Heat-moisture Treatments of Cowpea Flour and Their Effects on Phytase Inactivation

NICOLE S. AFFRIFAH, MANJEET S. CHINNAN, AND R. DIXON PHILLIPS

ABSTRACT: Samples of finely ground cowpea flour with moisture content adjusted to 10%, 25%, 35% (dry basis) were heated in sealed retort pouches at 70 to 95 °C for periods of 2 to 32 min. Phytase showed a high thermal resistance with residual activity ranging between 50% and 95%. Thermal inactivation of cowpea phytase was adequately described by a fractional conversion model based on a 1st-order rate equation. Overall, increasing temperature and initial moisture content resulted in increased enzyme inactivation. Estimated activation energies between 70 and 95 °C were 33.3, 37.9, and 43.4 kJ/mol at 10%, 25%, and 35% moisture, respectively. The kinetic models generated were successfully used to predict phytase activity in cowpea flour.

Keywords: phytase, cowpeas, inactivation kinetics, moisture content, fractional conversion

Introduction

Cowpeas are an important source of protein in the diets of many populations around the world providing a less expensive alternative to animal protein. Despite the high nutritional quality of cowpeas and other legumes, limitations such as the hard-to-cook defect decrease consumption and production. The basis of the defect is still under debate; however, a dual-enzyme mechanism involving phytase and pectin methyl esterase has been suggested (Jones and Boulter 1983). It is hypothesized that phytase acts on phytic acid in the cotyledon releasing inorganic phosphate, magnesium, and calcium ions while pectin methyl esterase hydrolyzes pectin to pectinic acid and methanol in the middle lamella (Mafuleka and others 1993). The calcium and magnesium ions move to the middle lamella forming calcium and magnesium pectates that subsequently restrict cell separation during cooking.

Several studies have examined the relationship between the development of the hard-to-cook defect and phytate content. Kon and Sanshuck (1981) reported a negative correlation between the phytic acid/calcium ratios and cooking time in dry beans. A low but significant correlation ($r = -0.716$) was observed between losses in phytate and cooking time in black beans suggesting that phytate is a contributor, but perhaps not the sole operating mechanism, in the hardening defect (Hincks and Stanley 1986). Longe (1983) also reported that phytic acid was the only chemical component among 4 others that showed a correlation with the cooking time of 13 varieties of cowpeas. Mafuleka and others (1993) recorded increased phytase activity and a strong positive correlation with cooked texture in decoricated Malawian red and white bean genotypes stored under different temperature, water activity, and time periods.

Phytases are a subfamily of high molecular weight histidine acid phosphatases that catalyze the hydrolysis of phytic acid to inositol and free orthophosphate (Liu and others 1998; Viveros and others 2000). The main end products of the reaction are phosphoric acid and myo-inositol with intermediate compounds such as phosphodiyl inositol representing various degrees of dephosphorylation from inositol hexakisphosphate to inositol (Liu and others 1998). The enzyme is widely distributed both in plants and animals as well as various fungi and bacteria species (Cosgrove 1966). Phytases are generally thermostable enzymes with pH optima between 4.0 and 5.6 (Dvorakova 1998). Although Jongbloed and Kemme (1990) previously showed that phytase was readily inactivated at temperatures of 70 °C or higher, Ma and Shan (2002) reported a high thermal stability in 3 cereal seeds. After heating for 1 h at 100 °C, rye2, wheat NEAU123, and triticale 5305 retained more than 80% of their original phytase activity.

Based on the reported relationship between phytase activity and cooking time of various legumes, we are hypothesizing that inactivating phytase could be used as a strategy in controlling the hard-to-cook defect. There is however a scarcity of information on the behavior of phytase following heat treatment. Thus understanding the inactivation kinetics would help in designing heat processes targeted at preventing or reducing the development of the hard-to-cook defect in legumes. The objectives of this study were to determine the effectiveness of heat treatments in inactivating phytase in cowpeas and to model and estimate the kinetics of thermal inactivation as a function of moisture content.

Materials and Methods

Freshly harvested cowpea seeds were obtained from SeedGrow, LLC (Meridian, Calif., U.S.A.) and stored at 4 °C until used. All chemicals used were of analytical grade and obtained from Sigma-Aldrich Chemical Co. (St. Louis, Mo., U.S.A).

