pH-stat Method to Evaluate the Heat Inactivation of Subtilisin Inhibitor in Legumes

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A pH-stat technique was used to evaluate the effect of heat treatments on removal of Subtilisin inhibitor in chickpea flour. Chickpea flours, that had been heated to different extents, were hydrolysed with a commercial protease, Alcalase 0.6 L. Initial rates of hydrolysis were determined at several enzyme concentrations. Useful information about enzyme inhibitor removal was obtained. Results indicated that pH-stat method has some advantages over other commonly used procedures.

Key words:
Subtilisin, pH-stat, protease inhibitors, chickpea, Alcalase, hydrolysis.

Introduction

Vegetable protein is being employed in ever increasing quantities in the food industry. Thus, legume seeds are used to manufacture nutritional protein hydrolysates. However, before using legume seeds, removal of inhibitors of digestive enzymes is recommended. Besides the digestive enzyme inhibitors, other protease inhibitors are present in legumes seeds. One of them is the Subtilisin inhibitor. Subtilisins are serine proteases, like digestive enzymes. They are the quantitatively most significant industrial proteases and particularly efficient in hydrolysing legume protein.[1–3] Then, removal of inhibitors of Subtilisin is also recommended when hydrolysis of legumes is carried out with Subtilisin.

Heat treatment is normally used to remove the protease inhibitors. Consequently, methods for determining the effect of the heat treatments on inhibitor removal are necessary.

The methods, most used at present for determination of inhibitor activity, consist of a prior extraction of inhibitor from seeds and a subsequent analysis through enzymatic hydrolysis: a given protein substrate (casein, BAPA, TAME,...) is hydrolysed by the enzyme which inhibitor is analysed in presence of some amount of extracted inhibitor.[4–6] Nevertheless, these methods need several hours to obtain results of inhibitor activity and the activity of enzyme for the assay has to be perfectly known.

The pH-stat method, usually used for controlling proteolytic reactions[7], has been also used to determine the effects of heat treatments on the removal of digestive enzymes, trypsine, in legumes.[8,9] Then, it may be useful in examining the effects of heat treatments on removal of Subtilisin inhibitor.

The purpose of this study is to check if the pH-stat method can be used as an alternative method to evaluate the effects of heat treatment on Subtilisin removal in legumes.

To prove this possibility, the hydrolysis of a heated and unheated legume, chickpea (*Cicer arietinum*), with a commercial enzyme used in the food industry, Alcalase, has been controlled by pH-stat method. Results obtained for Subtilisin inhibitor have been compared with these obtained for trypsin inhibitor in order to check the relationship between both effects.

Materials and methods

Chickpea substrate

Commercial chickpeas (*Cicer arietinum*), grown in Spain, served as starting material. The chosen chickpea was a small-seeded variety with a cream-coloured coat. The deshusked seeds were ground to flour and passed through a 350 μm mesh sieve.

Chickpea flour samples of 50 g were heated in 250 ml porcelain evaporating dishes at 140 °C for 1, 3, 6, 12 and 24 hours.

Moisture analysis

Moisture was determined in the unheated and heated flours by drying at 105 °C in air for 16 hours.[7] All moisture analyses were done on triplicate samples. Average standard deviation and average variation coefficient obtained from these experiments were 0.0026 and 0.0003, respectively.

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Protein analysis

Protein content (N x 6.25) was calculated from semi-micro Kjeldahl technique[7] in the heated and unheated flours. All protein analysis were done on triplicate samples. Average standard deviation and average variation coefficient obtained from these experiments were 0.3300 and 0.0014, respectively. Protein content remained constant in all treatments and was similar to that of unheated flour (0.227 kg · kg $^{-1}$ in dry basis).

Enzyme

Enzyme preparation used for pH-stat and Subtilisin inhibitor assays was Alcalase 0.6L (Novo Industri), a non-specific bacterial endo-peptidase from *Bacillus licheniformis*, with Subtilisin Carlsberg as its main component (specific activity = 6 · 10^2 AU · kg $^{-1}$, density of 1260 kg.m^{-3}). Alcalase is a food grade endo-peptidase which is particularly efficient in hydrolysing legume protein.[1–3]

Enzyme preparation, used for trypsin inhibitor assays, was Pancreatic Trypsin Novo (PTN), a serine protease derived from porcine pancreas, with trypsin as the major component.

