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Effects of Feeding a Carboxypeptidase Inhibitor from Potatoes to Newly Hatched Chicks[1] (41669)

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Abstract. For the first time, the effects on animal growth and protein digestion of a specific inhibitor of the pancreatic digestive exopeptidases carboxypeptidases A and B were studied. Carboxypeptidase inhibitor from potato tubers was fed to newly hatched chicks at a level equal to that present in a diet containing 50% raw potato solids, which was severely growth depressing. At this level the effect of the carboxypeptidase inhibitor on growth was insignificant but the following effects were noted: (1) increased fecal protein (the increase mainly consisting of low-molecular weight proteins); (b) poorer feed efficiency; and (c) a significant decrease in pancreatic digestive proenzyme levels, although no hypertrophy was noted. In addition, the inhibitor was not digested readily in the intestinal tract and increased in concentration in intestinal contents as it progressed down the tract. Potato Inhibitor II, a potent trypsin inhibitor, when fed to chicks, also at the level found in the diet containing 50% raw potato, was severely growth depressing. The inhibitor produced significantly increased fecal protein and caused pronounced pancreatic hypertrophy. Thus, the growth depressing effects of raw potato tubers is probably due in part to the trypsin inhibitor, with only a small contribution originating from the carboxypeptidase inhibitor.

Potatoes contain a variety of proteins that inhibit all five of the major pancreatic digestive endo- and exopeptidases that are responsible for protein degradation in the intestinal tracts of higher animals (1–4). The growth depressing effects on chick growth of a partially purified proteinase inhibitor fraction from Russet Burbank potato tubers has been reported (5). Upon autoclaving, this fraction became growth promoting, implying that the anti-nutrient inhibitor proteins, upon heat denaturation, became excellent food proteins (5).

Most of the inhibitors in potato tubers are destroyed rapidly by cooking. Huang *et al.* (6) demonstrated that two of the major endopeptidase inhibitors present in potato tubers were rapidly inactivated during three commonly used methods of cooking, i.e., boiling, baking, and microwaves. This research also demonstrated that a large percentage of a carboxypeptidase inhibitor (CPI) present in the tubers survived all three cooking methods. The unique stability of CPI may be at least partially due to its small size (mol wt 4100) and its stabilization by three disulfide linkages (3). The inhibitor has been extensively studied, physically, chemically, and kinetically. Its specificity is very broad and it inhibits virtually all metallocarboxypeptidases with specificities similar to mammalian pancreatic carboxypeptidases A and B (7).

The levels of CPI vary significantly among potato varieties (8). In a study of proteinase inhibitors in 106 commercial and experimental varieties, CPI concentrations were found from 0 μg/ml of tuber juice to 846 μg/ml (8). In the latter variety the inhibitor represents over 4% of the soluble proteins. The effects of a carboxypeptidase inhibitor in the diets of animals is unknown. To gain some initial knowledge of the effects of ingested carboxypeptidase inhibitor in animals, CPI was purified in large quantities and added to diets of newly hatched chicks to determine its effects on digestion and growth. The effects of the diets containing CPI were evaluated by comparing them with control diets, diets containing raw and cooked potatoes, and with a diet containing a potent trypsin inhibitor from potato tubers, called Inhibitor II (2).

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Methods and Materials. Protein was determined with Coomassie blue by the method of Bradford (9). Bovine serum albumin was employed as the standard.

Inhibitors were quantified using the technique of immunological radial diffusion in agar gels containing antibodies (10). Antisera to each of the inhibitors were pepared as described previously (10) and pure proteinase inhibitors were used to standardize the assays. Trypsin, chymotrypsin, carboxypeptidase A, and carboxypeptidase B were assayed spectrophotometrically as previously described (2, 3) using tosyl-L-arginine methyl ester, benzoyl-L-tyrosine ethyl ester, hippuryl-L-phenylalanine, and hippuryl-L-arginine as substrates, respectively. All enzymes and substrates were purchased from Sigma Chemical Company.

Chick carboxypeptidase A and B activities were assayed in extracts from chick pancreata. Chicks were sacrificed 10 days after hatching and the pancreata removed, weighed, and homogenized at 4°C in water (20% w/v). After centrifuging at 45,000 rpm at 4°C, the supernatants were activated with trypsin (0.2%) for 1 hr at 4° C and assayed spectrophotometrically as described above. Appropriate quantities of CPI were added to the enzyme in assay buffer and incubated for 2 min at room temperature before assaying.

