43

Assessment of Lectin Inactivation by Heat and Digestion

Arpad Pusztai and George Grant

1. Introduction

Proteins/glycoproteins from plants, particularly lectins, are more resistant to heat denaturation than animal proteins *(1,2)*. With legume seeds, whose lectin content is appreciable, this presents potentially serious problems in nutritional practice. Therefore, before they can be used safely, legume-based food/feeds usually require thorough and expensive heat processing to inactivate antinutritive components. Indeed, dry or moist heating of seeds at 70°C for several h has little or no effect on their lectin activity (Fig. 1) and treatment at much higher temperatures is needed to inactivate the biological and antinutritional effects of legume lectins *(1,2)*. The safety aspect is even more serious with some monocot lectins, such as wheatgerm agglutinin or a number of oilseed lectins, such as peanut agglutinin and many others because they are extremely heat stable and normal cooking or other conventional heat treatments may fail to inactivate them *(3)* Thus, the best way to avoid potential harmful effects of these heat-resistant lectins is to limit their dietary intake to a minimum.

A wide range of procedures have been used to eliminate lectin activity in legume-based or other plant products including dry roasting or toasting, autoclaving, microwaving, and infrared heating treatments *(4,5)*. However, these processes generally require expensive equipment and are more suited to large-scale processing units. Furthermore, to be effective, a number of variables including temperature, the duration of the heating, particle size, and the moisture content of the meal have to be precisely controlled This can be difficult when large quantities or numerous batches of material have to be processed

From *Methods in Molecular Medicine Vol 9 Lectin Methods and Protocols*
Edited by J M Rhodes and J D Milton Humana Press Inc , Totowa, NJ

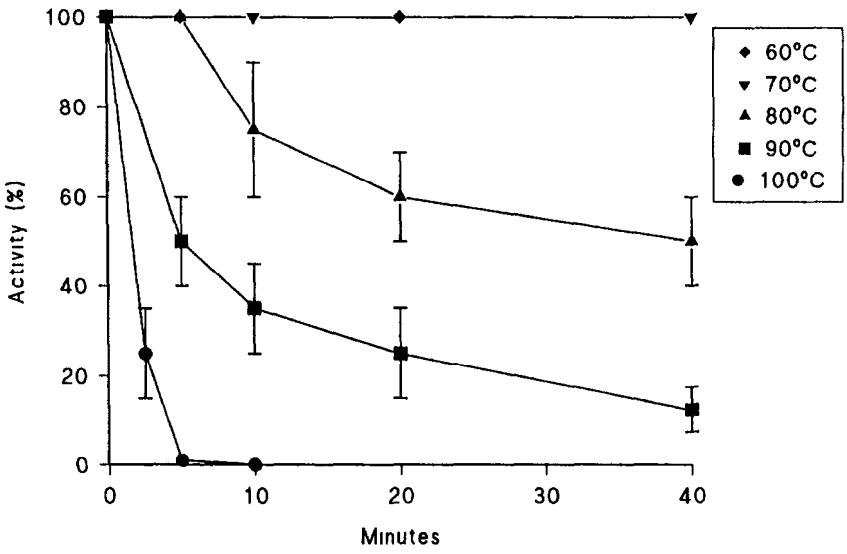

Fig. 1. Loss of lectin activity during aqueous heat treatment of soybean at various temperatures

and may explain why small but significant amounts of active lectins can frequently be detected in some processed seed products *(3,6)*.

The most practical, effective, and commonly used method to abolish lectin activity is aqueous heat treatment. Seeds are first fully soaked in water, and then heated in water at or close to 100°C Under these conditions, the lectin activity in fully hydrated soya beans *(Glycine max)* (Fig. 1), kidney beans *(Phaseolus vulgaris)*, faba beans *(Vicia faba)*, and lupinseeds *(Lupinus angustifolius)* could be eliminated by heating at 95°C for 1 h or at 100°C for 10 min *(2,7)*.

