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Utilization by Chicks of Half-Cystine from Native and Denatured Proteinase Inhibitor Protein from Potatoes[1] (40415)

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The excellent quality of cooked potato protein is ranked high among world food proteins (1). Although the protein content of potatoes is only about 2%, the high yields of tubers in many areas of the world indicate that the net protein production per hectare can surpass any other plant crop and suggests that potato protein may assume an increasingly important role in world food supplies (2).

The nutritional value of potato protein appears to be somewhat limited by the relatively low level of the sulfur amino acids methionine and cystine (1). If specific proteins in potato tubers that contained unusually high sulfur amino acid contents could be increased by genetic selection so that they represented a high percentage of the total protein, they might significantly increase the nutritional quality of the potato.

We have isolated and characterized a group of proteins and polypeptides from potatoes that are rich in the sulfur amino acid half-cystine (3–6). This group is comprised primarily of at least six heat-stable proteinase inhibitors that range in molecular weight from 4500 to 39,000 that have from two to 12 half-cystine residues per 9000 MW. These proteins and polypeptides are powerful inhibitors of animal and microorganism digestive proteinases. Although heat stable when pure, they apparently denature rapidly in the intact potato tuber when cooked.[2] These inhibitor proteins are present in tubers in relatively high concentrations (in some varieties over 10% of the soluble proteins (7)). Two of the major inhibitors, called Inhibitor I, a powerful inhibitor of bovine chymotrypsin and Inhibitor II, a potent inhibitor of both bovine chymotrypsin and trypsin, have been shown to be related to the total protein of the potato and are being used as genetic markers to select for high protein potatoes (8). Thus genetic selections based on the sulfur-rich inhibitor proteins to increase the protein content of potato tubers holds promise of significantly improving both the quantity and quality of potato proteins.

In order to determine whether such biologically active proteins could be nutritionally beneficial (or deleterious) when fed to animals, and whether the cysteine residues are available, experiments were designed in which the entire group of inhibitors, native and heat denatured, were fed to newly-hatched chicks maintained on diets limited in half-cystine. The results of these experiments are reported herein.

Materials and methods. Sixty grams of the heat-stable group of proteinase inhibitors was isolated from 600 lbs of Russet Burbank potatoes as previously described. This is the "crude inhibitor" fraction (3–5) from which several proteinase inhibitors have been isolated and characterized. From this fraction several proteinase inhibitor proteins with specificities directed toward animal and microorganism proteinases have been isolated including Inhibitor I (MW 39,000) (5), Inhibitor II (MW 21,000) (3), Carboxypeptidase Inhibitor (CPI,[3] MW 4,500) (6), Polypeptide Chymotrypsin Inhibitor (PCI, MW 5,000) (4), and at least one other polypeptide that inhibits trypsin.[4] The fraction contains about

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[3] Abbreviations: CPI, carboxypeptidase inhibitor; PCI, polypeptide chymotrypsin inhibitor; TAME, Tosyl-L-arginine methyl ester; ATEE, acetyl-L-tyrosine ethyl ester; and HPA, hippuryl-L-phenylalanine.

[4] The polypeptide fraction from potato juice contains several proteinase inhibitors of animal pancreatic enzymes. The entire complement of these polypeptides are presently under study.

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63% protein and a summary of its known components is presented in Table I.

Protein was determined by the Lowry method (9). Individual inhibitor contents were quantitated by the immunological radial diffusion method. Each pure inhibitor was injected into rabbits to obtain monospecific anti-inhibitor serum as described previously (10). Purified inhibitors were used as standards. Pure Inhibitor I, II, CPI, and PCI were prepared as previously described (3–6). Enzymic assays for trypsin, chymotrypsin, and carboxypeptidase A were determined as previously described (3, 6) using the substrates TAME, ATEE, and HPA respectively. All three enzymes were purchased from the Worthington Biochemical Co. and all substrates were from Sigma.

Amino acid content of the crude inhibitor fraction was determined using the method of Moore and Stein (11). One mg samples were hydrolyzed under nitrogen gas in sealed ampules at 110° for 24, 48, and 72 hr. An internal standard of 100 nmoles of norleucine was added to each sample before hydrolysis. The hydrolysates were dried under vacuum and were dissolved in 0.2 ml sodium citrate, pH 2.2, for analysis on a Beckman Model 120 C automatic amino acid analyzer. Separate 1.0 mg samples were performic acid oxidized, hydrolyzed, and analyzed separately to determine half-cystine and methionine content (12). Corrections for loss of threonine and serine were made by extrapolation to zero hydrolysis time, from which the amino acid content was calculated. Values for other amino acids were determined from averages of the three analyses.

