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Identification and stability of trypsin inhibitor isoforms in pea (*Pisum sativum* L.) cultivars grown in New Zealand

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Abstract

Trypsin inhibitor activity (TIA) of 17 spring-sown field pea cultivars grown in New Zealand ranged from 0.33 to 0.75 TIU/mg DM. These values were much lower than those reported for most European pea cultivars. After soaking and cooking, values fell by 42–91%, with an average reduction of 78% (0.07–0.19 TIU/mg DM). After heat treatment, the residual percentage of TIA was negatively correlated to the amount of TIA in the raw seed. Six to ten trypsin isoinhibitors were observed in each cultivar of the raw extracts, with isoelectric points ranging from 4.6 to 7.6. Only three of the isoinhibitors, with isoelectric points of 5.1, 5.9 and 7.6, remained after heat treatment.

Pea cultivars with high TIA levels in the raw seed may be more suitable as processing peas because cooking substantially reduces TIA levels, suggesting that adverse effects of consuming TIA would be limited. Conversely, raw peas with low TIA levels would be more suitable for use as animal feed. Cultivars that contain more unstable isoforms may require less heat treatment, therefore retaining their nutritional value be more suitable for human consumption and use as animal feed, and have reduced processing costs.

Keywords: Pea; *Pisum sativum*; Trypsin inhibitor; Isoform; Isoinhibitor; Stability

1. Introduction

Raw pea seeds are important in animal nutrition but the presence of endogenous trypsin (or protease) inhibitors limits their utilisation. The trypsin inhibitor content of peas is similar to that of the field bean (*Vicia faba*), but is only 10% of the level found in soybeans (*Glycine max*) (An, Bacon, & Fenwick, 1993; Hove & King, 1979; Valdebouze, Bergeron, Gaborit, & Delort-Laval, 1980). However, the levels found in some pea cultivars are of nutritional significance as they are linked to decreased protein digestibility and poor growth performance in animals (Jondreville, Grosjean, Buron, Peyronnet, & Beneytout, 1992; Le Dréan, Le Huërou-Luron, Philouze-Romé, Toullec, & Guilloteau, 1995). In France, it has not been permitted to register pea varieties with levels of trypsin inhibitors two units higher than the two official control cultivars since 1992 (Page, Quillien, & Duc, 2000), and financial penalties exist for selling pea varieties with high trypsin inhibitor levels (Al-Wesali, Lambert, Welham, & Domoney, 1995; Bacon, Lambert, Matthews, Arthur, & Duchene, 1995; Domoney, Welham, Ellis, & Hellens, 1994). However, protease inhibitors have been reported to play a role in protecting the plant against attack by microorganisms, insects and predators during growth (Ryan, 1990).

Pea protease inhibitors (PPI), or pea seed trypsin inhibitors (PSTI), are similar in structure to the well-character-

Abbreviations: TIA, trypsin inhibitor activity; TIU/mg DM, trypsin inhibitor units per milligram of dry matter; PPI, pea protease inhibitor; BBI, Bowman-Birk inhibitor; FPLC, fast protein liquid chromatography; IEF, isoelectric focusing; SDS–PAGE, SDS–polyacrylamide gel electrophoresis.

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ised Bowman-Birk inhibitors (BBI) in soybean. The molecular mass of PPI is 6.8–7.9 kDa (Ferrasson, Quillien, & Guéguen, 1997), similar to that of BBI. However, inhibitors with a molecular mass up to 12 kDa in peas have been reported (Frøkiær, Hørlyck, Sørensen, & Sørensen, 1993). cDNA (Domoney, Welham, & Sidebottom, 1993b) and protein (Ferrasson, Quillien, & Guéguen, 1995) sequencing have also shown that pea protease inhibitors belong to the Bowman-Birk family. The amino acid sequences show homology and a high cysteine content, unlike the Kunitz soybean trypsin inhibitor family. PPI and BBI both contain two active sites, which primarily inhibit the proteolytic enzymes, trypsin and chymotrypsin (Arentoft, Frøkiær, Sørensen, & Sørensen, 1994; Frøkiær et al., 1993). Monoclonal antibodies against BBI have shown cross-reactivity toward PPI (Arentoft, Frøkiær, Sørensen, & Sørensen, 1992). Although PPI are homologous to BBI, their sequences and behaviour in an in vitro digestion system have distinct characteristics (Al-Wesali et al., 1995; Domoney, Welham, Sidebottom, & Firmin, 1995). Like other trypsin inhibitors, PPI exist in several different isoforms, known as isoinhibitors, which can be differentiated by isoelectric focusing. Qualitative and quantitative differences in individual pea protease isoinhibitors have been reported. The number and relative proportion of isoinhibitors varied in 65 lines of *Pisum sativum* (Domoney, Welham, & Sidebottom, 1993a). Three to nine isoinhibitors have been detected in New Zealand cultivars (Howard, 1996).