Activity analysis

Subtilisin activity and subtilisin inhibitor activity were measured as described by *Bergmeyer*.[5] Five analyses were carried out per sample. Average standard deviation and average variation coefficient (standard deviation/mean value) obtained from these experiments were 0.1 and 0.04, respectively. One subtilisin unit (SU) is arbitrarily defined as an increase of 0.01 absorbance units at 280 nm under standard conditions of assay. Subtilisin inhibitor activity is defined as the number of subtilisin units inhibited (SUI).

Trypsin activity and trypsin inhibitor activity were assayed by the method of *Hamerstrand* et al.[6] Five analyses were carried out per sample. Average standard deviation and average variation coefficient obtained from these experiments were 0.1 and 0.01, respectively. One trypsin unit (TU) is arbitrarily defined as an increase of 0.01 absorbance units at 410 nm under standard conditions of assay. Trypsin inhibitor activity is defined as the number of trypsin units inhibited (TUI).

pH stat assays

Hydrolysis experiments were carried out in a 0.5 l batch jacketed reactor equipped with a burette for the addition of the base, a stirrer, a pH-electrode and a thermometer, for mixing, pH and temperature control in the reactor, respectively. In a previous paper apparatus was described in detail.[10]

Samples were prepared by suspending a given amount of chickpea flour (0.01–0.05 kg) in 0.4 l of hot water (50 °C). Solutions were then adjusted to pH 8.0 with NaOH 2 N l^{-1}. Alcalase was added (0.3–1.5 kg · m^{-3}) and stirred (300 rpm). Temperature and pH remained constant at 50 °C and 8, respectively. These were conditions of maximal activity and stability for Alcalase.[11] All hydrolysis experiments were done on duplicate samples. Average standard deviation and average variation coefficient obtained from these experiments were 0.03 and 0.02, respectively.

Hydrolysis degree

Hydrolysis was followed by the base consumption, NaOH 2 mol l^{-1}. The base required to keep pH constant is proportional to the hydrolysis:[7]

$$DH = \frac{V_B N_B}{m_p} \frac{1}{\alpha} \frac{1}{h_t} \quad (1)$$

where V_B is the consumption of base, N_B is the concentration of the base, α is the average degree of dissociation (0.885 for 50 °C and pH 8)[12], m_P is the mass of protein, and h_t is the total number of peptide bonds per mass unit (because of its similarity to soy meal, for chickpea protein an approximate α value of 7.8 eqv kg $^{-1}$ can be used).[7]

Enzymatic hydrolysis rate

The initial rate of the hydrolysis reaction was calculated from the DH and initial substrate concentration:

$$r_0 = \gamma_{s_0} \frac{dDH}{dt} \quad (2)$$

The reaction was run for ten minutes but only the initial, straight-line portion of the hydrolysis curves (5 minutes) was used to calculate rates of hydrolysis. All straight lines were fitted to the data by least squares.

Results and discussion

The chickpea flours used for the pH-stat experiments were either extensively heated or unheated.

To check the presence of Subtilisin inhibitor (SI) in unheated chickpea flour, hydrolysis rate was measured with 0.89 kg · m $^{-3}$ of Alcalase for different concentrations of sample. The increase of the hydrolysis rate with decreasing substrate concentration (Figure 1) suggests that some Subtilisin inhibitor is present in the unheated flour. This presence is supported by *Chavan* and *Hejgaard*,[13] *Chavan* et al.,[14] *Seidl* et al.,[15] and *Márquez* and *Fernández*,[16]

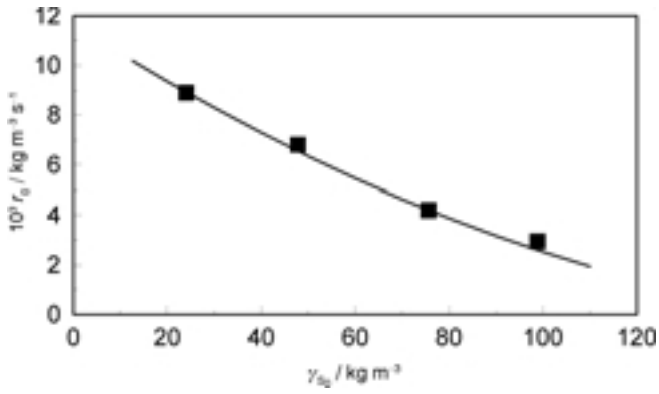

Fig. 1 – *Effect of substrate mass concentration (unheated chickpea flour) on hydrolysis rate with 0.89 kg · m^{-3} of Alcalase.*

