Vol. 127, No. 3, 1985 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 29, 1985 Pages 713-719

BACILLUS SUBTILIS CONTAINS MULTIPLE FORMS OF SOMATOSTATIN-LIKE MATERIAL

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Received January 10, 1985

Extracts of B. subtilis contain somatostatin-like immunoactivity (1-20 pg per g wet weight cells). Two major forms were detected, one with reactivity in both N-and C- terminal immunoassays similar to somatostatin-28 and a second form reactive only in the C-terminal specific immunoassay similar to somatostatin-14. Both forms were active in a bioassay and the bioactivity was neutralized in the presence of antibody to the central, biologically active part of somatostatin-14. Preconditioned medium contained no detectable somatostatin whereas conditioned medium had 80-380 pg per liter. © 1985 Academic Press, Inc.

Previous reports from our laboratory have described the presence of materials similar to vertebrate-type peptide hormones in unicellular eukaryotes and some bacterial strains (1-6). Most recently we reported the existence of materials in extracts of Escherichia coli resembling somatostatin (SRIF)(6). Two forms of SRIF-immunoactivity were found following HPLC purification: one form, somatostatin-28 (SRIF-28) was reactive in both N- and C-terminal specific immunoassays and somatostatin-14 (SRIF-14) that was only reactive in the C-terminal specific immunoasssay (6).

To determine whether the presence of somatostatin-like immunoactivity (SRIF-LI) is widespread amongst bacteria we have searched for the presence of SRIF-LI in Bacillus subtilis grown in defined, synthetic medium. This report describes the presence of both SRIF-28 and SRIF-14 immunoactivity in B. subtilis and in addition demonstrates that the HPLC purified material was active in a bioassay for somatostatin.

0006-291X/85 $1.50
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MATERIALS AND METHODS

Fermentation: Five batches of B. subtilis (no 61293, a gift of Dr. E. Freese, NIH, Bethesda) were grown in 50-100 liter fermenters in synthetic, defined medium which contained; K_2HPO_4 5mM, $NH_4(SO_4)$ 10mM, glutamic acid 20mM, glucose 20mM, tryptophan 50µg/L, methionine 20µg/L, Mg++ 2mM, Ca++ 0.7mM, Mn++ 50µM, Zn++ 1µM, Fe 5µM, Thiamine 2µM. At the end of the logarithmic growth phase cells were harvested by continuous centrifugation using a Sharples centrifuge.

Extraction: The cell paste obtained following centrifugtion was weighed and homogenized using a Manton Gaulin homogenizer. The homogenates were then extracted in 5-10 volumes (v/w) cold acid/ethanol (0.2N HCl/75% ethanol) as previously described 1,2,6). After overnight mixing at 4°C, this extract was centrifuged; the remaining supernatant concentrated by evaporation of the ethanol under nitrogen and then neutralized using 1.0N NaOH. The extact was boiled for 20 minutes, centrifuged, and trifluoroacetic acid (TFA) added to the remaining supernatant to a final concentration of 0.2%TFA. Unconditioned as well as conditioned medium from batches 2,3 and 5 were acidified using acetic acid and processed as for cells.

Sep-Pak C_{18} Purification: Cells and medium were applied to SepPak C_{18} (reverse phase) disposable 1 ml cartridges; one cartridge per 10 g. wet weight of cells and one cartridge per 100 ml. of medium. Elution of the material that adsorbed to the Sep-Pak column was performed using acetonirile in 0.1% TFA. The material that eluted with 25-50% acetonitrile was kept for further purification.

Biogel P-6 Chromatography: Further purification of the SRIF-LI eluting from the Sep-pak C_{18} columns was performed by Biogel P-6 column chromatography. Samples were applied and eluted in 0.01% formic acid; individual fractions were lyophilized extensively and tested for SRIF-LI (6).

High Performance Liquid Chromatography (HPLC): Fractions from the Biogel columns that contained SRIF-LI were combined and further purified by reverse phase HPLC using C_{18} µBondapak (Waters Association) at a flow rate of 1.0 ml per minute using isocratic elution with 28% acetonitrile in 0.01%TFA (6).