All legume products require moist heat treatment to inactivate protease inhibitors. Sayeed and Njaa (1985) found that moist heat treatment reduced protease inhibitor activity in peas by up to 86%. However, thermal processes also affect the nutritional value of legume seeds. Decreases in lysine, methionine and cysteine content occur during prolonged heat treatment (El-Refai, Gouda, & Ammar, 1987; Savage & Elliott, 1993).

Heat-labile antinutritional factors, such as trypsin inhibitors, are less important in human diets as cooking and processing are normally carried out before consumption (Savage & Deo, 1989). However, nutritional components are often degraded during prolonged processing methods. A long-term aim of germplasm development programmes is the selection of cultivars with high levels of heat-labile trypsin isoinhibitors. This may ensure that the growing plant is protected from invading pests and microbes, while subsequently providing as much digestible protein as possible for human consumption after the cooking of peas.

The aim of the work described in this paper was to quantify trypsin inhibitor activity in pea cultivars grown in New Zealand and identify individual isoforms before and after heat treatment to assess their stability.

2. Materials and methods

2.1. Materials

Sixteen pea (*Pisum sativum* L.) cultivars were grown in a replicated field trial (four replicates) in the 1996/1997 season at Crop & Food Research, Lincoln, New Zealand (Table 1). Cultivar Allure was grown at the same location but in the 1994/1995 season. The soil at the Lincoln site, situated 172°29′E 43°39′S, 11 m above sea level, is Temple-

Table 1
Characteristics of 17 pea cultivars grown in New Zealand

Cultivar	Sown	Aspect	Testa colour	Cotyledon colour	Flower colour
Freezer					
Bolero	Spring	Wrinkled	White	Green	White
Green					
CFR 4L25[a]	Spring	Smooth	White	Green	White
Crusader	Spring	Smooth	White	Green	White
Emerald	Spring	Smooth	White	Green	White
Hadlee	Spring	Smooth	White	Green	White
Prussian Blue	Spring	Smooth	White	Green	White
Rovar	Spring	Smooth	White	Green	White
Maple					
Courier	Spring	Dimpled	Mottled brown	Yellow	Purple
Crown	Spring	Dimpled	Mottled brown	Yellow	Purple
Mega	Spring	Dimpled	Dun	Yellow	Purple
Whero	Spring	Dimpled	Mottled brown	Yellow	Purple
Marrowfat					
Midichi	Spring	Angular	White	Green	White
Primo	Spring	Angular	White	Green	White
White					
Allure	Spring	Smooth	White	Yellow	White
Birte	Spring	Smooth	White	Yellow	White
Komet	Spring	Smooth	White	Yellow	White
Rex	Spring	Smooth	White	Yellow	White

[a] Crop & Food Research accession number.

ton silt loam soil. Its structure is poor, with low organic matter as a result of over-cropping. The soil has poor moisture holding capacity as a consequence and has to be watered frequently to maintain adequate moisture levels. Peas were harvested with a Wintersteiger Nursery Master at a 14% moisture content.

2.2. Sample preparation

After harvest and yield assessment, composite samples from each of the four replicates were thoroughly mixed to obtain a representative sample of peas from each cultivar. Samples were air-dried to a consistent moisture content prior to sampling and stored at room temperature until analysis.

To prepare heat-treated samples, 50 g dry peas were soaked for 18 h in 500 ml cold distilled water. The rehydrated peas were added to 250 ml water in a beaker and covered with aluminium foil to minimise evaporation. They were brought to boiling point and then cooked for a further 20 min, until tender and edible. After cooling, the seeds and liquid were frozen together and freeze-dried.

