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Potential of Vis/NIR spectroscopy in estimating ATP content per protoplast as an indicator of freshness of spinach

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Abstract: The possibility of using ATP content per protoplast as an indicator of freshness was studied in spinach (*Spinacia oleracea*). The ATP content per protoplast of spinach was determined and compared during 3 days of storage at 25 °C. ATP content increased rapidly at the early stages of storage, reaching a peak level after 24 h. After one day (24 h) of storage, the ATP content decreased with the storage time. Overall, the ATP content changed as a quadratic polynomial function of storage time. This phenomenon, which is likely to be an expression of the loss of protoplast viability, is a useful indicator, allowing the prediction of spinach deterioration during storage. In addition, the correlation between ATP content and Vis/NIR spectra during storage was excellent (R^2=0.8245 for transmittance at 681 nm, and R^2 =0.9008 for the first derivative of transmittance at 760 nm). These results demonstrate the possibility of evaluating the freshness of spinach through estimation of the ATP content per protoplast with Vis/NIR spectroscopy after approximately 30 hours of the harvested plant storage.

1. INTRODUCTION

Adenosine 5'-triphosphate (ATP) is produced as an energy source during the processes of photosynthesis and cellular respiration, and consumed by many enzymes and a multitude of cellular processes. Numerous studies have reported that ATP is automatically degraded during storage of meats and fishes, and it has been used to calculate the K value for estimating the freshness of meats and fishes (Bae et al., 2006; Shahidi et al, 1994). Many researches have also revealed the changes of ATP concentrations in fruits and plant seeds under different storage conditions (Peppelenbos and Oosterhaven, 1998; Siegenthaler and Douet-Orhant, 1994). Peppelenbos and Oosterhaven (1998) suggested that when ATP levels drop below levels needed for cell maintenance activities such as membrane repair, fruit disorders such as internal browning in pears may develop. Siegenthaler and Douet-Orhant (1994) demonstrated the possibility of using ATP content as an indicator of seed quality in onion seeds (*Allium cepa* cv. Wadenswil). Therefore, ATP level may be used as a potential indicator for estimating the freshness and quality of both animal and plant produces. However, so far little research has been reported regarding the cellular ATP level as well as its potential role in evaluating the freshness of vegetables during storage.

To utilize the ATP level as a potential indicator for evaluating the quality of agricultural product during storage, there is a demand for intensive sampling and measuring of the ATP levels in the product. Traditional methods of determining the ATP concentrations in plants have depended on destructive sampling to determine the ATP level through a series of complicated procedures. This is not only costly but also time-consuming (Gould and Subramani, 1988).

Recently, the potential of using visible and near-infrared (Vis/NIR) spectroscopy has been intensively examined for developing a non-destructive method to evaluate various quality properties of agricultural products. Due to its non-destructive characteristics and successful application for prediction of other quality indices (i.e., sugar content), spectroscopy has been considered in this study for predicting the ATP level in spinach protoplasts.

Spinach is one of the most highly perishable leafy vegetables. It is commonly consumed in Japan. Therefore, we selected it as the experimental material in the present study. The objectives of this study were: 1) to investigate the change of ATP content per protoplast in spinach during storage; and 2) to examine the correlation between the ATP content and the Vis/NIR spectra obtained during storage.

2. MATERIALS AND METHODS

2.1 Plant material

Spinach plants (variety *Cyclone*) were purchased 1 day after harvest from the Tsukiji Wholesale Market in Tokyo. Samples were selected for uniformity and stored in an acrylic chamber. The chamber with spinach was kept inside an incubator with 25 ℃ constant temperature for a storage period of 3 days. The chamber was ventilated once a day during storage. Before and during the storage (every 24 hours), ATP contents and Vis/NIR spectra for 4 leaves (3^{rd} or 4^{th} leaf from the outermost leaf of the plants) from the samples were measured.