Conditioning of cowpea flour

Cowpea seeds were finely ground in a Wiley laboratory mill (Model 4, Arthur H. Thomas Co. Philadelphia, Penn., U.S.A.) to pass through a nr 40 mesh sieve. The moisture content of the flour was adjusted by adding a pre-determined amount of water to the flour in a mixer. The amount of water required was calculated using the following equation:

$$W = \frac{A \times B \times C}{D},$$ (1)

where W is amount of water required (g/100 g), A is initial moisture content (g/100 g), B is dry matter in original sample (g/100 g), C is final moisture content (g/100 g), and D is dry matter in final sample (g/100 g).

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The experiments were performed at moisture contents of 10%, 25%, and 35% (dry basis). About 300 g flour was conditioned at each moisture content, sealed in high density polyethylene bags, and stored for 24 h at 4 °C to allow for equilibration. The sealed bags were kept at 4 °C until their use within a period of about 7 d. Moisture content was determined by a standard AOAC method (AOAC 1980).

Thermal inactivation experiments
A 6 × 5 factorial design with 6 levels of heating temperature (70 to 95 °C) and 5 levels of heating time (2 to 32 min) was used. Retort pouches (15.5 cm × 6.5 cm) obtained from CLP Packaging Solutions Inc. (Fairfield, N.J., U.S.A.) were used for the thermal inactivation study. About 3 g flour at the desired moisture content was thinly dispersed in the pouch to a thickness of 3.6 mm and vacuum sealed. The pouches were heated in an ethylene glycol bath with controlled temperature (Tdesired ± 3 °C). Thermal inactivation was carried out at heating temperatures ranging from 70 to 95 °C and exposure times between 2 to 32 min. The temperature in the pouch was estimated by inserting a 30 gauge k-type thermocouple with a 0.01-inch bead (Omega Engineering, Stamford, Conn., U.S.A.) in a similar pouch and recording the change in temperature over the heating time. The come-up time, which was the time required to reach 99% of the desired temperature, was between 10 and 20 s depending on the final temperature desired. The pouches were removed from the heating bath as a function of the inactivation time and were immediately cooled in a water-ice mixture for 5 min. The residual phytase activity in the flour sample was determined soon after using the method described below. A control sample that was not subjected to any heat treatment was included for comparison. The phytase activity in this control sample was considered as the initial phytase activity (A0). Thermal treatments of the flour were performed in triplicate.

Phytase activity assay
The phytase activity was measured by direct incubation with sodium acetate buffer according to the method described by Greiner and Egli (2003) with some slight modifications. The heat-treated sample (1 g) was suspended in 20 mL of 0.1 M sodium acetate buffer, pH 5.0, containing 100 μmol of sodium phytate preincubated at 45 °C. The reaction mixture was incubated at 45 °C for 30 min after which the amount of phosphorus released was determined using the method described by Eckhout and De Paepe (1994). A 2-mL portion of the reaction mixture was added to a test tube containing 2 mL of 10% trichloroacetic acid to arrest the reaction and then centrifuged at 10000×g for 5 min. One milliliter of the supernatant was then added to 1 mL of a color-forming reagent. The color reagent was a mixture of 4 parts of solution A (15 g ammonium heptamolybdate in 55 mL 36 N H2SO4, made up to 1 L) and 1 part of solution B (27 g of FeSO4·7H2O, a few drops of 36 N H2SO4, made up to 250 mL). The blue color formed was measured at 700 nm in a diode array spectrophotometer (Model 8451, Hewlett Packard, Palo Alto, Calif., U.S.A.) after centrifuging at 10000×g for 3 min to remove any cloudiness formed. A calibration curve was produced to cover the range of 1 to 4 μmol of phosphate and used to estimate the enzyme activity. The analysis was performed in triplicate and reported as units/kg flour. One unit of phytase activity was defined as the amount of phytase that liberates inorganic phosphorus from a 0.001-M Na-phytate solution at a rate of 1 μmol/min at pH 5 and 45 °C.

Kinetic data analysis
Inactivation of enzymes can be described by a 1st-order kinetic model and the inactivation rate constant (k) can be estimated using linear regression analysis (Wiley 1994; Whitaker 1994).

\[ A_t = A_0 \exp (-kt) \]  

where \( A_0 \) and \( A_t \) are the initial activity and residual activity at time \( t \), respectively.

