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In Vitro Solubility of Calcium, Iron, and Zinc in Rice Bran Treated with Phytase, Cellulase, and Protease

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Absorption of minerals is inhibited by phytic acid, fiber, and protein because of the chelates formed. Response surface method (RSM) was used in this study to evaluate the effect of application of commercial phytase, protease, and cellulase in rice bran on the *in vitro* solubility of calcium (IVCa), iron (IVFe), and zinc (IVZn). It is shown that IVCa and IVZn were significantly improved by the application of phytase and cellulase, and the models of two second-order polynomials are recommended for prediction, with coefficients at $R^2 = 0.86$ and $R^2 = 0.88$, respectively. Interaction between protease and cellulase also significantly affected IVCa and IVZn. Cellulase had more efficiency than phytase on IVCa. Differing in its effect on Ca and Zn, phytase had a significant linear correlation with IVFe, and none of the other processing parameters exerted a significant effect. The largest increment for IVFe, IVCa, and IVZn occurred in the treatment with applications of phytase at 2.5 U g^{-1} and protease and cellulase at 0.2% and 1%, respectively, which were 3.3, 3.6, and 4.3 times, respectively, that of the untreated material.

KEYWORDS: Rice bran; phytase; protease; cellulase; solubility; calcium; iron; zinc

INTRODUCTION

Rice is the most important staple food in China. Influenced by eating habits and preference, people in China seldom consume brown rice. Rice bran is the most quantity of coproduct in rice milling industry. It was roughly estimated that about 10 million tons of rice bran are produced as a byproduct of milled rice every year in China.

Rice bran contains many nutrients, such as protein, fat, minerals, dietary fiber, and antioxidants, and it has high potential as a raw material for functional foods or nutraceuticals (*1*). However, it is currently underutilized for human food and has traditionally been primarily used in animal feed. This may due to its high contents of phytic acid, polyphenols, and fiber, which affect the taste on one hand, and decrease bioavailability of other nutrients, especially micronutrients, on the other hand. Considering its health-promoting properties, some researchers have advocated introduction of rice bran into special human diets. Huang et al. suggested to improve the possible deficiency of the body's mineral metabolism by the consumption of rice bran (*2*). Domene et al. found that only about 11% of zinc (Zn) from rice bran was nutritionally available and that supplementing with calcium (Ca) was detrimental, indicating that rice bran is not a recommended source of minerals although it has a high concentration of zinc (*3*). Rice bran should be treated so that it can be nutritionally improved and reasonably utilized.

It has been reported that many factors affect the bioavailability of minerals, among which phytic acid and dietary fiber are considered as major factors causing impaired absorption of several essential minerals, such as Ca^{2+}, Zn^{2+}, Mg^{2+}, and Fe^{2+} (*4*). Application of enzymes is an effective approach to reduce the effect of phytic acid and fiber. Phytase was widely used in animal feed to degrade phytic acid to reduce its negative nutritional aspect, especially on minerals and protein, and for its environmental effect, mainly on phosphorus pollution (*5−7*). Cellulase is used to degrade dietary fiber and weaken its binding and/or sequestering effects on minerals, because in some diets, dietary fiber content negatively correlated to mineral availability (*8, 9*). In recent years, protein and peptides were also studied for their effect on bioavailability of minerals. Protein may behave positively or negatively, depending on the source of protein and composition of polypeptides and amino acids. If protein from soybeans is the major source of protein, up to 15 mg of dietary zinc may not be sufficient to meet the daily zinc requirement for adult human subjects (*10*). Kim et al. found that when using protein from casein or pork, intestinal solubility and absorption of Fe was improved (*11*). Liang et al. also found that in brown rice, decreasing the content of phytic acid was not sufficient for an increase of *in vitro* solubility of Zn and deduced that other components also influence the availability of minerals in rice (*12*). In addition, we also found that application of phytase

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Table 1. Design Matrix and Variable Combination[a]

		variable levels				
independent variable	level code	−1.68	−1	0	1	1.68
phytase (U g^{-1})/P	x_1	0.50	0.90	1.50	2.10	2.50
protease (alcalase, %)/A	x_2	0.03	0.10	0.20	0.30	0.37
cellulase (%)/C	x_3	0.16	0.50	1.00	1.50	1.84

[a] Enzyme addition based on the dry matter weight basis of rice bran ($w \times 100/w = \%$).

affected the availability of Ca, Fe, and Zn in rice bran significantly (*13*).

