A Nematode Growth Factor from Baker's Yeast[1]

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Abstract: An extract prepared from commercially available yeast supported maturation of the free-living nematode *Caenorhabditis briggsae*. The extract can be used to supplement a chemically defined medium or, after a limited dialysis, as a complete medium. Several biologically active fractions were prepared; those containing larger amounts of ribonucleic acid (RNA) had greater biological activity, the most active being a pellet resuspended after centrifugation at 30,000 × *g* for 30 min. This fraction could be substituted for serum in a medium which supports the maturation of the animal parasites *Trichinella spiralis* and *Hymenolepis nana*. Addition of protamine sulfate decreased the RNA content, leaving inactive protein fractions which could be reactivated by specific treatments that caused protein precipitation. It is postulated that biological activity is associated with protein sedimented with ribosomes. *Key Words:* Axenic culture, *Caenorhabditis briggsae*, Particle formation, Ribosomes.

The free-living nematode *Caenorhabditis briggsae* can be cultivated axenically in a chemically defined medium (3, 6, 15) supplemented with proteinaceous growth factor extracted from liver (14). This growth factor is biologically inactive at a 100 μg/ml protein level unless activated by freezing (7), mild heating (8, 9, 15) or addition of Ficoll (3). Activation in each case is dependent upon precipitation of the proteinaceous supplement (4).

More recently, supplements from yeast extracts have supported the reproduction of *C. briggsae* and several other nematodes (2). In the present study biological and biochemical properties of growth factor(s) from yeast extract are investigated and the increase of biological activity by precipitate formation is discussed.

MATERIALS AND METHODS

PREPARATION OF GROWTH FACTORS: Yeast extracted was prepared from baker's yeast cake (Fleischmann's) by homogenization either in a Waring blender or in a colloid mill as described by Buecher and Hansen (2). Briefly, the pertinent features of the method are as follows: a 2 lb yeast cake, suspended in 800 ml of 0.05 M potassium phosphate buffer (pH 6.5), was homogenized for 1 hr using 800 ml of Superbrite® (Minnesota Mining and Manufacturing Co.) glass beads, average diameter 120 μ. The homogenate was centrifuged for 20 min at 10,000 × *g* to remove unbroken cells and cell walls. The supernatant fluid was designated "yeast extract" and stored at a protein concentration of 50 mg/ml at –25 C.

Solid ammonium sulfate was slowly dissolved (by stirring) in yeast extract to the percent of saturation desired (refer to Table 1). The solution was placed at 4 C for 30 min, and the precipitate, collected by centrifugation at 27,000 × *g* for 20 min, was dissolved in 0.10 M potassium phosphate buffer, pH 7. Dialysis of dissolved ammonium sulfate precipitate continued until ammonia was no longer detectable with Nessler's reagent.

A freshly prepared 2% aqueous solution of protamine sulfate (Calbiochem) was slowly added to the yeast extract with stirring. When the specified concentration was reached (refer to Table 2) the precipitated extract was kept at 4 C for 10 min, and the precipitate collected by centrifugation at

Received for publication 27 June 1969.

[1] This investigation was supported by PHS Research Grant A1-07359 from the National Institute of Allergy and Infectious Diseases.

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30,000 × *g* for 15 min. All protamine sulfate precipitates were suspended in 0.05 M potassium phosphate buffer, pH 7.0, and dialyzed against this buffer.

Ribonuclease (bovine pancreatic, 5x-crystallized) (Calbiochem) was added to yeast extracts at 1% of the protein concentration and incubated at 23 C for 24 hr.

Protein concentrations were estimated spectrophotometrically on a Beckman DB-G spectrophotometer at 280 and 260 mμ using the method of Warburg and Christian (16). When large amounts of nucleic acids are present in crude protein preparations, protein concentrations determined at the above absorbancies are subject to error; therefore, when more accuracy was required, determinations were made colorimetrically with the Folin phenol reagent according to the method of Lowry (11). The values determined by this method are indicated by the subscript L. The percentage of RNA was based on the ratio of RNA determined by the orcinol method (5) to the protein determined by the Lowry method.

Protein solutions were subjected to electrophoresis at 100 volts for 17 min in veronal buffer of 0.06 ionic strength, pH 8.5 on a Millipore Phoroslide apparatus.