CPI was prepared by the method of Pearce and Ryan (11), which is based on the differential solubility of CPI in 80% ETOH with respect to other tuber proteins. A brief summary of the method is as follows: Russet Burbank potatoes (5 lb/run) were quartered and placed in a water bath at 80°C for 10 min. The tubers were rinsed with cold water and blended to a paste. Absolute ethanol was blended in slowly to give a final level of 80% ethanol and the mixture was filtered through Whatman No. 1 filter paper with the aid of a vacuum. The filtrate, containing CPI, was vacuum evaporated to remove the ethanol and dialyzed in a 2000-mol wt cut off dialysis bag against three changes of distilled water. The remaining extract in the dialysis bag was lyophilized; the solid material contained approximately 50% salt, but the protein present was almost exclusively CPI as judged by electrophoresis (12) and immunological analyses (10). The remaining salt could be dialyzed away by exhaustive dialysis, but to avoid losses of the small inhibitor, the fraction was employed as is. Although neither Inhibitor I nor Inhibitor II could be detected in the fraction by immunological techniques, a small quantity of trypsin inhibitor activity (less than 0.5%) was present, detected kinetically. It was presumably from contamination by the polypeptide trypsin inhibitor that is known to be present in potato tubers (4).

Proteinase Inhibitor II was purified by the method of Bryant *et al.* (2). Soybean trypsin inhibitor and enterokinase were purchased from Sigma Chemical Company. Russet Burbank potatoes were a gift of the Department of Horticulture and Landscape Architecture, of this university. Newly hatched male Hubbard chicks were purchased from Fors Farms of Puyallup, Washington.

Freeze-dried raw potato tissue was prepared by blending the tubers in a large blender and immediately freezing the slurry in stainless steel trays. The material was lyophilized with a Repp Industrial freeze drier and stored at room temperature. Cooked potatoes were prepared by autoclaving at 120°C for 1 hr and dried overnight in an air drier at 38°C. The dried potato solids were powdered with the aid of a Wiley mill with a 20-mesh screen. Autoclaving destroyed all of the chymotrypsin and trypsin inhibitor activities and over 70% of the CPI activity (cf. Fig. 1), determined by quantitative immunological techniques and verified by enzymic analysis.

The experimental diet employed contained all of the growth requirements for the chicks but allowed for 50% addition of either cooked or raw potato, glucose or glucose plus the in-

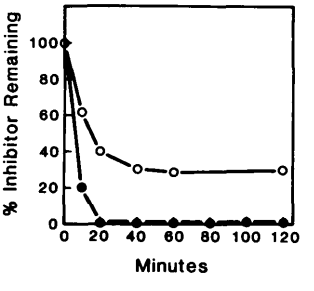

FIG. 1. Loss of Inhibitor II and CPI in autoclaved potato tubers. Russet Burbank potato tubers were cut into pieces (2 × 2 cm) and autoclaved at 120°C for the times indicated. Levels of Inhibitor II (●) and CPI (○) were assayed immunologically (10).

hibitor preparations (13). The basal diet consisted of soybean meal (dehulled) 30.6%, yellow corn 5.2%, fishmeal 2.8%, isolated soy protein 1.075%, dehydrated alfalfa 2.25%, meat and bone meal 4.00%, DL-methionine 0.15%, dicalcium phosphate 0.90%, NaCl 0.30%, mineral premix[3] 0.075%, fat (animal) 2.25%, and vitamin premix[3] 0.40%.

Results and Discussion. Supplementing chick diets with a protein fraction, isolated from raw Russet Burbank potato tubers, enriched in proteinase inhibitors, had previously been shown to severely depress growth (5). The inhibitor-rich fraction contained at least six well-characterized inhibitors of mammalian pancreatic digestive proteinases trypsin, chymotrypsin, elastase, and carboxypeptidases A and B (5). Since the fraction contained an array of proteinase inhibitors it was not known if the carboxypeptidase inhibitor was responsible for any of the growth-depressing activity. Previous data from a number of laboratories have indicated that trypsin inhibitors are the most deleterious to growth and that chymotrypsin inhibitors, when fed alone, are not so toxic (14). In no case, however, was the presence of a carboxypeptidase inhibitor reported or the possible effects of such an inhibitor considered.

