JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Near-Infrared Reflectance Spectroscopy-Based Methods for Phytase Registration in Feed Industry

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The application of biotechnological products in the feed industry has undergone explosive growth in recent years, and phytase from microorganism accounts for one-third of the entire feed enzyme market. In this study, some differences in the composition of protein and denaturation temperature between two commercial phytases were determined by HPLC and differential scanning calorimetry, which were derived from the same origin of *E. coli*. At the same time, we found that it was advantageous for near-infrared reflectance spectroscopy (NIRS) to display the protein differences in the commercial phytase, which is most important for ensuring the traceability of biotechnological products in feed and food safety control. Furthermore, NIRS could track the changes in phytase during the spray-drying process and the change of enzyme activity during storage of phytase. Our experiments proved that the information from NIRS could describe well the individual characteristics of the commercial phytase, which indicated that near-infrared reflectance spectra could be exploited to use in the registration system of commercial phytase.

KEYWORDS: Near-infrared reflectance spectroscopy; phytase; registration; feed control

INTRODUCTION

The feed industry is the major application field of biotechnological products, especially for genetically modified plants and microbial enzymes. Due to mineral efficiency and environmental protection, phytase produced by a wide range of plant, bacterial, fungal, and yeast sources has been widely supplemented in diets for food animals to improve phosphorus efficiency and to reduce phosphorus pollution from animal excrement (*1*–*3*). The annual sales value of commercial phytase, especially from *A. niger* and *E. coli*, was estimated at US$ 500 million within the decade, taking approximately one-third of the entire feed enzyme market (*4*). Now, more and more commercial phytases are developed by genetic engineering based on the gene sequences and protein structures of phytase (*5*, *6*).

In order to maintain a high level of protection for human health, animal health, and the environment, some new regulations for biotechnological products have been implemented recently (*7*). Regulation 1829/2003/EC on genetically modified food and feed requires that the application shall be accompanied by a detailed description of the manufacturing method and scopes of the application, monitoring plans, detection methods, and labelling proposals (*8*). Furthermore, regulation 1830/2003/EC lays down a harmonized framework for the traceability of genetically modified organisms, and regulation 65/2004/EC lays down a system for the development and assignment of unique identifiers for genetically modified organisms (*9*, *10*). It is well-known that maintaining traceability is most important in supervising biotechnological products, and the collection of individual registration information is the foundation for the establishment of a specific traceability system for feed and food. To make a strong impact on the behalf of operators and consumers, the debate concerning potential registration style and application content of biotechnological products is a matter of the utmost concern for feed operators, regulatory authorities, scientists, and consumers (*11*, *12*).

With the landmark paper of Norris and colleagues in 1976, NIRS has been used to measure almost every parameter routinely tested in a conventional forage and feedstuff laboratory, such as moisture, protein, fiber, lipid, carbohydrate, digestibility, energy content, antiquality components, and silage fermentation characteristics (*13*–*16*). NIRS is a secondary method that can be as accurate as the reference method, and is simpler to operate once calibrated. With ongoing development in equipment and implementation of current and new calibrations, NIRS analysis of forages and feeds will continue to be

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10.1021/jf071241u CCC: $37.00
Published on Web 08/23/2007

widely adopted in research, teaching, and industry. It should be noted that a major advantage of NIRS is that it can supply immediate multianalyte information simultaneously that is closely related to sample origin (*17, 18*). The maximal information from NIRS has more advantages in describing the product than some individual parameters obtained with traditional chemical methods, such as proteins, sugars, and organic acids. NIRS has been used in a number of studies to determine species composition, which concluded that an NIRS procedure was often preferred over chemical or physical procedures for estimation of species composition. In one study, species composition was predicted by NIRS in mixtures that contained bahiagrass, aeschynomene, and phasey bean (*19*). NIRS has also been adopted to discriminate among the different types of coffees, vinegar samples, and woods from different origins (*20, 21*). In this regard, NIRS has a good potential application in registration and supervision of biotechnological products in the feed industry.

It is well-known that the expression systems and the process parameters, such as culture medium, centrifugation, and filtration, and any changes to them could contribute to the specific catalytic activity, thermostability, and the different composition of the microbial enzyme products (*5*). So, it is necessary to monitor the process with more efficient techniques. Over the past decade, NIRS has been successfully utilized within the fermentation stages of bioprocesses for online measurements, including monitoring of organic acids, recombinant proteins, and hormones derived from the submerged bioprocesses, where primary measurement data from NIRS can be used to diagnose the process for early recognition of process disturbances, and for appropriate remedial action (*22, 23*). For efficiently ensuring the traceability and high level of safety of biotechnological products, we carried out this study to investigate the utility of NIRS within the bioprocess except for the fermentation stages of commercial phytase, and to investigate the potential valuable information obtained from NIRS to describe the individual characteristics of commercial phytase, as well as to exploit the NIRS-based methods for the registration of biotechnological products.

MATERIALS AND METHODS

Phytase Samples. Three hundred and sixty individual phytase samples from *E. coli* were collected from two companies in Beijing (these samples have passed the quality control in the phytase industry and are acceptable for sale in the supermarket to be sold); the phytase samples were divided into four different groups A, B, C, and D. These samples were collected every week for 3 months and were analyzed shortly after collection. In addition, liquid phytases from one feed company was collected to carry out spray-drying experiments and were stored at 4 °C. Phytase R was a reference phytase from *A. ficuum* (Sigma-Adrich, Switzerland).

