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Effect of heat, rutin and disulfide bond reduction on *in vitro* pepsin digestibility of Chinese tartary buckwheat protein fractions

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Abstract

Protein fractions (albumin, globulin, prolamin and glutelin) were extracted from defatted tartary buckwheat flour. The *in vitro* pepsin digestibilities of the four protein fractions were different, and albumin was more susceptible to pepsin hydrolysis. The native structure of the four protein fractions may be destroyed by heat treatment, and the digestibilities were all improved significantly ($P < 0.05$). Adding rutin to the digestion mixture of the four fractions did not cause a decrease in pepsin digestibility, although it did cause a significant increase in certain instances ($P < 0.05$). Treatment with β-mercaptoethanol (2-ME) only caused a higher initial proteolysis rate and did not increase the final digestibility distinctly except for prolamin. After pepsin digestion, the remaining proteins of unhydrolyzed albumin, globulin, prolamin and glutelin (untreated) shared some similarities. They also exhibited a minor band at 20,000 Da and a broad band at 10,000–14,000 Da.

Keywords: Tartary buckwheat; Protein fractions; *In vitro* pepsin digestibility; SDS–PAGE

1. Introduction

The genus *Fagopyrum* has about 15 species distributed in different parts of the world (Tahir & Farooq, 1988). Among these species, only two types of buckwheat are used as food around the world: common buckwheat (*Fagopyrum esculentum*) and tartary buckwheat (*Fagopyrum tataricum*) (Bonafaccia, Gambelli, Fabjan, & Kreft, 2003). Recently, the physiological properties of common buckwheat protein have also been studied. Rat feeding experiments, have proved that common buckwheat protein has hypocholesterolemic effects (Kayashita, Shimaoka, Nakajoh, Yamazaki, & Kato, 1997), anticonstipation activity (Kayashita, Shimaoka, Yamazaki, & Kato, 1995), and suppression activity in mammary carcinogenesis (Kayashita, Shimaoka, Nakajoh, Kishida, & Kato, 1999) and colon carcinogenesis (Liu et al., 2001).

Plant foods are critical for global human nutrition, supplying about 65% of food protein. Food protein quality evaluation takes into account three important parameters: (a) essential amino acid composition, (b) protein digestibility and (c) the ability to supply essential amino acids in the amounts required by humans (Henley & Kuster, 1994).

Buckwheat protein has a high biological value, but its digestibility is relatively low (Ikeda & Kishida, 1993). The factors that contribute to the protein digestibility may be divided into two broad categories: exogenous factors and endogenous factors. Exogenous factors include the protein interactions with polyphenols, phytates, carbohydrates, lipids and protease inhibitors (Duodu, Taylor, Belton, & Hamaker, 2003; Ikeda, Oku, Kusano, & Yasumoto, 1986). Endogenous factors refer to the protein structural characteristics, such as the tertiary and quarternary structure, and the structure may be partly destroyed by heat

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and reduction treatment (Deshpande & Damodaran, 1989a; Ikeda, Sakaguchi, Kusano, & Yasumoto, 1991; Vaintraub, Seliger, & Shutov, 1979). The effects of dietary fibre, tannin, phytate and protease inhibitor on the *in vitro* pepsin–pancreatin digestibility of common buckwheat protein has been studied (Ikeda et al., 1986).

Tartary buckwheat is commonly taken as a diet in eastern Asian countries (Kawakmi, Kayahara, & Ujihara, 1995). In China, tartary buckwheat is mainly grown in some mountainous regions, such as the Liang Shan Yi Autonomous region in Sichuan province and Jing Zhou in Gui Zhou province (Li & Zhang, 2001). It is well known that tartary buckwheat has a high rutin content, a flavonol glycoside compound (Ujihara, 1994). Our previous study showed that *in vitro* pepsin digestibility of tartary buckwheat protein was lower than that of other edible proteins, such as soybean and wheat germ proteins (Guo & Yao, 2006). The main purpose of the present investigation was to determine the effects of rutin, heat treatment and breakage of disulfide bonds on *in vitro* pepsin digestibility of tartary buckwheat protein fractions.

2. Materials and methods

2.1. Materials

Tartary buckwheat flour was obtained from the milling factory for minor crops in the Liang Shan region in Sichuan province. Flour was defatted for 24 h with *n*-hexane under continuous stirring, air-dried at room temperature, and stored at 4 °C until used. The electrophoretic chemicals were purchased from Sigma Chemical Co (St. Louis, USA). Molecular weight markers were purchased from Shanghai Institute of Biochemistry (Shanghai, China). Pepsin (1:3000, from porcine stomach mucosa) was purchased from Deyang Biochemical Company (Deyang, China). All other chemicals used were of analytical grade.