Before embarking on a feeding study with CPI, it was important to establish if the inhibitor was indeed an inhibitor of chick carboxypeptidases A and B. Extracts of chick pancreata, containing considerable carboxypeptidase A and B activities, were obtained (see Methods and Materials) and the inhibition activities by CPI were demonstrated (Fig. 2). The inhibition of carboxypeptidase A and B activities by CPI was linear, suggesting that the mode of inhibition of the chick enzymes is similar to the inhibition of homologous mammalian enzymes (3, 7). The potent inhibition of the chick carboxypeptidases by CPI is not surprising since the inhibitor has a broad

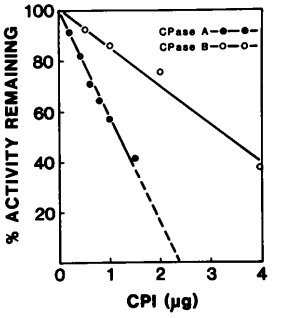

FIG. 2. Inhibition of chick pancreas carboxypeptidases A and B by potato CPI.

specificity toward metallocarboxypeptidases and has been shown to potently inhibit all animal carboxypeptidases A and B tested to date including metallocarboxypeptidases of insects and microorganisms (7).

Having established that CPI in the CPI-enriched fraction strongly inhibited chick pancreas carboxypeptidases A and B, the fraction was fed to baby chicks to establish whether the inhibitor exerted any effects on growth and protein utilization. Preparations of freeze-dried raw and cooked (autoclaved) potato tubers were also added to diets in order to establish basal data for comparisons with the diet containing CPI. In Figs. 3A and B the effects of the raw and cooked potato tubers are shown with respect to their percentage of the total dietary solids. The raw potatoes significantly decreased growth rates, even when added as 10% of the total diet. The effects of raw potatoes became more severe as their percentage of the diet increased (Figs. 3A and B). The raw potatoes caused both a decrease in weight gain and a lower feed efficiency. Severe diarrhea was observed with chicks fed the raw potato diets. Although not quantified, it appeared that a correlation existed between the dietary levels of raw potato solids and the severity of the diarrhea. No diarrhea was observed with chicks fed cooked potatoes.

The CPI enriched preparation was then supplied to chicks at a level equal to the level found in the 50% raw potato diet (cf. Fig. 3A), which was severely growth depressing. Also fed were two pure trypsin inhibitors, potato Inhibitor II, which potently inhibits both trypsin and chymotrypsin, and soybean trypsin inhibitor (SBTI), also a potent trypsin–chy-

[3] Mineral premix at 0.075% of the diets provides the following: Zn^{+}, Mn^{2+}, and Fe^{3+} at 75 ppm; Ca^{2+} at 45 ppm; Co^{2+} at 7.5 ppm, and I at 2.25%. Vitamin premix at 0.40% of diet provides, per kilogram: vitamin A, 5500 IU; vitamin D_3, 1650 ICU; vitamin E, 6.4 IU; thiamin, 1 mg; riboflavin, 3.3 mg; pyridoxine, 2 mg; vitamin B_1, 0.011 mg; pantothenic acid, 9.05 mg; niacin, 22 mg; biotin, 0.05 mg; and ethoxyquin, 62.2 mg.

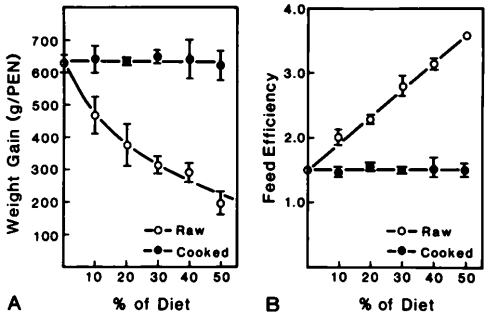

FIG. 3. (A) Effects of freeze-dried raw and cooked potatoes *on* the growth of newly hatched chicks. Chicks were fed diets with increasing percentages of potato solids as described in Methods and Materials. Each value represents an average of 3 pens, 5 chicks per pen, fed each diet for 10 days. (B) The relationships of feed efficiency to the percentage of raw or cooked potato solids in diets. The data represent the feed efficiency for the experimental points in (A). Feed efficiency is the feed consumed (grams) per weight gain (grams).