Orally administered native, undenatured lectins are extremely resistant to proteolytic breakdown by pancreatic and small intestinal proteases during passage through the mammalian digestive tract *(8)* As most of these lectins can be recovered from the feces, they must also be resistant to bacterial proteases in the large intestine. It has been suggested that this resistance is mainly the result of stabilization of the conformation of the lectin molecule by its avid binding to carbohydrate moieties of gut epithelial membrane glycoconjugates and this may also shield peptide bonds, which in the absence of the saccharides would be open to protease attack *(9)*. However, as some lectins, like the mannose-specific snowdrop *(Galanthus nivalis)* bulb agglutinin (GNA), which do not bind to the gut wall can also be recovered in high amounts, binding induced stabilization is not always essential for stability against proteolytic breakdown *(8)*.

The high stability of lectins to proteolysis can be fully abolished by denaturation with appropriate heat treatments. Thus, the substantial improvement in the nutritional quality of legume meals after processing is most likely owing not only to the inactivation of lectin activity by denaturation, but also to the increased digestibility of the lectin protein whose component amino acids can then be absorbed in the gut and fully utilized by the body.

1.1. Indirect Noncompetitive ELISA

ELISA assay methods *(10)* are illustrated with the description of the protocols used for the measurement of the lectin content in kidney bean lectin, PHA.

In this assay, the antigen, PHA, is first immobilized on the solid-phase, ELISA microtiter plate, and then reacted with a suitably diluted specific rabbit antilectin antibody solution. The antigen–antibody complex formed is detected by a goat immunoglobulin preparation specific for the rabbit antibody, and labeled with ExtrAvidin peroxidase, whose quantity is measured by a colorimetric reaction. The reaction rate, i.e., the increase in absorbance per unit time, A/t, is directly related to the quantity of antigen present in the sample. The first step in this method is to establish the optimum dilution of the rabbit antilectin antibody. For this, the wells in each horizontal row are coated with a serially diluted PHA solution followed by the addition of different dilutions of the rabbit antibody so that the antibody concentration is halved in the wells of each successive horizontal row. From the results of the color reaction obtained at different dilutions of the antibody, calibration curves are drawn on a semilog graph paper. The curve showing the best sigmoidal shape (high sample dilution = low value of A/t, and low sample dilution = high value of A/t, S-shaped) indicates optimal parameters.

1.2. Indirect Competitive ELISA

This assay is based on the competitive inhibition of the reaction between a known amount of antigen coated to the plate and its specific antibody by free antigen present in test or calibration samples.

2. Materials

2.1. Inactivation by Heat

1 Seed samples should be purchased from a reliable source to ensure that the individual seeds are from the same cultivar. This is particularly important with kidney beans, in which the lectin content of different cultivars may differ considerably. To obtain seed meals containing uniform size particles and to minimize exposure to heat, seeds are ground in a high precision hammermill, such as a Glen Creston Hammermill (Glen Creston, Stanmore, Herts, UK) fitted with a 1-mm mesh, taking care that the mill is not overheated but remains at room temperature

2. Phosphate-buffered saline solution, pH 7 6 (PBS) 8 0 g/L NaCl, 0 2 g/L KCl, 1 15 g/L Na_2HPO_4, 0 2 g/L KH_2PO_4
3 Saline: 9 g/L NaCl in water
4 Collect blood into a heparinized tube (30 U/mL blood), quickly dilute 20-fold with saline, and store at 1°C The cells are stable for up to 1 wk if kept at this temperature This diluted and untreated blood is used directly in the assays
5 Trypsin-treatment of blood cells increases the sensitivity of the hemagglutination assay. Centrifuge diluted rat or cattle blood at $500g_{max}$ for 5 min and wash the cells twice with saline Resuspend the cells in saline to their original diluted volume, add trypsin (0 1 mg/10 mL of diluted blood cells) and incubate for 45 min at 25°C Centrifuge the trypsin-treated cells, wash four times with saline, and finally resuspend in saline to their original diluted volume. Store cells at 1°C, but do not keep for more than 1 d.