Group A was formed from 120 spray-dried phytase samples, and Group C was formed from 60 attach-dried phytase samples, which were from one feed company.

Group B was formed from 120 spray-dried phytase samples, and Group D was formed from 60 attach-dried phytase samples, which were from another feed company.

SDS-PAGE Analysis of the Commercial Phytase. The commercial phytases and the reference phytase, obtained in powder form, were resuspended with acetic acid–sodium acetate buffer at pH 5.5, and centrifuged at 12000 rpm for 5 min to remove any insoluble particles. Sufficient aliquots from the three preparations were concentrated by ultrafiltration using 50 kDa MWCO polyethersulfone membrane columns from Sigma-Aldrich. Subsequently, 20 μL of the commercial phytase was analyzed by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis).

Table 1. Methods Used for Calculating the Calibration Equations

steps	methods
pathlength correction	measured, MSC, SNV (alone), SNV (detrend), normalize
data preparation	mean center, variance scale
calibration type	quantitative: PLS-1, PLS-2, PCR qualitative: PCA, discriminate
diagnostic type	self prediction, cross validation, leverage validation
derivative	Savitzky-Golay first, Savitzky-Golay second, gap first, gap second

HPLC Analysis of the Commercial Phytase. The pretreatment of the commercial phytases was the same as the previous SDS-PAGE analysis, but 10 μL of a solution containing 3 mg/mL of the commercial phytase was injected each time. The high performance liquid chromatography (HPLC) system (Agilent 1100, Böblingen, Germany) consisted of a quaternary pump, an autosampler, a degasser, an automatic thermostatic column compartment, and a computer with a chemstation software (Analyst 1.4, Applied Biosystems Inc., USA). The analytical column was an Agilent Zorbax 300 SB-C18 column (5 μm, 150 $\times$ 4.6 mm).

The mobile phases were A (water containing 0.1% trifluoroacetic acid) and B (acetonitrile and water containing 0.09% trifluoroacetic acid, 80:20, v/v). The mobile phases were degassed automatically using the electronic degasser system. With a flow rate of 1 mL/min at ambient temperature, phytase was eluted with a gradient of acetonitrile and quantified by peak area monitored at 210 nm, which was detected by a variable wavelength detector. The gradient elution timetable for B was 0%, 21%, 39%, 42%, 46%, and 54%, respectively, when the elution times were 0, 21, 23, 30, 31, and 45 min.

Differential Scanning Calorimetry. Thermal properties of phytase were analyzed by differential scanning calorimetry (DSC 6200, Seiko Instrument Inc., Japan). Ten milligrams of spray-dried phytase samples were transferred into preweighed DSC aluminium pans (30 μL, pans BO14–6118, cover BO14–6650, Perkin Elmer, USA), and were stored in open pans for 8 h to equilibrate at room temperature. The pans were hermetically sealed and placed into the sample holder of the DSC. Triplicate samples of each material were analyzed. An empty pan was used as a reference. The indium standard was used for temperature and heat flow calibration. The samples were then heated in the DSC from 25 to 150 °C at a heating rate of 5 °C/min. Onset temperature and peak temperature were determined for each endotherm using *Pyris Manager* data processing software.

Near Infrared Spectroscopy. An NIRS Systems (Perten DA7200, Perten Instrument AB, Sweden) equipped with a ceramic plate as reference was used. The window is made of quartz, with a surface area of 25 cm^2, with measuring reflectance in the near infrared zone of 950–1650 nm. Operation of the spectrophotometer and the collection of spectra were performed using*Simplicity* software. The spectra were stored in optical density units log(1/*R*), where *R* represents the percent of energy reflected. The recording of spectra was carried out at intervals of 2 nm, performing 32 scans for both the reference and all samples. The phytase sample was poured into a cup to the rim, and the surface was leveled with a knife. Thereafter, the cup was placed on the rotating sample platform so that the light from the source directly struck the sample and the diffusely reflected light was returned back. To minimize sampling error, all samples were analyzed in triplicate.

NIRS Data Processing. A commercial program (*Grams PLSplus IQ*, Thermo Galactic, USA) for multivariate spectral data analysis was used to process the spectral data. A principal component analysis (PCA) was run and the generalized Mahalanobis distances (*H*) were computed for each spectrum. All samples having an *H* value above 3 were considered outliers.

Different calibration models of phytase activity for one kind of commercial phytase were developed successively by comparing the different data processing methods (**Table 1**), in which 100 samples from group A were assigned to the calibration data set and 20 samples from group A were assigned to the validation data set. The optimal

process was confirmed with three criteria: First, a good model should have a low square error of prediction (SEP), a low square error of calibration (SEC), a high correlation coefficient, and a small difference between SEP an SEC. Second, a relatively low factor was desirable in order to avoid inclusion of signal noise in the modeling, and the minimum prediction residual error sum of squares (PRESS) was used to determine the optimal number of factors. Third, multiple calibration techniques influenced the result as well (*24–26*). Comparing different data preparation, path length correction, derivative, calibration type, diagnostics, and outliers eliminating methods in processing steps, model with multiplicative scatter correction (MSC), mean center, cross validation, second derivative spectra, and PLS-1 showed the best results in both calibration and prediction results for phytase activity prediction.