The crude inhibitor fraction was utilized for feeding studies either directly as prepared (native) or after autoclaving for 2 hr in solution (denatured). To prepare the denatured inhibitor fraction 50 g of the native inhibitor fraction was dissolved in 750 ml water and autoclaved at 121° for 2 hr. After autoclaving the inhibitors were lyophilized and analyzed for proteinase inhibitor activity and immunological cross reactivity with native inhibitors. The composition of the native fraction is shown in Table I. After autoclaving only the carboxypeptidase inhibition exhibited any immunological cross reactivity against antisera prepared against each pure inhibitor,

TABLE I. COMPOSITION OF CRUDE PROTEINASE INHIBITOR FRACTION FROM POTATO TUBERS.

Total protein, % of crude fraction	63.2%
Inhibitor I, % of crude fraction	11.9%
Inhibitor II, % of crude fraction	18.2%
PCI, % of crude fraction	11.1%
CPI, % of crude fraction	8.1%
Inhibitors, % of crude fraction	49.4%
Inhibitors, % of total protein	78.2%

indicating that all inhibitors except CPI were destroyed. Although not completely destroyed, CPI had dropped from 8.1% (Table I) to 1.6% of the total protein in the denatured preparation.

The same results were obtained enzymically (Table II). The trypsin and chymotrypsin activities present in the native protein was completely destroyed by autoclaving. The carboxypeptidase activity was reduced by over five-fold by this treatment, confirming the immunological data.

The autoclaving treatment also caused some losses of amino acids as determined by amino acid analyses (Table III). The final percentages of half-cystine added to the diets were calculated from the values shown in Table III. As can be seen, 44% of the half-cystine in the proteins was destroyed by autoclaving.

Preparation of the basal diet. The composition of basal diet was as follows: glucose, 21.9%; ground dry pea seeds, 40.0%; gelatin, 5.0%; casein, 7.0%; dehydrated alfalfa, 3.0%; limestone, 0.5%; dicalcium phosphate 1.7%; vitamin premix,[5] 1.0%; mineral mix,[6] 0.10%; iodized salt, 0.3%; animal fat, 4.0%; meat and bonemeal, 4.0%; and ground corn meal, 11.6%. The basal mix contained 20.13% protein. The diet was formulated to be slightly deficient in methionine (0.41%) and significantly deficient in half-cystine (0.13%). The requirement for newly hatched chicks is

[5] Vitamin premix at 1.0% of the diet supplies the following per kg of the diet: vitamin A, 22,000 I.U.; vitamin D_3, 6,600 I.C.U.; vitamin E, 17.6 I.U.; riboflavin, 13.2 mg; calcium pantothenate, 17.6 mg (or pantothenic acid, 16.20 mg); niacin, 88 mg; choline chloride 2.31 g; vitamin B_{12}, 0.044 mg; and ethoxyquin, 2.49 g.

[6] Mineral premix at 0.10% of the diet supplies the following per kg of the diet: Mn, 100 mg; Fe, 100 mg; Cu, 10 mg; Zn, 100 mg; I, 3.0 mg; Ca, 120 mg; and Co, 1.0 mg.

0.46% methionine and 0.40% half-cystine (13).

Preparation of the experimental diets. Twelve diets, 3 kg each, were prepared from the basal diet by adding various levels of half-cystine and methionine as the free amino acids or by adding protein half-cystine and methionine present in the native or denatured inhibitor protein fractions. The half-cystine and methionine contents of these diets is shown in Table IV.

Brolier type chicks were hatched under standard incubating conditions and the newly hatched chicks were placed in holding boxes without food for 24 hr after hatching. The chicks were then fed the basal diet for 5 days and weighed. For experiments, chicks were randomly selected from groups of 80 males and 60 females, previously segregated by sex. Fifteen chicks, nine male and six female, were utilized for each experiment. They were randomly segregated into three groups of five chicks per cage. The chicks were weighed, fed experimental diets for 7 days, and weighed again. Food consumption was also monitored over the 7 days.

Results and discussion. Naturally occurring proteinase inhibitors have been suspected for years of contributing to growth depression of animals when present in foods in their native, but not denatured forms (14). As shown in Tables I and II, the majority of proteins present in the heat stable crude inhibitor fraction obtained from Russet Burbank potato tubers are potent inhibitors of animal pancreatic proteinases and thus are potentially toxic substances. These results also indicate that the inhibitory and immunological properties of the inhibitors can be destroyed by autoclaving.

The amino acid analysis of the crude proteinase inhibitor fraction indicated that it exhibited an unusually high amount of sulfur containing amino acids, particularly of half-cystine (about 5% of the protein amino acids). The availability of the half-cystine in the native and denatured protein fraction to newly hatched baby chicks was determined to help assess the potential nutritive value of the inhibitor proteins, which are being genetically enriched in certain high protein potato selections (8).