Both raw and heat-treated samples were ground using an IKA-Universalmühle M20 grinder (Janke and Kunkel, Staufen, Germany) equipped with cooling system to prevent heating of samples.

2.3. Extraction

Ten grams of pea flour was homogenised in 50 ml acidified water (pH 2.4, adjusted with HCl) (An et al., 1993) for 2 min using a Kinematica GmBH homogeniser (Brinkmann Instruments, Canada). Samples were stirred constantly at room temperature for 30 min, centrifuged at 3800*g* for 4 min at 4 °C and the supernatant was collected. The remaining pellet was resuspended in 50 ml acidified water and centrifuged at 3800*g* for 4 min at 4 °C (Valdebouze et al., 1980). The supernatant was collected and pooled. Extractions were performed in triplicate. Immediately prior to assays, supernatants were centrifuged at 9800*g* for 10 min at 4 °C to obtain a clear solution.

2.4. Determination of trypsin inhibitor activity

Trypsin inhibitor activity was determined in triplicate by the Kakade enzymic assay (Kakade, Rackis, McGhee, & Puski, 1974) and trypsin inhibitor units were calculated according to Gaborit, Quillien, and Guéguen (1993), where one trypsin inhibitor unit (TIU) is defined as a decrease of 0.01 absorbance units at 410 nm per 10 ml assay solution (Kakade et al., 1974).

2.5. Purification and separation of isoinhibitors

Crude pea extract (20 ml) was loaded on to a Sephadex G-50 (G-50-150, Sigma Chemical Co., St. Louis, MO) size exclusion column (100 × 2.5 cm) and eluted at a flow rate of 2 ml/min using FPLC (Pharmacia Biotech, Uppsala, Sweden) with distilled water as the elution buffer; 44 × 9 ml fractions were collected. Absorbance was measured at 280 nm. Fractions containing trypsin inhibitor activity, collected from the Sephadex G-50 column, were pooled together and loaded on to a 20 × 1.5 cm anion exchange column containing DEAE-Sepharose Fast Flow (DFF-100, Sigma Chemical Co., St. Louis, MO). The column was eluted with a linear gradient of 0–500 mM NaCl in 50 mM Tris buffer, pH 8.8 (Ferrasson et al., 1997) at a flow rate of 2 ml/min. Fractions of 10 ml each were collected and absorbance was measured at 280 nm. Fractions were assayed for trypsin inhibitor activity by the Kakade enzymic assay.

2.6. Determination of isoelectric point and molecular mass

Crude extracts of 10 of the 17 cultivars were selected for isoelectric focusing (IEF) on an Ampholine PAGPlate (pH 3.5–9.5) (Pharmacia Biotech, Uppsala, Sweden) using an LKB Multiphor II apparatus (Pharmacia Biotech, Uppsala, Sweden). Gels were stained using a negative staining procedure specific for trypsin inhibitors (Chavan & Hejgaard, 1981). Fractions of cultivar Primo exhibiting TIA obtained from anion exchange chromatography, plus pooled fractions, from both size exclusion and anion exchange chromatography, were also focused to determine isoelectric points. These fractions were also resolved by SDS–PAGE (Laemmli, 1970) using a Bio-Rad Mini-PROTEAN II cell (Bio-Rad Laboratories, Hercules, CA) to determine molecular mass. Samples were concentrated and desalted using Millipore Ultrafree-15 Biomax-5 centrifugal filter units (Millipore Corp., Bedford, MA) to enable detection.

3. Results and discussion

3.1. Trypsin inhibitor activity in raw peas

Trypsin inhibitor activity in 17 pea cultivars grown in New Zealand ranged from 0.33 to 0.75 TIU/mg DM with a mean TIA of 0.57 TIU/mg DM (Table 2). Bolero, a wrinkled-seeded freezer cultivar, displayed the lowest TIA of 0.33 TIU/mg DM. Besides Bolero, the maple (purple-flowered) cultivars in general exhibited low TIA (mean 0.46 TIU/mg DM) compared to the other cultivars (mean 0.63 TIU/mg DM). These results concur with other studies linking phenotypic traits and TIA. In general, wrinkled-seeded cultivars have lower TIA than those that are smooth-seeded (Cousin, Tomé, & Gaborit, 1993; Valdebouze et al., 1980), and purple-flowering cultivars have lower TIA than those with white flowers (Grosjean, Bourdon, Kiener, Castaing, & Gatel, 1991; Pisulewski, Pisulewska, Hanczakowski, & Ernest, 1983). The marrowfat cultivars, Midichi and Primo, were both in the upper range of TIA (0.66 and 0.73 TIU/mg DM, respectively). Other researchers have also observed higher TIA levels in