At the same time, PCA was performed on the spectra to examine qualitative differences among the four commercial phytase varieties, and to track the changing of commercial phytase when it was stored under different levels of storage moisture. PCA is a method of data reduction that constructs new uncorrelated variables, known as principal components (PCs), which are linear combinations of the original ones. The PCs account as much as possible for the variability in the original variables, and PCA was usually used to examine the possible grouping of samples and the presence of outliers (*27, 28*).

Spray-Drying Experiment. The liquid phytase was supplemented with 25% (w/v) cornstarch. Prior to spraying, the phytase suspension (450 mL) was homogenized with the high-pressure homogenizer (NS 2006, L PONP, NIRO-SOAVI, Italy). All solutions were spray-dried in a mini-spray-dryer Büchi model 191 (Büchi Labortechnik AG, Switzerland). A magnetic stirrer was applied to the suspensions to maintain a homogeneous solution during spraying. In studying the effect of various temperatures, all conditions were fixed (atomizing air pressure 5 Kgf/cm^2, flow rate of the peristaltic pump 10 mL/min, nozzle diameter 1 mm). The inlet and outlet air temperatures were 140, 150, 155, 160, and 170 °C and 70, 75, 80, 85, and 90 °C. The microencapsulation of phytase under different conditions was performed in triplicate. The spray-dried materials were collected immediately in closed desiccators with silica gel and stored at room temperature.

Storage of Phytase Samples. The same batch of phytase was separated into 5 treatments: 4 replicates were included in each treatment, and 60 g phytase sample was used in every replicate. These samples were put into 5 sealed plastic containers (Antibiotic Airtight Container, 27 cm × 11 cm × 11 cm) loaded with the saturated salt solutions of NaBr, $CuCl_2$, NaCl, KCl, and $BaCl_2$ respectively, which gave a series of relative water vapor pressures of 53.17%, 61.28%, 74.68%, 82.32%, and 89.90%. Then, the containers were placed into the thermostat box (MCO-175, Sanyo, Japan) at a temperature of 40 °C for 10 weeks. In the period of storage, these samples were scanned with the spectrophotometer every week, and 2 g phytase from each sample was utilized to determinate the phytase activity after NIR scanning.

Phytase Activity Assay. Phytase activity was determined by the ferrous sulfate-molybdenum blue method (*29*). 50 μL of enzyme solution was incubated with 950 μL of substrate solution (4 mM sodium phytate in 0.25 M sodium acetate buffer, pH 4.5) at a temperature of 37 °C for 30 min. The reaction was stopped by adding 1 mL of 10% (w/v) trichloroacetic acid. The released inorganic phosphate was analyzed by adding 2 mL of a coloring reagent C (1% ammonium molybdate, 3.2% sulfuric acid solution, and 7.2% ferrous sulfate solution), and the absorption was measured at 700 nm. One unit of phytase activity was defined as the amount of enzyme that releases 1 μmol of phosphate per minute at a temperature of 37 °C.

RESULTS AND DISCUSSION

Composition of the Commercial Phytase Products. The commercial phytase products were measured with SDS-PAGE and HPLC. In fact, SDS-PAGE is a very effective analytical tool to achieve fractionation of protein mixtures, to analyze purity, and to estimate molecular weight. On the other hand, HPLC resolves proteins with differing hydrophobic properties and primary structure changes that alter these properties. **Figure 1** showed that the molecular weights of phytase A and phytase B were subtle different, both displaying a molecular weight of approximately 45 kDa on SDS-PAGE, and the molecular weight of the reference phytase was showed as 68 kDa, which was in close agreement with studies performed by several other researchers (*30, 31*). As such, it was evident that the two commercial phytases were all derived from *E. coli*, but were glycosylated to different degrees for the obvious difference displaying in the HPLC profile of phytase protein (**Figure 2a**,**b**). Previous studies have shown that the glycosylation of the phytases was highly variable in different expression systems, and glycosylation may have some effect on the properties of an enzyme, such as stability and pI of a protein (*32*).

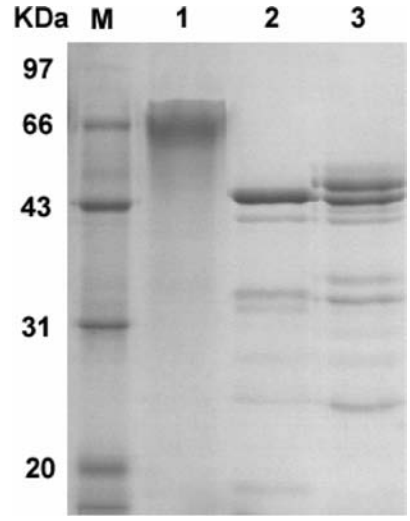

Figure 1. SDS-PAGE analysis of phytase samples.1: the reference phytase. 2: phytase B. 3: phytase A.

