizing oxygen radical-mediated toxicity (Yoluk and Marshall, 2003). PPO was the principal enzyme responsible for quality deterioration in browning process in most fruits and vegetables. It catalyzed the formation of highly active oxidizing group that reacted with amino or sulfhydryl groups in proteins or enzyme (Mayer and Harel, 1979). The mechanism for browning involved the interaction of polyphenolic substrates with PPO in presence of oxygen. The PPO catalyzed two reaction: one, hydroxylation reaction was relatively slow and the other, oxidation of diphenols to quinine. The hydroxylation reaction was relatively slow and resulted in colorless products, while the oxidation

reaction was relatively rapid and the resultant oxidized were colored. Subsequent reactions of the oxidized led to melanin accumulation which caused the brown or dark pigment in plant tissue (Markakis, 1974).

In normal situation, to investigate these 3 genes expression profiles based on common real-time RT-PCR method, it is required to have not only expensive real-time PCR device but also the corresponding reagents and 4 probes (three for biomarkers and one for housekeeping) and technical expertise to maneuver. These limit the application to only well equipped facilities. Biosensor is an alternative solution to cover those limitations.

In order to apply biosensor to perform RNA analysis, it requires a system to convert RNA signals to DNA signals. In this study, reverse transcription polymerase chain reaction (RT-PCR) is still a basic platform applied. Four RT-PCR systems based on the amplification of specific target ATP synthase, SOD, and PPO genes and housekeeping 18S rRNA gene were established. Plasmid clone of cDNAs corresponding with these target genes and 18S rRNA gene were applied as reference DNAs. When the target genes and the 18S rRNA gene were amplified, expected DNA fragments of 154, 202, 208 nt and 207 nt respectively, without non-specific products, could be obtained (Fig. 1). These specificities should meet the minimum requirement of signal amplification with no false signals, because only true signals needed to be detected.



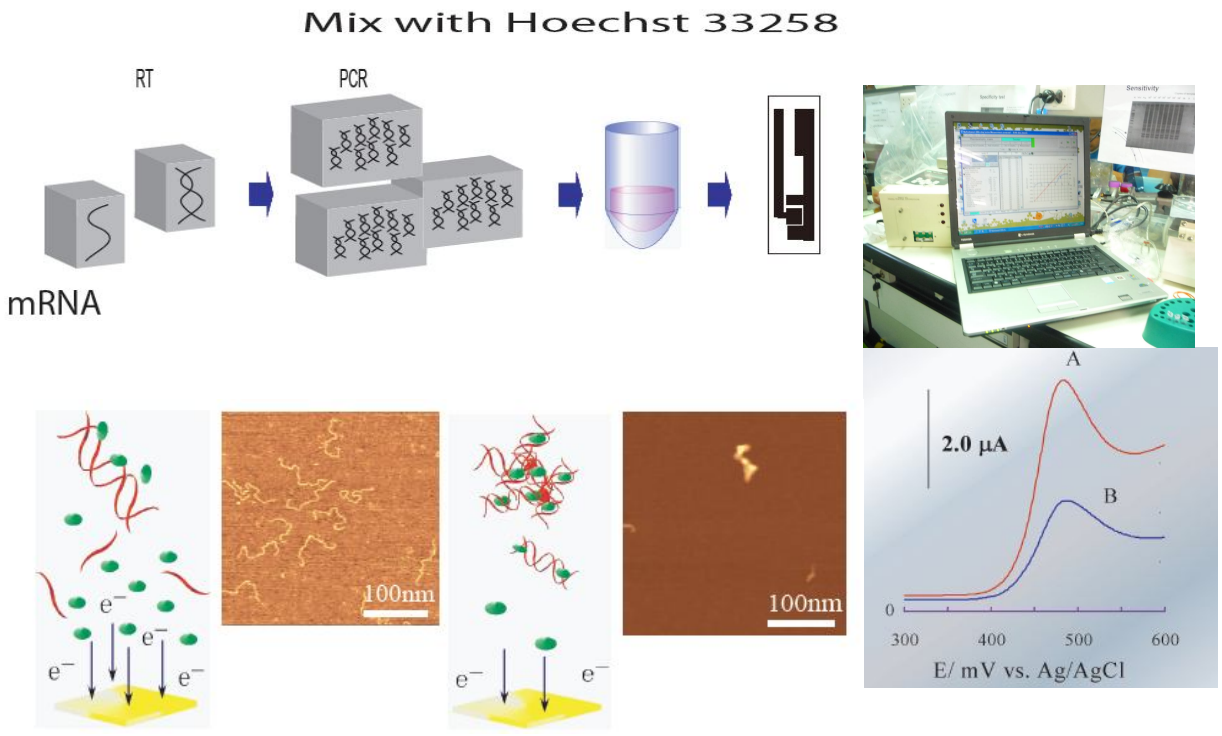
lane 1 ; ladder 1 Kbps, lane 2; The PCR product size of ATP synthase 154 bps, lane 3; The PCR product size of Superoxide dismutase 208 bps, lane 4; The PCR product size of Polyphenol oxidase 202 bps, lane 5; The PCR product size of 18SrRNA 100 bps

