

Purification and activity of human fetal hepatic stimulating substance

Wei-Jian Hou, Su-Yuan Liu, Wei Xing, Zhong-Cheng Pan, Jin-Lan Liu, Dian-Hong Wang

Wei-Jian Hou, Su-Yuan Liu, Wei Xing, Zhong-Cheng Pan, Dian-Hong Wang, Department of Biochemistry, School of Basic Medical Sciences, China Medical University, Shenyang 110001, Liaoning Province, China

Jin-Lan Liu, Department of Gastroenterology, First Clinical College, China Medical University, Shenyang 110001, Liaoning Province, China

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Correspondence to: Dr. Dian-Hong Wang, Professor, Department of Biochemistry, School of Basic Medical Sciences, China Medical University, Shenyang 110001, Liaoning Province, China

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Abstract

AIM: To purify human fetal hepatic stimulating substance (hHSS) and to determine its activity.

METHODS: hHSS was purified by ion exchange chromatography, nondenaturing polyacrylamide gel electrophoresis (PAGE), and ISCO (*In situ* chemical oxidation) concentrator electrophoresis. The molecular weight was determined by sodium dodecyl sulfate (SDS)-PAGE and the activity of hHSS was examined by both ³H-thymidine incorporation assay and enzyme linked immunosorbent assay (ELISA).

RESULTS: The purity of hHSS was increased along with the purification process. After many steps, the major band of nondenaturing PAGE showed a high activity and purity, and two bands were revealed by SDS-PAGE.

CONCLUSION: The molecular weight of hHSS is about 70 kDa, and it consists of two subunits with molecular weights of 18 kDa and 53 kDa, respectively. The activity of hHSS was assayed by both ³H-TdR incorporation assay and ELISA, and good consistence was seen.

Key words: Liver stimulating; Substance; Molecular weight

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INTRODUCTION

Hepatic stimulating substance (HSS) stimulates specifically DNA synthesis in liver cells both *in vivo* and *in vitro*^[1]. It has organ specificity but no species specificity. Although HSS purification has been studied using rat livers, only crude human fetal hepatic stimulating substance (hHSS) was used for clinical treatment and research. The purification of human HSS remains to be further developed. In this study, hHSS was purified to near homogeneity by ion exchange chromatography, nondenaturing polyacrylamide gel electrophoresis (PAGE) and ISCO (*In situ* chemical oxidation) concentrator electrophoresis.

MATERIALS AND METHODS

Animals

Kunming mice were purchased from the Department of Experimental Animal of China Medical University.

Instruments and reagents

RC5C centrifuge was purchased from Dupont Company, United States; 2023 MinicoldLab from LKB Co., Sweden; and UV-260 spectrophotometer from Shimadzu Co., Japan. Liquid Scintillation system model 1801 was obtained from Beckman Co., United States; EL-309 Microplate Reader from BIO-TEK Co., United States; and ³H-thymidine (³H-TdR) (20 Ci/mmol) from the Institute of Atomic Energy of China. Antibody of hHSS was from the Department of Immunology, General Hospital of Chinese PLA (Nanjing, China). Cell culture reagents and Williams' Medium E were purchased from Sigma Co., United States

Purification of hHSS

A fresh fetal liver (from a mouse aged 5 mo) was excised and immediately homogenized in ice-cold 0.9% NaCl containing EDTA (Ethylenediaminetetraacetic acid) 10 mmol/L and PMSF 0.09 mmol/L with a polytron. Then it was centrifuged at 400 r/min for 20 min. The supernatant was heated at 95 °C for 15 min and centrifuged at 45000 g for 20 min, then 40% cold ethanol was added to the supernatant and stirred at 4 °C for 2 h^[1]. After centrifugation, the precipitate was dissolved in 0.02 mol/L Tris-HCl buffer, and centrifuged at 12000 g for 20 min.

The supernatant was purified by chromatography on a DEAE-Sephadex A50 ion exchange column in Tris-HCl (0.02 mol/L, pH 7.8) buffer and eluted with a continuous gradient of 0-0.6 mol/L NaCl. The active peak of DEAE-Sephadex A50 was further purified by nondenaturing gradient PAGE. Following the electrophoresis, the major protein band was sliced and homogenized. Elution and concentration were done by the ISCO concentrator. The recovered protein was tested for hHSS activity using both enzyme linked immunosorbent assay (ELISA) and ³H-TdR incorporation assay. The molecular weight was determined by sodium dodecyl sulfate-PAGE (SDS-PAGE).

Table 1 Assay of human fetal hepatic stimulating substance activity by ³H-TdR incorporation method

Sample	Sample volume (μL)	Concentration of protein (mg/mL)	cpm (mean ± SD)	IS	Specific activity (IS/mg protein)	Fold of purification
Control (0.9% NaCl)	25	-	1275 ± 203	-	-	-
Homogenization	25	52.600	3162 ± 329	2.48	1.89	1.00
ETOH-ppt hHSS	25	8.280	3683 ± 401	3.03	14.64	7.74
DEAE A50 peak V	25	3.470	2920 ± 251	2.29	26.40	13.97
Nondenaturing PAGE major band	25	0.169	2039 ± 114	1.60	326.89	172.76

Any value > 1.3 is significant; *P* = 0.05; Nondenaturing PAGE utilized DEAE-Sephadex A50 peak V as a starting material. Its purity had been improved obviously. hHSS: Human fetal hepatic stimulating substance; PAGE: Polyacrylamide gel electrophoresis; DEAE: Diethylaminoethyl; ETOH: Ethyl alcohol; IS: Index of stimulation.