Ly-Nguyen and others (2002) described a special case of the 1st-order model known as the fractional conversion model that was applied in this study. Their article provides an outline of the basis and details of the model, as shown in the following summary. Fractional conversion \( f \) accounts for the nonzero activity \( (A_n) \) after prolonged heating and can be expressed as

\[ f = \frac{(A_0 - A_n)}{(A_0 - A)} \]  

For most irreversible 1st-order reactions, \( A_n \) approaches zero, and Eq. 3 can be reduced to

\[ f = \frac{A_0 - A}{A_0} \]

A plot of the logarithm of \((1-f)\) against time results in a straight line with a rate constant expressed by the negative slope value:

\[ \ln(1-f) = -kt \]

Thus, when \( A_n \) approaches zero, Eq. 5 and 2 are identical.

To account for the nonzero activity after prolonged heating, the following form of the fractional conversion is employed:

\[ \ln(1-f) = \ln\left(\frac{A_0 - A_n}{A_0 - A}\right) = -kt \]

Rearranging Eq. 6 yields Eq. 7:

\[ A_t = A_n + (A_0 - A_n) \exp(-kt) \]

By plotting \( A_t \) against inactivation time at constant temperature conditions, the inactivation rate constant, \( k \), and the remaining activity, \( A_n \), can be estimated using nonlinear regression analysis. The nonlinear estimation function in STATISTICA (StatSoft, Tulsa, Okla., U.S.A.) was used with the sum of squares of the relative error between measured and calculated enzyme activity used as the optimization criterion.

The rate constants determined above were replotted in Arrhenius plots at the moisture contents studied and the activation energy \( (E_a) \) calculated from the slope according to Eq. 8 (Anthon and Barrett 2002).

\[ \ln(k) = \left(\frac{E_a}{RT}\right) + c \]

Results and Discussion
Phytase proved to be fairly heat stable. The enzyme retained almost 63% of its initial activity after subjecting to the highest temperature (95 °C) and time (32 min) combination at the lowest
Thermal inactivation of cowpea phytase...

moisture content (10%). The reduction in activity was determined to a large extent by the moisture content of the flour prior to heat treatment. Overall, the reduction in phytase activity relative to initial activity ranged between 5% to 37% for samples heated at 10% moisture content whereas heating at 25% and 35% moisture resulted in 20% to 56% and 29% to 64% reduction in phytase activity respectively. Other studies have also reported that plant phytase (dry flour) showed a high heat resistance. According to Ma and Shan (2002), wheat and rye phytase retained 89.47% and 104.64%, respectively, of their activity after heating at 100 °C for 1 h. All factors considered in the present study (moisture content, temperature, and time) had significant effects on the residual phytase activity following the heat treatments (Table 1). Increasing temperature has a general effect of increasing enzyme activity up to an optimum after which the activity decreases as the enzyme is progressively inactivated (Parkin 1993). It was therefore expected that residual phytase activity would decrease with increasing temperature. Additionally, the sensitivity of an enzyme to thermal inactivation is dependent on the water content of the environment such that enzymes in dry or semi-moist food systems tend to be more heat stable (Parkin 1993). This is because water is needed to promote the unfolding of proteins during thermal denaturation.

A plot of residual phytase activity as a function of heating time resulted in nonlinear curves irrespective of heating temperature or initial moisture content (Figure 1). The thermal inactivation of phytase in cowpea flour could not be adequately described by a simple 1st-order kinetic model. Generally, the plots showed an initial drop in phytase activity during the 1st few min of heating at each temperature. Beyond this, there appeared to be a gradual leveling in activity as the residual activity apparently stabilized. The results suggest that the decrease in phytase activity may be more dependent on heating temperature with the effect of heating time not being as pronounced especially for samples treated at high temperatures (80 to 95 °C). None of the treatment combinations studied resulted in complete inactivation of the enzyme. Although it is not known if isozymes of cowpea phytase exist, multiple enzymes have been reported for some plant seeds including lettuce (Shannon 1968), lupine (Greiner 2002), and soybean (Hamada 1996). The use of whole flour rather than a purified enzyme fraction could have introduced isozymes that could account for the deviation from simple 1st-order kinetics. It has also been reported that there is an association between phytase and a non-specific acid phosphatase also capable of hydrolyzing phytic acid (Lolas and Markakis 1977).