2.2. Inactivation by Proteolysis In Vivo

1 ELISA 96-well microtiter plates (TITERTEK, Flat Bottom, Labsystems, Basingstoke, UK). Incubator set at 37°C, pipet tips (Labsystems Finntips, 0 5–300 µL; Labsystems); microdensitometer (ELISA reader, Uniskan II, Labsystems)
2. Coating buffer (stored at 4°C in the refridgerator). 0 05*M* carbonate-bicarbonate buffer, pH 9 8, 2.69 g $NaHCO_3$, and 1 91 g Na_2CO_3 dissolved in 1 L distilled water
3. Blocking solution (prepared fresh) 0 5% (w/v) gelatin in PBS diluted 10X with distilled water Dissolve 0 15g gelatin in 30 mL of PBS with heating to 45°C and stirring, then dilute to 300 mL with distilled water
4. Washing solution (prepared fresh). 0 1% (v/v) Tween-20 in PBS To 1000 mL PBS add 1 mL Tween-20
5 Dilution buffer· T-G-PBS (prepared fresh) To 1000 mL PBS add 1 mL Tween-20 and 0 5 g gelatin
6 Substrate working buffer solution 0.05*M* citric acid-Na_2HPO_4 buffer, pH 5 0
   a Solution A 0 1*M* citric acid, 2 10 g citric acid monohydrate dissolved in 100 mL water
   b Solution B 0 2*M* Na_2HPO_4; 2 84 g Na_2HPO_4 dissolved in 100 mL water Stock substrate buffer 0 1*M* citric acid-Na_2HPO_4, pH 5 0, is made up by mixing 48 5 mL of solution A with 51 5 mL of solution B For the substrate working buffer, the stock substrate buffer is diluted 1 1 with distilled water
7 OPD-H_2O_2 reagent, 20 mg *o*-phenylenediamine free base (1 tablet, Sigma) is dissolved in 50 mL substrate working buffer and 20 µL 30% (v/v) H_2O_2 is added 10 min before use
8 Stopping solution. 3*M* sulfuric acid
9 Antilectin (rabbit) antibodies are obtained from commercial sources (e g , Sigma) or raised in rabbits locally
10 Kit reagents for ELISA rabbit extravidin peroxidase staining kit Extra-3 (Sigma) containing 1 vial biotinylated goat antirabbit immunoglobulin, affinity-purified in buffer containing preservative (diluted 1·1000), and 1 vial extravidin peroxidase in buffer containing preservative (diluted 1 500)

3. Methods

3.1. Inactivation by Heat

For whole seeds, carry out steps 1–8. For seed meals, use steps 9–10.

1 Sort seed samples visually to ensure purity
2 Soak seeds in water (1·4 w/v) at 20°C for up to 16 h (a minimum of 8 h)
3. Remove any seeds floating on the surface of the water and discard
4 Pour off the soaking water and discard
5 Sort the seeds visually and discard any that are not fully hydrated (*see* Note 1)
6 Pour the seeds into a container (cooking pot or waterbath) of preheated water (300 g wet wt/L) and boil for 10 min In localities in which the boiling temperature of water is <100°C, the cooking time needs to be extended accordingly (*see* Note 2)
7 Pour off the cooking water and wash the seeds with fresh water, drain, and check for uniform softness Discard abnormal seeds (*see* Note 3)
8. The seeds may then be safely consumed without further treatment or be added to other dishes. Alternatively, they can be dried, ground, and tested by hemagglutination or by rat feeding experiments to establish the degree of inactivation of the seed lectin
9 The recommended method for the heat treatment of seed meals is simpler Weigh suitable amounts of ground seed meal into a stoppered flask, followed by the addition of 3–4 vol of water There is no need for presoaking, place the stoppered flasks in a preheated waterbath and keep at the appropriate temperature for the required time After the heating, recover the contents of the flasks by freeze-drying and test by hemagglutination and/or rat feeding experiments
10 For heat treatment of purified lectins, the procedure is similar to that recommended for seed meals Dissolve the lectins in PBS and heat in stoppered flasks at appropriate temperatures for the required time and test the degree of inactivation by hemagglutination (*see* Note 4)