Figure 1: The corresponding size of PCR products

For the electrochemical studies, the main target tests were based on the application of signal transduction induced by the aggregation of target biomarkers in term of corresponding cDNA molecule with Hoechst 33258 without any post PCR cleaning. In principle, when there were amount of RNAs expressed in the tissue, corresponding amount of target cDNA products were obtained. The more cDNA in the system, the more binding of Hoechst 33258 resulted in the less free electro-active electrons on the surface of electrode.

Hoechst 33258 is a well defined and commonly used staining dye in cytological study of the chromosome (Kobayashi et al., 2004). It binds well with the AT rich domain of the minor groove DNA but do not or less bind to a short oligonucleotide (below 40 nt) and even not bind with RNAs (Squire et al., 2000). This made Hoechst 33258 a perfect choice for use in expression study as an electro-active indicator for DNA detection due to the available oligonucleotides and RNAs in the RT-PCR system would not interfere with the measurement. Anodic current peak measurement by Hoechst 33258 directed to correct cDNA signals, leaving the leftover RNAs and oligonucleotide primers in RT-PCR reaction out of the signal detection pathway.

In practical, RT-PCRs for each target gene were carried out. Only target cDNA was amplified and its products were mixed with Hoechst 33258 before applying on electrode. Measurement of voltammetric anodic current peak and typical LSV curves of Hoechst 33258 (25 µM) at room temperature in the absence and the presence of target biomarker DNA are shown in Fig. 2. In the absence of cDNA, unbinding of Hoechst 33258 resulted in maximum free electron at surface of electrode and maximum anodic redox current. However, when the biomarker gene was expressed, Hoechst 33258 binded with target cDNAs and induced DNAs aggregation which in turn resulted in less free anodic redox current. This then generated a reduction of anodic current peak.



Target mRNA was reverse transcribed and amplified specifically via RT-PCR. Then the cDNA products was mixed with Hoechst 33258, applied directly on the surface of electrode and measured via linear sweep voltammetry (upper). After binding with DNAs, Hoechst 33258 induced DNAs aggregation (lower) which in turn resulted in the reduction of free anodic redox current, from high peak (red) to the low one (blue).

Figure 2. Schematic steps for signal detection via electrochemical biosensor.

In the experiment, anodic current peaks average obtained from the samples were shown in Table 1. The results of redox current measured were the first demonstration of the aggregation phenomenon of target cDNAs in the presence of Hoechst 33258 on the investigation of all three target biomarkers's expressions. It was also the first application of electrochemical biosensor in fresh-cut pineapple study.

Table 1. Anodic current peak (µA) of the representative ATP synthase gene after storage at 25 °C for 72 hours

Stage	Anodic peak (uA)							
Gene	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
ATP	1.27	1.31	1.53	1.57	1.81	1.88	2.1	2.25
PPO	2.3	2.28	2.1	1.78	1.61	1.48	1.81	1.91
SOD	1.27	1.24	1.2	1.19	1.21	1.18	1.47	1.64
18S	1.23	1.16	1.19	1.21	1.48	1.73	1.77	2.35

stage1 =0 hour, stage 2= 3 hours, stage 3= 6 hours, stage 4= 9 hours, stage 5= 12 hours, stage 6=24 hours, stage 7= 48 hours, stage 8= 72 hours