BIOLOGICAL ASSAY: The hermaphroditic nematode *Caenorhabditis briggsae* was used in all assays. Yeast extract supplements, sterilized by passage through a 0.3 μ Millipore filter, were added to the *C. briggsae* chemically defined medium (CbMM) (obtained from Grand Island Biological Co. at 2× concentration, pH 6). The biological activity of the proteinaceous supplement was determined on the basis of the time required for maturation of three newly hatched larvae inoculated into each of two tubes containing 0.25 ml of medium. The number of days required for a complete reproductive cycle is designated "F_1 time." The "relative response" is the ratio of the 2.8-day F_1 time of *C. briggsae* cultured on *E. coli* to the assay F_1 time × 100.

Yeast extract preparations were treated by methods used to activate growth factor isolated from liver: freezing of the complete medium (7), inclusion of 7.7% w/v Ficoll in the medium (3), heating of the factor at 53 C for 5 min (15), addition of γ-globulin-cardiolipin (CL) micells (4), and addition of polystyrene latex beads (PSL), 250 mμ in diameter (Dow Chemical Co., Midland, Michigan) (4). Activation was also accomplished by aseptically changing the pH of the chemically defined medium to 7.4.

RESULTS

YEAST EXTRACT AS A COMPLETE MEDIUM: Yeast extract could be used as the complete medium for the maturation of *C. briggsae* if it was first dialyzed for more than 2 but less than 6 hr against water or buffer. Two ml of yeast extract were dialyzed aseptically against 1 litter of 1 mM potassium phosphate buffer, pH 7. Without dialysis, the extract failed to support maturation, whereas in extracts dialyzed for 2, 3, and 6 hr maturation occurred in 4 days and good populations developed. After prolonged dialysis, the yeast extract was no longer effective as a complete medium.

YEAST EXTRACT AS A SUPPLEMENT: Dialyzed yeast extract was satisfactory as a supplement to the chemically defined medium (CbMM). Yeast extracts dialyzed 24 hr against 0.005 M or 0.05 M potassium phosphate buffer, pH 7, gave a relative response of 50% at 250 μg protein/ml.

PREPARATION OF YEAST FACTOR: Ammonium sulfate precipitation was the first step in the purification of yeast extract. Several ammonium sulfate precipitated fractions were prepared; their biological activities are compared in Table 1. At approximately 250 μg protein/ml, the 0–40% ammonium sulfate fraction gave the best relative response

TABLE 1. Biological activity of ammonium sulfate precipitated fractions of yeast extract used as supplement to CbMM.

Ammonium sulfate % sat.	Protein level tested μg/ml	Relative response[a] %
0	210	57
0–40	250	74
40–60	250	46
60–70	250	0
70 supernatant	250	0
0–70	312	41

[a] The ratio of the 2.8 day maturation time of *C. briggsae* cultured with *E. coli* to the F_1 time expressed as a percentage.

(74%). The 40–60% fraction was less active, whereas the 60–70% fraction and the 70% supernate fraction were inactive. When the 0–40% fraction was placed on hydroxylapatite and eluted with 0.15 M and 0.5 M potassium phosphate buffer at pH 7, no activity was recovered.

The untreated extract and the 0–40% fraction both retained activity after freezing, but both were grossly precipitated, making further fractionation impossible. Although the 0–70% fraction lacked high biological activity, it did not precipitate after being frozen and thawed several times. Frozen storage of samples is essential because proteolytic activity can occur at 4 C; therefore, the 0–70% fraction was chosen for further investigation. This fraction will be referred to as "yeast factor."

ACTIVATION OF YEAST FACTOR: Determinations were made to see whether the various treatments essential for the activation of liver growth factor would affect the activity of the yeast factor assayed at 300 μg protein/ml. When yeast factor, usually at a phosphate level of 0.15 M, was dialyzed against 0.05 M potassium phosphate, the relative response was enhanced from 41% to 80%. When the pH of the complete medium was brought from 6.1 to a final pH of 7.42, the relative response increased to 93%. Titration at this pH to 100 μg protein/ml gave a relative response of 80%. Heating of the concentrated factor at 53 C for 5–10 min increased the relative response to 93%.

Visual turbidity was increased both by heat activation and pH activation of yeast factor, suggesting that, as with liver growth factor (4), precipitation of yeast factor enhances biological activity.

When dialyzed yeast extract was added to the defined medium, at least 24 hr elapsed before precipitation was noticeable. This raised the question whether nematodes transferred immediately into the clear medium would respond in the same manner as those placed in the precipitous medium.