Table 2
The effects of boiling on trypsin inhibitor activity in peas grown in New Zealand (mean of duplicate observations on triplicate extractions on a representative sample)

Cultivar	TIA, raw (TIU/mg DM)	TIA, boiled (TIU/mg DM)	Residual TIA[a] (%)
Freezer			
Bolero	0.33	0.19	57.6
Green			
CFR 4L25	0.60	0.07	11.7
Crusader	0.63	0.10	15.9
Emerald	0.49	0.10	20.4
Hadlee	0.46	0.14	30.4
Prussian Blue	0.75	0.07	9.3
Rovar	0.71	0.11	15.5
Maple			
Courier	0.44	0.18	40.9
Crown	0.38	0.13	34.2
Mega	0.41	0.08	19.5
Whero	0.62	0.13	21.0
Marrowfat			
Midichi	0.66	0.12	18.2
Primo	0.73	0.13	17.8
White			
Allure	0.58	0.11	19.0
Birte	0.62	0.14	22.6
Komet	0.57	0.08	14.0
Rex	0.71	0.10	14.1

[a] Residual TIA (%): the percentage of the TIA remaining in the seeds after heat-treatment.

marrowfat cultivars (Griffiths, 1984; Leterme, Beckers, & Théwis, 1990; Sayeed & Njaa, 1985).

3.2. Effect of heat-treatment on trypsin inhibitor activity

After soaking and boiling, the TIA decreased to 0.07–0.19 TIU/mg DM (9–58% of original TIA), with a mean of 0.12 TIU/mg DM (22% of original TIA) (Table 2). The wrinkled-seeded cultivar, Bolero, which had the lowest TIA in the raw seed (0.33 TIU/mg DM), showed the smallest reduction in TIA upon heat treatment (42% reduction in TIA to 0.19 TIU/mg DM). This suggested that the more heat-stable isoinhibitors represent a greater proportion of the total isoinhibitors in this cultivar. The green pea cultivar Prussian Blue, which exhibited the greatest levels of TIA in the raw seed (0.75 TIU/mg DM), also exhibited the greatest reduction in TIA in the cooked seed (91% reduction; 0.07 TIU/mg DM). A negative correlation ($r = -0.801$) was observed between the residual percentage of TIA in the cooked seeds (log-transformed) and TIA in the raw seed (Fig. 1). Residual TIA (%) is defined here as the percentage of the TIA remaining in the seeds after heat-treatment. This result agrees with those of Sayeed and Njaa (1985) who observed a negative correlation ($r = -0.900$) between these two parameters after soaking peas overnight and cooking for 45 min. It appears that boiling destroyed TIA more in cu1tivars with higher levels of TIA than in those with lower levels of TIA. This suggests that peas with high levels of TIA may contain a higher proportion of the more heat-labile isoinhibitors and vice versa.

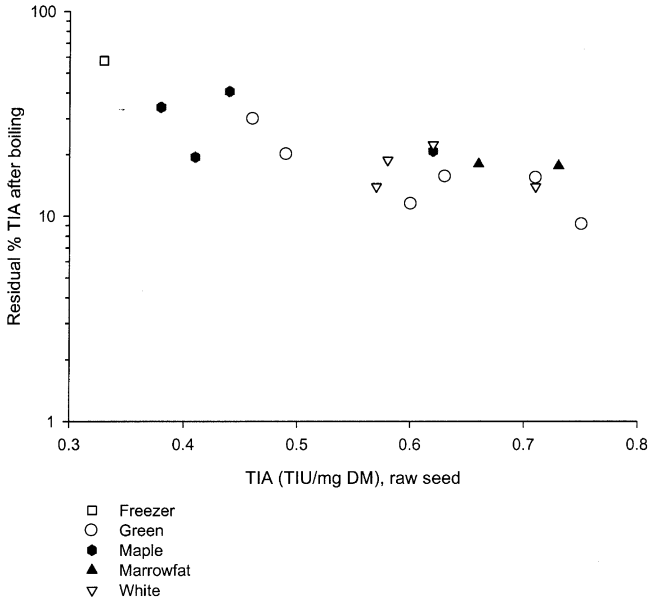

Fig. 1. The relationship between trypsin inhibitor activity in raw seeds and the residual fraction in the heat-treated seeds.