2.2. Protoplast preparation

For measuring ATP content, metabolically competent protoplasts were prepared from leaves of spinach according to the method described below: First, the epidermis was scraped off the spinach leaves with a pair of fine tweezers. The leaves were then cut into segments of 0.5 - 1 cm in size, and soaked in an enzyme solution. The enzyme solution consisted of 0.5 M mannitol, 1% cellulase R-10 (Yakult Honsha Co.), and 0.2% macerozyme R-10 (Yakult Honsha Co.). The leaf slices or pieces in a 9 cm-diameter dish containing the enzyme solution were incubated for 3 hours at 25 ℃, covered with a plastic cover. After the incubation, the cell wall was digested by the enzymes and removed from the cells. The solutions were then filtered through nylon mesh (70 μm pore size) to remove vascular tissue and undigested material. The filtered solutions were centrifuged at 900 rpm for 2 minutes. After the supernatants were carefully removed and discarded, 0.5 M mannitol solution was added to the pellet, and then the solution was centrifuged again. The protoplasts were collected after centrifugation of the washes for 3 times and the supernatant was aspirated and discarded.

2.3. Determination of ATP content

ATP content per protoplast in spinach was analyzed by the firefly luciferase bioluminescence technique (Gould & Subramani, 1988), using the luciferine-luciferase test kit (Toyo B-Net Co., Ltd., Japan). In order to obtain an accurate average of ATP content per protoplast, the number of protoplasts in 2μl prepared protoplast suspension was first counted with the microscope (Leica DFC 320, Leica Microsystems Wezlar GmbH, Germany). While in ATP assay, 100μl protoplast suspension was used, thus the number of protoplasts contained in the measured protoplast suspension was multiplied by 50 times in order to calculate the average of ATP content per protoplast. In the experiments, the number of protoplasts contained in 100μl protoplast suspension ranged from 100 to 1700. ATP in protoplasts was first extracted by LL100-2・ATP extraction kit (Toyo B-Net Co., Ltd., Japan). After 10 seconds of extraction, the LL100-1・ATP luminescence kit (Toyo B-Net Co., Ltd., Japan) was added to the sample, and the light output from the luciferine-luciferase reaction was measured by a luminometer (Luminescencer -PSN AB-2200/-R, Atto Corporation, Japan). An ATP standard curve was obtained by measuring the Relative Light Unit (RLU) of the properly diluted standard solutions (with known ATP concentrations). It was then used to calculate the ATP contents in samples based on the RLU recorded during measurement.

2.4. Measurement of Vis/NIR spectra

Before preparation of protoplasts, Vis/NIR spectra were obtained for leaf samples, using spectrometer FQA-NIR GUN (Fruit Quality Analyzer) (FANTEC Research Institute, Japan). The spectrometer covers the 610-950 nm wavelength range with a spectral resolution of 2 nm. The spectrometer is connected to a computer, and the spectral data is recorded in the computer and can be further analyzed with the attached GUN-CONTROL and CA-maker software (FANTEC Research Institute, Japan). For each of the leaf samples, 4 different points along the leaf edge were measured and the average of these 4 points was used as the spectral data for the leaf.

2.5. Data analysis

Statistical analysis of the data was mainly performed in Excel 2003 (Microsoft Corporation, USA). Spectral data including transmittance, and the 1st derivatives of transmittance were obtained by the GUN-CONTROL software. The correlation analysis between Vis/NIR spectra and ATP content per protoplast was performed in CA-maker software.

3. RESULTS AND DISCUSSION

3.1. Change of ATP content per protoplast during storage

Fig. 1 shows that before storage the ATP content per protoplast was at a low level. However, the ATP level increased rapidly during the first day of storage (up to 24 h). After reaching the peak, the ATP content decreased during the later two days of storage (from 48 to 72 h). This pattern of ATP content in protoplast was similar with our previous experiment with a different spinach variety (*Planton*). The overall trend of decrease in ATP content during storage can be attributed to the lack of continued supply of nutrients after harvest. Nevertheless, the increase in ATP level in spinach protoplasts during the early stage of storage may result from the increased respiration and its concomitant oxidative phosphorylation, in order to maintain the cell viability through increased ATP synthesis.

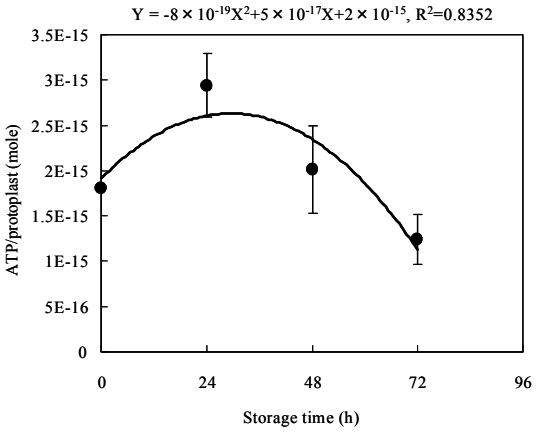

Fig. 1 ATP content per protoplast in spinach as a quadratic polynomial function of storage time. The error bar indicates the standard error of the mean.