2.2. Extraction and fractionation of tartary buckwheat flour protein

Albumin, globulin, prolamin and glutelin were sequentially extracted with distilled water, 1 M NaCl, 55% 1-propanol and 0.05 M NaOH. The protein content of the fractions was determined by the Kjeldahl method ($N \times 6.25$).

2.3. In vitro pepsin digestibility

In vitro pepsin digestibility was determined according to the method of Rick and Fritsch (1974), but with a slight modification.

For heat treatment, 0.1 g samples were heated prior to the addition of pepsin in a 100 ml Erlenmeyer flask containing 5 ml of water, for 30 min in a boiling water bath.

For reductive cleavage of disulfide bonds, 0.1 g samples were heated prior to the addition of enzyme in a 100 ml Erlenmeyer flask containing 5 ml of 5% (v/v) β-mercaptoethanol (2-ME) for 30 min in a boiling water bath.

A suitable amount of rutin was suspended in 20 ml of pH 1.0 HCl, prior to the addition of enzyme, and incubated for 20 min in a shaking water bath at 37 °C.

Final digestion conditions: the pH values of the samples (treated or untreated) were adjusted to 1.0 and the total volume made up to 20 ml, 10 mg of pepsin were added to the sample, and the flask was incubated in a shaking water bath at 37 °C for 3 h. One hundred microliter aliquots of digestion samples were taken in triplicate at appropriate time intervals, and were added to 100 μl of trichloroacetic acid (10%, w/v). The solution was allowed to stand for 10 min, and centrifuged at 4000*g* for 20 min. A blank was prepared by the same method, using 10 mg of pepsin without substrate. The supernatant obtained was assayed for peptide.

Digestibility was calculated on the basis of the measurement of α-amino nitrogen in the supernatant by the ninhydrin method (Troll & Cannan, 1953). Leucine was used as the standard.

2.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

After pepsin digestion, the undigested protein of samples (untreated) was precipitated by trichloroacetic acid (final concentration 10%), washed three times with acetone, and then used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out using the discontinuous system (15% separating/4% stacking gel) with reduction of the protein by 2-ME. The following buffer systems were used: pH 8.8 Tris–HCl, 0.1% (w/v) SDS for the separating gel, pH 6.7 Tris–HCl, 0.1% (w/v) SDS for the stacking gel, 0.025 M Tris–HCl, 0.192 M glycine, 0.1% (w/v) SDS (pH 8.3) for the running buffer, and pH 6.7 Tris–HCl, 20% (v/v) glycerol, 1% (w/v) SDS, 5% (v/v) 2-ME and 0.05% bromophenol blue as sample buffer. All samples were heated at 100 °C for 3 min, centrifuged at 4000*g* for 10 min, and the supernatants were used to load the gels. Electrophoresis was conducted at a constant current of 20 mA for about 3 h. The gels were stained in Coomassie brilliant blue R-250. Molecular weights of protein subunits were calculated using the following markers: phosphorylase (97,400), bovine serum albumin (66,200), rabbit actin (43,000), bovine carbonic anhydrase (31,000), trypsin inhibitor (20,100), and hen egg white lysozyme (14,400).

2.5. Statistical analysis

Statistical analyses were performed using the SPSS 11.5 software package. Results were given as means ± SD and were compared with one-way analysis of variance (ANOVA) with a 95% confidence interval.

