possess no detectable phosphorylating mechanism, although they show some phosphatase activity (*4*). The high concentration of phosphorylase in the guard cells suggests that the enzyme may play a part in the osmotic changes of the cells and therefore in the movement of stomata. Experiments along this line are now in progress.

Closer observations reveal that the phosphorylase activity is exclusively localized in the chloroplasts. It is especially clear in cells which have been incubated for but a short time before too much starch has been accumulated (Fig. 1). No activity is found in the nucleus and cytoplasm (*1*). Each chloroplast possesses one or more active loci. As the reaction proceeds, these loci enlarge and merge so that the whole plastid appears to be filled with starch. The localization of phosphorylase in plastids is further corroborated by observations on the chloroplasts of the mesophyll cells, the chromoplasts of Tropaeolum flower, and the leucoplasts of germinating seeds (*4*). The phenomenon is therefore quite general and confirms the anticipation of Hanes (*2*) that starch formation in the plastids is due to the phosphorylase mechanism. Unlike other intracellular enzymes, phosphorylase apparently bears no relation to the nucleus (*1*) or the mitochondria (*3*).

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## Inadequacy of Proteolytic Enzyme Inhibition as Explanation for Growth Depression by Lima Bean Protein Fractions


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Discovery of the presence of trypsin inhibitors in soybeans (*3, 7*) and other legumes (*2*) and the ability of concentrates of these factors to inhibit growth in rats (*11*) and chicks (*8*) have stimulated interest in the mechanism involved in the poor nutritional values of the proteins of raw legumes.

An obvious hypothesis is that these bean fractions exert growth-inhibiting effect through their ability to inhibit normal enzymatic protein hydrolysis in the intestinal tract. This report concerns experiments with hydrolyzed proteins which have given results contradictory to that hypothesis.

Desikachar and De (*6*) have recently reported that active extracts of the soybean trypsin inhibitor had a depressing effect on the biological value of papain-digested soybean meal essentially equal to their effect on undigested meal. The two values reported, 45.9 and 44.7, respectively, were quite low, and presumably much lower than a value for undigested soybean meal in the absence of the inhibitor fraction would have been if data on such a positive control had been obtained. Although these data suggest that the low biological value of raw legumes is not due primarily to inhibition of enzymatic digestion, it must be pointed out that several studies (*1, 4, 9, 12*) have shown that papain, at a maximum, hydrolyzes only one-half to two-thirds of the peptide bonds in casein and other proteins. Also, solubility of 91.6% of the nitrogen of the papain digest in 7% trichloroacetic acid, used by Desikachar and De to estimate completeness of hydrolysis by papain, does not preclude the existence of some soluble peptides (*1, 5*).

Hence, the possibility has remained that the low biological value found for the papain-digested meal was due to inhibition of hydrolysis of residual bonds. As a part of our continuing study of the growth inhibitors in legumes, we have carried out experiments that eliminate this possibility in so far as trypsin-inhibiting activity obtained from lima beans is concerned.

Lima bean protein fractions that contained high *in vitro* trypsin-inhibiting activity and also high growth-inhibiting activity in normal diets were fed to rats on diets containing completely acid-hydrolyzed casein as the sole source of nitrogen. In addition to nitrogen-contributing components, the diets contained, in per cent: cottonseed oil, 5; U.S.P. cod-liver oil, 2; salt mixture (McCollum's No. 185 plus trace elements), 4; dried liver extract (Wilson), 0.4; protein-free yeast extract equivalent to 2% yeast, and, as mg%, α-tocopherol, 5; choline chloride, 150; inositol, 100; thiamin hydrochloride, 0.5; riboflavin, 1.0; pyridoxin, 0.5; nicotinic acid, 1.0; calcium pantothenate, 2.5; *p*-aminobenzoic acid, 7.5; 2 methyl-1,4-naphthoquinone, 0.2; biotin, 0.01; and sufficient equal-weight mixture of corn starch and cane sugar to make 100%. All diets contained 15/6.25% = 2.4% nitrogen, made up in diets 1 to 5 from 0.4% *dl*-tryptophan, 0.6% *l*-cystine, and 20% casein hydrolysate and in diets 6 and 7, from commercial casein, plus, in both cases, the nitrogen contributed by the lima bean fraction when present. The lima bean fraction was added to the diet without an equivalent deduction in amount of casein-hydrolysate nitrogen in order to eliminate the possibility that the inhibiting effect would be even partly due to a lowered concentration of available amino acid or protein nitrogen in the diet.

