PURIFICATION OF BOVINE SOMATOMEDIN [1,2]

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Received October 8,1975

SUMMARY: A procedure for the purification of bovine somatomedin (SM [4]) is presented. The purification scheme utilizes ultrafiltration through membranes of nominal mol. wt. cutoffs, molecular sieve chromatography and finally isoelectric focusing. Two peaks of SM activity, measured by the in vitro stimulation of ^{35}S-Na_2SO_4 and ^{3}H-thymidine uptake by costal cartilage, were present after focusing; an acidic component having a pI of 6.0 - 6.7 and a basic component having a pI in the range of 7.8 - 8.3. The acidic component comprised 2% of the initial activity and was 120,000-fold purified: the basic component comprised 10% of the initial activity and was 350,000-fold purified relative to the starting material. These components are similar in molecular size and pI to SM-A and SM-C isolated from human plasma.

INTRODUCTION: It has become increasingly evident that there exists in plasma a family of growth promoting substances (1-4). These peptides, which have insulin-like activities, share common traits with the so-called serum growth factors necessary for the proliferation of cells in tissue culture.

A number of growth promoting substances has been isolated to varying degrees of purity from serum of different species. Among these are SM-A and SM-B isolated by Hall and Uthne (3,5) and SM-C isolated by Van Wyk and colleagues (4). Both groups used a Cohn IV fraction from outdated human plasma as the starting material. SM-A is a neutral peptide (pI ≈ 7), SM-B is acidic (pI 4 - 5.2) and SM-C is a basic peptide (pI 8.6 - 9.4). Other substances include NSILA-S from a Cohn II fraction (1) and MSA from calf serum (2). The purification of SM from bovine blood is presented in this communication. The majority of activity appears similar to that obtained by Van Wyk et al. using human plasma (4).

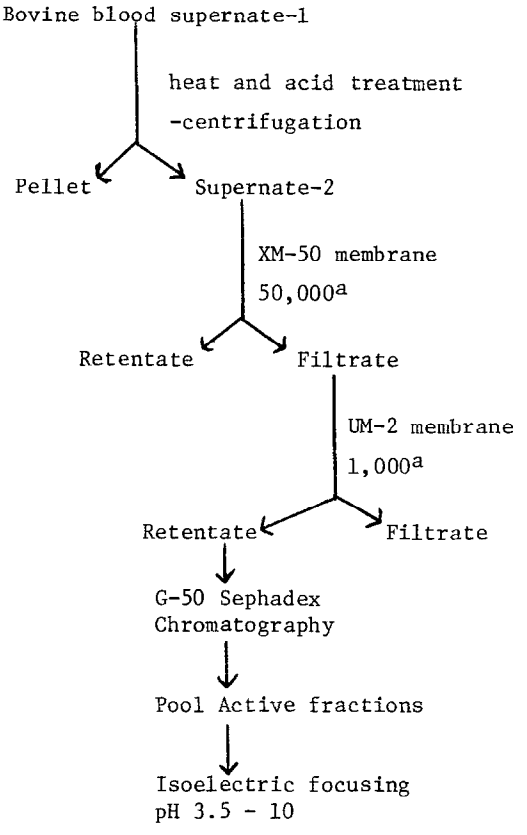
[1]Supported by NIH grant AM-16457.
[2]A portion of this work has been presented at the Endocrine Meetings, New York (7).
[3]Recipient of Research Career Development Award, K 04-AM00125.
[4]Abbreviations: SM, Somatomedin; MSA, multiplication stimulating activity; NSILA-S, non-supressible insulin-like activity-soluble.