Radioimmunoassay: At each step of the purification process, SRIF-LI material was measured by a C-terminal specific immunoassay as previously described (4,6). Limits of detectability were reproducibly 1-2 pg per assay tube with 50% displacement of 10-15 pg per tube (6). Following HPLC purification SRIF-LI

as also measured by a N-terminal specific immunoassay as described (6). Lower imit of detectability in this assay was 40 pg per assay tube and 50% displaceient was 200 pg per tube.

omatostatin Bioassay: HPLC purified SRIF-LI was tested for biological ıctivity in an established bioassay for somatostatin (7). Briefly, the effect ›f somatostatin on guinea pig oxyntic cell hydroxyl ion production is measured ›y quantitative cytochemistry. Under the assay conditions somatostatin ;timulates hydroxyl ion production. The hydroxyl ions are trapped by cobalt ıydroxide which, in the presence of H_2S forms a brown/black pigment of cobalt ;ulfide. Cobalt sulfide is then quantified by scanning microdensitometry. The ıssay is sensitive to 10^{-14}M somatostatin (7), and there is a linear lose-dependent increase in staining with concentrations of somatostatin from .05 through 50 pg/ml somatostatin -14. The biological activity of somatostatin -14 and -28 are indistinguishable and can be neutralized with an antiserum lirected towards the mid-portion of the somatostatin -14 molecule.

RESULTS

3iogel Purification: The SRIF-LI recovered from Sep-Pak C_{18} columns, when :hromatographed on Biogel P-6 columns eluted a broad peak (Figure 1). 3ecoveries from each column were between 75-85% of the SRIF-LI applied to the

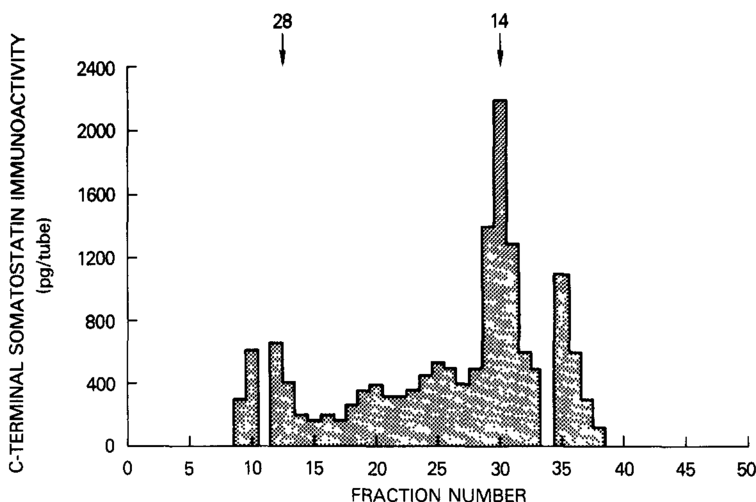

Figure 1. Biogel P-6 Chromatography of Sep-Pak C_{18} purified extracts. Aliquots were applied to a 48 ml column (52cm x 0.9cm) and eluted with 0.01% formic acid. Individual 1.5 ml fractions were lyophilized, reconstituted in distilled water and tested for SRIF-LI using the C-terminal specific SRIF assay. SRIF-28 and SRIF-14 standards were run separately (after the samples) under similar conditions. The arrows represent peak elution fractions for the synthetic standard preparations.

Table 1

Somatostatin-related Immunoactivity in Bacillus subtilis

Batch	Wet Weight	Immunoactive somatostatin C Terminal Assay: Elution off Sep-Pak pg/G wet weight	Biogel pg/G	HPLC pg/G	Medium: Unconditioned pg/Liter	Medium: Conditioned pg/Liter
1	150	11.2	NT	NT	NT	376.9
2	270	1.35	1.09	NT	NT	89.8
3	630	2.12	0.81	0.23	<41.4*	211.4
4	900	0.72	1.86	0.81	NT	NT
5	600	37.3	21.6	20.3	< 4.2*	214.5

NT=Not tested
*Less than the assay detection limits.

column, and blank runs had no detectable SRIF-LI. Between 0.8 and 22 pg SRIF-LI/g wet weight cells was recovered. Unconditioned medium had no detectable SRIF-LI, whereas conditioned medium had 80-380 pg/liter (Table 1).