At the same time, the HPLC peak profile showed some imperceptibile differences between the proteins in two commercial phytases. As in the analysis of SDS-PAGE, the elution chromatographs for the reference phytase and the commercial phytase A (**Figure 2a**,**c**) showed that they mainly contained one protein. This conclusion was consistent with the finding of Wyss and co-workers that *A. ficuum* phytase as well as *E. coli* phytase were considered monomeric protein (*32*). It is interesting that the hydrophobic properties are more similar than the molecular weights between the reference phytase from *A. ficuum* and the commercial phytase A from *E. coli*, for which the HPLC peak profiles were so conformable (**Figure 2a**,**c**). Although the commercial phytases A and B in this study were both from *E. coli*, their HPLC peak profiles were different (**Figure 2a**,**b**). The chromatograph of commercial phytase B showed that it contained several proteins, despite the fact that the contents were much lower than phytase and they were easily eluted at 3–22 min.

It is well-known that the expression systems and the process parameters, such as culture medium, centrifugation, and filtration conditions, largely contribute to the different compositions of the phytase products. So, it is necessary to supervise the composition changes of commercial phytase for quality and safety control. The near infrared reflectance spectra contain information concerning the relative proportions of C–H, N–H, and O–H bonds (*33*), which are the primary structural components of biotechnological products. So, changes in the composition of commercial phytase could be tracked by the NIRS spectra in the same way as determination with HPLC or SDS-PAGE, while the operation of NIRS is simpler than that of HPLC or SDS-PAGE.

Characteristics of Thermal Denaturation of Phytase Products. Heat treatment is a common process during feed

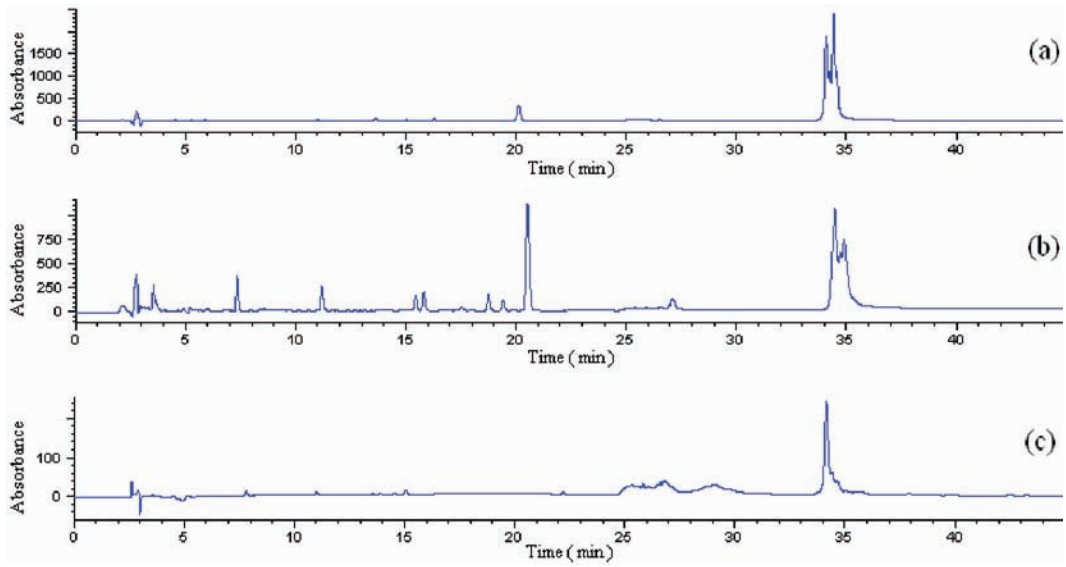

Figure 2. HPLC chromatogram relative to phytase samples. (a) Phytase A. (b) Phytase B. (c) The reference phytase.

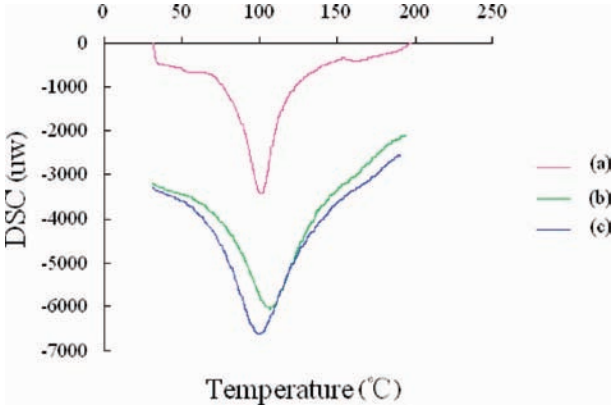

Figure 3. Differential scanning calorimetric thermograms. (a) The reference phytase. (b) Phytase A. (c) Phytase B.