For the physiological changes at 25°C of storage, results of fresh-cut pineapple deterioration including weight loss, color (a* b* L* hº), total soluble solid, firmness, titratable acidity, ethylene production, respiration rate and ascorbic acid content were analyzed. These physiological changes were used as the index for fruits senescence. The weight loss increased with time. Of all treatments showed, an increasing of weight loss in term of percentage compared with original fresh-cut pineapple was varied from first hour 99.98% to 66.50% at the final. The respiration rate in term of carbondioxide released in fresh-cut pineapples being stored during 0-6 hours were in a 1200-2500 CO_2/Kg.hr range. After that, the respiration increased until reaching maximum at 12 hours and decreased rapidly after then. The ethylene production rate had changed a little over the period of storage. The firmness showed fresh-cut pineapple remained fresh until at 12 hours after that rapidly decreased.

The pH value of all treatments had almost no change with value stable at 4 over period of storage. On the contrary, the titratable acidity had nearly stable at during 0-12 hours, when time of storage is more, it increased rapidly and highly at 72 hours of storage.

The ascorbic content of fresh-cut pineapple found more change during time of storage by time of storage more than, it decreased follows in the time. The leakage of ion a slightly change until the 48th hours and changed in the most at 72th hours. The color changing of fresh–cut pineapples including a* b* L* and h angle were found to change very slight (Fig. 3.)

For study of gene expression, the sample of surface slide of fresh-cut pineapples in varies hours of storage (0 3 6 9 12 24 48 and 72) were extracted for total RNA and cDNA with ATP synthase, SOD,PPO and 18S rRNA genes were synthesized by RT-PCR technique. Resulting PCR products of these genes were further analyzed for its size by agarose gel electrophoresis separation. The expected size of PCR products were founded as 154, 208, 202 and 100 respectively. For gene expression study, the products of these genes as abundance were observed through the DNA band appeared on the gel.

Figure 3. Quality changes of fresh-cut pineapple during storage (Contd…..)