HPLC Purification: SRIF-LI resolved into two major peaks on HPLC using an isocratic elution with 28% acetonitrile in 0.01% TFA (Figure 2). The early peak eluted with a retention time similar to SRIF-28 and was immunoactive in both the C-terminal as well as the N-terminal specfic immunoassays. The later peak eluted with a retention time similar to SRIF-14 and was only reactive in the C-terminal specific immunoassay (Figure 2). Recoveries of SRIF-LI from columns were 85-95% and blank runs before and after application of the samples contained no detectable SRIF-LI.

Bioactivity: Synthetic SRIF-14 stimulated hydroxyl ion production by guinea pig gastric oxyntic cells in a dose dependent manner from .05 to 50 pg/ml. Serial dilutions of HPLC purified material from Bacillus also stimulated hydroxyl ion production (Figure 3). Both the early HPLC peak ("SRIF-28", figure 2) as well as the later peak ("SRIF-14") demonstrated reasonably parallel biological activity which was similar to that of the synthetic somatostatin-14 standard preparation. In addition the biological activity of each sample was inhibited almost completely by the presence of (C-terminal specific) anti-somatostatin antiserum (Figure 3).

DISCUSSION

Originally isolated from extracts of mammalian hypothalamus, SRIF is found widespread throughout vertebrate tissues and more recently in

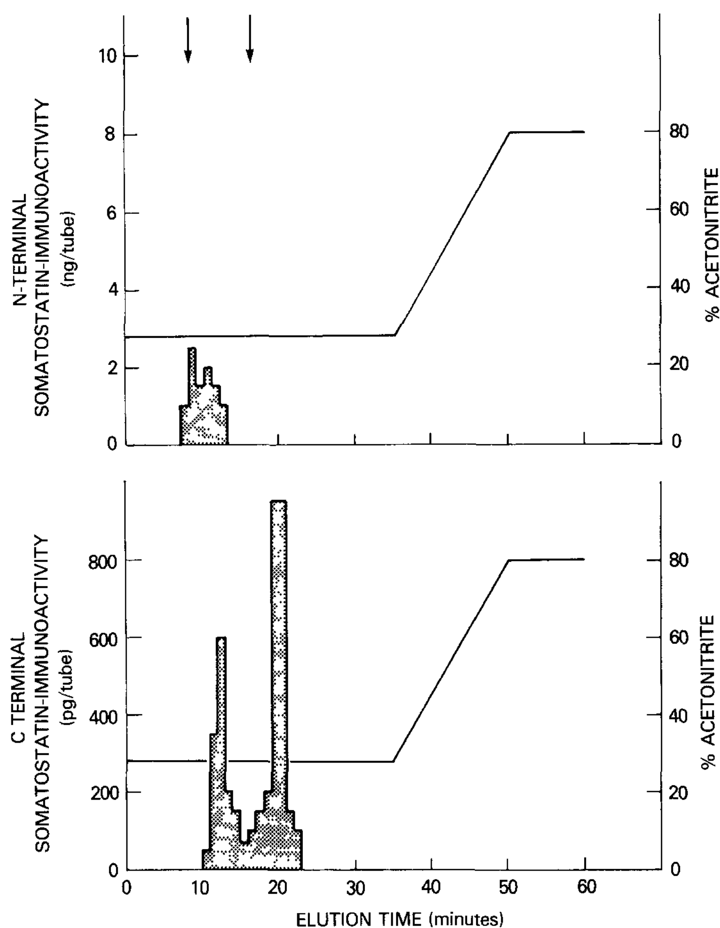

Figure 2. Following Sep-Pak and Biogel chromatography batches 3-5 were further purified on HPLC. Batch 5 alone is represented. The aliquots were applied to a μBondapak reverse phase column. Solvent A contained 0.01% TFA and solvent B contained 98% acetonitrile in 0.01% TFA. Flow rate was 1.0 ml per minute. Initial elution was carried out for 40 min under isocratic conditions using 28% acetonitrile and then the gradient was increased to 80% acetonitrile. Fractions were collected, lyophilized, reconstituted in water and assayed for SRIF-LI using the C-terminal (upper panel) as well as the N-terminal assay (lower panel). SRIF-28 and SRIF-14 standards were run separately and blank runs were devoid of SRIF-LI.