Yeast extract was dialyzed for 24 hr against 0.05 M potassium phosphate buffer at pH 7 and was assayed in CbMM at 250 μg protein/ml. Initially, the absorbance at 550 mμ was 0.005 O.D. units. Nematodes inoculated at this time had a relative response of 48% (F_1 time = 5.8 days). When the medium was preincubated at 20 C for 5 days prior to inoculation, the absorbance increased to 0.030 O.D. units and the relative response was 80% (F_1 time = 3.5 days).

Since biological activity was increased in the presence of precipitation it was of interest to test the higher molecular weight material. Yeast extract was centrifuged at 30,000 × *g* for 30 min; the pellet was resuspended in 10 ml of 0.05 M potassium phosphate buffer, dialyzed against 0.05 M potassium phosphate buffer, and assayed after sterile filtration. This preparation contained approximately 1 part RNA to 3 parts $protein_L$ and was highly active; at 68 μg $protein_L$/ml, untreated media gave a relative response of 93%. Titration to 5 μg $protein_L$/ml gave a relative response of 30% after freeze activation.

The 30,000 × *g* supernatant fraction also exhibited biological activity. At 68 μg $protein_L$/ml untreated media had a relative

response of 25%, whereas freeze activated media had a relative response of 72%.

REMOVAL OF RNA FROM YEAST EXTRACT AND YEAST FACTOR: Since both yeast extract and yeast factor had a high RNA content, it seemed advisable to remove it prior to protein fractionation. When ribonuclease was added to either yeast extract or yeast factor at a final concentration of 1%, a precipitate formed which increased with incubation at 20 C. After ribonuclease treatment for 24 hr, the precipitate was removed by centrifugation; the supernatant fluid was dialyzed and examined for RNA content. The absorbance ratio 280/260 mμ increased from 0.65 to 1.2 with a five-fold loss in absorbance at 260 mμ. However, no biological activity was found in these preparations. When this treatment was repeated under sterile conditions and the precipitate retained, there was good biological activity.

Another experimental approach for the removal of RNA from extracts is precipitation with protamine sulfate (12). The 30,000 × *g* supernatant fluid, which exhibited biological activity but still contained considerable RNA was further fractionated with protamine sulfate. Supernatant and resuspended pellet fractions were dialyzed in 0.05 M potassium phosphate buffer, pH 7, and assayed at 250 μg protein/ml.

Biological activity and an estimate of the RNA content of the protamine sulfate fractions are shown in Table 2. By the stepwise addition of protamine sulfate, the 280/260 mμ ratio increased from 0.68 to 1.60 and the RNA content decreased to 9%; however, the biological activity decreased (see Fraction C). Only fractions A and D were active without prefreezing of the medium. Fractions E and F were totally inactive and fractions B and C were active after prefreezing of the complete medium.

ACTIVATION OF PROTAMINE SULFATE FRACTIONS B AND C: As shown in Table 2, the two protamine sulfate fractions with the lowest RNA content, fractions B and C, could be activated by prefreezing the complete medium. Other treatments were also effective in activating these fractions (Table 3). Both fractions were activated by heating at 53 C for 5 min and by adding Polystyrene latex beads; the addition of Ficoll activated only fraction B.

TABLE 2. Biological activity of protamine sulfate (PSO_4) precipitated fractions of yeast extract used as a supplement to CbMM at 250 μg protein/ml.

Fraction	Method of preparation	Relative response[a] (%) Untreated	Treated[b]	280/260	RNA[c] %
SUPERNATANT	30,000 × g	28	72	0.68	22
–A	0–.06% PSO_4	24	62	0.72	26
–B	.06–.12% PSO_4	0	47	1.02	11
–C	.12–.18% PSO_4	0	42	1.60	9
PELLET					
–D	0–.06% PSO_4	72	35	0.52	80
–E	.06–.12% PSO_4	0	0	0.50	83
–F	.12–.18% PSO_4[d]	0	0	0.67	–[e]

[a] The ratio of the 2.8 day maturation time of *C. briggsae* cultured with *E. coli* to the F_1 time expressed as a percentage.
[b] The complete medium prefrozen –25 C for 24 hr.
[c] Ratio of RNA to total protein.
[d] Low recovery from sterile filtration.
[e] Not determined.

TABLE 3. Biological activity of two protamine sulfate supernatant fractions after treatment by several "activation" methods.