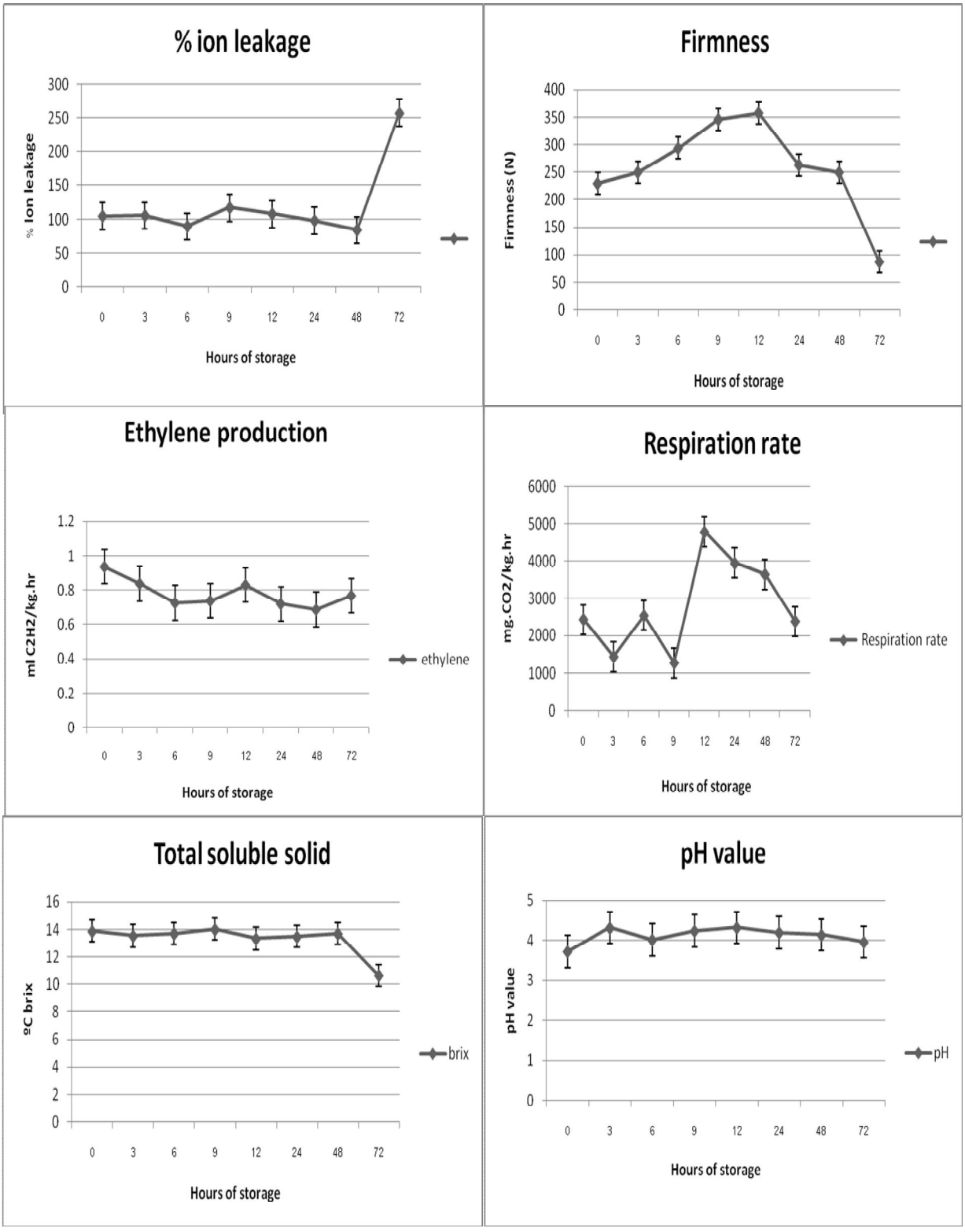
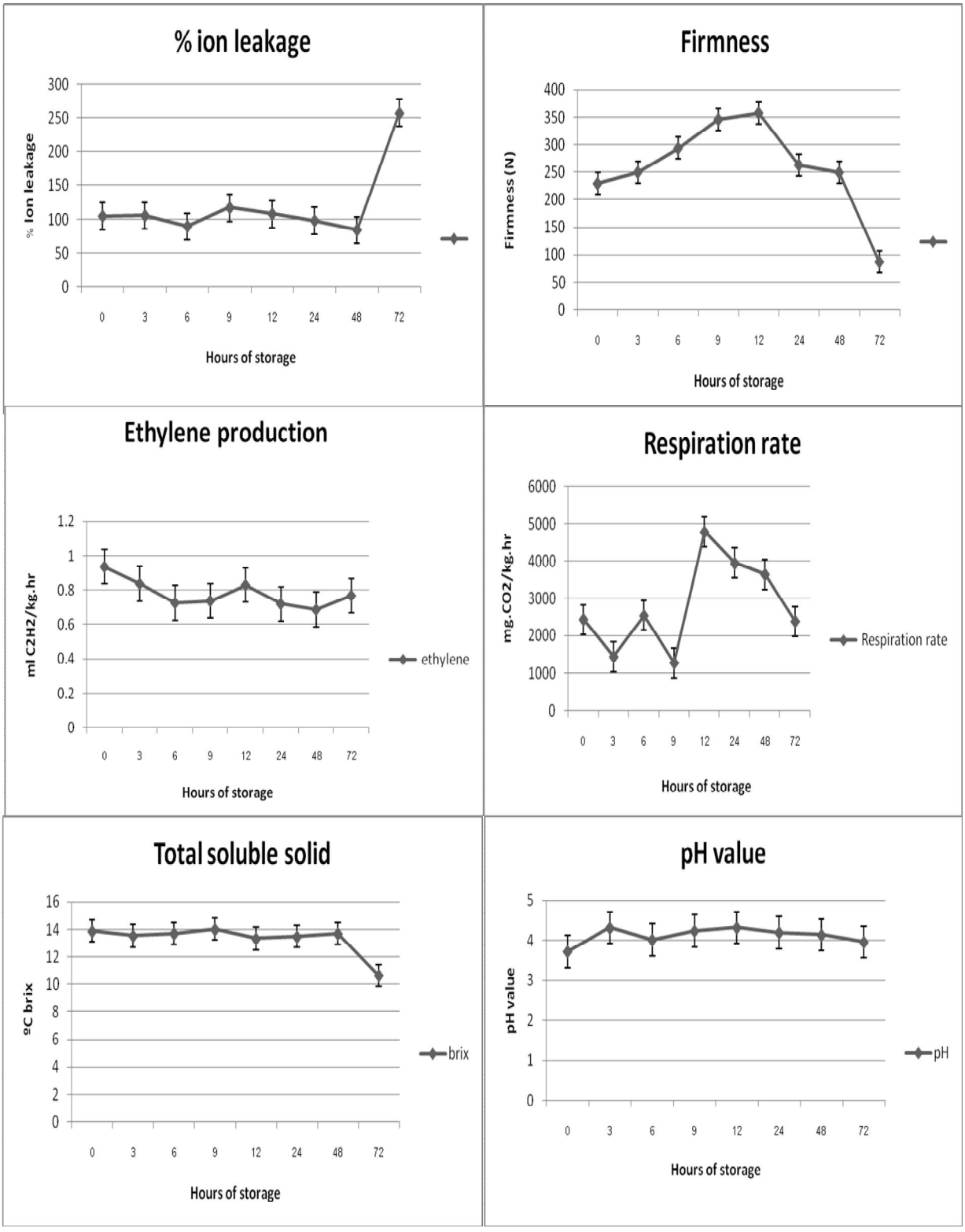