3.2. Inactivation by Proteolysis in the Gut

1. Feed rats with 1 mL of lectin solution (10 mg/mL) by intragastric intubation
2 Kill by halothane overdose precisely 1 h later
3. Ligate stomach and small intestine, excise, and rinse with PBS containing aprotinin (1000 kIU/mL) to recover free, unbound lectins from the lumen of these tissues
4. Homogenize the washed stomach and small intestinal tissues with PBS (10 mL), also containing aprotinin and the monosaccharide (1 g/L) appropriate for the specificity of the lectin (Table 1), in a Janke-Kunkel homogenizer (20,000 rpm, 30 s)
5 Estimate the amounts of immunoreactive lectins in both the luminal washings and tissue homogenates by a suitable technique, such as ELISA
6. Calculate the degree of survival as percentage of the lectin originally administered (*see* Note 5)

Table 1
Survival and Binding of Pure Lectins to the Small Intestinal Mucosa

Lectins	Specificity	Binding	Recovery, %
PHA *(Phaseolus vulgaris)*	Complex	+ + +	> 90
Con A *(Canavalia ensiformis)*	Man/Glc	+	> 90
GNA *(Galanthus nivalis)*	Man	–	> 90
SNA–I *(Sambucus nigra)*	α-2,6-NeuAc–Gal	+	50–60
SNA–II	GalNAc	+ + +	> 60
SBA *(Glycine max)*	GalNAc/Gal	+ +	40–50
LEL *(Lycopersicon esculentum)*	GlcNAc	+	40–50
WGA *(Triticum vulgare)*	GlcNAc	+ +	50–60
PSL *(Pisum sativam)*	Man/Glc	±	30–40
VFL *(Vicia faba)*	Man/Glc	±	20–30
DGL *(Dioclea grandiflora)*	Man/Glc	±	18–20

The results are taken from ref *8* Rats were intragastrically intubated with 10 mg of individual lectins The amounts of lectin surviving in the stomach and small intestine were estimated from luminal washings and supernatants of the tissues homogenized with 0 1*M* solution of the appropriate specific carbohydrate in phosphate-buffered saline, pH 7.6 The strength of binding is marked on an arbitrary scale + + +, strong binding, –, represents no binding at all

7 Fecal samples are freeze-dried, ground to a fine powder, extracted with PBS containing 0 02% (w/v) NaN_3 (feces.PBS ratio is 1 10, w/v), and centrifuged (50,000g max for 30 min), the clear supernatants are used for measurement of lectin concentration by ELISA

3.3. Hemagglutination Assay

1 Twelve small tubes (possibly 24 or 36 if treated cells are used) each containing 150 µL of saline are set up for each sample
2 Add 150 µL of sample to the first tube, and mix the contents well
3. Remove 150 µL from this first tube and transfer to tube 2 Continue this serial dilution to the last tube, mix the diluted samples with 150-µL aliquots of blood cells, and leave for 2–3 h at room temperature
4. Resuspend the cells by agitation, and assess the degree of agglutination by eye or, preferably, by a microscope
5 The degree of clumping is expressed as follows $3+^{v}$, large clumps visible by eye, 3+, 80–100% of cells are clumped (by microscope), 2+, 40–60% of cells are clumped (by microscope), 1+, 10–20% of cells are aggregated (by microscope) and tr, < 10% of cells are clumped (by microscope) (*see* Note 6)
6 One unit of hemagglutinating activity (HU) is defined as the amount of material (µg/mL) in the last dilution in which 50% of the cells are agglutinated (*see* Notes 7 and 8)