Treatment	Relative response[a] (%) at 250 μg/ml Fraction B	Fraction C
None	0	0
Heated 53 C for 5 min	55	41
7.7% FICOLL added	20	0
γ-globulin-cardiolipin added	0	0
Polystyrene latex beads added	32	20

[a] The ratio of the 2.8 day maturation time of *C. briggsae* cultured with *E. coli* to the F_1 time expressed as a percentage.

ELECTROPHORETIC PATTERNS OF YEAST PREPARATIONS: The electrophoretic patterns of purified liver growth factor shows a major band in the β-globulin region. Dialyzed yeast extract, the resuspended 30,000 $\times$ *g* pellet, and protamine sulfate-precipitated fractions B and C all contained a protein band in this position, whereas fractions D, E, and F had a group of bands of high electrophoretic mobility, near the albumin band.

BIOLOGICAL ACTIVITY OF *E. COLI* 70S RIBOSOMES: Since the greatest activity was associated with a pellet which probably contained denatured ribosomes, *E. coli* 70S ribosomes[3] were assayed in CbMM at 250 μg protein$_L$/ml. *C. briggsae* matured in 5.8 days, the relative response being 48%. By prefreezing the complete medium, the relative response at 250 μg protein$_L$/ml increased to 88%. At 100 μg protein$_L$/ml, the relative response in the prefrozen medium was 80%; this activity was retained through 7 serial sub cultures.

CULTURE OF ANIMAL PARASITES: The 30,000 $\times$ *g* resuspended pellet, one of the most active yeast preparations, was tested for its ability to grow animal parasites. The fraction was dialyzed against 0.005 M potassium phosphate, pH 7 to avoid possible phosphate toxicity. The following results were obtained in collaboration with A. K. Berntzen (personal communication). When the yeast fraction at 1 mg protein$_L$/ml was substituted for serum in Berntzen's basal medium (1), it supported the growth of the cysticercoid stage of *Hymenolepis nana* and the larvae of *Trichinella spiralis*. At 250 μg protein$_L$/ml in the presence of serum, the yeast preparation enhanced both the development of *H. nana* eggs to the cysticercoid stage, and the growth of *T. spiralis* larvae.

DISCUSSION

The availability of a new source of nematode growth factor from yeast extracts provides a useful supplement for culture of free-living nematodes, and may be of value in the culture of other organisms. This proteinaceous material can be used either as a supplement or as a complete medium, if properly prepared. Large quantities can be obtained by homogenizing commercially available baker's yeast. The procedures for isolating the active fractions of yeast extract are relatively simple and have produced a fraction which is more active than liver growth factor and nearly as active as bacteria in supporting the growth of *C. briggsae*. In addition, several parasitic species are supported or enhanced in growth by the addition of this fraction of yeast extract.

Hitherto, nematode growth factor was isolated from liver by ammonium sulfate precipitation followed by hydroxylapatite chromatography. Similar methods applied to yeast extracts failed to yield an active preparation.

As with growth factor isolated from liver, the biological activity of the yeast fractions is associated with a precipitate in the medium. Yeast extracts form a precipitate after being added to the chemically defined medium while the higher molecular weight fractions, which can be suspended in buffer and filtered, aggregate during frozen storage. Biological activity can be increased in these yeast preparations at 100 μg protein/ml levels by treatments that enhance precipitate formation.

When the RNA was removed from yeast extra by the stepwise addition of protamine sulfate, the ability of the yeast extract to precipitate decreased and it became necessary to use activation methods to obtain biological activity. The necessity of activat-

[3] A preparation of 70S ribosomes from *E. coli* K-12 grown in a basal salts medium (M-9) supplemented with glucose was a generous gift of David Goodman, University of California at Berkeley.

ing these yeast fractions by treatments which precipitate proteins makes it difficult to interpret their specific activity. Any variation in presentation of material to the nematode could alter the apparent biological activity of the supplement.

Liver growth factor has good biological activity after activation even though it contains little or no RNA. If yeast factor and liver growth factor are chemically similar, the RNA content of the yeast factor must be non-essential. If the protein is the essential component, the question arises whether the biological activity is due to basic proteins precipitating with the denatured ribosomes, to ribosomal structural proteins, or to proteins being made on the ribosomes but still attached. Since purified 70S ribosomes were biologically active, it appears that the growth factor(s) is (are) protein(s) associated with the ribosomes. The next step would be to prepare yeast ribosomes and to determine their activity. If most of the activity were found there, proteins could be eluted (13) and the nature of the yeast factor(s) further defined.

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