Figure 3. Quality changes of fresh-cut pineapple during storage



The results of RNA abundance via cDNA products compared among ATP synthase, SOD and PPO genes with 18S rRNA expression revealed the DNA level of ATP gene started decreasing at Stage3 (6 hours) until it could not observed at Stage6 (24 hours). The DNA level of SOD gene was found DNA level was similar in all stages except after Stage7 (48 hours). The PPO gene did not express at Stage1 to 2, but it was slightly found at the third and fourth stage and reached maximum at Stage 6, and then decreased rapidly. Whereas, the level gene expression of 18S rRNA was found with strong level at beginning and started decreasing after Stage5 (12 hours). Expression of ATP synthase was halted at Stage 8 (72 hours) (Fig 4).

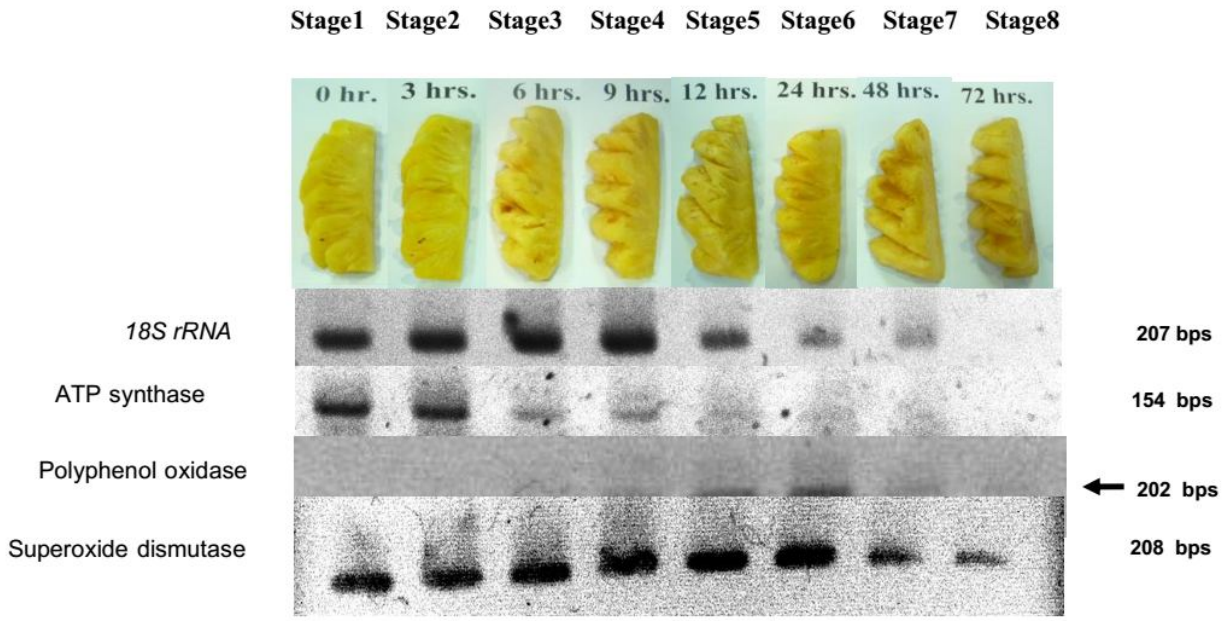


Figure 4 Genes comparing of 18S rRNA, ATP synthase and Polyphenol oxidase from dense DNA band in 8 stages

Moreover, since all target biomarker genes were amplified under the same condition, these allow the test to be performed simultaneously.