3. Results and discussion

3.1. Digestibility of tartary buckwheat protein fractions

3.1.1. Digestibility of albumin (untreated or treated)

As shown in Fig. 1, heat treatment significantly improved the pepsin digestibility of albumin from 82.6 to 132 mg Leu/g protein ($P < 0.05$). During heating, the tertiary and quarternary structures of albumin may be partly destroyed, which made it more susceptible to pepsin hydrolysis. Adding rutin to the digestion mixture also increased the digestibility to 129 mg Leu/g protein ($P < 0.05$) (Fig. 1). Flavonoids and phenolic acids contain hydroxyl groups and may interact with proteins which negatively affect protein digestibility. However, there are conflicting views that such interactions cause a decrease in protein digestibility (Elkin, Freed, Hamaker, Zhang, & Parsons, 1996; Sathe & Sze-tao, 1997; Venkatachalam & Sathe, 2003). Venkatachalam and Sathe (2003) have reported that none of the tested phenolic compounds adversely affected phaseolin hydrolysis by pepsin. The protein digestibilities of sorghum cultivars with similar tannin contents may show great variability (Elkin et al., 1996). Our data showed that rutin addition increased the *in vitro* digestibility of albumin, which is consistent with the previous finding (Neucere, Jacks, & Sumrell, 1978). The investigation showed that, when arachin was exposed to catechol or pyrogallol, it was more susceptible to pepsin hydrolysis *in vitro*. Treatment with 2-ME improved the pepsin digestibility of albumin from 60.3 to 74.7 mg Leu/g protein after 2 h of digestion time. However, the final digestibility was 85.9 mg Leu/g protein which was only a little higher than that of untreated albumin (82.6 mg Leu/g protein). Disulfide bonds play an important role in stabilizing the tertiary structure of the proteins (Kowalski et al., 1974). Disulfide bond breakage with reducing agents disrupted the tertiary structure and increased the digestibility of chickpea albumins (Alfonso et al., 2000). Hamaker et al. (1987) reported that cooking sorghum flour in water containing a reducing agent increased *in vitro* pepsin digestibility and attributed this to the formation of disulfide bonds which resulted in toughening at the surface and interior of the protein bodies. Our results showed that treatment with 2-ME only caused a higher initial proteolysis rate which could be explained by the location of disulfide bonds. Scanning electron microscopy showed that albumin was digested by pitting from the outer surface to the inner part (Guo et al., accepted), so we concluded that disulfide bonds were mainly located at the outer surface of albumin.

3.1.2. Digestibility of globulin (untreated or treated)

Heat treatment clearly improved the pepsin digestibility of globulin from 35.8 to 62.7 mg Leu/g protein ($P < 0.05$) (Fig. 2). Adding rutin to the digestion solution increased the digestibility to 33.2 mg Leu/g protein after 1 h of digestion time, and this value approximated the final digestibility of globulin (untreated) which showed that adding rutin caused an increase in the initial proteolysis rate. It is apparent that rutin addition should not adversely affect the digestibility of globulin. This is in disagreement with the earlier report, which concluded that tannic acid and catechin exhibited a significant inhibitory effect on the *in vitro* peptic and pancreatic digestion of common buckwheat globulin (Ikeda et al., 1986). Treatment with 2-ME had no effect on *in vitro* pepsin digestibility of globulin (Fig. 2), which indicated that globulin had lower levels of disulfide bonds.

3.1.3. Digestibility of prolamin (untreated or treated)

The effect of heat treatment on the digestibility of prolamin was significant ($P < 0.05$), and the digestibility was increased from 26.9 to 68.2 mg Leu/g protein (Fig. 3). Adding rutin to the digestion solution also increased the digestibility to 40.5 mg Leu/g protein ($P < 0.05$). Treatment with 2-ME improved the digestibility to 36.1 mg Leu/g protein ($P < 0.05$), which was a significantly greater increase than those of albumin and globulin. This suggested that the reduction of inter- and intramolecular disulphide bonds of prolamin caused the structure disruption and enhanced the accessibility of sites susceptible to digestion. Boonvisut

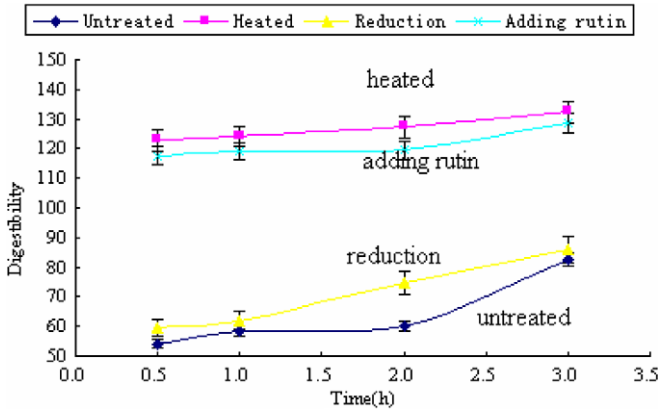

Fig. 1. Digestibility (mg of Leu/g of protein) of albumin (untreated or treated). Each value is expressed as means ± SD ($n = 3$).