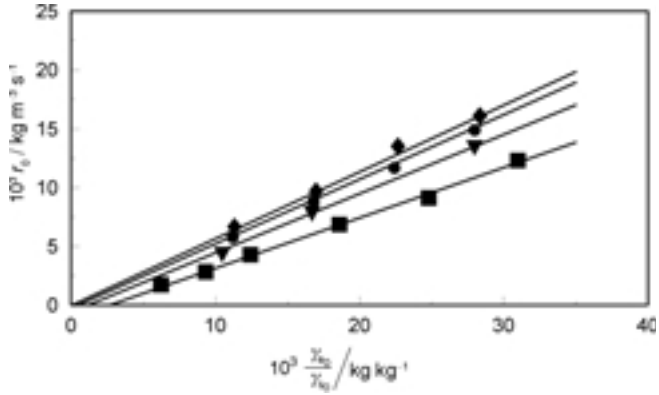

Fig. 2 – *pH stat assays at several Alcalase concentrations comparing samples of unheated and heated chickpea flour (■ heating time = 0 hours, ▼ heating time = 3 hours, ● heating time = 6 hours, ◆ heating time = 12 hours).*

Table 1 – *Chemical analyses of unheated and heated samples*

Heat treatment (h)	(SUI/kg flour*)·10^{-6}	(TUI/kg flour*)·10^{-6}
0	4.28	11.75
1	3.25	10.83
6	0.97	3.99
12	0.17	1.14
24	0.01	0.57

*dry mass basis

who found Subtilisin inhibitor in chickpea. The proof of the SI presence in the unheated sample (Table 1) was obtained by directly measuring the inhibitor activity with the accepted chemical method of *Bergmeyer.*[5]

The Subtilisin inhibitor removal was obtained by heating the flour for several time periods at 140 °C. This treatment was chosen because the inactivation of the inhibitor is then reached slowly and a better observation of the process is possible.

Each line in figure 2 is the result of pH-stat assays at one heat treatment and for several Alcalase concentrations. When the lines are extrapolated to zero rates of enzymatic hydrolysis, the intercept on the enzyme axis is a measure of the Subtilisin inhibitor amount for the amount of substrate.

Since mass relationship between Subtilisin and Subtilisin inhibitor is not perfectly known, we have preferred to use mass (kg) of Subtilisin inhibited by mass (kg) of flour as a measure for inhibitor. Further, the purity of the Subtilisin used in the assay will affect the quantity of Subtilisin inhibitor found when a factor is used to convert Subtilisin inhibited to Subtilisin inhibitor.

One important problem in this kind of analysis is that unfortunately most commercial preparations of Subtilisin are far from pure, so that it becomes necessary to establish the purity of the Subtilisin being used. Due to this problem, when inhibitor activity was assayed by the chemical method of *Bergmeyer*,[5] inhibitor units were expressed in function of absorbance units (Table 1), and comparison of Subtilisin inhibitor obtained using pH-stat and Bergmeyer method was carried out from "remaining inhibitor activity" (RIA) or percentage of inhibitor non-inactivated. Figure 3 shows pH-stat gave RIA values which had a good correlation with RIA values obtained from Bergmeyer assay.

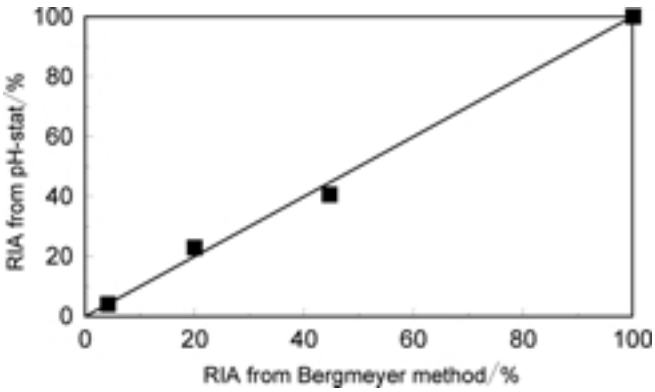

Fig. 3 – *Comparison of remaining inhibitor activity (RIA) for Subtilisin using pH-stat and Bergmeyer assay.*

However, in this study it is not necessary to know the purity of Subtilisin in Alcalase, because a comparative analysis is proposed and then we only want to determine the treatment for which Subtilisin inhibitor is not present in the chickpea (line $r_o/(e_o/s_o)$ crossing the coordinate origin). Thus, Subtilisin inhibitor presence has been measured as weight of Alcalase inhibited per amount of dry flour.