invertebrates (8,9,10). We have reported its presence in the unicellular protozoan Tetrahymena pyriformis and more recently in Escherichia coli (4,6). The presence in Bacillis subtilis of material closely resembling somatostatin adds support to the concept that somatostatin-like substances may be widespread among unicellular eukaryotes and prokaryotes.

The SRIF-LI immunoactivity recovered from the B. subtilis resolved into two peaks on HPLC, resembling SRIF-28 and SRIF-14. Both peaks were bioactive in a typical somatostatin bioassay which is characteristic of vertebrate

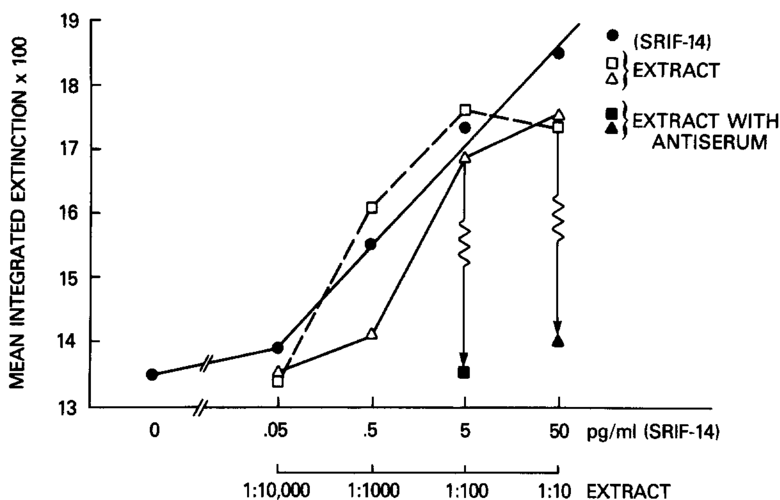

Figure 3. Hydroxyl ion production by guinea pig oxyntic cells was measured as the intensity of staining with cobalt sulfide detected by microdensitometry and is presented as the mean integrated extinction times 100 as an index of this activity. Synthetic somatostatin -14 (●—●) was used as the standard and (□—□) represents the early HPLC peak ("SRIF-28-like") whereas (△—△) represents the late HPLC peak ("SRIF-14-like") from figure 2.

SRIF-28 and SRIF-14 (11,12). It is interesting to speculate that a precursor-product relationship similar to that in vertebrate species may be present in B. subtilis. However such a relationship has not been demonstrated by the present studies.

Quantities of SRIF-LI in Bacillus cells ranged widely probably reflecting marked variations in recoveries of endogenous SRIF-LI during multiple purification procedures. Alternatively SRIF-LI production may vary with growth conditions, which were not optimized in our cultures. Contamination of the extracts by exogenously added SRIF-LI was excluded by the absence of SRIF-LI in unconditioned medium processed in an identical manner. In addition blank runs were devoid of SRIF-LI and recoveries from chromatography columns were always slightly less than 100%.

It is apparent that the SRIF-LI identified by molecular sieve chromatography contained material with MW similar to somatostatin -28 and somatostatin -14. However, there was also significant immunoactive somatostatin eluting in other regions which may represent other molecular species of somatostation (13).

Final confirmation of these findings will require isolation and sequencing of the gene(s) coding for SRIF in the prokaryotes.

ACKNOWLEDGMENT

Supported by a grant from the NIADDK AM 27077.

Thanks to Violet Katz for excellent secretarial assistance and Michael Berelowitz, M.D. for gifts of antisera.

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