For target biomarker cDNA quantifications, the amount of target cDNA in samples was calculated based on the measurement of signal transduction induced by aggregation of DNA molecules that were amplified from samples with known copy number ranging from 10^0 to10^7 copies. The mitochondrial 18s rRNA gene was employed because it was well characterized, it exhibited less variance in expression across a variety of treatment conditions. Moreover, 18s rRNA was so abundant, it amplified rapidly during RT-PCR (deLeeuw et al., 1989).

Dilution of cloned chlorophyllase and 18s rRNA genes were carried out similarly to one performed in real-time PCR quantification (Anonymous, 2003). The measurement of anodic current signals were based on total binding of Hoechst 33258 molecules to the released products and measuring at last step similarly to RT-PCR system using SYBR Green™ at last round (Zhang et al., 2003).

As described earlier, Hoechst 33258 molecules had a very limited efficiency in binding short double strand DNAs such as non specific primer dimer, binding single strand DNA such as primers lefted in RT-PCR, and binding the remaining

template RNA molecules in the RT-PCR tube. False signals due to trace of these products would be cut off. (Chaumpluk et al., 2007). But, if the reactions were either in saturation stage or with some non-specific contamination in the DNA products, incorrect signal values would result. Thus the non-specific products of RT-PCR of the target genes needed to be eliminated strictly by adjusting the RT-PCR reaction conditions. Here the annealing temperature suitable for each amplifications were set separately. Signal detection during quantification was also manipulated only for 30 cycles of operation to detect PCR products just before the saturation threshold. In the experiment, the relationship between ATP synthase gene dose (concentration via copy number/50µL reaction in log) and an anodic peak (Y; µA) was then concluded in Fig. 6. Estimation copy number of the ATP synthase in each sample was drawn based on the relationship $Y^{ATP} = -0.2242X + 2.3008$ with $R^2 = 0.9915$, where Y^{ATP} was an anodic peak, µA, from each sample and X was a ATP synthase gene concentration (log copy number) as described (Kuribara et al., 2002; Chaumpluk et al., 2006). Similar calibrations were also carried out for polyphenol oxidase, superoxide dismutase and 18S rRNA gene with relationship; $Y^{PPO} = -0.2229X+2.305$ with $R^2 = 0.9914$, $Y^{SOD} = -0.2388X+2.3583$ with $R^2 = 0.9932$, and $Y^{18S} = -0.2374X+2.3833$ with $R^2 = 0.9937$ (data not shown). Anodic measurements for all 4 genes, the ATP synthase, polyphenol oxidase, superoxide dismutase, and the 18S rRNA genes and their corresponding copy numbers (anti log), calculated from anodic current peaks and relationship in terms of ATP synthase, polyphenol oxidase, superoxide dismutase, and 18S equations for fresh cut pineapple samples, in conjunction with the expression pattern during quality deterioration were described in Fig. 5.

Anodic current peaks of the ATP synthase gene among samples varied between 1.27 to 2.25µA. ATP synthase gene expression at beginning was at 39600 copies. Decreasing of the gene expression was observed after storage. Rapid drop of the expressions from 26260 copies to 2742 copies were observed after 6 hours after storage and nearly all were terminated after 12 hours. These reflexing the freshness of the cells of fresh cut pineapple at 25°C storage especially at the fresh cut surface. Expression of 18S rRNA, a house keeping gene, was at 72118 copies at beginning. It increased expression to a maximum at 142202 copies after 6 hour. The expression was then decreased rapidly and nearly terminated after 12 hours after storage. This was agreed with the status of the cells as predicted from the ATP expression levels. Expression of PPO was, in contrast, increasing from 36089 copies at beginning and reached maximum at 78051 copies after 9 hours after storage. This indicated the response of cells to the deterioration in term of program cell death by browning. The expression of SOD was surprisingly at high level compared among the genes studied. It expression was nearly approach that levels of the house keeping 18s rRNA. The high level of this gene expression indicated the factor of important of this gene to the deterioration. The level of SOD expression was dropped after stage 6 (24 hours after storage). At this moment, results implied that cells were not anymore in good conditions.