Casein hydrolysates were prepared by autoclaving commercial casein with four parts of 9N $H_2SO_4$ at 15 lbs of pressure for 18 hrs. The sulfuric acid was removed with barium hydroxide to a pH of 4 to 5, and the resultant filtrate was concentrated *in vacuo* and dried from the frozen state. The completeness of hydrolysis was verified by amino nitrogen analyses, which indicated 71–74% of total nitrogen present as amino nitrogen (*13*).

A modification of Kunitz' (*10*) method of isolating the

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the globulin-trypsin inhibitor from soybeans was used to prepare a lima bean protein fraction with a high trypsin-inhibiting activity. Ground lima beans were extracted with dilute acid, and the active factor in the extract adsorbed on bentonite, eluted wih aqueous pyridine, dialyzed free of pyridine, precipitated by ammonium sulfate, dialyzed salt-free, and dried from the frozen state. *In vitro* tests with crystalline trypsin and casein substrate indicated that this preparation produced 95% or more inhibition of one-fifth of its weight of crystalline trypsin protein.

The experimental diets were fed to groups of 10 albino rats (5 males and 5 females per group, with an average initial weight of 80 gm) for 6 days, during which daily records were kept of food consumption per group and weight of the individual rats. Experiments of this nature, carried for as long as 7 weeks in this laboratory, have conclusively demonstrated that results after 5 days are completely indicative of the relative values after 6 weeks or longer. Litters were divided between groups so that comparison between litter mates was possible; however, results on this basis did not differ significantly from those based on average weight gain per group.

The results of two experiments are presented in Table 1. Experiments A and B were run with different batches of casein hydrolysate and with different amounts of added tryptophan, which may explain the relatively minor quantitative differences in some cases. The lima bean fraction produced a marked depression of growth when fed at both the 1.6 and 3.2% levels. Growth rates on diets containing the heated, and hence inactivated, lima bean fractions were essentially equal to the rate on the basal diet. The growth-depressing effect of the lima bean fraction in the hydrolysate diet was equal to, or greater than, the effect in the diet containing the unhydrolyzed protein. These results are consistent with the hypothesis that lima bean protein fractions that have exceptionally high trypsin-inhibiting activity exert their major growth-inhibiting effect through some mechanism not directly related to the inhibition of the normal enzymatic hydrolysis of dietary protein. A more extensive study of the growth-inhibiting effect of crude and purified lima bean protein fractions will be published at a later date.

TABLE 1

GROWTH INHIBITION PRODUCED BY LIMA BEAN PROTEIN FRACTIONS IN RATS ON CASEIN HYDROLYSATE DIETS

| Group | Nitrogen source | Feed consumption (gm/rat/day) A* | Feed consumption (gm/rat/day) B | Weight gain (gm/rat/day) A | Weight gain (gm/rat/day) B | Gain in weight (gm/gm N consumed) A | Gain in weight (gm/gm N consumed) B |
|---|---|---|---|---|---|---|---|
| 1 | Casein hydrolysate + tryptophan + cystine | 8.5 | 9.3 | 3.3 | 2.5 | 16.1 | 11.2 |
| 2 | " + lima bean fract., 1.6% | 7.2 | ... | 1.7 | .. | 8.6 | ... |
| 3 | " + lima bean fract., 1.6%, heated at 15 lbs steam for 30 min | 8.0 | ... | 3.4 | .. | 15.8 | ... |
| 4 | " + lima bean fract., 3.2% | 6.7 | 7.6 | 1.1 | 0.3 | 6.0 | 1.1 |
| 5 | " + lima bean fract., 3.2%, heated at 15 lbs steam for 30 min | 8.4 | 10.0 | 4.1 | 3.3 | 18.2 | 11.2 |
| 6 | Commercial casein | 9.0 | 11.1 | 3.5 | 4.4 | 16.2 | 16.5 |
| 7 | " " + lima bean fract., 3.2% | 7.3 | 8.8 | 1.1 | 1.5 | 5.0 | 5.7 |

* Experiment A, 0.4% tryptophan, 0.6% cystine; Experiment B, 0.25% tryptophan, 0.8% cystine.


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