TABLE II. PROTEINASE INHIBITOR ACTIVITIES OF NATIVE AND AUTOCLAVED CRUDE POTATO INHIBITOR FRACTIONS.

Enzymes	Enzyme inhibited (mg/mg crude fraction)[a] Native	Autoclaved
Trypsin	0.033	0
Chymotrypsin	0.300	0
Carboxypeptidase A	0.116	0.025

[a] Calculated at 50% inhibition.

TABLE III. AMINO ACID ANALYSES OF NATIVE AND HEAT DENATURED POTATO PROTEINASE INHIBITOR FRACTIONS.

Amino acid residues	Native g/100 g	Denatured Crude inhibitor
Aspartic acid	7.3	5.9
Threonine	3.3	2.8
Serine	3.6	2.7
Glutamic acid	6.8	6.4
Proline	3.6	3.1
Glycine	3.8	2.9
Alanine	3.2	3.2
Half-cystine	*5.0*	*2.8*
Valine	4.0	3.2
Methionine	*0.63*	*0.33*
Isoleucine	2.7	1.9
Leucine	3.8	2.5
Tyrosine	2.8	1.6
Phenylalanine	2.3	1.6
Lysine	5.6	5.2
Histidine	1.7	1.6
Arginine	3.1	2.3

The protein contents of native and denatured crude inhibitor fraction were determined by the Lowry method. The Kjeldahl method was not used because the purified inhibitors gave anomolously high protein values using $6.25 \times N$ as the conversion factor. Therefore the Lowry assay was employed using as a standard a mixture of inhibitor proteins as shown in Table I. The value of 63% protein for the native crude inhibitor fraction agreed well with that calculated from the amino acid analysis of 58%, which did not include tryptophan or free ammonia. The protein content of the denatured crude inhibitor fraction, 56.1%, was also calculated from the same standards. The amino acid analysis also showed some losses in several amino acid residues and some additional losses may have been introduced in denaturing the inhibitors. Carbohydrate analyses are not included because of the large errors encountered in selecting standards. It is assumed that the majority of the non-protein fraction of crude inhibitor fraction is carbohydrate material.

TABLE IV. WEIGHT GAINS AND FEEDING EFFICIENCIES OF FIVE-DAY-OLD CHICKS FED A HALF-CYSTINE LIMITED DIET SUPPLEMENTED WITH HALF-CYSTINE AND NATIVE AND DENATURED PROTEIN INHIBITOR FRACTIONS FROM POTATOES.

Diet	Source of half-cystine	Added to Basal Diet		Amt. Wt. Gain/Chick[b] (g)	Feeding Efficiency (F/G)[c]
		Half-cystine[a]	Methionine[a]		
1	Basal diet	0	0	73.8 ± 1.6	1.99 ± 0.02
2	Free	0.030	0.003	83.1 ± 2.8[d]	1.89 ± 0.05
3	Native inhibitor protein	0.022	0.003	30.8 ± 0.8[e]	3.33 ± 0.05
4	Denatured inhibitor protein	0.014 0.028	0.002 0.005	83.0 ± 7.2 86.6 ± 2.4[d]	1.92 ± 0.11 1.86 ± 0.12

[a] % of total diet.

[b] Average of 15 chicks. Mean ± SEM of three groups of five chicks over a 1-week period. Chicks were five days old and had been maintained on the basal diet prior to the experiments.

[c] Feed intake (F) per weight gained (G) ± SE.

[d] Significant weight gain ($P < 0.05$) from control value.

[e] Significant weight loss ($P < 0.05$) from control value.

The availability of the half-cystine in the native and denatured crude inhibitor protein fractions was compared with results from control experiments in which half-cystine was fed in increasing increments as the free amino acid in the presence of 1/10 its concentration of free methionine (Fig. 1). Methionine was added since it is also present in the inhibitor protein fractions (Table III) and its possible effect on the results of the experiments was important to establish. Methionine, when added alone to the basic diet only partially supplemented half-cystine, even when present up to 0.06% of the diet. At this level it promoted 42% of the growth as that promoted by a mixture of 0.15% half-cystine and 0.015% methionine.