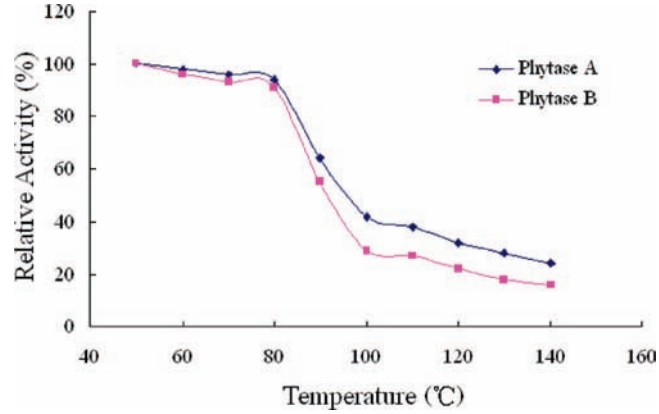

Figure 4. Thermal stabilities of commercial phytases A and B.

preparation. Therefore, it is necessary for commercial phytases to have sufficient thermal stability during the feed-pelleting and expansion processes. In this study, the thermal stability characteristics of phytases were assessed and compared by differential scanning calorimetry. It was shown that the denaturation temperature of the reference phytase from *A. ficuum* was 101.5 °C, that for commercial phytase A was 127.8 °C, and that for commercial phytase B was 118.5 °C (**Figure 3**). This is in agreement with previous study indicating the phytase from *E. coli* has better thermal stability than that of the phytase from fungi (*34*). In contrast to the reference phytase, there was similarity in the widths of DSC peaks between the two commercial *E. coli* phytase samples, and the DSC profiles do not differ much from each other. However, the denaturation temperature of commercial phytase A was higher than that of commercial phytase B. Moreover, thermostability studies also revealed that commercial phytase A was more thermostable than commercial phytase B (**Figure 4**); in the studies, commercial phytase samples were diluted with 5 mM sodium acetate buffer of pH 5.5, and heated to relevant temperature for 5 min, then placed on ice for 30 min, and residual phytase activity was assessed using the standard assay procedure at 37 °C.

As the measurement with HPLC discussed above illustrated, there were some differences in the compositions of protein between the two commercial phytases, which may contribute to the different denaturation temperatures between them. Commercial phytase A was more purified than product B and more resistant to heat denaturation than commercial phytase B. This is in agreement with the observation by Martin et al. (*35*). These authors indicated that, when phytase in nonpurified form was heated, less refolding was observed, which may be due to other components present in the sample that may have inhibited proper refolding of phytase. On the other hand, heat treatment could cause certain changes on the surface of the protein molecule, such as hydrophobic or free sulfhydryl groups becoming accessible and participating in intermolecular interactions, forming soluble aggregates through noncovalent and disulfide bonds (*36, 37*). These interactions between proteins could contribute to the characteristic of thermal denaturation of phytase products.

Spray-Drying Temperature and near Infrared Reflectance Spectra of Phytase. The powder commercial phytases are composed of constituents possessing functional groups such as C—H, O—H, N—H, S—H, and C═O that are selective absorbers of NIRS radiation, and the spectra produced by the NIRS give an indication of chemical and physical properties of a sample (*33*). While water absorbs strongly at specific wavelengths and usually exhibits a broad band, the spray-drying phytases in this study were kept in the sealed plastic boxes containing saturated salt solutions for 48 h, so as to minimize the differences in water contents at different spray-drying temperatures. **Figure 5** showed that the spectra were collected from phytases prepared with different spray-drying temperatures, and the higher curve was for the sample heated at lower

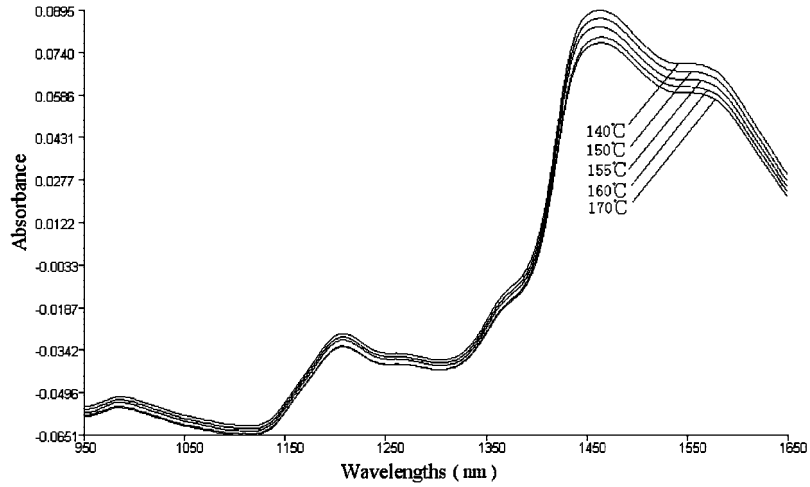

Figure 5. Near infrared reflectance spectra of phytase prepared at difference temperatures.

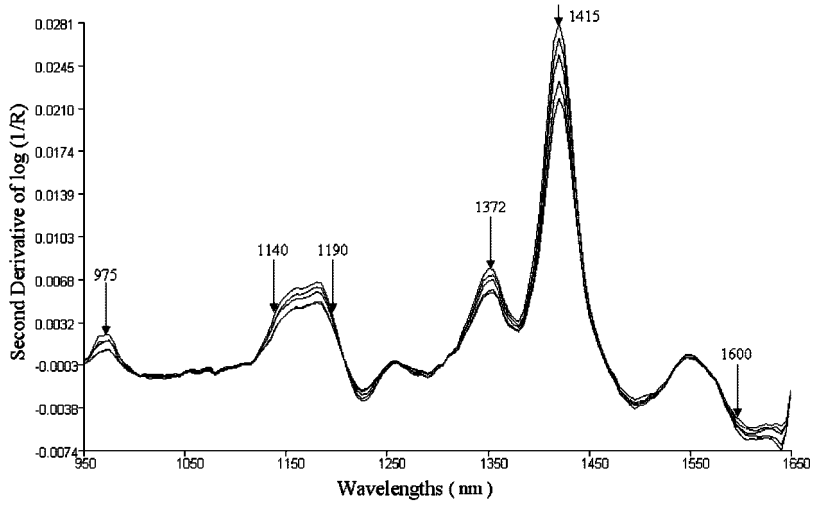

Figure 6. Second-derivative transformation of spectra of phytase prepared at difference temperatures.

temperatures, whereas the lower curve applied to the higher temperatures.