motrypsin inhibitor. Inhibitor II was also fed at the level found in 50% raw diets (cf. Fig. 3A). SBTI was fed at twice the level of Inhibitor II. The contents of the diets are presented in Table I. The effects of these diets on the weight gain, feed efficiency, fecal protein, and mortality of newborn chicks is presented in Table II. The effects of CPI were small in comparison with the severe effects of raw potatoes, Inhibitor II, and SBTI. The feed efficiency of the CPI diet was only slightly elevated from the control diets and the fecal protein was moderately elevated. Potato Inhibitor II caused a significant decrease in feed utilization, significant decreases in weight gain and increases in fecal protein equal to those resulting from feeding raw potato solids. The results of these experiments indicate that if chick carboxypeptidases A and B are being inhibited by CPI in the intestine, then this inhibition is probably not enough to severely affect chick growth. These results also demonstrate that the trypsin Inhibitor II from potatoes significantly depresses chick growth and may be a major cause of growth depression by the raw potato solids.

Because trypsin and chymotrypsin inhibitors are well known as promotors of pancreatic hypertrophy and hyperplasia, we determined the weights and the contents of activatable pancreatic digestive enzymes in pancreata at the termination of all the experiments to assess the effects of the diets on these parameters. Pancreata were weighed and extracts were prepared and assayed for activities of trypsin, chymotrypsin, and carboxypeptidases A and B, after activating their zymogens with either trypsin or enterokinase.

Diets containing raw potato solids inhibited total growth much more than pancreas growth. The larger pancreata with respect to body weight (Table III) is apparently caused by a hypertrophic condition induced by the raw potatoes. Chicks fed CPI exhibited little growth inhibition and no measurable pancreas enlargements. The pancreata from chicks fed trypsin inhibitors were also larger than expected on a body weight basis. The effects of diets containing raw potatoes and trypsin inhibitors included striking decreases in levels of some pancreatic preproteinases but not of others. CPI did not affect hypertrophy but caused a reduction in levels of both chymotrypsin and trypsin. The differential effects seen in Table III are not understood but must

TABLE I. COMPOSITION OF DIETS CONTAINING COOKED AND RAW POTATOES, CPI, INHIBITOR II, AND SBTI[1]

	Percentage of total diet					
				Inhibitors		
Diet	Basal diet[1]	Glucose	Potato solids[1]	CPI	Inhibitor II	SBTI
1. Control	50	50	0	0	0	0
2. Autoclaved potato	50	0	50 Cooked	0	0	0
3. Raw potato	50	0	50 Raw	0	0	0
4. CPI	50	49.7	0	0.033	0	0
5. Inhibitor II	50	49.7	0	0	0.098	0
6. SBTI	50	49.8	0	0	0	0.20

[1] See Methods and Materials.

TABLE II. SUMMARY OF EFFECTS OF VARIOUS DIETS CONTAINING PROTEINASE INHIBITORS ON THE WEIGHT GAIN, FEED EFFICIENCY, FECAL PROTEIN, AND MORTALITY OF NEWLY HATCHED CHICKS[1]

Diet	Weight gain[2] (g/pen)	Feed efficiency[2,3]	Fecal protein[4] (mg/g)	Mortality[5] (%)
1. Control	675	1.46	65	0
2. Autoclaved potato	572	1.46	64	0
3. Raw potato	197[6]	4.82[6]	153[6]	20
4. CPI	595	1.55	80[6]	0
5. Inhibitor II	494[6]	1.82[6]	135[6]	0
6. SBTI	514	1.80	135	0

[1] Chicks fed diets described in Methods and Materials for 10 days.
[2] Average of three pens, five chicks per pen, except SBTI, which was only one pen.
[3] Feed efficiency equals the feed consumption per weight gain (g/g).
[4] Average of three samples per pen; three pens. Except SBTI diet, three samples, one pen.
[5] Total from all chicks in the experiments.
[6] Significant difference ($P < 0.05$) from control values. No statistical analysis done on SBTI diet.

reflect variations in the feedback mechanisms that are operating in response to the various inhibitors. CPI may simply block the further degradation of trypsin–chymotrypsin–elastase-generated fragments causing a feedback mechanism to shut down synthesis of trypsin and chymotrypsin. A lack of hypertrophy under these conditions would support this mechanism. It is possible that an extended feeding of CPI might cause severe hypotrophy and might further depress growth of the chicks.