Table 2 Assay of human fetal hepatic stimulating substance by enzyme linked immunosorbent assay method

Sample	Abs at 630 nm	Abs at 280 nm	Abs at 630 nm/Abs at 280 nm	Fold of purification
Control (0.9% NaCl)	0.077	0	-	-
Homogenization	0.141	2.790	0.051	1.00
ETOH-ppt hHSS	0.159	0.964	0.165	3.24
DEAE A50 peak V	0.284	0.236	1.200	23.52
Nondenaturing PAGE major band	0.197	0.019	10.368	203.29

Abs at 280 nm is absorbent value of samples at 280 nm, which represents the concentration of the samples. Abs at 630 nm represents the hHSS content in samples. hHSS: Human fetal hepatic stimulating substance. ETOH: Ethyl alcohol; DEAE: Diethylaminoethyl; PAGE: Polyacrylamide gel electrophoresis.

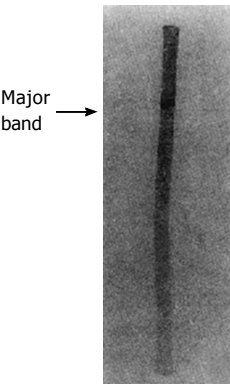


Figure 1 Nondenaturing gradient PAGE. Sample: DEAE-Sephadex A50 peak V.

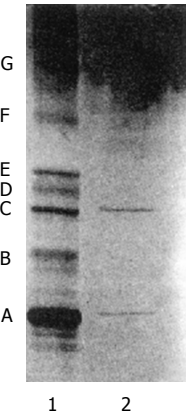


Figure 2 The sample was prepared in 0.1% SDS and SDS-PAGE was run. Silver staining was performed. Lane 1 is molecular weight standards: A = 18 kDa; B = 34 kDa; C = 53 kDa; D = 76 kDa; E = 116 kDa; F = 170 kDa and G = 212 kDa. Lane 2 is major bands of nondenaturing PAGE.

Assay of hHSS activity

³H-TdR incorporation assay: This assay was performed according to the methods described by LaBrecque^[5] and Schwarz^[6]. An index of stimulation (IS) was then determined as: IS = Count per minute (cpm) for experimental hepatocytes/cpm for control hepatocytes.

Assay of hHSS by ELISA

hHSS- antibody diluted to 1:1000 with coating buffer was added to a 96-well microplate (0.2 mL/well) and kept at 4 °C overnight. After absorption, blocking solution was added to the wells at 0.4 mL/well and set at room temperature for 4 h. After the blocking solution was removed, 0.1 mL of sample was added to the coated wells and allowed to react at 37 °C for 1 h. After washing 5 times, 0.1 mL of peroxidase-conjugated anti-rabbit IgG secondary antibody

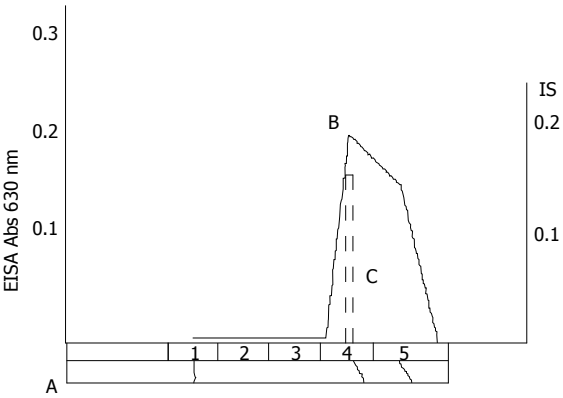


Figure 3 After ISCO concentrator electrophoresis, the activity showed: A: the protein bands on the tube gel; B: the content of hHSS by ELISA method; C: the value of activity (IS) by ³H-TdR incorporation method.

was added to the testing wells and incubated at 37 °C for 1 h. After washing, the wells were subjected to chromogenic reaction.

RESULTS

Purification

A combination of the above steps produced an almost 200-fold purification of hHSS. The results of purification are shown in Table 1 (by ³H-TdR incorporation method) and Table 2 (by ELISA method).

Nondenaturing gradient PAGE

Peak V of DEAE-Sephadex A50 was loaded on a nondenaturing gradient polyacrylamide tube gel (Figure 1). After electrophoresis, three bands were shown on the tube gel. Assay of hHSS activity showed that the major band had a very high specific activity.

Determining the molecular weight of hHSS by SDS-PAGE

The major band of nondenaturing PAGE was subjected to SDS-PAGE, and the molecular weight of hHSS was shown as two bands with molecular weights of 18 kDa and 53 kDa, respectively (Figure 2).

DISCUSSION

hHSS is able to initiate and/or stimulate hepatic cell growth and regeneration. Many techniques of purification and assay were studied in many laboratories^[1-7], but few were concerned with the purification of hHSS. From the process of isolation, it was shown that large amounts of contaminated protein was removed after heating treatment and ISCO concentrator electrophoresis (Figure 3). SDS-PAGE showed that only two bands with molecule weights of 18 kDa and 53 kDa were found. So the molecular weight of hHSS may be 70 kDa, and it

consists of two subunits with molecular weights of 18 kDa and 53 kDa, respectively.

³H-TdR incorporation assay is a standard method for the assay of HSS activity, but it is difficult to control when a large number of samples are assayed simultaneously. In 1991, Tsubouchi used ELISA for measuring HSS in blood^[7]. In the present study, we utilized ELISA for measuring the hHSS content in every step of the purification, and compared the measured values with those by ³H-TdR incorporation method. The values measured by both methods were basically parallel, and the ELISA method is easier to perform and is suitable for measuring a large number of samples simultaneously.

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