Furthermore, the differences in absorbance related to the heat treatment were observed at different wavelengths with the spectra that were subsequently scatter-corrected using SNVD (standard normal variate and detrend) and second-derivative treatments. Systematic differences in absorbance related to the heat treatment were observed at different wavelengths throughout the derivative-treated spectra where the characteristic absorption peaks were more clearly separated. The second-derivative spectra of the spray-drying phytases shown differences in absorbance related to the heat treatment at different wavelengths throughout the spectra, and these differences were more evident between 1140 and 1190 nm and 1600 and 1650 nm where the main absorbance bands of proteins occurred (**Figure 6**). Spectral changes upon heat treatment should be related to the changes in secondary structure due to the denaturation of proteins and changes in the state of water.

Change of Reflectance Spectra under Different Levels of Storage Moisture. Enzyme activity is influenced by the environmental conditions, especially ambient humidity (*38, 39*). In our study, when commercial phytases were stored under high ambient humidity at the temperature of 40 °C, phytase activity decreased significantly with increasing of ambient humidity after ten weeks (**Figure 7**). Furthermore, the near infrared reflectance spectra were collected along with the storage, and were processed by principal component analysis (PCA), which usually

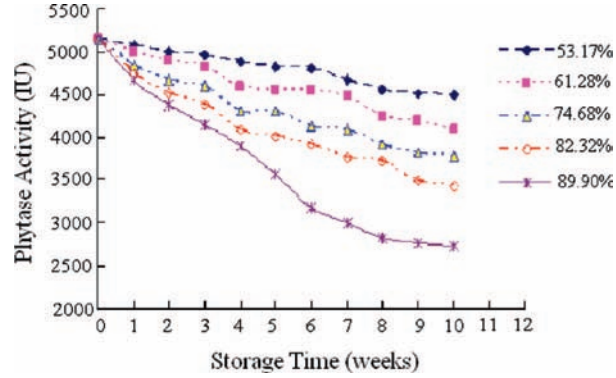

Figure 7. Effect of storage conditions on phytase activity.

was used to develop a study of the spectral population before developing the predictive models. This analysis eliminates redundant information resulting from high correlations between absorbencies in different regions of the spectra. Therefore, a linear combination of variables is defined, in a benchmark system in which the axes are linearly independent. Thus, the initial information is synthesized, by reducing the number of variables, explaining the same variability practically. Once the PCA was carried out on each sample presentation, the center of the spectral populations was determined in order to detect anomalies and samples with the characteristic behavior (*40, 41*).

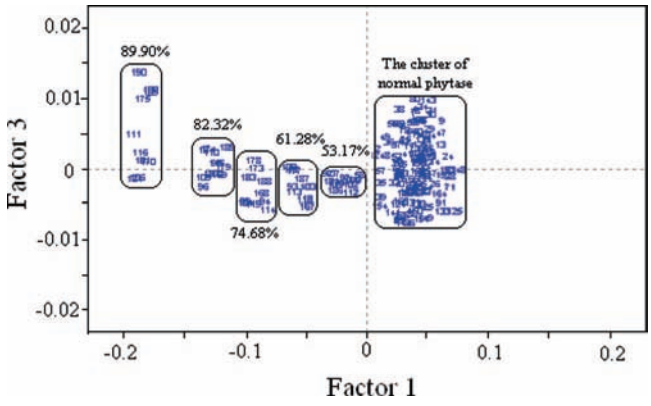

Figure 8. Near infrared reflectance spectra affected by ambient humidity.

The spectra of the commercial phytase stored under different ambient humidity levels were clearly separated in the score plot of factor 1 and factor 3 (**Figure 8**). All of them were separated from the normal data cluster, but they assembled into five new clusters with different ambient humidities. The data clusters of higher ambient humidity were more disperse, and were farther away from the normal data cluster. The NIRS data cluster of phytase stored under 53.17% ambient humidity was more condensed, and the water content of commercial phytase may contribute to the separation from the normal data cluster. However, the NIRS data cluster of phytase stored under ambient humidity of 89.90% was more disperse, possibly due to the change of phytase activity. In conclusion, the change of near infrared reflectance spectra could not only reflect the change of water content, but also include the fluctuating information of phytase activity influenced by storage conditions.

Phytase Activity Assay Using NIRS. Near infrared reflectance spectra used for a calibration model should cover a range as wide as possible to minimize the error of prediction. The experiment was designed to cover phytase samples that were suitable to put into market, and contain variations of one kind of commercial phytase for the company. The descriptive statistics in **Table 2** showed that the 100 samples from group A assigned to the calibration data set and the 20 samples from group A assigned to the validation data set had similar ranges and variation in phytase activity. These data indicate that the two sets contain enough diversity to develop calibration equations and that the external validation set is representative of the range of variation found in the overall samples.