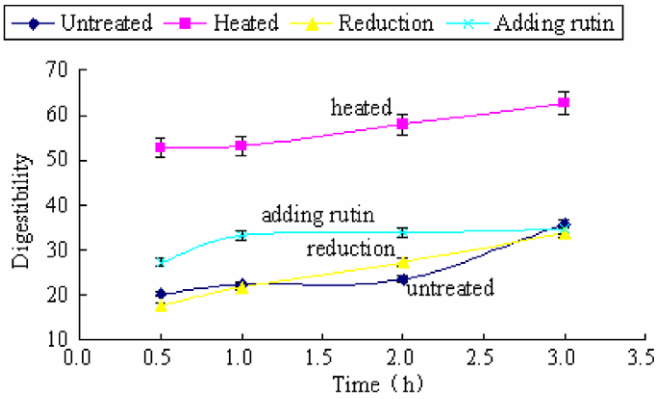

Fig. 2. Digestibility (mg Leu/g protein) of globulin (untreated or treated). Each value is expressed as means ± SD ($n = 3$).

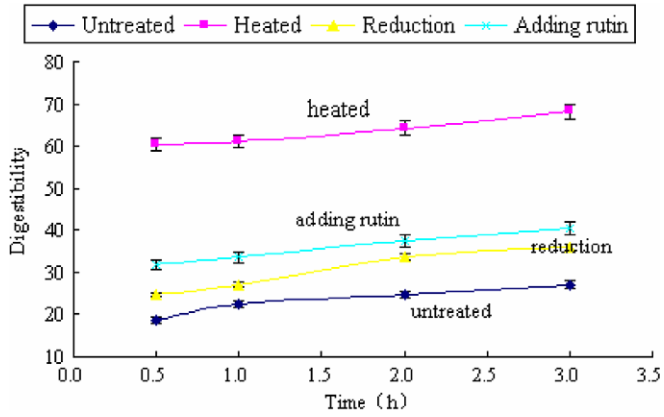

Fig. 3. Digestibility (mg Leu/g protein) of prolamin (untreated or treated). Each value is expressed as means ± SD ($n = 3$).

and Whitaker (1976) reported that cleavage of disulfide bonds increased the *in vitro* digestibility of the soybean proteins. Compared with albumin and globulin (untreated), the digestibility of prolamin (untreated) was lower. The lower digestibility of prolamin may be related to the compact structure and native conformation which hinder the susceptibility to proteolysis. On the other hand, prolamin is a hydrophobic protein, while enzymes function in an aqueous environment. Therefore prolamin may be less accessible to the enzyme and less digestible than albumin and globulin.

3.1.4. Digestibility of glutelin (untreated or treated)

Heat treatment with glutelin clearly improved the pepsin digestibility from 31.2 to 75.8 mg Leu/g protein ($P < 0.05$) (Fig. 4). Although the *in vitro* pepsin digestibilities of the four protein fractions were significantly increased by heat treatment, their final digestibilities were different. This may be related to the sequence of the protein, amino acid composition, molecular size and steric impediment. Adding rutin to the digestion solution also increased the digestibility to 41.8 mg Leu/g protein ($P < 0.05$) (Fig. 4). The increased digestibility found in assayed protein fractions

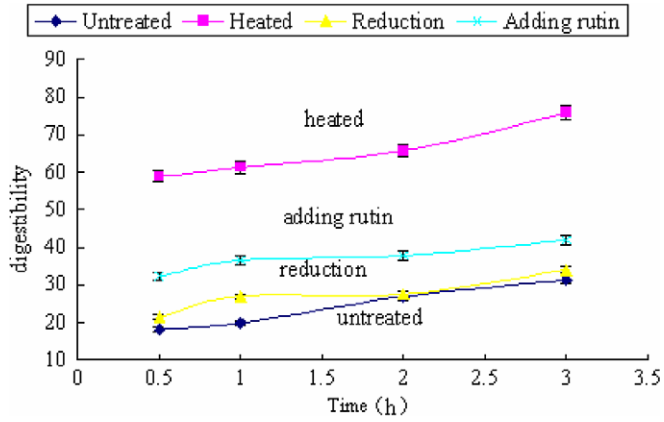

Fig. 4. Digestibility (mg Leu/g protein) of glutelin (untreated or treated). Each value is expressed as means ± SD ($n = 3$).