To assess direct effects of the diets on fecal protein, which appeared to be significantly increased in the presence of all diets containing proteinase inhibitors, protein in fecal matter from chicks fed the experimental diets (Table I) for 10 days were analyzed by electrophoresis (Fig. 4). The electrophoresis was carried out in 1% sodium dodecyl sulfate (SDS) in which the proteins are separated by size (molecular weights). Electrophoresis of feces from either a control diet (no inhibitors or potato solids) or a diet containing cooked potato solids revealed little protein material (lanes 1 and 2). Feces from diets containing raw potato solids (lane 3), potato Inhibitor II (lane 5), or SBTI (lane 6), contained large amounts of both high- and low-molecular weight proteins, indicating

TABLE III. PANCREATA WEIGHTS AND ACTIVITIES OF CHYMOTRYPSIN, TRYPSIN, AND CARBOXYPEPTIDASES A AND B IN EXTRACTS OF ENZYME-ACTIVATED CHICK PANCREATA AFTER 10 DAYS ON DIETS CONTAINING POTATO SOLIDS OR PURE PROTEINASE INHIBITORS CPI, INHIBITOR II, OR SBTI

			Enzyme activities (units/g pancreas)[2]			
					Carboxy-peptidases[5]	
Diet[1]	Body wt (g/chick)	Pancreas g/100 g Body wt	Tyrpsin[4]	Chymotrypsin[5]	A	B
1. Control	203	0.501	158.0	188.2	83.9	29.5
2. Autoclaved potato	166	0.529	165.2	139.0	95.2	22.1
3. Raw potato	80	0.859[3]	51.0[3]	38.7[3]	31.2[3]	11.8[3]
4. CPI	152	0.479	104.3[3]	70.0[3]	69.5	19.7
5. Inhibitor II	133	0.604[3]	146.2	60.6[3]	46.2[3]	17.2
6. SBTI	131	0.653[3]	151.0	88.4[3]	70.8	19.6

[1] See Table I and Methods and Materials.
[2] One unit is defined as that quantity of enzyme that hydrolyzes 1 μmole substrate/min at 25°C. See Methods and Materials for details of activations and assays. Values are an average of six pancreata for all diets, except five for SBTI.
[3] Significant difference ($P < 0.05$) from control value.
[4] Activated by enterokinase.
[5] Activated by trypsin.

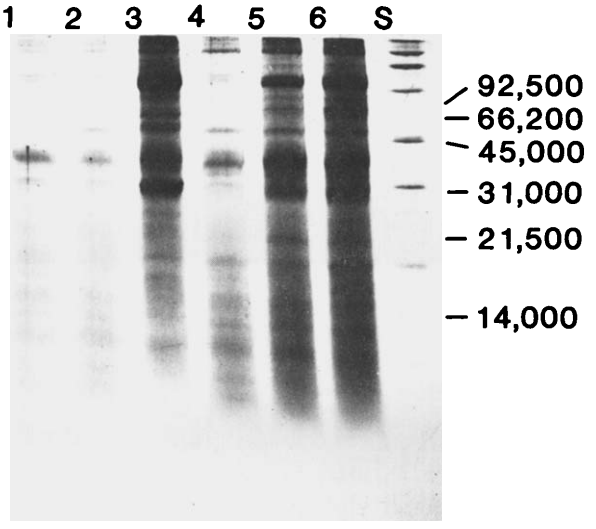

FIG. 4. Electrophoresis of feces obtained from chicks after 10 days feeding on various diets containing (1) control diet; and control diets plus (2) cooked potato solids, (3) raw potato solids, (4) CPI, (5) Inhibitor II, (6) SBTI, and (7) standards. Equal quantities of fecal matter (400 μg/track) were applied. Details of diets are in Methods and Materials.

