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Stimulatory Effects of Peptides on Growth of the Free-Living Nematode *Caenorhabditis briggsae*[1] (38615)

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Recent work on ageing and genetics of higher organisms using nematodes as model systems has focused attention on the need for completely defined axenic culture media for these organisms. The free-living nematode *Caenorhabditis briggsae* requires the addition of a proteinaceous factor to a medium containing amino acids, vitamins, minerals, nucleotides, heme and a sterol (1–4). Proteins from widely different sources have been found to be active (5–7).

A recent paper (8) has proposed that such proteins derive their sole activity from facilitation of uptake of the essential nutrient heme, possibly by stimulation of phagocytosis. This theory is supported by the activating effect of precipitation on proteins and on heme itself. The evidence presented, however, has not been supported by experimental work in our own laboratory and we feel alternative modes of action of protein growth factors should be considered. The possibility that such growth factors may derive their activity from peptide fragments produced by gut hydrolysis was investigated and evidence for this is presented here. Peptides are already known to be of considerable importance in the nutrition of bacteria (9) and mammals (10, 11), however this is the first indication that they may play a role in the nutrition of invertebrate metazoa.

Materials and Methods. The assay method used was a modification of the mass culture method of Tomlinson and Rothstein (12), in which nematodes were grown in 1.0 ml of medium in 13 mm diameter tubes rotated at 1.0 rpm at 20°. The basal medium consisted of *C. briggsae* Maintenance Medium (CbMM, Grand Island Biological Company), β-sitosterol (50 μg/ml) and cytochrome C (50 μg/ml). Peak populations of 10^5 nem/ml are routinely achieved in casein-supplemented medium using this method. Casein was dissolved in 0.1 *M* KOH and adjusted to pH 6.4 and all supplements were sterilized by autoclaving 8 min at 15 psi, before addition to the basal medium. To reduce carryover of nutrients in the inoculum, *C. briggsae* was cultured in unsupplemented CbMM/β-sitosterol/cytochrome C for 14 days prior to inoculation.

Results and Discussion. Casein and its commercial acid hydrolysate, casamino acids, were found to support consistently high levels of growth of *C. briggsae* in CbMM/β-sitosterol/cytochrome C over an extended period of culture (7 mo, 11 subcultures).

The response of *C. briggsae* to casein, casamino acids, commercial enzymatic hydrolysate (DIFCO casitone) and soy peptone is shown in Fig. 1. At concentrations up to 2.5 mg/ml, commercial hydrolysates of casein appeared more active than the protein itself. Amino acid analysis of the acid hydrolysate of casein indicated that up to 20% of amino acid residues were still bound in peptide form. A mixture of free L-amino acids reconstituted in the same proportions as in the acid hydrolysate had little or no activity (Fig. 1). The higher activity of the hydrolysates at low levels, therefore, appears to be due to the presence of peptides.

At higher concentrations, the acid hydrolysate inhibited growth. This inhibition was unrelated to the high level of salt present (36% dry wt). A similar but less marked inhibition was observed in cultures supplemented with high levels of a mixture of free amino acids (Table I), indicating that high concentrations of free amino acids may be inhibitory.

Progressive hydrolysis of low levels of casein should result first in activation, then a fall off in activity as hydrolysis nears

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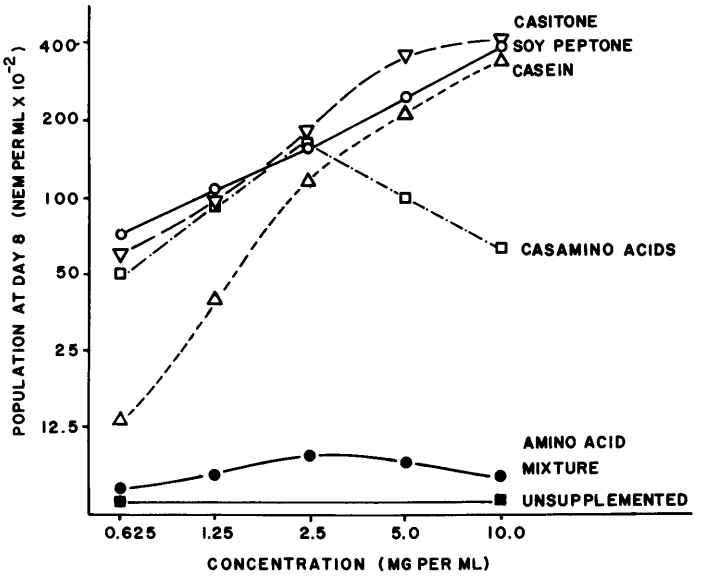

FIG. 1. Growth response of *C. briggsae* to casein and casein hydrolysates in sterol and heme supplemented *C. briggsae* Maintenance Medium. ▽ - - - - - ▽, enzymatic hydrolysate (DIFCO casitone); ○——○, soy peptone; △ - - - - - △, casein; □—·—□, acid hydrolysate (DIFCO casamino acids); ●——●, reconstituted mixture of amino acids; ■——■, no addition.

completion if peptide fragments of casein are more active than the protein itself and free amino acids show little or no activity. Results obtained with casein hydrolyzed under vacuo in 6 *N* HCl at 110° for increasing lengths of time are shown in Table I. The 1, 4 and 12 hr hydrolysates showed progressive activation, while activity of the 24 hr hydrolysate was diminished. These data suggest that protein growth factors may derive all or part of their activity from peptide fragments produced by incomplete hydrolysis in the gut of the nematode. The high activity of the 12 hr hydrolysate suggests that fairly small molecular weight peptides are active.