The data were adequately described by a fractional conversion model in the temperature range studied (70 to 95 °C). This suggests the presence of a stable enzyme fraction that was apparently not significantly affected by the processing conditions under study. This behavior was observed at all the 3 moisture contents studied. The fitting of this model to the experimental data is shown in Figure 1. The fractional conversion model was used to estimate the kinetic inactivation parameters: namely, the initial phytase activity (A₀), the inactivation rate constant (k), and the amount of enzyme remaining after treatment (Aₐ). These parameters were estimated by fitting individual data points to the model using nonlinear regression and are summarized in Table 2.

The initial phytase activity estimated using nonlinear regression was found to be between 81.4 and 84.0 units/kg. This was comparable to the average initial activity (82.3 units/kg) measured in the samples. The fraction remaining after the longest heating time varied with the heating temperature and initial moisture content. The values ranged between 32 and 72 units/kg representing approximately 38% and 88% of the initial activity, and decreased with increasing temperature and moisture content (Table 2). The high values obtained are indicative of the high thermal stability of the phytase enzyme. The variation in A₀ values implies that the susceptibility of the stable enzyme fraction to heat inactivation is dependent on treatment combinations. This further suggests that under the appropriate conditions of heating temperature and moisture content, it might be possible to completely inactivate the enzyme.

### Table 1—ANOVA summary table for phytase inactivation

<table>
<thead>
<tr>
<th>Factor</th>
<th>DF</th>
<th>F-ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>2</td>
<td>90515.20a</td>
</tr>
<tr>
<td>Temperature (Temp)</td>
<td>5</td>
<td>13065.00a</td>
</tr>
<tr>
<td>Time</td>
<td>4</td>
<td>2733.65a</td>
</tr>
<tr>
<td>Moisture × Temp</td>
<td>10</td>
<td>440.76a</td>
</tr>
<tr>
<td>Temp × Time</td>
<td>20</td>
<td>8.01a</td>
</tr>
<tr>
<td>Moisture × Time</td>
<td>8</td>
<td>105.96a</td>
</tr>
<tr>
<td>Moisture × Temp × Time</td>
<td>40</td>
<td>26.76a</td>
</tr>
</tbody>
</table>

*aSignificant at P < 0.01*

### Figure 1—Thermal inactivation of phytase in cowpea flour as a function of heating time at various temperatures and moistures
or reduce it to negligible levels. The effect of temperature on the inactivation rate constants was described using the Arrhenius equation and is shown in Figure 2. The inactivation rate constants increased with increasing temperature as expected. This temperature dependence of the inactivation rate constant is explained by the concept of activation energy (Morales-Blancas and others 2002). The calculated activation energies, $E_a$ in the temperature range 70 to 95 °C were 33.3 ± 0.4, 37.9 ± 0.6, and 43.4 ± 0.3 kJ/mol, respectively at 10%, 25%, and 35%. Peers (1953) reported an inactivation energy value in the temperature range 55 to 65 °C of 171.5 kJ/mol for partially purified phytase in wheat. The typical range for inactivation energy for enzymes is 209 to 628 kJ/mol (Parkin 1993).

Enzyme activity relative to the unheated control was significantly reduced by heating moistened cowpea flour. Increasing moisture content generally facilitated the thermal inactivation of phytase. The variation of the inactivation rate constant and inactivation energy with moisture content are shown in Figures 3 and 4, respectively. There was a general increase in the rate constant with increasing moisture content confirming the hypothesis that the heat stability of enzymes increases at low moisture contents. At higher moisture contents, the increased availability of solvent water results in an increased opportunity for denaturation of the enzyme. The thermal stability of phytase at different initial moisture content according to $k$ values would therefore be as follows: 35% < 25% < 10%. The activation energy, $E_a$ was highly correlated with the moisture content ($R^2 = 0.972$). This dependence on the moisture content is also obvious from Figure 4 where increasing moisture content resulted in an increase in $E_a$. Because $E_a$ decreases at the lower moisture contents, it suggests that the susceptibility of phytase to thermal inactivation decreases at the lower moisture content. Several results in literature have also shown this dependence of the activation energy for deteriorative reactions on the moisture content of the particular food product (Labuza 1980; Buera and others 1984). The nonlinear curves obtained for the inactivation of phytase could have resulted from thermal inactivation being a water-catalyzed reaction dependent upon successive