When we plotted the intercept of the abscissa (mass of Alcalase inhibited by amount of dry flour)

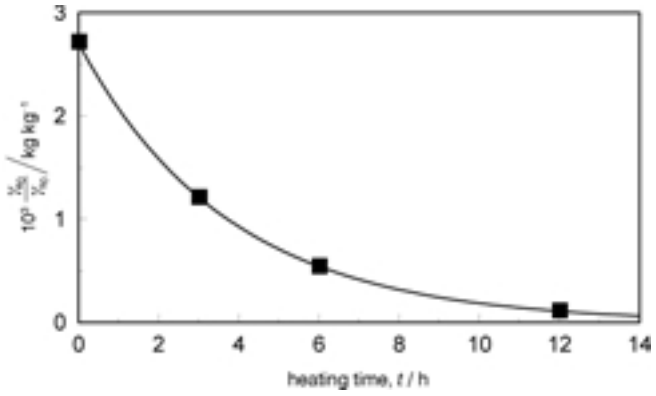

Fig. 4 – *Relationship between heating time and weight of Alcalase inhibited per amount of flour.*

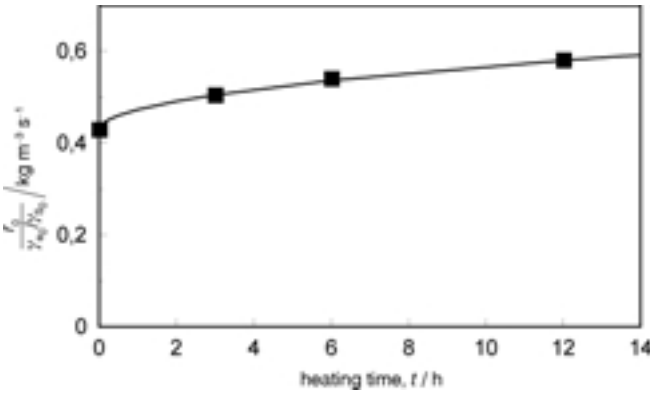

Fig. 6 – *Relationship between heating time and the slope of the lines in Figure 2.*

against heating time an exponential curve resulted (Figure 4), which can be fitted to:

$$\frac{\gamma_{e_0}}{\gamma_{s_0}} = e^{-(0.267t+5.913)} \quad r^2 = 0.999 \qquad (3)$$

This curve shows how the heat treatment of chickpea results in a decrease of Subtilisin inhibitor activity. This decrease of Subtilisin inhibitor activity is similar to decrease of trypsin inhibitor activity (TI) obtained for the same samples (Table 1). To check the relationship between SI and TI removal, "remaining inhibitor activity", or percentage of inhibitor non-inactivated, was determined (Figure 5). Then, the Subtilisin inhibitor removal seems indicative of a simultaneous removal of inhibitors of digestive enzymes.

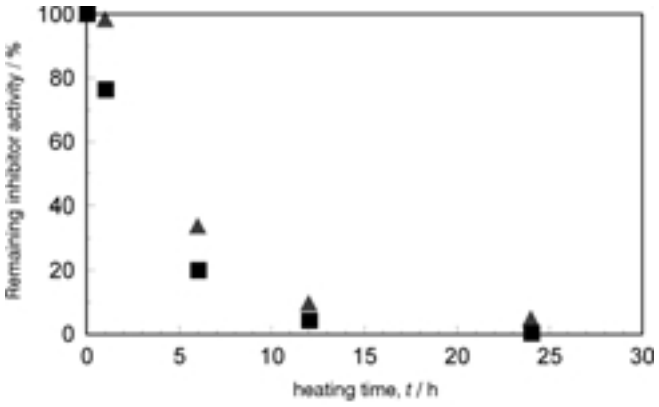

Fig. 5 – *Remaining inhibitor activity for Subtilisin ■ and trypsin ▲.*

The slopes of the lines for rates vs amounts of inhibited Alcalase in Figure 2 seem also related to heat treatment. As the heat treatment decreases, the slope decreases, indicating less susceptibility to hydrolysis. The representation of these slopes versus heating times (Figure 6) fits to:

$$\frac{r_0}{\gamma_{e_0}/\gamma_{s_0}} = 0.428 + 0.045\,t^{0.491} \quad r^2 = 0.999 \qquad (4)$$

It is well-known, that for legume proteins heat treatments result in: i) inactivation of protease inhibitors, and ii) denaturation of the storage proteins. Both effects result in an increased susceptibility to hydrolysis. Figure 6 shows how the heat treatment of chickpea results in an increase of the susceptibility to hydrolysis.

Using equations (3) and (4), data for 1 and 24 hours heated samples were determined. As can be seen from table 2, experimental data fit perfectly theoretical predictions.