MATERIALS AND METHODS: Bovine blood was collected at a local slaughter house and transported to our laboratory in plastic buckets. The blood was allowed to clot at room temperature. After 4-5 hours, the fluid surrounding the retracted clot was decanted and its volume measured. The procedures described below are for 4 - L portions. Four L of the supernatant were heated in a water bath. When the temperature reached 52°, 20 ml of glacial acetic acid and 100 ml of 1 M Na acetate pH 5.5 were added. The mixture was maintained between 53° and 57° and it was stirred continuously. After 30 min., the mixture, which appeared dark brown and had a great quantity of coagulated material, was cooled in an ice water bath. The mixture was centrifuged for 15 min. at 5000 x g and 10° and the precipitate was discarded. The supernatant of this step was ultrafiltered at room temperature through a 50,000 mol. wt. cutoff membrane (Amicon, Lexington, Mass.) with stirring and at 30 psi. The filtrate was concentrated by ultrafiltration through a 1000 mol. wt. cutoff membrane at 50 psi at room temperature. The 1000 mol. wt. retentate was chromatographed on a (3.18 x 61 cm) column of Sephadex G-50 medium. Eluates of peaks having biological activity were pooled, concentrated using a 1000 mol. wt. cutoff membrane and subjected to isoelectric focusing in the pH range of 3.5 to 10. Most of the ampholyte was removed from the focused fraction by chromatography on G-50 Sephadex followed by ultrafiltration through a 1000 mol. wt. cutoff membrane. The retentates were assayed for SM activity.

ASSAY: Hypophysectomized, male rats (100-110 g) were obtained from Zivic-Miller Labs, Allison Park, Pa., and were used 7-10 days post-operation. The procedures for the in vitro uptake of various precursors (carrier-free ^{35}S-Na_2SO_4 or ^{3}H-thymidine purchased from New England Nuclear Corp.) by costal cartilage segments have been described elsewhere (6,7). The reference standard (bovine serum) and each test preparation were assayed at 2 or 3 dosages. The test preparations were diluted with saline to be equivalent in volume to the initial serum. When ^{35}S-Na_2SO_4 uptake was determined, the incubation reaction was stopped by boiling, washed overnite with tap water, air dried and weighed. Cartilage segments were treated similarly for the ^{3}H-thymidine uptake experiments except that after boiling, the cartilage was soaked for 4 h in 5% trichloacetic acid. The cartilage segments were digested with formic acid and counted in a Beckman scintillation spectrometer. Results are expressed as c/m/mg cartilage. SM units were obtained from a 4-or 6-point symmetrical comparison with the standard reference serum using classic bioassay procedures (8). One SM unit is defined as the activity present in 1 ml of the reference standard. Protein was determined by the method of Lowry with bovine serum albumin as the standard (9).

RESULTS: The purification procedure devised is presented in Table I. Initially, bovine serum was prepared by centrifugation prior to the acid/heat step. However, it was found that decantation of the supernate (supernate-1) surrounding the retracted clot provided an equally suitable preparation. Thus, the SM activity of the supernate-1 was comparable to that of serum. Centrifugation of the acid/heat treated supernate yielded a dark brown fluid (supernate-2) which possessed ca. 30% protein and 45% of the SM activity of supernate-1. Ultrafiltration of supernate-2 was performed at room temperature in a stirred cell fitted with a 2 L reservoir. The flow rate was 1 ml min^{-1} initially and decreased to half that when the volume of the retentate was reduced by 70%.

TABLE 1: PURIFICATION SCHEME FOR BOVINE SOMATOMEDIN



[a] Approximate mol. wt. cutoff of membrane

The filtrate exhibited 1360 units of SM as measured by the uptake of $^{35}S\text{-}Na_2SO_4$ and ^{3}H-thymidine and had a specific activity of 2.66. The 50,000 mol. wt. filtrate (1.7 L) was concentrated to 19 ml using a 1000 mol. wt. cutoff membrane. The initial flow rate was 1.8 ml min^{-1}. The concentrate (18.5 ml) was placed on a column of G-50 Sephadex and eluted with 0.02 M NaCl. Five ml fractions were collected, pooled as indicated (Fig. 1) and assayed for their ability to stimulate $^{35}S\text{-}Na_2SO_4$ and ^{3}H-thymidine uptake. SM activity was widely distributed with the greatest amount residing in fractions D and E. Fractions D and E were concentrated with a 1000 mol. wt. cutoff membrane and