The relationship between ATP content per protoplast and storage time can be expressed with a quadratic polynomial function, as shown in Fig. 1. The coefficient of determination (R^2) of the calibration model was 0.8352, showing a significant correlation between ATP content per protoplast and storage time. Based on the function, it could be derived that the ATP per protoplast in spinach reaches the

maximum level (2.6568×10^{-15} mole) at approximately 30 hours after storage. This result indicates the potential of using the model to estimate the ATP level in spinach protoplasts with the storage time.

3.2. Relationship between ATP content per protoplast and Vis/NIR spectra

Fig. 2 illustrates the average spectra of the transmittance and the first derivatives of transmittance for the samples measured at 4 different times before and during storage. A rapid change of transmittance was observed in the 660-770 nm wavelength range. This range falls within the so-called "red edge" region, which represents the region of abrupt change in leaf spectra. It is generally recognized that this abrupt change in leaf spectra is caused by the combined effects of strong chlorophyll absorption in the red wavelengths and high reflectance in the NIR wavelengths due to leaf internal scattering (Gates et al., 1965; Horler et al., 1983). Numerous studies have shown that this region have high information content for vegetation spectra (Collins, 1978; Horler et al., 1983).

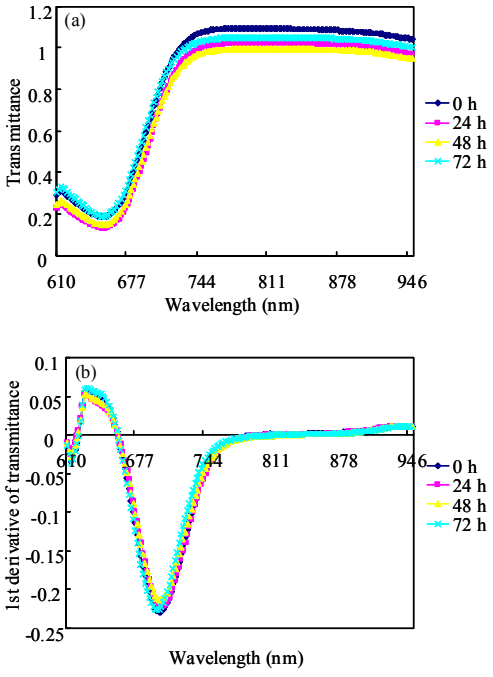

Fig. 2 Average spectra of spinach samples obtained at 4 different times before and during storage. (a) transmittance and (b) the 1st derivative of transmittance.

Analysis of the correlations between the ATP per protoplast and the average spectra at each wavelength for the samples obtained at different storage times was performed and Pearson correlation coefficients were evaluated. Fig. 3 shows the correlation coefficients obtained with different average spectra. It was found that the transmittance of the 610-700 nm wavelength range was significantly correlated with the ATP per protoplast ($r>0.9$). A sharp decrease in the degree of correlation between the ATP content and the transmittance of the wavelengths greater than 700 nm was observed. These results indicate the significant correlation between visible bands and the ATP content in protoplasts. In plants, ATP is synthesized in thylakoid membrane of the chloroplast during the light-dependent reactions of photosynthesis. As chlorophyll is vital for photosynthesis, which allows plants to obtain energy from light, there is a potential correlation between the ATP content and chlorophyll in plant protoplasts. Many previous studies have demonstrated the correlation between visible bands and chlorophyll (Yoder & Pettigrew-Crosby, 1995; Gitelson et al., 1999). Therefore, those visible bands which are correlated with chlorophyll may also have a correlation with the ATP content in plant protoplasts. The significant correlation between the ATP content and the spectra of visible bands (610-700 nm) obtained in this study confirmed this hypothesis.

In contrast, the first derivative of transmittance demonstrates a pattern of several wavelength ranges with a high correlation with the ATP content. Among these wavelength ranges, the 710-770 nm wavelength range shows a stable correlation with the ATP content. This wavelength range, which belongs to the longer wavelength portion of the "red edge" region, demonstrates a significant correlation with the ATP content in terms of the first derivative of transmittance. The first derivative was calculated by dividing the difference between successive spectra values by the wavelength interval separating them. Therefore, the derivatives describe the rate of change in spectral values between successive wavelength bands. The above results indicate that the first derivative of transmittance in the 710-770 nm (NIR) wavelength region are also useful for estimating the ATP content in plant protoplasts.