The objective of this study is to evaluate the effect of treatment variables and interaction between enzymes on *in vitro* solubility of Ca (IVCa), Fe (IVFe), and Zn (IVZn) in rice bran. Response surface methodology (RSM) was selected in this study for analysis and a prediction model of different parameters, because RSM is an effective statistical method that uses a minimum of resources, and quantitative data from an appropriate experimental design to determine and simultaneously solve a multivariate equation.

MATERIALS AND METHODS

Rice Bran. White rice bran was provided by Hunan Jinjian Cereals Industry Co. Ltd. (Hunan, China) in July 2007. It was kept at −18 °C before use.

Phytase. A fungal phytase was obtained from DSM (Delft, The Netherlands), released only for scientific purposes. The activity is 6000 U g^{-1}, and the suggested dose, based on application in animal feeds, was 500 U kg^{-1} dry matter (optimum pH 2.5−5.5).

Protease and Cellulase. Protease (Alcalase 2.4 L FG, activity 20000 U g^{-1}, optimum pH 6.8−8.5) and cellulase (Celluclast 1.5 L, activity 700 EGU g^{-1}, optimum pH 5.0−7.0) were provided by Novozymes (China) Biotechnology Co., Ltd. (Tianjin, China) for scientific purposes only.

Experimental Design. In this study, a central-composite experimental design for $K = 3$ factors was used to evaluate the effect of three different enzymes used in rice bran on solubility of Ca, Fe, and Zn in a quadratic function. The variables were the applied concentrations of enzymes (phytase: 0.5−2.5 U g^{-1}; protease: 0.1−0.3%; cellulase: 0.5−1.5%, on dry-based rice bran). The design generated 20 observations which were distributed as follows: eight kernel points, six star points, and six replications at the central point. The design matrix and combination of variables is presented in **Table 1**.

Experimental Processing. Rice bran was ground (HY-04B, Beijing Xinhuanya, China) until passing through with a 0.5 mm sieve. About 30 g (accuracy 0.01 g) of rice bran powder was mixed with deionized water (1/5, w/v) and adjusted the pH to 6.0 using 1 M HCl, followed by enzymes addition. The mixture was incubated at 37 °C in a water bath shaker for 2 h (150 rpm). All experiments were carried out in duplicate. Treated rice bran with liquids was freeze-dried and stored in sealed plastic bags at −18 °C for further analysis.

***In Vitro* Digestion for Soluble Ca, Fe, and Zn.** The *in vitro* digestion procedure described by Kiers et al. was used with small modification (*14*). About 5 g (accuracy 0.0001 g) treated samples were digested in two steps using pepsin and pancreatin to simulate gastrointestinal conditions in human body. After digestion, the mixture was centrifuged (5000*g*) at 4 °C for 15 min, and the supernatants were filtered with a 0.22 μm membrane for analysis of Ca, Fe, and Zn. The *in vitro* digestions were carried out in duplicate. *In vitro* soluble mineral is defined as mineral in samples transferred into enzymatic solutions after enzyme extraction.

Contents of Ca, Fe, and Zn. Contents of minerals were analyzed by inductively coupled plasma atomic emission spectrometry (ICP-AES) after microwave-accelerated digestion. Particularly, approximately 0.3 g (accuracy 0.0001 g) of dried powder was mixed with 4 mL of nitric acid and digested in a microwave-accelerated closed vessel digestion system (Mars 5, CEM Corporation, Rockville, Maryland). Then, the concentration of Ca, Fe, and Zn in digested solutions was determined by ICP-AES (Optima 2000 DV, Perkin-Elmer Life & Analytical Sciences Inc., Massachusetts) at wavelengths of 317.933, 238.204, and 206.200 nm, respectively. The amounts of minerals were expressed as mg 100 g^{-1} dry matter. Analyses were in duplicate, and the average contents were used to calculate the solubility of minerals.