In our study, the model with multiplicative scatter correction (MSC), mean center, cross validation, second-derivative spectra, and PLS-1 showed the best results in both calibration and prediction results. **Table 3** showed the statistical equations designed to predict phytase activity both in calibration and in validation (cross and external).The coefficients of determination (R^2) selected for both calibration and validation were over 0.9 in all the equations. Shenk and Westerhaus reported that any equations with values achieved that were equal to or higher than this value were extremely accurate (*42*). So far, from the statistics (SEC, SEP, RPD, and SECV) used to evaluate the models, we could see that the samples presented models with lower typical errors (SEC, SECV, and SEP) and higher relative predictive determination (RPD) values, and were therefore more robust models. The models have a good predictive power based on internal cross validation ($r^2 = 0.954$), external validation ($R^2_{EV} = 0.951$), and RPD (RPD = 3.84). From the above information, it may be deduced that the NIRS technique is a good alternative for the determination of phytase activity.

Phytase Spectral Data Analysis. The raw spectra (no pretreatment) of 360 phytase samples from different sources are shown in **Figure 9**. The near infrared spectra of commercial phytase were complex, and it was not possible to assign separate peaks to each component in the raw spectra. To obtain more characteristic information for different commercial phytase, the spectra data were used in a chemometric analysis with different data processing methods from the *Grams PLSplus IQ*.

Figure 10 showed the second derivative of mean spectrum of four commercial phytase samples. Important wavelengths were noted throughout the 950–1650 nm range, indicating that absorption in the visible region and absorption arising from O—H (950 and 1400 nm), C—H (1200, 1400, and 1650 nm), and N–H (1050 and 1500 nm) overtones contributed to classifications. There were visible differences in the regions 950–1020, 1230–1260, 1350–1380, 1500–1540, and 1600–1630 nm. In the visible band, there was one particularly noticeable band, within the 965–1020 nm range; an absorption band in the transition area of the visible to near infrared zone is detected. This band is most likely due to water, related to the third overtone (*43*). The regions around 950–1020 and 1500–1540 nm are primarily associated with the O–H stretch of protein, and the differences in these regions were probably connected with the protein compositions of two commercial phytases. The spectral features at 950–1020, 1350–1380, and 1600–1630 nm were mainly due to the absorption of O–H and C–H stretching bands of cornstarch. So, the differences in the **Figure 10** for commercial phytases might be explained by the differences in the ratio of phytase protein and other protein and the differences in cornstarch for different commercial phytases. The final important band was observed at around 1450 nm. Shenk related this region to combination bands of the C–H bond (CH_2 at 1440 nm and aromatic structure at 1446 nm), O–H stretch first overtone and C═O stretch third overtone (1450 nm), and N–H stretch first overtone ($CONH_2$ at 1463 nm) (*44*). In this study, this band has been linked with water because of to the O–H bond.

With the above collected foundation data of near infrared reflectance spectra, qualitative analysis of phytase spectra could be carried out by principal component analysis (PCA) to acquire the individual characteristics of phytase. The PCA projects the spectra into new axes: principal components (PC), which represent the main variations of the samples (*45*). The spectra of the four commercial phytases were clearly separated in the score plot of factors 1 and 2 (**Figure 11**). In this plot, four commercial phytases were widely separated from each other. The same phenomena occurred for the commercial phytases of other manufacturers. In a word, near infrared reflectance spectra could exactly distinguish commercial phytases into classes for different products. On the other hand, the calibration model could be used to determine whether a commercial phytase is counterfeit for a factory, which could show clearly the problem of detecting counterfeit product in the market. Furthermore, the calibration model should also be well-applied in an individual factory where an established product spectra library can act as a basis for detecting suspicious specimens among a more restricted range of products.

Conclusion. In this study, NIRS approaches showed a great potential to obtain rapid information about the related compositions, preparation properties, and phytase activity of commercial phytases in powder form. If NIRS data were introduced in the registration procedures of commercial phytase, the success of a traceability system could be constructed on the basis of NIRS technology, which will help

Table 2. Reference Values of the Calibration and Validation Sets

	calibration set				validation set			
parameter	no.	mean	range	RSD (%)[a]	no.	mean	range	RSD (%)
phytase activity (IU)	100	4869	4422–5964	10.5	20	4793	4512–5884	9.6

[a] RSD: relative standard deviation.

Table 3. Calibration Statistical Description for the NIRS Determination of Phytase Activity

					validation set				
			calibration set		cross		external		
parameters	pretreatment	PLS factor	SEC (%)	R^2	SECV (%)[b]	r^2	RPD	SEP (%)	R_{EV}^2
phytase activity (IU)	MSC and D2	9	3.28	0.946	3.52	0.954	3.84	3.32	0.951

[b] SECV: square error of cross validation.