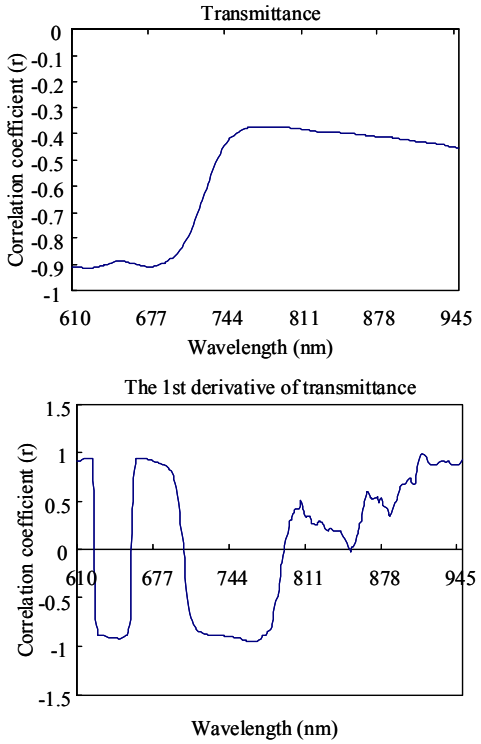

Fig. 3 Correlations between ATP per protoplast and average spectra in spinach based on averages of data samples. (a) transmittance and (b) the 1st derivative of transmittance.

As the 610-700 nm wavelength range for the transmittance and the 710-780 nm wavelength range for the first derivative of transmittance demonstrate a stable correlation with the ATP content, the best correlated wavelength (681 nm and 760 nm) for each of these two wavelength ranges were selected for model development. Fig. 4 shows the scatter plots of the ATP content versus the transmittance at 681 nm (Fig. 4a), and the ATP content versus the first derivative of transmittance at 760 nm (Fig. 4b). Simple linear regression analyses between the ATP content and the spectra at the selected two wavelengths were performed, and the regression statistics (R^2, slope and intercept) obtained with different models were illustrated in Fig 4. The model based on the transmittance at 681 nm achieved a R^2 of 0.8245.

Greater prediction accuracy (R^2=0.9008) was obtained with the model based on the first derivative of transmittance at 760 nm, suggesting the greater relevance of the first derivative of transmittance at 760 nm in estimating the ATP content per protoplast in spinach with Vis/NIR spectroscopy.

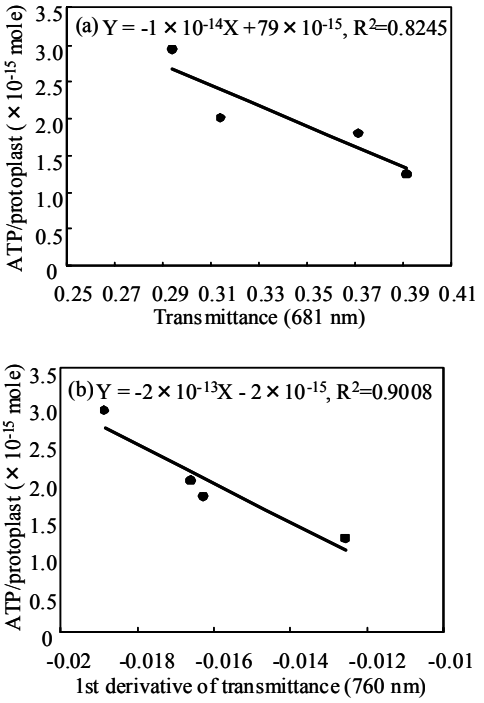

Fig. 4 Models based on averages of data samples: (a) transmittance at wavelength 681 nm, and (b) the 1st derivative of transmittance at wavelength 760 nm.