3.4. ELISA Method

3.4.1. Indirect Noncompetitive ELISA

1 To the first well in each horizontal row of a 96-well ELISA plate add 200-μL aliquots of a solution of PHA (500 μg/mL) (in coating buffer), and into all others add 100 μL coating buffer.
2 Serially dilute the PHA solution in a horizontal direction so that the amount of PHA-antigen decreases from 50 μg in the first well to 0 025 μg in the last
3. Incubate the plate for 60 min at 37°C.
4 Remove the unbound PHA by draining and washing the wells three times with washing solution and twice with distilled water (250 μL each time)
5 Add 200 μL of blocking solution to each well and incubate the plate for a minimum of 30 min at 37°C.
6 After the removal of the blocking solution, wash the plate three times with washing solution and twice with distilled water (250 μL each time).
7 Since the conditions of the immune reaction are best optimized in a checkerboard design, add 100 μL of anti-PHA antibody in twofold dilutions in dilution buffer in each successive row from top to bottom of the plate Thus, although the dilution of the antibody is constant for the 12 wells in each horizontal row, overall it changes from 1·250 in the first to 1.32,000 in the eighth row
8 Cover the plate with parafilm and aluminium foil and incubate for 60 min at 37°C in a humid atmosphere
9 Wash the plate three times with washing solution and twice with distilled water (250 μL each time) and dry by turning upside down and banging it against filter paper
10 Pipet 100 μL of prediluted (1 1000) conjugate of biotinylated goat antirabbit immunoglobulin into all wells and incubate the plate for 60 min at 37°C in a humid atmosphere
11. Drain the wells, wash three times with washing solution and twice with distilled water (250 μL each time) and dry
12 Pipet 100 μL of 1.500 diluted extravadin peroxidase into each well and incubate for 60 min at 37°C in a humid atmosphere.
13 Wash the plate five times with washing solution (250 μL each time) and dry.
14. Develop the color reaction by adding 100 μL OPD reagent to each well, leaving the plate to stand for 16 min and stopping the reaction by adding 50 μL stopping reagent
15 Read the color with an ELISA reader at 492 nm.

3.4.2. Indirect Competive ELISA

1 Two plates are used Coat the first plate with a known optimal concentration of purified PHA, 100 μL, dissolved in the coating buffer, incubate at 37°C for 60 min
2 Remove the unbound PHA, wash three times with washing solution, and twice with distilled water (250 μL each time) and dry.
3. Incubate the second plate with 200 μL blocking solution overnight at 37°C

4 Wash the plate three times with washing solution and twice with distilled water (250 µL each time) and dry
5 Into all wells, except those in the first vertical column which contain 200 µL of a PHA standard solution, add 100 µL of dilution buffer in which the PHA solution is serially diluted in a horizontal direction
6 Transfer aliquots of the serially diluted PHA solutions (50 µL each) from wells on plate 2 to equivalent wells on the first plate, then add 50 µL of suitably diluted anti-PHA antibody (previously determined to be optimal by indirect noncompetitive ELISA)
7. Cover the plate with parafilm and aluminium foil, incubate at 37°C for 60 min in a humid atmosphere
8. Drain the plate and wash three times with washing solution and twice with distilled water (250 µL each time) and dry
9 Immunoreaction, color development and reading at 492 nm are done as before

4. Notes

1 It is important to ensure that seeds are fully hydrated Thus, at 100°C, the cooking time required to eliminate lectin activity in partially hydrated seeds can be more than sixfold greater than that necessary for fully hydrated seeds
2 The aqueous heating procedure described can effectively eliminate the lectin activity from most plant materials consumed in human or animals diets. However, wheatgerm agglutinin or gluten-associated lectins are not inactivated under these conditions *(11)* Taro tuber lectin is also reported to require prolonged heating at high temperature *(12)*
3 Many beans develop hard-shell or hard-to-cook characteristics during long-term storage under conditions of high humidity and temperature *(13)* and cooking times necessary to eliminate antinutritional factors, such as lectins become very extended regardless of whether dry or moist heating is used If at stages 2, 5, or 7 of the aqueous heat treatment procedure a high proportion of the seeds have to be thrown away, this is a clear indication that there are problems with that batch of seeds and it would be wise to reject all seeds in the batch
4. Purified lectins in aqueous solution, in plant extracts or seed meal suspensions appear to be inactivated under the same temperature conditions as those required for intact seeds *(2)*
5. The degree of total lectin survival is estimated by measuring the amounts of immunoreactive lectins in the feces of rats and comparing them to the total dietary input over the entire period of feeding To allow for slow stomach emptying and increased intestinal passage time owing to the presence of lectins in the diet, collection of fecal samples is continued for at least 48 h after the last meal containing lectins The extent of the survival of lectins during small intestinal passage is always significant but quite variable An example is given in Table 1.
6 Occasionally in hemagglutination assays with enzyme-treated cells, the resuspended cells exhibit an unacceptably variable degree of background clumping. If this is the case, the stock suspension of the cells should be left to stand for 20–30

min at room temperature to allow the majority of the clumps to settle out. The upper layer is then decanted and used in the assay.