Statistical Analysis. Experimental data were analyzed using the Stat-Ease Design Expert 7.1.4 Trail Program. A second-order polynomial model was recommended to establish the relationship between the response (*Y*) and the variables (*X*) as follows:

$$Y = b_0 + \sum_{1}^{3} b_i X_i + \sum_{1}^{3} b_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} b_{ii} X_i X_i$$

where b_0 is a constant and can be interpreted as the estimated value of Y at the central point, b_i is a linear effect coefficient, b_{ij} is an interaction effect coefficient, and X_i is an independent variable (i, j = 1, 2, 3), given as the deviation from its mean value. The fitted polynomial equations were expressed in a 3D response surface, where the response is presented on the vertical axis and two factors at the two horizontal perpendicular axes. The proportion of variance explained by the polynomial models obtained is given by the multiple coefficients (R^2) of determination. The significance of each coefficient was also determined.

RESULTS

The concentration of calcium (Ca), iron (Fe), and zinc (Zn) in untreated rice bran was high, up to 45.7 mg 100 g^{-1}, 7.8 mg 100 g^{-1}, and 3.3 mg 100 g^{-1}, but the availability of minerals was very low, 16.1%, 6.4%, and 8.4%, respectively. Response values of different treatments are presented in **Table 2**. The polynomial equations fitted to experimental data using the Stat-Ease Design Expert 7.1.4 Trail program and the evaluated linear regression coefficients are presented in **Table 3**.

Impact of Processing Parameters on IVCa. Compared with untreated rice bran, IVCa of all treatments increased significantly (16%). The highest (57%) was obtained from treatment no. 10 (with the highest phytase addition) and the lowest (33%) from treatment no. 9 (with the lowest phytase addition). Phytic acid is the major factor inhibiting bioavailability of Ca.

RSM yielded a regression equation, which is an empirical relationship, between IVCa and the variables of applied of enzymes, $R^2 = 0.86$.

$$Y_1 = -9.02 + 23.82X_1 + 46.31X_2 + 50.87X_3 + 16.20X_1X_2 - 1.92X_1X_3 - 69.08X_2X_3 - 6.01X_1^2 - 19.24X_2^2 - 10.95X_3^2$$

Response of IVCa from the effect of enzymes and the interactions among the three enzymes are shown in the 3D graphic surface (**Figure 1**). It is clear that in this case, *P*, *C*, *AC*, and C^2 are significant model terms, with an increasing order: AC, C^2, P, C. Application of phytase and cellulase affected IVCa significantly at levels of $P < 0.01$ and $P < 0.001$, respectively

Table 2. Response for *in Vitro* Solubility of Ca, Fe, and Zn (mean of replicates (±SD), on dry basis)