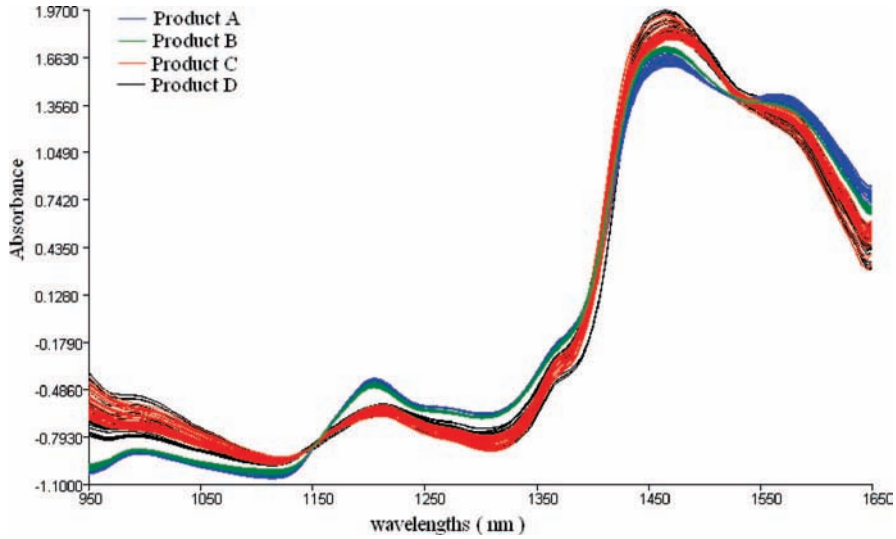

Figure 9. Near infrared reflectance spectra of spray-drying phytase samples: blue lines are product A, green lines are product B, red lines are product C, and black lines are product D.

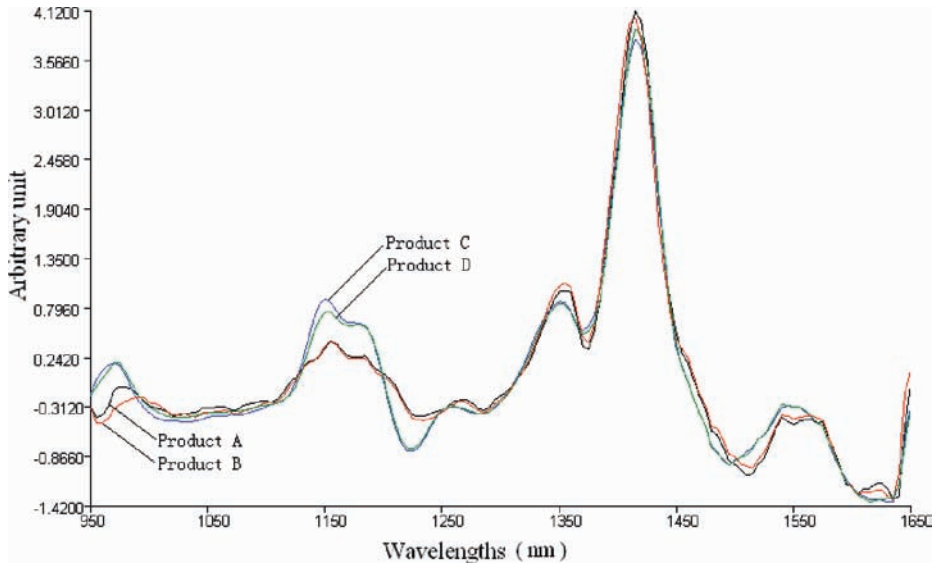

Figure 10. Relevant absorption features of the mean spectrum of four commercial phytase samples.

the administrators guarantee the safety of biotechnological products with a high level of confidence. At the same time, with the construction of authenticated NIR spectra library files, the phytase producers and administrators could conveniently improve product quality, enhance production speed, and carry out the hazard analysis and critical control point (HACCP) or good manufacturing practice (GMP). In conclusion, because it is more suitable to monitor fermentation processes, to minimize risk of contamination, to enhance reliability and ease of operation and maintenance, and to improve rapidity of analysis, and with the advances of computers and software developments, NIRS would benefit

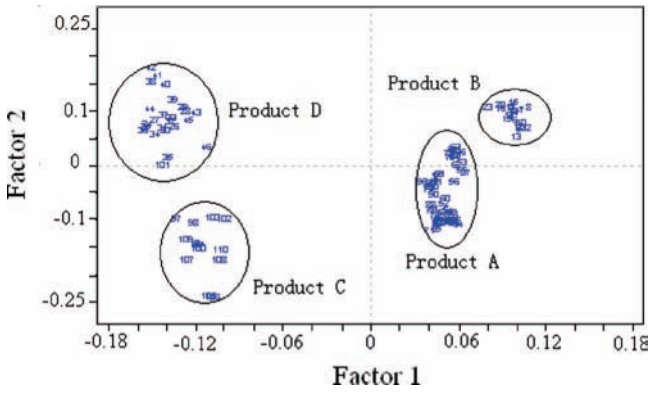

Figure 11. Score plot of principal component analysis (PCA) factors 1 and 2 for phytase spectral data from two companies.

the manufacturers, the clients, and the consumers, while lifting the burden on regulatory authorities and allowing them to concentrate surveillance efforts on referred suspicious specimens.

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Received for review April 26, 2007. Revised manuscript received July 30, 2007. Accepted July 30, 2007.

JF071241U