These results corresponded with the results obtained using RT-PCR with gel visualization (Fig. 4). Expression level agreed well with physiological changes in terms of weight loss, respiration rate, ion leakage, and their firmness loss (Fig.3). SOD was the most obvious change and this was accompanied by loss in proteins accumulation in membrane.

Here we revealed that expression induction could be detected using the developed biosensor as early as the first hour after storage with the level comparable with that of housekeeping. By applying similar approach, majority of genes involved in senescence, that expressed in postharvest could be easily investigated. Although the measurement by biosensor could be made via anodic current measurement, its application for quantification was still at semi conservative level. This was because the involved processes were still relied on reverse transcription step, and PCR amplification which might involving with error in their amplification efficiency that might affect the electrochemical measurement. However, if these were customed properly, this electrochemical biosensor approach could be used for gene expression study.

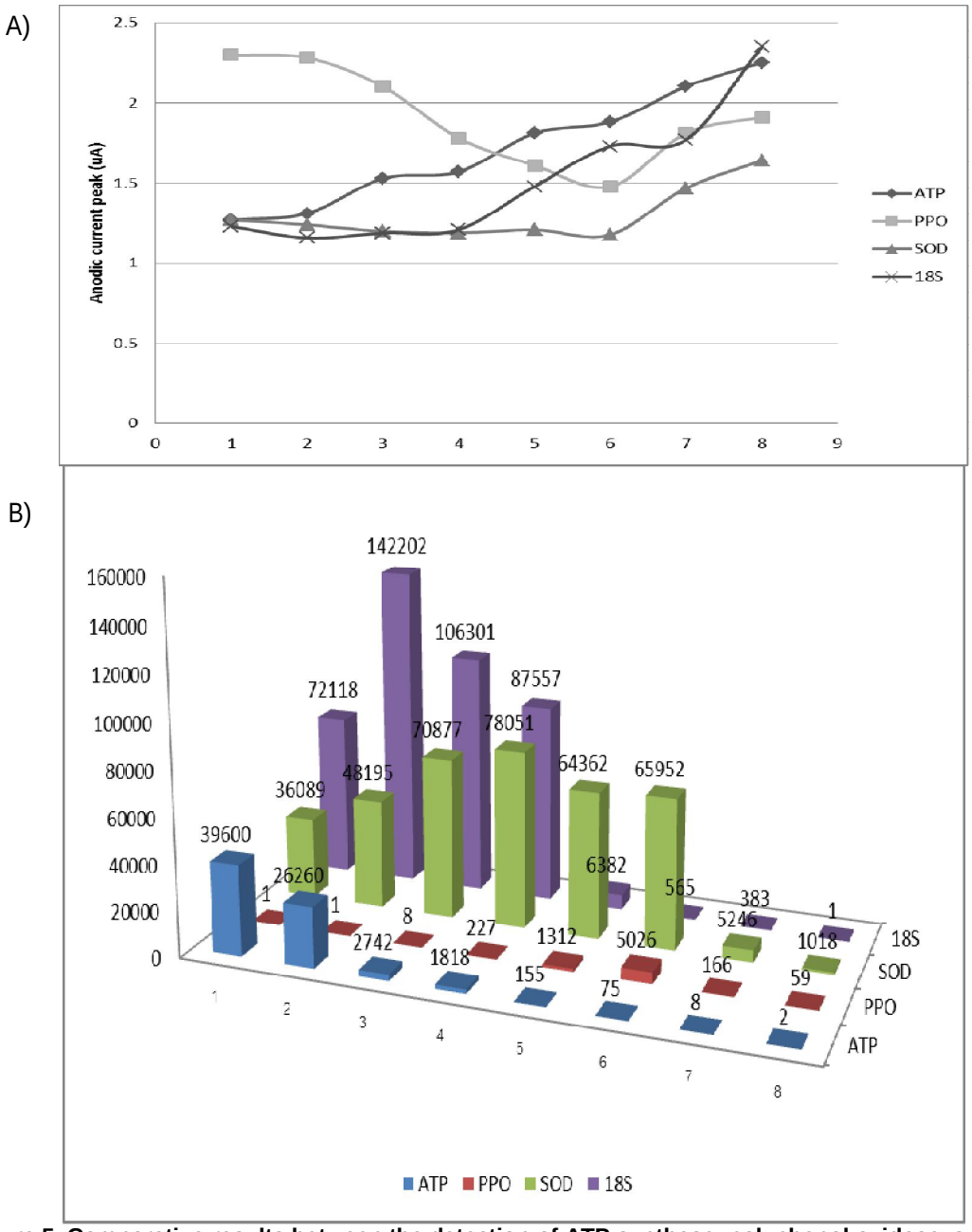


Figure 5. Comparative results between the detection of ATP synthase, polyphenol oxidase, super oxide dismutase cDNA and 18S rRNA gene through anodic current peaks measurement during storage at 25 °C (A) (see also Table 1), and the quantitative analysis of their gene expression (copy numbers), based on standard curve analysis, compared with that of 18S rRNA gene (B). Copies number of gene was calculated from each equation and make it round number. No. 1 to 8 was repreented the stage of fruit tested.

The measurement of this would allow a quantitative analysis based on comparative ratio between copy numbers of any target gene and 18S rRNA of house keeping genes which providing merit in gene activity comparison. Results revealed an increase in SOD gene expression on the first 6 to 12 hours at 50.04 % (36089, SOD/72118, 18SrRNA) and 33.89 %

(48195, SOD /142202, 18SrRNA) comparing with those of the housekeeping gene before reaching a maximum of 89.14% (78051, SOD /87557, 18SrRNA), respectively . This was the first time that target gene expression could be illustrated together with the level of gene expression of housekeeping without using real-time PCR. Demonstration in this way helps better understand the gene expression profiling which is crucial for the study on cell function (Wang et al., 2007).