treatment code	level codes			IVCa(%) Y_1	IVFe(%) Y_2	IVZn(%) Y_3
1	−1	−1	−1	34.53(±4.42)	9.31(±0.63)	12.95(±0.82)
2	1	−1	−1	38.13(±0.60)	7.96(±1.78)	18.95(±3.08)
3	−1	1	−1	35.23(±0.43)	7.65(±1.34)	11.26(±1.28)
4	1	1	−1	42.20(±0.40)	12.39(±0.93)	23.67(±1.09)
5	−1	−1	1	55.42(±3.60)	9.66(±1.69)	12.70(±2.69)
6	1	−1	1	56.25(±1.83)	16.13(±2.86)	25.30(±0.90)
7	−1	1	1	41.82(±3.36)	11.19(±0.63)	14.64(±1.10)
8	1	1	1	46.99(±4.11)	14.46(±0.81)	22.60(±1.21)
9	−1.68	0	0	32.74(±5.67)	9.85(±0.54)	16.13(±3.03)
10	1.68	0	0	57.22(±2.80)	21.01(±3.93)	35.75(±2.28)
11	0	−1.68	0	47.58(±3.87)	13.92(±0.37)	17.07(±0.64)
12	0	1.68	0	53.33(±2.67)	11.16(±1.85)	20.91(±6.39)
13	0	0	−1.68	33.32(±3.14)	11.55(±1.22)	16.05(±1.73)
14	0	0	1.68	53.19(±7.81)	14.14(±1.88)	23.43(±3.19)
15	0	0	0	51.51(±0.96)	11.76(±0.19)	12.97(±1.21)
16	0	0	0	49.75(±1.72)	9.04(±0.98)	10.55(±2.02)
17	0	0	0	49.77(±2.71)	11.30(±0.51)	11.58(±0.83)
18	0	0	0	47.30(±4.54)	9.25(±0.86)	11.43(±1.96)
19	0	0	0	50.84(±0.79)	12.27(±0.16)	13.97(±2.22)
20	0	0	0	48.95(±3.95)	8.21 ±(1.14)	10.43(±1.02)

Table 3. Coefficients of the Variables in the Model and Their Corresponding R^2

coefficient[a]	IVCa(%)	IVFe(%)	IVZn(%)
b_0 (constant)	−9.02	16.51	34.69
P	23.82[c]	−10.46[c]	−27.24[d]
A	46.31	−12.03	−54.04
C	50.87[d]	−2.45	−11.40
PA	16.20	6.07	3.72
PC	−1.92	2.67	0.90
AC	−69.08[b]	−7.28	−9.48
P^2	−6.01	3.50	11.48[d]
A^2	−19.24	21.80	160.83
C^2	−10.95[b]	1.30	7.49[b]
R^2	0.86	0.66	0.88

[a] *P* = coefficient for phytase addition, *A* = coefficient for protease addition, *C* = coefficient for cellulase addition. [b−d] Significant at p <0.05, p <0.01, and p <0.001, respectively. Data reported in this table are the measured values of the coefficients b_0, b_i, and b_{ij} in the quadratic equation.

(**Table 3**). Moreover, the interaction between protease and cellulase was also found to be significant ($p < 0.05$); however, protease alone had no significant effect on the IVCa level. These results were inconsistent with our single factor experiments previously (data not shown).

Impact of Processing Parameters on IVFe. IVFe levels in enzyme-treated rice bran fell in the range of 7.6−21%, which were 1.2−3.3 times higher than that in untreated ones (6.4%). IVFe showed a large variation (**Table 2**) with treatment, which is more perceptible at the central point of the design where nine replications were performed (treatment no. 15−20 in **Table 2**); the same phenomena was also found in Kayode's research (*15*). Like that for IVCa, the highest value of IVFe was also from treatment no. 10, but the lowest was from treatment no. 3 (0.9 U g^{-1} phytase, 0.3% alcalase, and 0.5% celluclase addition).

From the 3D graphic surface (**Figure 2**), we can clearly see that there are no apparent interactions among the three factors. Concentration of phytase was the only processing parameter affecting IVFe and had a significant linear correlation. Higher content of phytic acid strongly contributes to a lower availability of iron. The second-order polynomial model (R^2 = 0.66) was not strongly recommended. In addition, IVFe tended to decrease when being treated with protease.

Impact of Processing Parameters on IVZn. IVZn varied from 10.4 to 35.8%, significantly higher than for the level of untreated rice bran (8.4%). When addition of phytase reached the highest, IVZn reached the maximum. From **Figure 3**, it was also found that IVZn in samples from the central point (treatments no. 15−20 in **Table 2**) were lower than in other treatments.