3.3. Trypsin inhibitor isoforms

Between 6 and 10 isoinhibitors with isoelectric points (pI) ranging from 4.6 to 7.6 were observed, as clear white bands against a pink-red background, in the crude extracts of the 10 pea cultivars (Table 3). The number and range of pI varied between cultivars, but was similar to that observed in previous research (Arentoft et al., 1994; Ferrasson et al., 1997; Frøkiær, Hørlyck, Sørensen, & Sørensen, 1994; Mortensen, Olsen, Sørensen, & Sørensen, 1995).

The majority of cultivars had seven isoinhibitors with pI of 4.6, 4.8, 5.1, 5.4, 5.9, 6.8 and 7.6. The marrowfat cultivar Primo exhibited the greatest number of isoinhibitors of all the cultivars, with 10 isoforms in total. The three extra bands, observed in the centre of the gel, were distinct bands in close proximity to each other, with pI of 6.1, 6.3 and 6.5. These bands were not detected in any of the other cultivars assayed. Cousin et al. (1993) suggested that the presence or absence of a particular isoinhibitor may determine the level of TIA. However, in this study the number of isoinhibitors or a particular isoinhibitor did not appear to correlate with levels of TIA, and no patterns could be established within or between the pea classifications.

Since TIA levels in peas appear to be genetically controlled (Domoney et al., 1994), progeny with a common parent may have the same number and type of inhibitors. Crusader and Hadlee, both of which are progeny of OSU442-15, exhibited the same isoinhibitors. However, and more importantly, they both clearly exhibited an isoinhibitor with pI 7.2, which was unable to be detected in the other cultivars assayed.

Table 3
Identification of trypsin isoinhibitors in crude extracts of pea cultivars grown in New Zealand

Isoelectric point	Freezer	Green		Maple			Marrowfat		White	
	Bolero	Crusader	Hadlee	Courier	Crown	Mega	Midichi	Primo	Birte	Rex
4.6	+	−	−	+	+	+	+	−	+	+
4.8	+	−	−	+	+	+	+	+	+	+
4.9	−	−	−	−	−	−	−	+	−	−
5.1	+	+	+	+	+	+	+	+	+	+
5.4	+	+	+	+	+	+	+	+	+	+
5.9	+	+	+	+	+	+	+	+	+	+
6.1	−	−	−	−	−	−	−	+	−	−
6.3	−	−	−	−	−	−	−	+	−	−
6.5	−	−	−	−	−	−	−	+	−	−
6.8	+	+	+	+	+	+	+	+	+	+
7.2	?	+	+	?	?	?	?	?	?	?
7.6	+	+	+	+	+	+	+	+	+	+
Number of isoforms	7	6	6	7	7	7	7	10	7	7

[+]Isoinhibitor present.
[−]Isoinhibitor absent.
[?]Isoinhibitor possibly present at low levels.

Following purification and separation, only four isoinhibitors (pI 4.9, 5.9, 6.3, 6.8) were detected in the marrowfat cultivar Primo compared with 10 isoinhibitors in the crude pea extract (Table 4). Other researchers have also indicated a reduction in the number of isoforms during the purification process (Frøkiær et al., 1994; Gaborit, Delort-Laval, Thanh, & Paraf, 1989).

Two prominent isoinhibitor bands were detected in fractions 20–21 (a 20 ml fraction from anion exchange chromatography (AEC) that exhibited high TIA), with isoelectric points of 4.9 and 6.3, respectively. Although these isoinhibitors were also observed in fractions 22–27 (a 60 ml fraction from AEC), these bands were much less intense. All isoinhibitors detected in the individual fractions were detected in the pooled fractions collected from size exclusion and anion exchange chromatography.

From the fractions assayed, it appears that the trypsin isoinhibitors from cultivar Primo were eluted from the anion exchange column in the following order: 5.9 and 6.3 simultaneously, 4.9, and 6.8. Further separation of pI 5.9 and 6.3 using anion exchange chromatography would establish their elution order.