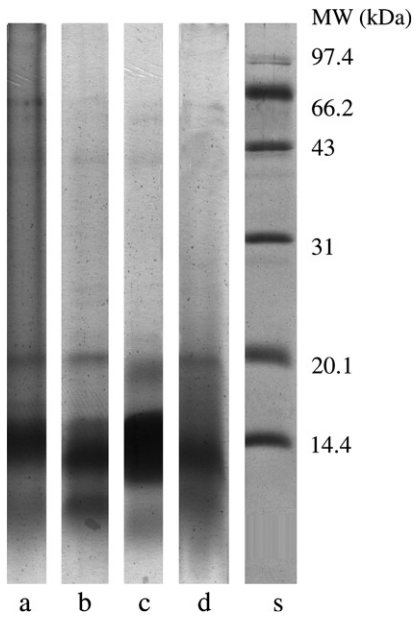

Fig. 5. SDS–PAGE of residual protein fractions (untreated) under reductive conditions: a, albumin; b, globulin; c, prolamin; d, glutelin; s, molecular weight marker.

may be due to a change in their tertiary structure that enhances further pepsin hydrolysis (Martinez & Moyano, 2003). Treatment with 2-ME improved the pepsin digestibility of glutelin from 19.8 to 26.9 mg Leu/g protein after 1 h of digestion time, and the final digestibility was increased to 33.9 mg Leu/g protein after 3 h. The result showed that treatment with 2-ME caused a higher initial proteolysis rate and slight improvement in the final digestibility of glutelin. The effect of disulfide bond breakage on the digestibility of glutelin was similar to that of albumin, which indicated that the disulfide bonds of glutelin were mainly located at the surface of the protein bodies.

3.2. SDS–PAGE of residual protein fractions

Under reductive electrophoretic conditions, the remaining proteins of unhydrolyzed albumin, globulin, prolamin and glutelin (untreated) shared some similarities (Fig. 5). After pepsin digestion, the residual protein of four fractions showed diffused electrophoretic bands. They also exhibited a minor band at 20,000 Da and a broad band at 10,000–14,000 Da. In addition, globulin shared a band at 9000 Da.

4. Conclusions

The present study showed that the *in vitro* pepsin digestibilities of the four protein fractions were different. The native structure of the four protein fractions may be destroyed by heat treatment, and the digestibilities were all improved significantly. Adding rutin to the digestion mixture of the four fractions caused a significant increase in pepsin digestibility, except for globulin, which suggested

that rutin addition did not adversely affect protein hydrolysis. Treatment with 2-ME only caused a higher initial proteolysis rate and did not increase the final digestibility clearly except for prolamin, which may be related to the location of disulfide bonds and their content. After pepsin digestion, the remaining proteins of unhydrolyzed albumin, globulin, prolamin and glutelin (untreated) shared some similarities. They also exhibited a minor band at 20,000 Da and a broad band at 10,000–14,000 Da.

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References

Alfonso, C., Javier, V., Raul, S. V., Justo, P., Juan, B., & Francisco, M. (2000). Factors affecting the *in vitro* protein digestibility of chickpea albumins. *Journal of the Science of Food and Agriculture, 80*, 79–84.

Bonafaccia, G., Gambelli, L., Fabjan, N., & Kreft, I. (2003). Trace elements in flour and bran from common and tartary buckwheat. *Food Chemistry, 83*, 1–5.

Boonvisut, S., & Whitaker, J. R. (1976). Effect of heat, amylase and disulfide bonds cleavage on the *in vitro* digestibility of soybean proteins. *Journal of Agricultural and Food Chemistry, 24*, 1130–1135.

Deshpande, S. S., & Damodaran, S. (1989a). Heat induced conformational changes in phaseolin and its relation to proteolysis. *Biochimica et Biophysica Acta (BBA) – Protein Structure and Molecular Enzymology, 998*, 179–188.

Duodu, K. G., Taylor, J. R. N., Belton, P. S., & Hamaker, B. R. (2003). Factors affecting sorghum protein digestibility. *Journal of Cereal Science, 38*, 117–131.

Elkin, R. G., Freed, M. B., Hamaker, B. R., Zhang, Y., & Parsons, C. M. (1996). Condensed tannins are only partially responsible for variations in nutrient digestibilities of sorghum grain cultivars. *Journal of Agricultural and Food Chemistry, 44*, 848–853.

Guo, X. N., & Yao, H. Y. (2006). Fractionation and characterization of tartary buckwheat flour proteins. *Food Chemistry, 98*, 90–94.

Guo, X. N., Yao, H. Y. & Chen, Z. X. *In vitro* digestibility of Chinese tartary buckwheat protein fractions: the microstructure and molecular weight distribution of their hydrolysates. *Journal of Food Biochemistry* [accepted].