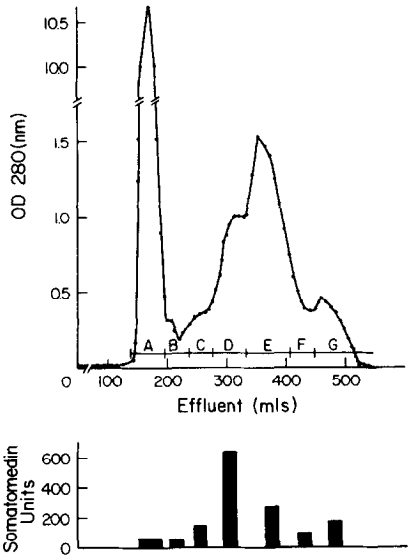


Fig. 1: Molecular sieve chromatography of bovine somatomedin.
UPPER PANEL - 19 mls of the 1000 mol. wt. retentate was applied to a Sephadex G-50 column and eluted with 0.02 M NaCl. The flow rate was 1.2 ml. min^{-1} and 5 ml fractions were collected. Chromatography was done at 4° C.
LOWER PANEL - Fractions were pooled as indicated and the biologic activity of each was determined as described in Methods.

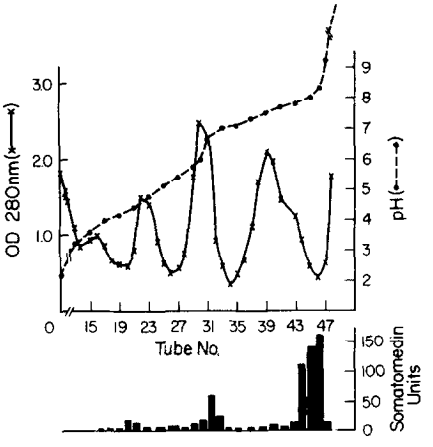


Fig. 2: Isoelectric focusing of Bovine Somatomedin.
UPPER PANEL - Isoelectric focusing between pH 3.5 and 10 was performed in a 110 ml LKB ampholine column with a 1% ampholyte solution. Fractions D and E of G-50 (Fig. 1) were concentrated and placed in the light solution. Sucrose gradients were formed with an LKB gradient mixer. The cathode (-) was 0.25 M NaOH in water and the anode (+) was 1.3 N H_2HO_4 in sucrose. Focusing was done at 4° for 63 hr. at ca. 600 V.
LOWER PANEL - SM activity was measured as described in Methods section.

TABLE 2: THE RECOVERY AND PURIFICATION OF SOMATOMEDIN FROM 4 L of BOVINE SERUM

Preparation	Protein (mg)	Units*	Recovery %	Units/mg	Purification
1) Supernate-1	3.44×10^5	4000	(100)	0.0116	(1)
2) Supernate-2 [Acid/heat treatment of (1)]	1.03×10^5	1800	45	0.0175	1.5
3) 50,000 mol. wt. filtrate of (2)	511	1360	34	2.66	229
4) Eluates of G-50 Sephadex fractions of (3)	2.26	920	23	407	35×10^3
5) Eluates of isoelectric-focused fractions of (4)					
A) pH 6.0 to 6.8	0.057	80	2	1404	12×10^4
B) pH 7.9 to 8.3	0.098	400	10	4082	35×10^4

* 1 unit defined as SM activity present in 1 ml of Supernate-1

subjected to isoelectric focusing. The results (Fig. 2) show that the majority of SM activity was located in 2 regions: one in the pH range 6.0 to 6.8 and one between pH 7.9 and 8.3. A minor peak of activity was noted in the 4.2 to 4.4 pH range.

A tabulation of the progress of the purification is presented in Table 2. The isoelectric fraction in the pH range 6.0 to 6.8 contained 2% of the initial SM activity and had a specific activity which was 120,000 times greater than native serum. The basic component (pH range 7.9 to 8.3) possessed 10% of the original SM activity and was 350,000 times more pure than native serum.