7 A lectin standard is included in each assay. For comparison purposes, the results are best expressed as HU/100 g bean meal or as lectin equivalents/100 g bean meal *(1)*

8 Some examples: A kidney bean cultivar of high lectin content, e g., "Processor" using native blood cells gave the following values 1 HU = 49 μg or 2040×10^6 HU/100 g meal A low lectin kidney bean cultivar, Pinto III, using native blood cells gave· 1 HU = 12,500 μg or 8×10^3 HU/100 g meal. The titers estimated with trypsin-treated cells are far higher

References

1 Pusztai, A (1991) *Plant lectins* Cambridge University Press, Cambridge, UK

2 Grant, G and van Driessche, E. (1993) Legume Lectins. physicochemical and nutritional properties, in *Recent Advances of Research in Antinutritional Factors in Legume Seeds* (van der Poel, A. F. B., Huisman, J , and Saini, H. S , eds), Wageningen Pers, Wageningen, The Netherlands, pp. 219–234

3 Nachbar, M S and Oppenheim, D. J (1980) Lectins in the United States diet a survey of lectins in commonly consumed foods and a review of the literature *Am J Clin Nutr* **33,** 2338–2345

4 Melcion, J -P and van der Poel, T F B (1993) Process technology and anti-nutritional factors principles, adequacy and process optimization, in *Recent Advances of Research in Antinutritional Factors in Legume Seeds* (van der Poel, A. F B , Huisman, J , and Saini, H S , eds), Wageningen Pers, Wageningen, The Netherlands, pp 419–434

5 Rackis, J J , Wolf, W J , and Baker, E C. (1986) Protease inhibitors in plant foods content and inactivation, in *Nutritional and Toxicological Significance of Enzyme Inhibitors in Foods* (Friedmann, M , ed), Plenum, NY, pp 299–347

6. Ryder, S D , Smith, J A , and Rhodes, J M (1992) Peanut lectin· a mitogen for normal human colonic epithelium and human HT29 colorectal cancer cells *J Natl Cancer Inst* **84,** 1410–1416.

7 Grant, G , More, L J , McKenzie, N H , and Pusztai, A (1982) The effect of heating on the hemagglutinating activity and nutritional properties of bean *Phaseolus vulgaris* seeds *J Sci Food Agric* **33,** 1324–1326

8 Pusztai, A , Ewen, S W. B , Grant, G , Peumans, W. J , Van Damme, E J M , Rubio, L., and Bardocz, S (1990) The relationship between survival and binding of plant lectins during small intestinal passage and their effectiveness as growth factors *Digestion* **46(Suppl. 2),** 308–316.

9 Pusztai, A , Begbie, R , Grant, G., Ewen, S W B , and Bardocz, S (1991) Indirect effects of food antinutrients on protein digestibility and nutritional value of diets, in *In Vitro Digestion for Pig and Poultry* (Fuller, M , ed.), CAB International, Wallingford, Oxon, UK, pp 45–61

10 Tijssen, P (1985) Practice and theory of enzyme immunoassays, in *Laboratory Techniques in Biochemistry and Molecular Biology,* 5th ed. (Burdon, R H and Van Knippenberg, P H , eds), Elsevier, Amsterdam, The Netherlands, pp. 329–355

11 Liener, I E (1986) Nutritional significance of lectins in the diet, in *The Lectins* (Liener, I. E., Sharon, N , and Goldstein, I. J. eds), Academic, NY, pp 527–552.
12 Seo, Y -J , Satsuki, U , Tsukamoto, I., and Miyoshi, M (1990) The effect of lectin from taro tuber *(Colocasia antiquorum)* given by force-feeding on the growth of mice. *J Nutr Sci Vitaminol* **36,** 277–285
13 Aguilera, J M and Stanley, D W (1985) A review of textural defects in cooked reconstituted legumes–the influence of storage and processing *J Food Proc Preserv* **9,** 145–169