The following second-order polynomial model with $R^2 = 0.88$ resulted from the use of the Stat-Ease Design Expert 7.1.4 Trail program:

$$Y_3 = 34.69 + 27.24X_1 + 54.04X_2 + 11.40X_3 + 3.72X_1X_2 - 0.90X_1X_3 - 9.48X_2X_3 - 11.48X_1^2 - 160.83X_2^2 - 7.49X_3^2$$

Application of phytase was the one processing parameter affecting IVZn, and its linear and quadratic terms were found to be significant (P < 0.01, **Table 3**). Application of cellulase was another factor; its quadratic terms affected IVZn in a significant level at p < 0.05 (**Table 3**).

Adequacy of the Model. The coefficient (R^2) being calculated for confidence of the variation in the response could be explained by the model. Higher R^2 relating to the regression model can be used with more confidence for prediction of response values. In this study, the coefficients of determination of IVCa and IVZn were $R^2 = 0.86$ and $R^2 = 0.88$ indicated that the proposed model explained 86% and 88% of the observation of IVCa and IVFe, respectively. However, the coefficient of determination of IVFe was lower (R^2 = 0.66), which meant the observations did not fit the model very well. A liner model was suggested for IVFe using Stat-Ease Design Expert 7.1.4 Trail Program. Furthermore, we plotted the experimental data against the values by the model (**Figure 4**). The points are scattered favorably around the straight line, which indicated that the data fitted the model.

DISCUSSION

The introduction of enzymatic treatment in the processing of rice bran brought significant improvements in mineral nutritional quality. IVCa, IVFe, and IVZn in rice bran were improved after the application of phytase, protease, and cellulase. The largest increment for IVFe, IVCa, and IVZn was 3.3, 3.6, and 4.3 times that for the untreated material, respectively, and better availability in the human body is expected and indicates that enzymatic treated rice bran could have potential as a good source of minerals. Correlation of IVCa and IVZn

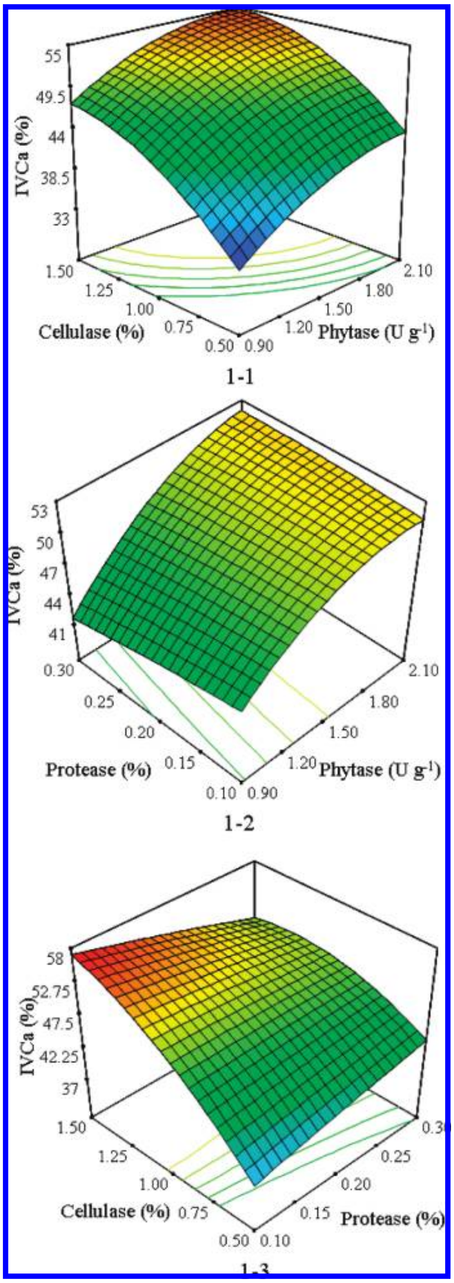

Figure 1. 3D graphic surfaces on the effect of phytase, protease, and cellulase addition on in vitro solubility of Ca.