Snell and coworkers, investigating the stimulatory effects of protein hydrolysates for bacteria, found that hydrolysates could be replaced by a number of single di- and tripeptides (13, 14). Such peptides were active in mixtures of amino acids when uptake of free essential amino acids was inhibited by a competing amino acid, or the free amino acid, having been taken up, was unavailable to the cell (15). Peptides have since been shown to be transported independently of amino acids and hydrolyzed intracellularly in both bacteria and mammalian gut (9, 10), thus becoming, in many cases, more efficient sources of amino acids than the amino acids themselves (11, 16).

C. briggsae maintenance medium has high levels of nonessential as well as essential amino acids based on the ratios present in *E. coli* (2). Competition for uptake of amino acids, as has been reported in cestodes by Read *et al.* (17, 18), may limit availability of one or more essential amino acids. The growth inhibition at high levels of casamino acids and the amino acid mixture which has been observed in these experiments may be due to such competition. Uptake of such a limiting amino acid in a more readily available peptide form could be expected to stimulate growth, and a mixture of peptides, such as is present in an incomplete hydrolysate, may satisfy requirements for several limiting amino acids. Different peptides containing a single limiting residue would then have partial activity, but no single peptide would have the full activity of the protein hydrolysate. Moreover, a wide range of proteins would be expected to show growth factor activity.

A number of individual di- and tripeptides

TABLE I.[a]

Expt	Supplement	Level (mg/ml)	8-Day population (nem/ml)	15-Day population (nem/ml)
I	Unsupplemented	0.0	550	900
	Casein	0.625	640	4900
	1 hr hydrolysate	0.625	1400	10,500
	4 hr hydrolysate	0.625	2700	20,000
	12 hr hydrolysate	0.625	5500	29,000
	24 hr hydrolysate	0.625	1100	11,800
II	Unsupplemented	0.0	750	1400
	Acid Hydrolysate of Casein (DIFCO Casamino Acids)	2.00	3700	56,000
	Casein	2.00	3700	18,000
	L-leucyl-L-phenylalanine	0.25	1000	4600
		0.50	2100	9400
		1.00	4600	18,000
		2.00	6700	21,000
	L-leucine + L-phenylalanine	1.00	630	1900
		2.00	1000	2400
	L-leucine + L-phenylalanine 1.00 L-leucyl-L-phenylalanine 1.00	2.00	4300	17,000
III	Precipitated hemin	200[b]	810	840
		100[b]	900	1300
		50[b]	880	1800
		25[b]	1400	2300
		12.5[b]	800	1400
	Soluble hemin	50[b]	710	740
	Soluble hemin Casein	50[b] 10[b]	6700	9000

[a] The basal medium in Expts I and II comprised CbMM, β-sitosterol (50 μg/ml) and cytochrome C (50 μg/ml). Precipitated hemin in Expt. III was prepared according to Van Fleteren's method (5). Stock hemin chloride solution was precipitated by dilution to 400μ g/ml in 0.01 *N* HCl. The suspension was serially diluted in the ratio 1:1 with CbMM/β-sitosterol at two times final concentration (50 μg/ml β-sitosterol). pH at 200 μg/ml was 5.0.

[b] Microgram/milliliter.

were assayed to see if such partial stimulatory activity could be observed (Table I, Expt. II). Di- and tripeptides containing phenylalanine, particularly l-leucyl-l-phenylalanine stimulated growth, while an equimolar mixture of the free amino acids showed no activity. Histidine containing peptides were less active, and glycine containing peptides were inactive.

Van Fleteren (8) has postulated that protein supplements are active solely as vectors of hemin, an essential nutrient for *C. briggsae* (3). He showed considerable growth in basal medium containing sterol and precipitated hemin in the absence of a protein supplement and concluded that the presence of particles stimulated the uptake of hemin. However, we have been able to obtain only slight stimulation of growth with precipitated hemin, both with the large-scale assay method used in these experiments (Table I, Expt. III) and with the larval assay method used by Van Fleteren (8). Neither precipitated hemin nor soluble hemin (50 μg/ml, pH 5.2) supported growth or maturation of newly hatched larvae, although casein at 6.3 mg/ml and soluble hemin supported maturation and reproduction to a population of 500 nematodes in 15 days.

Conclusion. In the light of the above results, we feel that the protein growth factor for *C. briggsae* cannot be considered solely as a vector of heme absorption. Evidence

presented here suggests that the protein itself provides nutrients in forms which are more readily available to the nematode. The possibility that peptide bound amino acids can be utilized preferentially to the free form has not been investigated in invertebrate metazoa, although considerable work has been done on competitive amino acid transport in cestodes by Read and colleagues (17, 18). The occurrence of other cases (19) where the culture of organisms requires the presence of polypeptides indicates that peptide transport may be a widespread phenomenon in the animal kingdom.

Summary. Evidence is presented that peptide products of hydrolysis of casein, including some di- and tripeptides, but not the constituent amino acids, can stimulate growth of *C. briggsae* in defined basal medium supplemented with cytochrome C and β-sitosterol. Peptide activity may arise from amino acid imbalances in the medium which cause competitive inhibition of uptake of essential amino acids. Such activity precludes facilitation of heme transport as the sole function of growth factor in *C. briggsae*. This is the first report of a nutritional role of peptides in invertebrate metazoa.

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