Table 2 – *Inhibited Alcalase, calculated and experimental reaction rate*

Heat treatment (h)	$\gamma_{e_0}/\gamma_{s_0}$ 10^3 kg kg^{-1}	$r_{o\ calculated} \cdot 10^3$ kg m^{-3} s^{-1}	$r_{o\ experimental} \cdot 10^3$ kg m^{-3} s^{-1}	Relative Error %
1	18.56	8.78	8.92	1.6
24	17.65	11.31	11.48	1.5

Conclusions

pH-stat can be used to determine the effects of heat treatment on the removal of Subtilisin inhibitor in legumes. The measurement of enzymatic inhibitor inactivation by pH-stat method has some inherent advantages over other commonly used procedures. This method allows a direct analysis of inhibitor activity from legume: previous inhibitor extraction is not necessary and assay substrate is the same legume protein. Moreover, the knowledge of the activity of enzyme for the assay is not essential. Thus, the results are obtained quickly: an average experimental time of about 5 minutes for an assay is

adequate. In comparison, the typical methods require several hours to carry out the extraction of inhibitor and the analysis of its activity.

If enzymes and operating conditions, used for the pH-stat analysis, are the same as the industrial process for manufacturing nutritional protein hydrolysates, effects of heat treatment on inhibitor removal in substrate and kinetic aspects of food protein hydrolysis can be simultaneously obtained.

Finally, the similar effects of heat treatment on Subtilisin inhibitor and on trypsin inhibitor could allow to use the removal of Subtilisin inhibitor as indicative of the removal of digestive inhibitors.

Nomenclature

DH – hydrolysis degree, dimensionless

h_t – total number of peptide bonds in a protein, mol · kg $^{-1}$

m_p – mass of protein, kg

N_B – concentration of the base, kmol · m $^{-3}$

r_o – initial reaction rate, kg.m $^{-3}$.s $^{-1}$

RIA – remaining inhibitor activity, %

t – hydrolysis time, s

TI – trypsin inhibitor

SI – Subtilisin inhibitor

V_B – base consumption, m^3

α – degree of dissociation of the α-amino group

γ_{e_0} – initial enzyme concentration, kg · m $^{-3}$

γ_{S_0} – initial substrate concentration, kg · m $^{-3}$

References

1. *Clemente, A., Vioque, J., Sánchez-Vioque, R., Pedroche, J., Bautista, J., Millán, F.,* Food Chem. **67** (1999) 269.
2. *Olsen, H. S.,* A survey on recent developments in industrial processing of Vicia faba protein. In Bond D. A. (Ed.), Vicia faba: feeding value, processing and viruses, ECSC, EEC, EAEC, Bruxelles, 1980, pp 233-255.
3. *Adler-Nissen, J.* (Novo Industri). US Patent 4,100,024 (1978).
4. *Kakade, M. L., Simons, N., Liener, I. E.,* Cereal Chem. **46** (1969) 518.
5. *Bergmeyer, H. U.* (Ed), Methods of Enzymatic Analysis, Vol. 2., Academic Press, New York, 1974, pp. 1018-1021.
6. *Hamerstrand, G. E., Black, L. T., Glover, J. D.,* Cereal Chem. **58** (1981) 42.
7. *Adler-Nissen, J.*, Enzymic Hydrolysis of Food Proteins, Elsevier, New York, 1986.
8. *Stinson, C. T. & Snyder, H. E.,* J. Food Sci. **45** (1980) 936.
9. *Hill, B. S., Snyder, H. E., Wiese, K. L.,* J. Food Sci. **47** (1982) 2018.
10. *Márquez, M. C., Fernández, V.,* Chem. Biochem. Eng. Q. **12** (3) (1998) 121.
11. Novo Industri, A/S, Alcalase Calidad Alimentaria. B-318, Novo Industri A/S, Bagsvaerd, Denmark, 1991.
12. Novo Industri, A/S, Use of Food Grade Alcalase or Neutrase for the Controlled Enzymatic Hydrolysis of Proteins, B163, Novo Industri A/S, Bagsvaerd, Denmark, 1980.
13. *Chavan, J. K. & Hejgaard, J.,* J. Sci. Food and Agric. **32** (1981) 857.
14. *Chavan, J. K., Kadar, S. S., Salunkhe, D. K.*, CRC Crit. Rev. Food Sci. Nut. **25** (1986) 119.
15. *Seidl, D., Lorenzo, P., Pinelli, B. E., Tovar, J.* Biol. Chem. Oppe-Seyles **369** (1988) 69.
16. *Márquez, M. C., Fernández, V.,* Process Biochem. **28** (1993) 481.