Hamaker, B. R., Kirleis, A. W., Butler, L. G., Axtell, J. D., & Mertz, E. T. (1987). Improving the *in vitro* protein digestibility of sorghum with reducing agents. *Proceeding of the National Academy of Science of the United States of America, 84*, 626–628.

Henley, E. C., & Kuster, J. M. (1994). Protein quality evaluation by protein digestibility-corrected amino acid scoring. *Food Technology, 4*, 74–77.

Ikeda, K., & Kishida, M. (1993). Digestibility of proteins in buckwheat seed. *Fagopyrum, 13*, 21–24.

Ikeda, K., Oku, M., Kusano, T., & Yasumoto, K. (1986). Inhibitory potency of plant antinutrients towards the *in vitro* digestibility of buckwheat protein. *Journal of Food Science, 51*, 1527–1530.

Ikeda, K., Sakaguchi, T., Kusano, T., & Yasumoto, K. (1991). Endogenous factors affecting protein digestibility in buckwheat. *Cereal Chemistry, 68*, 424–427.

Kawakmi, A., Kayahara, H., & Ujihara, A. (1995). Properties and elimination of bitter components derived from tartary buckwheat (*Fagopyrum tataricum*) flour. *Nippon Shokuhin Kagaku Kogaku Kaishi, 42*, 892–898.

Kayashita, J., Shimaoka, I., Nakajoh, M., Kishida, N., & Kato, N. (1999). Consumption of a buckwheat protein extract retards 7,12-dimethylbenz(α)anthracene-induced mammary carcinogenesis in rats. *Bioscience, Biotechnology, and Biochemistry, 63*, 1837–1839.

Kayashita, J., Shimaoka, I., Nakajoh, M., Yamazaki, M., & Kato, N. (1997). Consumption of buckwheat protein lowers plasma cholesterol and raises fecal neutral sterols in cholesterol-fed rats because of its low digestibility. *The Journal of Nutrition, 127*, 1395–1400.

Kayashita, J., Shimaoka, I., Yamazaki, M., & Kato, N. (1995). Buckwheat protein extract ameliorates atropine-induced constipation in rats. *Current Advance Buckwheat Research, 2*, 941–946.

Kowalski, D., Leary, T. R., Mckee, R. E., Sealock, R. W., Wang, D., & Laskowski, M. Jr., (1974). In *Proteinase inhibitors. Bayer symposium V* (pp. 311). Springer-Verlag.

Li, S. Q., & Zhang, Q. H. (2001). Advances in the development of functional foods from buckwheat. *Critical Reviews in Food Science Nutrition, 41*, 451–464.

Liu, Z., Ishikawa, W., Huang, X., Tomotake, H., Kayashita, J., Watanabe, H., et al. (2001). A buckwheat protein product suppresses 1,2-dimethylhydrazine-induced colon carcinogenesis in rats by reducing cell proliferation. *The Journal of Nutrition, 131*, 1850–1853.

Martinez, T. F., & Moyano, F. J. (2003). Effect of tannic acid on *in vitro* enzymatic hydrolysis of some protein sources. *Journal of the Science of Food and Agriculture, 83*, 456–464.

Neucere, N. J., Jacks, T. J., & Sumrell, G. (1978). Interactions of globular protein with simple polyphenols. *Journal of Agricultural and Food Chemistry, 26*, 214.

Rick, W., & Fritsch, W. P. (1974). Pepsin. *Methods of enzymatic analysis* (Vol. 2). New York: Academic Press.

Sathe, S. K., & Sze-tao, K. W. C. (1997). Effects of sodium chloride, phytate and tannin on *in vitro* proteolysis of phaseolin. *Food Chemistry, 59*, 253–259.

Tahir, I., & Farooq, S. (1988). Review article on buckwheat. *Fagopyrum, 8*, 33–53.

Troll, W., & Cannan, R. K. (1953). A modified photometric ninhydrin method for the analysis of amino and imino acids. *Journal of Biological Chemistry, 200*, 803–811.

Ujihara, A. (1994). The usage of buckwheat in the world. *New Food Industry, 36*, 11–16.

Vaintraub, I. A., Seliger, P., & Shutov, A. D. (1979). action of pepsin on the reserve proteins of some leguminous seeds. *Nahrung, 23*, 15–21.

Venkatachalam, M., & Sathe, S. K. (2003). Phaseolin *in vitro* pepsin digestibility: role of acids and phenolic compounds. *Journal of Agricultural and Food Chemistry, 51*, 3466–3472.