3.4. Molecular mass

Molecular masses for the crude extract and purified fractions of the pea cultivar Primo were determined. The crude extract exhibited proteins of nine different molecular masses of approximately 6.5, 14, 16, 21, 31, 35, 45, 50 and 52 kDa. After separation and purification by size exclusion and anion exchange chromatography, the pooled fractions of these two processes exhibited two bands at ~6.5 and ~21 kDa. Upon separation of anion exchange fractions, these two protein bands were observed in all fractions assayed, except fractions 49–51 (a 20 ml fraction from AEC), which exhibited no protein. In decreasing order, the majority of protein (representing trypsin inhibitor protein) occurred in fractions 20–21, 22–27 and 14–19 (a 60 ml

Table 4
Identification of trypsin isoinhibitors in purified fractions of pea cultivar Primo

Isoelectric point[a]	Pooled SEC[b]	Pooled AEC[c]	2–4	14–19	20–21	22–27	49–51
4.8	−	−	−	−	−	−	
4.9	+	+	−	−	+	−	−
5.1	−	−	−	−	−	−	−
5.4	−	−	−	−	−	−	−
5.9	+	+	+	+	+	+	−
6.1	−	−	−	−	−	−	−
6.3	+	+	+	+	+	+	−
6.5	−	−	−	−	−	−	−
6.8	+	+	−	−	+	+	−
7.6	−	−	−	−	−	−	−
Number of isoforms	4	4	2	2	4	3	0

[+]Isoinhibitor present.
[−]Isoinhibitor absent.
[a] All isoelectric points observed in crude pea extract of cv. Primo are listed.
[b] Pooled fractions from size exclusion chromatography.
[c] Pooled fractions from anion exchange chromatography.

fraction from AEC), which corresponds with results from anion exchange chromatography and isoelectric focusing.

Pea protease inhibitors characteristically have a molecular mass of 6–8 kDa (Ferrasson et al., 1997). The heavy protein band at ~6.5 kDa is likely to represent these inhibitors. The band observed at ~21 kDa may represent trimers of these inhibitors. Other trypsin inhibitor (BBI) molecules have also been observed to self-associate in aqueous solution to give dimers and trimers (Birk, 1987). Oxidation of the cysteine residues during electrophoresis leads to multiple forms and thus an overestimated molecular mass (Ferrasson et al., 1997). An electrophoresis gel run under reducing conditions may prevent this from occurring. However, Ferrasson et al. (1997) indicated that this could not be prevented, even after a preliminary reduction of the disulfide bridges. Compounds with greater molecular masses (12–16, 29–30 kDa) have been previously reported (Howard, 1996; Tomé, Dhoye, Gaborit, Kozlowski, & Valdebouze, 1981; Weder & Hory, 1972). Abnormal migration has previously been reported for trypsin inhibitors (Wu & Whitaker, 1990).

3.5. Heat-stable trypsin inhibitor isoforms

In all cultivars, only the three isoinhibitors with isoelectric points of 5.1, 5.9 and 7.6 remained after heat treatment, indicating greater stability of these three isoforms. Again, no differences between the cultivars were observed. Isoinhibitors with pI 5.1 and 7.6 were the most prominent isoinhibitors in the crude extracts, possibly indicating greater stability than pI 5.9.

4. Conclusions

Combined soaking and boiling is an effective method of reducing TIA. In this study, the greater the TIA level in the raw pea seed, the greater the proportional reduction or inactivation of TIA after heat treatment.

Pea cultivars with high TIA levels in the raw seed may therefore be more suitable as processing peas. The high TIA content in the raw seed will protect the plant against pests and disease, whereas the low TIA present after cooking indicates that adverse effects would be limited when consumed. Peas with low TIA levels in the raw seed would be more suitable as animal feed, which is often processed without heat treatment.

By identifying heat-stable isoinhibitors, breeders may be able to select cultivars without these isoforms in order to achieve maximum trypsin inhibitor inactivation during heat treatment. However, in this study the same three isoinhibitors remained after heat treatment in all cultivars tested. A greater range of genotypes needs to be screened to see if the heat-stable isoinhibitors present vary. Cultivars that contain more unstable isoforms may require less heat treatment and would be more suitable for consumption by humans and animals. They may also retain their nutritional value since less intensive processing would be required.

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