The above models were developed with the average spectra obtained at different storage times. In view of the difference in the ATP content between different sample leaves measured at the same storage time, all individual data samples were used to investigate the correlation between the ATP content and the Vis/NIR spectra, regardless of the storage time. The results show a similar pattern of correlations between the ATP content and the Vis/NIR spectra with those obtained with the average spectra, though the correlation coefficients decreased due to the data differences between individual samples (Fig. 5 and Fig. 6). This indicated that there is a consistent relationship between the ATP content and the Vis/NIR spectra obtained for individual leaves. Therefore, the non-destructive Vis/NIR spectroscopy approach has a considerable promise in estimating the ATP content in spinach, and thus can be used as a potential tool for measuring the freshness of spinach in the future.

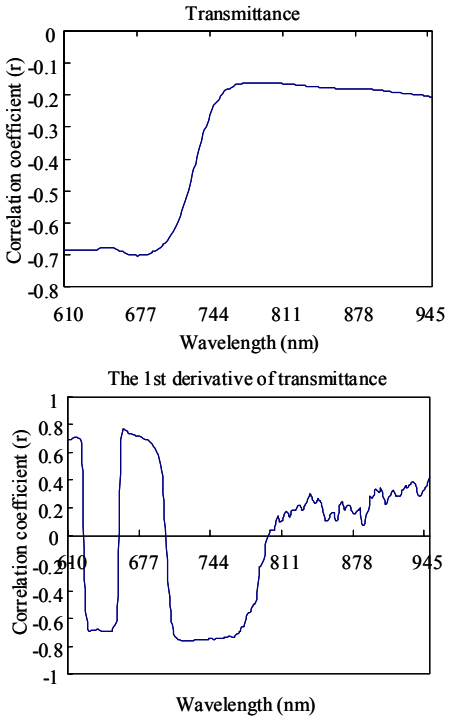

Fig. 5 Correlations between Vis/NIR spectra and ATP per protoplast in spinach based on individual data samples. (a) transmittance and (b) the 1st derivative of transmittance.

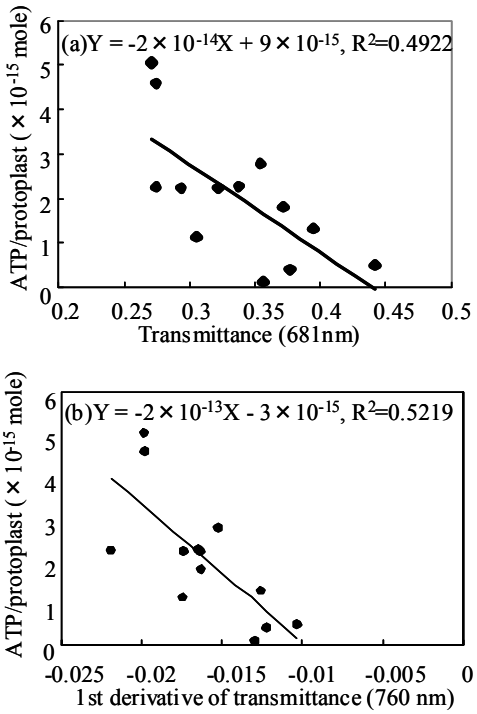

Fig. 6 Models based on individual data samples: (a) transmittance at wavelength 681 nm, and (b) the 1st derivative of transmittance at wavelength 760 nm.

4. CONCLUSION

In order to estimate the freshness of spinach, a new parameter, the ATP content per protoplast, was proposed and its relationship with the Vis/NIR spectra was examined in the present study. The ATP content per protoplast in spinach was determined by the firefly luciferase bioluminescence technique, and the Vis/NIR spectra were obtained using the spectrometer FQA-NIR GUN. A quadratic polynomial relationship was found between the ATP content in protoplasts and the storage time. The ATP level increased at the earlier stages of storage (up to 24 h), and decreased at the later stages of storage (48-72 h). We ascribe the increase of the ATP level at the earlier stages of storage to the increased respiration rates and its concomitant oxidative phosphorylation, which is automatically activated to maintain the cell viability through increased ATP synthesis. Correlation between the ATP content and the Vis/NIR spectra was subsequently analyzed for examining the possibility of predicting the ATP level based on the obtained Vis/NIR spectra. A correlation coefficient of more than 0.9 was achieved by the selected wavelengths at 681 nm and 760 nm for the transmittance and the first derivative of transmittance, respectively. The developed calibration models in this study need to be validated in future work. The effective wavebands also need a better definition to make it feasible for implementation in a real operation system. Notwithstanding its limitations, this study does show the potential of freshness evaluation on spinach using Vis/NIR spectral information after approximately 30 hours of plant storage.

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