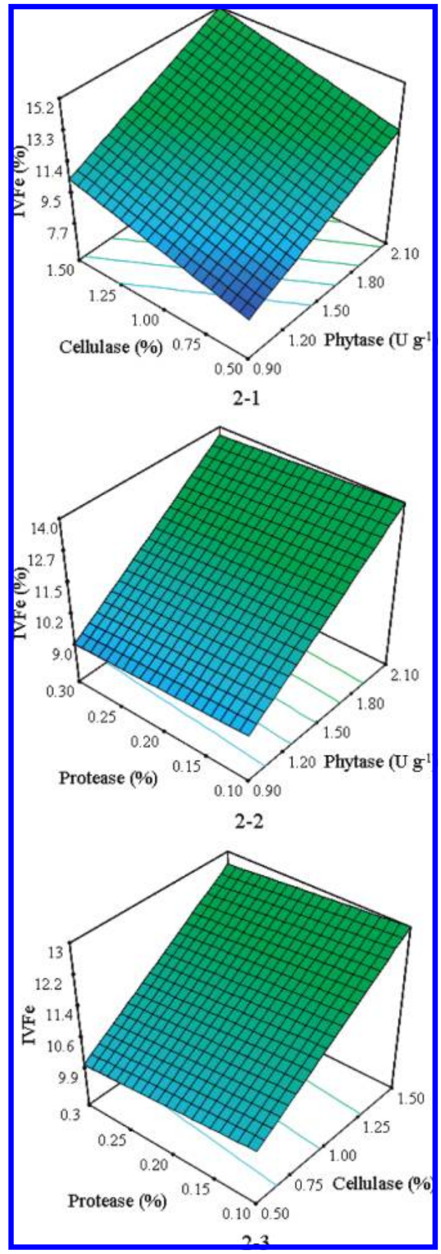

Figure 2. 3D graphic surfaces on the effect of phytase, protease, and cellulase addition on in vitro solubility of Fe.

improvement to addition of enzymes fitted the second-order polynomial models with a good coefficient, as analyzed with the Stat-Ease Design Expert 7.1.4 Trail program, and the maximum improvement occurred in the same treatment where the phytase is applied and at the highest concentration.

It was reported that minerals present as a complex with various components in foods (*16*) and the bioavailability of minerals will be affected by their forms and those components. Phytic acid is one of the most important inhibitors, which can form insoluble complexes with essential minerals such as Ca, Fe, and Zn at physiological pH and has a negative effect on their availability (*17*). In general, solubility of the mineral−phytate complex is dependent on environmental pH, types of mineral, and ratios of mineral and phytate, as well as the presence of other minerals. The average content of phytic acid in rice bran was 4.85%, which was almost 5-fold higher than that of brown rice as reported, and more than ten times higher than that of commercial white rice (*18*). To improve the availability of minerals from rice bran, phytic acid is the main factor that has to be considered. Phytic acid is degraded into biologically active forms through the action of phytase (*19*), and as a result, minerals are released from the mineral−phytate complex and result in a higher solubility. Our previous study also found that phytate decreases to an undetectable level after 2 h incubation with microbial phytase; however only 18% of the phytate degraded without addition of phytase (*13*). This may explain why IVCa, IVFe, and IVZn all had a positive correlation with application of phytase. Many reports supported this point. Studies about the effect of pea flours on rat growth showed that the digestive utilization of iron from raw peas could be negligible, while utilization of iron from pea flour treated with phytase was significantly improved (*20*). The same tendency was found in soybean infant formula. Significantly improved iron absorption was found after the enzymatic degradation of phytate by addition of microbial phytase (*4*).