that the endopeptidase inhibitors were severely inhibiting protein digestion in the intestinal tract. The addition to diets of the exopeptidase inhibitor, CPI, caused an increase in the low-molecular weight proteins of the feces (lane 4) compared with control diets. This indicated

TABLE IV. LEVELS OF INHIBITOR II AND CPI IN CONTENTS OF INTESTINAL TRACT SEGMENTS OF NEWLY HATCHED CHICKS FED DIETS FOR 10 DAYS CONTAINING INHIBITOR II AND CPI[1]

Intestinal segment	Dry weight (mg)		Protein (μg/mg dry wt)		Inhibitors (% protein)	
	Inh. II	CPI	Inh. II	CPI	Inh. II	CPI
Gizzard	230	218	126	131	0.32	0.30
Duodenum	167	225	443	362	0.14	0
Lower intestine Segment						
No. 1	103	127	369	277	0.32	0.18
No. 2	112	137	275	222	0.62	0.24
No. 3	113	132	185	159	1.24	0.45
No. 4	113	120	152	143	1.84	0.71
No. 5	195	125	142	99	2.60	1.37
No. 6	108	107	198	93	2.32	0.99
Cecum	125	88	303	260	3.07	0.96
Colon	137	192	158	118	1.52	1.36
Feces	n.d.	n.d.	135	80	2.37	1.20

[1] See Methods and Materials for diet compositions. The tracts were removed from the chicks and the contents of the gizzard, duodenum, cecum, and colon were recovered. The remainder of the intestine was cut into six equal segments (approximately 10 cm each segment) and the contents recovered by washing with 10 ml H_2O. Contents were freeze-dried and ground in a Wiley mill through a 60-mesh screen. An appropriate sample (25 mg) was dissolved in 0.5 ml of 0.5 *M* NaOH and incubated for 2 hr prior to protein analysis. A similar sample (50 mg in 1 ml H_2O) was heated to 60°C for 10 min, centrifuged, and assayed immunologically (10) for Inhibitor II and CPI concentrations. The data represent the average value of segments from six chicks. Fecal samples were freeze-dried and treated in a similar manner.

that CPI has indeed significantly prevented the complete breakdown of proteins in the intestinal tract, but the severity was minimal compared to the endopeptidase inhibitors.

An experiment was performed to determine whether CPI and Inhibitor II were present in the digestive tract after feeding either the CPI diet or Inhibitor II diet for 10 days. Inhibitor II and CPI are heat stable and can be easily separated from the enzymes they are complexed with in the gut by heat denaturation of the enzymes. The inhibitors were assayed immunologically in the resulting solutions. It can be seen in Table IV that the inhibitors were present throughout the entire length of the intestinal tract, increasing in concentration with respect to the total contents all the way from the stomach to the colon. The inhibitors were somewhat decreased in the feces, perhaps degraded by enzymes secreted by the bacterial flora.

In summary, this is the first report of the inclusion of a carboxypeptidase inhibitor in the diets of animals, of its effects on the digestion and growth of animals and its fate in the intestinal tract. From this study we can conclude that the carboxypeptidase inhibitor from potatoes has some measurable effects on protein digestion and feed efficiency of newly hatched chicks when supplied at levels up to 0.03% of the total diet. However, the level of CPI that caused these effects is much higher than levels that would be consumed by humans under any reasonable circumstances. Even if it potently inhibited human carboxypeptidases A and B, the low levels of CPI found in potatoes (about 0.03% of fresh weight) is considered insignificant as an antinutrient in human diets.

On the other hand, raw potatoes contain a broad spectrum of proteinase inhibitors. Inhibitor II, a potent trypsin inhibitor, is shown in this study to possess significant antinutrient properties. The CPI, while not a toxic compound alone, might contribute to the antinutrient properties of raw potato proteins when present with other inhibitors. CPI may be part of the array of antinutrient proteinase inhibitors that are apparently involved in plant protection as potential deterrents of plant pests. However, all of the proteinase inhibitors in potatoes other than CPI are rapidly destroyed when cooked, and are highly nutritious. As a group, they contain high levels of lysine and sulfur-containing amino acids (8). Their presence in cooked potatoes could be highly beneficial to humans in many parts of the world where potato tubers provide an important source of dietary proteins.

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