### Table 2—Kinetic parameters for phytase inactivation in cowpea flour

<table>
<thead>
<tr>
<th>Moisture content (%)</th>
<th>Temp. (°C)</th>
<th>$A_o$ (units/kg)</th>
<th>$A_o/\ln(1557)$ (units/kg)</th>
<th>k (min$^{-1}$)</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>70</td>
<td>81.95 ± 0.19a</td>
<td>72.66 ± 0.17</td>
<td>0.169 ± 0.012</td>
<td>0.937</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>82.24 ± 0.15</td>
<td>71.80 ± 0.08</td>
<td>0.229 ± 0.007</td>
<td>0.978</td>
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<tr>
<td></td>
<td>80</td>
<td>81.56 ± 0.19</td>
<td>68.60 ± 0.17</td>
<td>0.237 ± 0.029</td>
<td>0.915</td>
</tr>
<tr>
<td></td>
<td>85</td>
<td>81.78 ± 0.37</td>
<td>65.68 ± 0.37</td>
<td>0.240 ± 0.019</td>
<td>0.977</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>81.97 ± 0.22</td>
<td>60.24 ± 0.27</td>
<td>0.345 ± 0.016</td>
<td>0.951</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>82.22 ± 0.19</td>
<td>57.03 ± 0.39</td>
<td>0.407 ± 0.016</td>
<td>0.926</td>
</tr>
<tr>
<td>25</td>
<td>70</td>
<td>81.84 ± 0.18</td>
<td>58.96 ± 0.20</td>
<td>0.421 ± 0.024</td>
<td>0.938</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>83.06 ± 0.24</td>
<td>56.43 ± 0.29</td>
<td>0.466 ± 0.023</td>
<td>0.934</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>81.43 ± 0.17</td>
<td>46.76 ± 0.19</td>
<td>0.604 ± 0.011</td>
<td>0.975</td>
</tr>
<tr>
<td></td>
<td>85</td>
<td>81.53 ± 0.17</td>
<td>42.89 ± 0.25</td>
<td>0.663 ± 0.032</td>
<td>0.951</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>83.98 ± 0.17</td>
<td>39.90 ± 0.23</td>
<td>0.782 ± 0.021</td>
<td>0.966</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>82.32 ± 0.17</td>
<td>38.65 ± 0.16</td>
<td>1.104 ± 0.023</td>
<td>0.981</td>
</tr>
<tr>
<td>35</td>
<td>70</td>
<td>82.18 ± 0.17</td>
<td>51.67 ± 0.16</td>
<td>0.678 ± 0.022</td>
<td>0.986</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>82.32 ± 0.16</td>
<td>47.20 ± 0.12</td>
<td>1.136 ± 0.024</td>
<td>0.942</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>82.34 ± 0.16</td>
<td>41.14 ± 0.09</td>
<td>1.290 ± 0.016</td>
<td>0.980</td>
</tr>
<tr>
<td></td>
<td>85</td>
<td>82.37 ± 0.00</td>
<td>36.00 ± 0.02</td>
<td>1.596 ± 0.068</td>
<td>0.988</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>82.37 ± 0.00</td>
<td>33.49 ± 0.02</td>
<td>1.849 ± 0.083</td>
<td>0.988</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>82.38 ± 0.00</td>
<td>31.57 ± 0.03</td>
<td>2.052 ± 0.125</td>
<td>0.994</td>
</tr>
</tbody>
</table>

*aAsymptotic standard error.

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![Figure 2—Thermal dependence of rate constant for thermal inactivation of cowpea phytase](image1)

![Figure 3—Effect of moisture content on loss of phytase activity in heated cowpea flour](image2)
Phytase from cowpea flour exhibits a high heat resistance between moisture contents of 10% and 35% (dry basis). The thermal inactivation of the enzyme is highly dependent on the heating temperature and moisture content of the medium. Although the inactivation did not follow typical 1st-order kinetics, a modified 1st-order model using the same factors was used to successfully describe and predict the residual phytase activity in cowpea flour.

This study was carried out to provide information on the factors that significantly impact the inactivation of cowpea phytase and further apply this knowledge in developing heat processes for cowpea seeds. Overall, the results indicated that the highest degree of inactivation was achieved under conditions of high temperature, high moisture content, and within the 1st 4 min of exposure to heat. To strike a balance between achieving a sufficient degree of phytase inactivation (to affect its contribution to cowpea hardening), and retaining as much of the physicochemical characteristics of the treated cowpeas; the intermediate moisture content (25%) used in this study would be selected for further study. Additionally, the cowpea seeds would be briefly exposed to heat treatment because the decrease in phytase activity occurs fairly quickly.

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Thermal inactivation of cowpea phytase...