DISCUSSION: The purification procedure described in this report results in the appearance of two components which exhibit SM activity. The slightly acidic component that focused in the 6.0 to 6.8 pH range contained 57 μg of protein and represented a recovery of 2% of the initial SM activity. This com-

ponent was 120,000-fold more pure than the initial material based on the Lowry protein analysis. The basic component, focused between pH 7.9 and 8.3 was 350,000 times purified and accounted for 10% of the initial activity. Because of the minute amounts of SM in blood, the number of fold purification of SM can be misleading. Thus, assuming a mol. wt. of 7000 and a blood level of 10 ng/ml, the 98 µg of SM (350,000 fold purified material) contains 4% of pure SM. The activities determined in the course of the purification were the stimulation of ^{35}S-Na_2SO_4 and ^{3}H-thymidine uptake by costal cartilage segments. Both exhibited parallel activities throughout the purification steps. The basic component resembles SM-C isolated by Van Wyk's group (4), although theirs is slightly more basic (pI 8.6 - 9.4). The distribution of SM activity noted in the eluates of Sephadex chromatography suggests multiple forms of the hormone. Evidence has accumulated which indicates that in blood SM exists in at least two forms: one which is unbound (free) and the other which is bound to a transport molecule (3,10,11). The bound form has a mol. wt. of > 50,000 and the 'free' has a mol. wt. of ca. 7000 (11). In other laboratories (3,4) the material is treated with formic acid prior to chromatography. Presumably, this treatment dissociates SM activity from the larger species. Furthermore, chromatography is carried out in the presence of 1% formic acid which prevents reassociation during this procedure. Under these conditions, most of the SM activity is recorded in a single peak. It may be that the concentration of acetic acid used in our procedures was too low to dissociate SM from larger molecules effectively or to minimize reassociation during chromatography. Nonetheless, the majority of SM activity noted after chromatography resided in components having a mol. wt. in the range of 5- to 12000. This size is similar to that observed with SM from human serum (3-5) as well as other growth promoting substances (1,2). No insulin, as measured by radioimmunoassay, could be detected in the Sephadex fractions having the majority of SM activity. A small percent of the SM activity was noted in the effluent which corresponded to substances having mol. wts. of < 1500. This is similar to an activity which

we had observed previously (10) when purifying SM by different means. Although we have no direct evidence of its nature, experiments indicate it is not reduced or oxidized glutathione, serine, glutamate, or the nucleotide triphosphates. It is interesting to note that Pickart and Thaler (12) isolated a tripeptide from human serum which stimulates DNA, RNA, and protein synthesis in rat liver cells.

Both MSA and SM activities appear to be growth hormone dependent polypeptides (13). It is not known whether NSILA-S, MSA, and the SM's are distinct naturally occurring molecules or products of a single compound arising from the different purification procedures. Some of the confusion which exists can be attributed to the different bioassay systems with which the hormonal response is measured during the purification. Thus, SM-A activity was determined by a chick cartilage assay (14), whereas MSA activity has been assessed in chick embryo fibroblasts in monolayer culture (2). Lastly, SM-C was assayed using the costal cartilage of hypophysectomized rats (15). These peptides exhibit similar responses; viz, insulin and purified SM possessed MSA activity and increased glucose uptake (16); insulin and SM compete for binding sites of isolated fat cells and liver membranes (17). NSILA-S and insulin augment uptake of $^{35}S\text{-}Na_2SO_4$ by rat and chick cartilage (4). At this point, it is possible that the material we isolated and refer to as SM could be MSA or NSILA-S. However, because we have employed an assay of biologic activity which is similar to that of Van Wyk's and because the majority of the activity focuses in the basic region, we tentatively have assigned SM-C to our preparation.

ACKNOWLEDGEMENTS: The technical expertise of Yolanda P. Lyles and Lynne Banta is gratefully acknowledged.

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