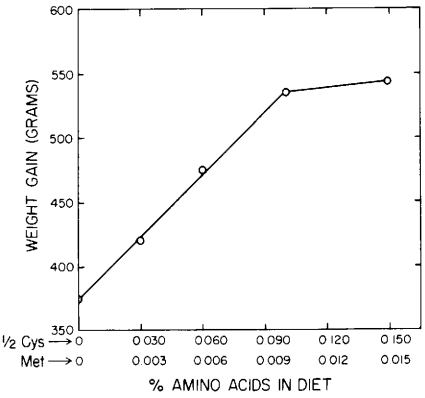

FIG. 1. The relationship of average chick growth, in grams per pen (5 chicks per pen, 3 pens) per seven days, to the quantity of a mixture of half-cystine and methionine added to the basic diet. Details are in the text.

Supplementing the basic diet with native and denatured inhibitor protein fractions produced dramatically differing results. The native protein produced severe growth depression in the chicks. Table IV shows that the average weight gain produced by the diet containing native protein was 30.8 ± 0.8 g/chick while the basal diet produced a weight gain of 73.8 ± 1.6 g/chick. The free half-cystine, having approximately the same concentration of half-cystine in the diet as the native protein produced a weight gain of 83.1 ± 2.8 g/chick. On the other hand the autoclaved denatured inhibitor protein fraction was an excellent source of half-cystine, producing weight gains of 83.0 ± 7.2 and 86.6 ± 2.4 g/chick at half-cystine levels of 0.014% and 0.028%, respectively. These values are comparable to the growth produced by the additions of half-cystine at percentage equivalent to those of the denatured protein, extrapolated from Fig. 1. These results demonstrate that the half-cystine present in denatured potato proteinase inhibitor proteins is available to chicks for growth.

The severe growth depression caused by the native inhibitor fraction indicated that its effects are not simply that it is unavailable to the chicks. The indications are that some components of the native preparations are severely toxic. The toxic substances are apparently destroyed upon heating and may be the proteinase inhibitors since they represent the major percentage of the protein. It had

been demonstrated earlier (15) that Inhibitor I, a potent chymotrypsin inhibitor, had much less effect on the pancreatic secretion of rats than soybean trypsin inhibitor when fed in the diet. However, the presence in the native inhibitor protein fractions of a wide spectrum of proteinase inhibitors may have produced the pronounced growth depression by severely arresting protein digestion.

From the data reported herein, it appears that the proteinase inhibitor proteins in the crude inhibitor fraction, which are utilized as a basis for selecting high protein potatoes, can effectively provide half-cystine for animal growth, but only in their denatured form.

Summary. In a proteinase inhibitor protein fraction obtained from the soluble proteins of Russet Burbank potatoes, over 78% of the total half-cystine rich proteins are inhibitors of the pancreatic proteases, trypsin, chymotrypsin, and carboxypeptidase A. This protein fraction was utilized to supplement a half-cystine limited chick diet to determine the availability of half-cystine residues in native and autoclaved (denatured) inhibitor proteins. Chicks fed diets supplemented with native inhibitor protein showed a severe growth depression. Weight gain of chicks fed autoclaved inhibitor protein were equivalent to chicks whose diets were supplemented with half-cystine. The experiments indicated that high protein potato selections based on high inhibitor contents should provide nutritionally available half-cystine, provided the potatoes are cooked before consumption.

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1. Schuphan, W., *in* "Proteins as Human Food" (R. A. Lawrie, ed.), pp. 245, Avi Publishing Co. (1970).
2. Kaldy, M. S., Economic Botany **26**, 142 (1972).
3. Bryant, J., Green, T. B., Gurusaddaiah, T., and Ryan, C. A., Biochemistry **15**, 3418 (1956).
4. Hass, G. M., Venkatakushnan, R., and Ryan, C. A., Proc. Nat. Acad. Sci. **73**, 1941 (1976).
5. Melville, J. C., and Ryan, C. A., J. Biol. Chem. **247**, 3445 (1972).
6. Ryan, C. A., Hass, G. M., and Kuhn, R., J. Biol. Chem. **249**, 5495 (1974).
7. Ryan, C. A., Kuo, T., Pearce, G., and Kunkel, R., Amer. Potato J. **43**, 443 (1976).
8. Ryan, C. A., and Pearce, G. L., Amer. Potato J. **55**, 351 (1978).
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).
10. Ryan, C. A., Anal. Biochem. **19**, 434 (1967).
11. Moore, S., and Stein, W., Methods Enzymol. **6**, 819 (1963).
12. Moore, S., J. Biol. Chem. **238**, 235 (1963).
13. Committee on Poultry Nutrition, "Nutrient Requirements of Poultry," National Academy of Sciences, Washington, DC (1971).
14. Leiner, I. E., and Kakade, M. L., *in* "Toxic Constituents of Plant Foodstuffs" (I. E. Leiner, ed.), pp. 8, Academic Press, NY (1969).
15. Green, G. M., and Lyman, R. L., Proc. Soc. Expt. Biol. Med. **136**, 649 (1971).

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