The measurement using fabricated sensor based on screen print carbon and Hoechst 33258 in this paper cost less than US$2.0 per measurement. From the experiment, estimation of target gene expression could simply be determined from the quantitative analysis of signal transduction through anodic changes induced via the binding of target RT-PCR products to Hoechst 33258 molecules. This is the first demonstration of gene expression study using electrochemical biosensor based on Hoechst 33258 induction in pineapple for its post harvest study. Similar applications can be implemented if cDNA amplification system could be developed. Although this quantitative detection was not a real-time estimate, our method demonstrated several merits in using electrochemical approach over the standard methods and real-time PCR, especially on its rapidity and simplicity in performing experiments, on the characteristic of quantification which required no sophisticated and expensive devices, and on its cost effectiveness (less reliable on expensive chemical reagents). Its results on quantification could also expedite the need for answering any field analysis on numeral basis which has never before been applied to the determination of gene expression in plant tissues. Potential of MP pineapples during 20 days of storage at 4ºC

CONCLUSION

A technique for the detection of deterioration biomarkers via gene expression based on electrochemical biosensor was developed. By using DNA aggregation induced via Hoechst 33258 binding, anodic current peak on linear sweep voltammetry could be measured. Anodic peaks for chlorophyllase varied between 1.16-2.35 µA among samples. These anodic peak changes were correlated with amount of each cDNA of target gene in tissue, directly indicating its gene expression. The expression of ATP synthase, polyphenol oxidase, superoxide dismutase gene at 25 ^{o}C was detected as early as 6 hours after storage. SOD expression level having more abundance than the rest studied might play key role in quality deterioration in pineapple. Semi-quantitative studies on gene expression revealed the increase in patterns of gene expression especially that of SOD up to 89.14% comparing with that of a housekeeping gene. These results provided gene expression configuration in responding to postharvest conditions of fresh cut pineapple. The technique has several merits on its rapidity and simplicity in performing the test and its cost effectiveness since no expensive instruments and reagents are needed.

ACKNOWLEDGEMENTS

This work was supported by Laboratory of Plant Transgenic Technology and Biosensor, Department of Botany, Faculty of Science, Chulalongkorn University.

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