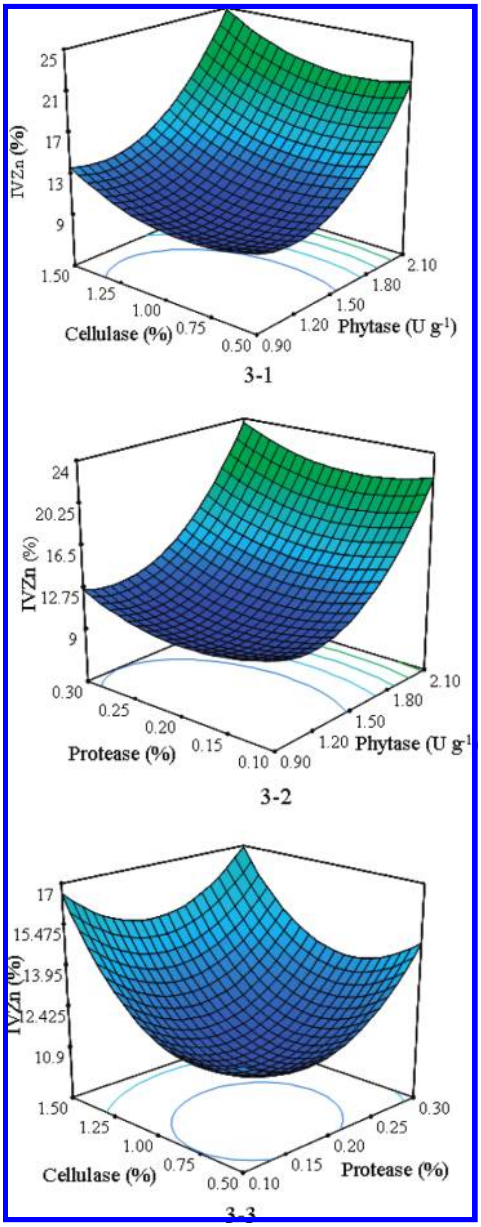

Figure 3. 3D graphic surfaces on the effect of phytase, protease, and cellulase addition on in vitro solubility of Zn.

Dietary fiber affected bioavailability of minerals and trace elements positively or negatively, while it differed with fractions of dietary fiber (*21*). When cellulase is added, elements may be released from the fiber molecule and become soluble, which means that higher absorption can result in the small intestine or that fractions of dietary fiber may be changed and the lower part of the digestive tract can behave as a putative site for mineral absorption. It is also possible that calcium was released from the calcium−fiber complex because of competitive interactions between calcium and other minerals in the fiber (*22*). In this study, cellulase also showed efficiency on IVCa similar to that of phytase, which means dietary fiber may be as important as phytic acid in the inhibition of Ca absorption from rice bran. This is different from Kamchan's results, which pointed out that fiber was not the most important factor for bioavailability of calcium from plant food, since some plants with low dietary fiber were also low in calcium dialysibility (*23*). All the points mentioned above serve to interpret the effect of cellulase on IVCa. For IVFe and IVZn, the situation is

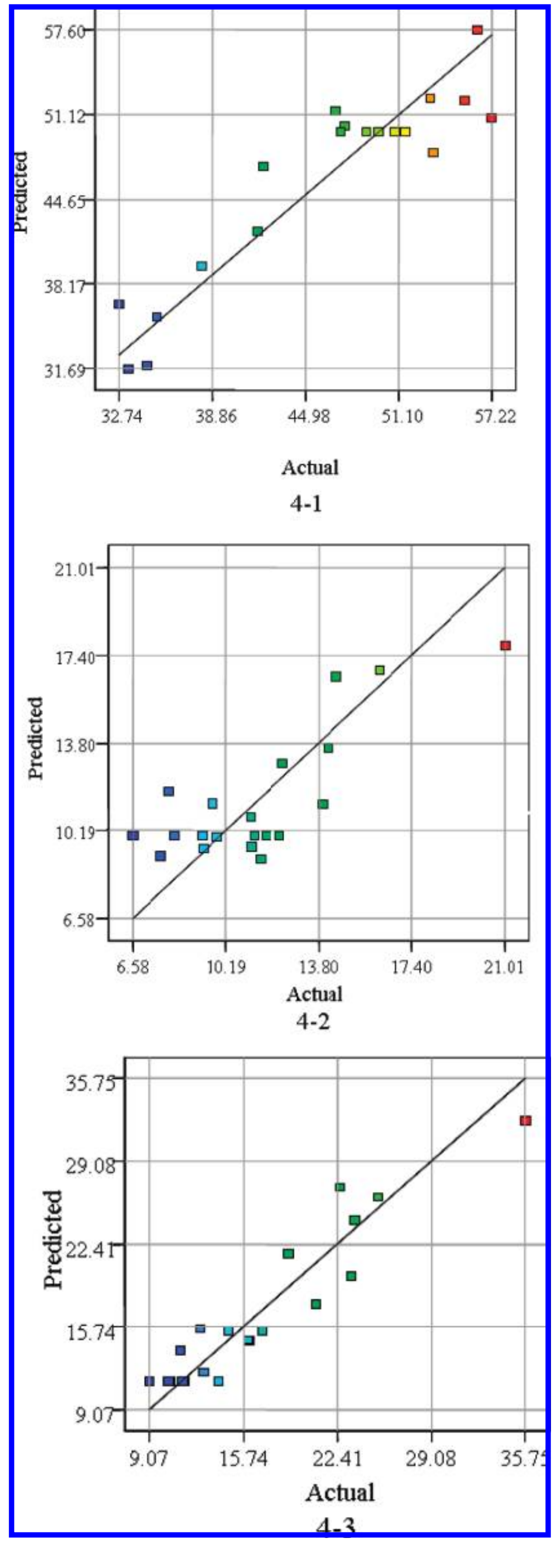

Figure 4. Response trace plots of experimental data against predicted value (%): (4−1) IVCa; (4−2) IVFe; (4−3) IVZn in rice bran.

different. Only phytase exhibited an important limiting effect on IVFe, and for IVZn, phytase showed more efficiency than cellulase. This is inconsistent with the report by Franz et al., which concluded that phytate rather than fiber is more important on limitation of the absorption of zinc from a diet rich in both phytic acid and dietary fiber (*24*). The difference in *in vitro* solubility of minerals from enzyme treatment may due to the binding capacity of the minerals. It was reported that cation capacity in binding with phytic acid is in the order $Cu^{2+} > Zn^{2+} >$

$Fe^{2+} > Ca^{2+}$, and the stability of the mineral−phytate complex is in the order $Zn^{2+} > Cu^{2+} > Ca^{2+}$ (*25*). The Ca−phytate complex has a weaker binding power than Zn−phytate, so phytic acid has less effect on Ca than Fe and Zn. Zn is the most sensitive element when considering the effect of phytic acid in rice bran.

Except for minerals, proteins exhibit the highest association to the fiber matrix. Studies on legumes showed that the protein was insoluble with dietary fiber and 23−43% of the total protein was associated to the insoluble fiber fraction (90%) (*26*). When protease is applied to protein, the bond between protein and fiber is broken, protein degrades to peptides and becomes soluble, which makes the fiber structure loosen, and calcium is released or calcium ion can chelate with soluble peptides or amino acids. As a result, the solubility of calcium increases. However, we also found that the application of protease decreased IVFe and IVZn slightly. This may be caused by the occurrence of nondigestible polypeptides during protease treatment and their interaction with minerals, leading to a peptide−cation complex and, consequently, decreased mineral absorption (*27*). Furthermore, an *in vivo* study of soy protein isolates indicated that the reduction in bioavailability of zinc is related to the formation of a protein−zinc−phytic acid complex resistant to hydrolysis (*28*).

The interaction between different minerals, such as calcium−zinc, calcium−iron, zinc−iron, and zinc−copper, has been reported to affect the availability of minerals (*15, 29*). For example, Fe can be in the ferric phytate (Fe-phytate) or the calcium iron phytate (Ca−Fe−phytate) form. These interactions may negatively affect iron bioavailability, but the influence of Ca on Fe absorption has not been elucidated (*30*). Domene et al. also found that Ca depresses zinc uptake in rats fed rice bran (*3*). This suggests that the relative concentrations of minerals present in the rice bran influence their bioavailability, but the hypotheses need further study.

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