A SIMPLIFIED METHOD FOR PURIFICATION OF AN ANTITUMOR ACIDIC GLYCOPROTEIN FROM *STREPTOCOCCUS PYOGENES* (Su STRAIN) BY IMMUNOADSORBENT CHROMATOGRAPHY

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A simplified method for purification of an antitumor acidic glycoprotein (SAGP) from *Streptococcus pyogenes* (Su strain) by immunoaffinity chromatography is described. A cell-free crude extract prepared from the cocci was applied to the anti-SAGP IgG coupled Sepharose column, and elution was conducted with an alkaline buffer. The material eluted was confirmed to be homogeneous and identical with SAGP as demonstrated by both relative mobility on the SDS-polyacrylamide gel column and the antigenicity on the double diffusion agar plate. The cell-growth inhibitory activity of SAGP prepared by the present method was almost the same as that of SAGP purified by the previous time-consuming method. Since this simplified method provides a higher yield of SAGP, it will be useful in further studies on the biological properties of SAGP.

We have purified previously streptococcal acid glycoprotein (SAGP) from the cell-free crude extract (CE) of *Streptococcus pyogenes* (Su strain) by a multi-step procedure[1]. SAGP is an acidic glycoprotein with the molecular weight of about 150,000 daltons and is composed of identical subunits of molecular weight 50,000 daltons. One of the carbohydrate moieties of SAGP was determined to be D-allose (1.5% w/w). In the antitumor experiments, SAGP prolonged the life span of mice bearing Ehrlich ascites carcinoma cells[1] and inhibited the growth of some cell lines in culture, including transformed hamster embryonic lung cells (THEL)[1], murine embryonic cells (BALB/3T3)[2], murine leukemic L1210 cells (L1210), B16 melanoma and Meth A sarcoma cells (unpublished data). On the other hand, KANAOKA *et al.*[3] reported that SAGP, which was purified from *S. pyogenes* Su by a slightly modified procedure, inhibited the growth of several tumor cell lines *in vitro* and exhibited the antitumor effect on mice inoculated ip with Meth A sarcoma cells or mice implanted im with sarcoma 180 tumor cells. For more extensive studies on the biological properties of SAGP, it was necessary to obtain greater amounts of SAGP. The previous method for purification of SAGP was time-consuming and consisted of thermal treatment, streptomycin precipitation, ammonium sulfate fractionation, and some liquid chromatographic procedures[1]. In this paper, we describe a simplified and improved method for purification of SAGP by immunoaffinity chromatography.

Materials and Methods

Preparation of Cell-free CE

The cell-free CE was prepared as described previously[1]. Briefly, *S. pyogenes* (Su strain) cells grown in 30 liters of WOOD and GUNSALUS broth[4] were collected by continuous centrifugation and washed with 0.01 M Tris-HCl buffer, pH 7.5 containing 0.01 M magnesium acetate. After mechanical disruption of the cells in a Vibrogen cell mill (Edmund Bühler, Tübingen), a water-soluble fraction

was obtained by centrifugation at 105,000×*g* for 2 hours and dialyzed against the 1 mM Tris-acetate buffer, pH 8.0 containing 0.14 M KCl and 0.02% sodium azide (starting buffer). CE was subjected to immunoadsorbent chromatography as the starting material for the purification of SAGP.

Antisera

One mg of SAGP purified by the previous method[1] was emulsified in complete FREUND's adjuvant. Rabbits were im inoculated at several sites of the body. One week later, the same dose of SAGP in complete FREUND's adjuvant was injected into the rabbits. For booster injection, 0.5 mg of SAGP in FREUND's incomplete adjuvant was administered into the rabbits 4 weeks later. Rabbits were bled by cardiac puncture 4 weeks after the last injection, and the antisera were prepared by precipitation with 35% saturated ammonium sulfate, followed by purification on DE-52 cellulose column according to the method of LEVY and SOBER[5]. The IgG fraction was concentrated to approximately 10 mg/ml with an UK-10 ultrafilter (Toyo Roshi, Tokyo, Japan).

Preparation of Immunoadsorbent

The anti-SAGP IgG was dialyzed against the coupling buffer; 0.5 M NaCl in 0.1 M $NaHCO_3$, pH 8.3. Then IgG (10 mg/g of Sepharose) was coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to manufacture's instructions. The immunoadsorbent was packed into a glass column (1.6×10 cm), and equilibrated with the starting buffer.

Assay of Cell-growth Inhibitory Activity

Biological activity of obtained fractions was determined by the assay on cell-growth inhibition. THEL and L1210 cells were used as the target cell lines. THEL cells were routinely maintained in EAGLE's minimal essential medium supplemented with 12.5% of horse serum, 2.5% of fetal calf serum (FCS), and antibiotics (benzylpenicillin, 100 U/ml; streptomycin, 100 μg/ml), and L1210 cells in Roswell Park Memorial Institute Tissue Culture Medium 1640 supplemented with 10% FCS and antibiotics. Inhibition of cell-growth was assayed as described previously[1]. Cells (5×10^4/plate) were inoculated into 5 ml of the medium containing an appropriate concentration of sample to be tested and incubated at 37°C for 72 hours in a humidified 5% CO_2 atmosphere. The number of the cells was determined by a Coulter counter (model ZBl, Coulter Electronic, Inc.) and the growth rate was expressed as percentage of cell-number in the control plates.

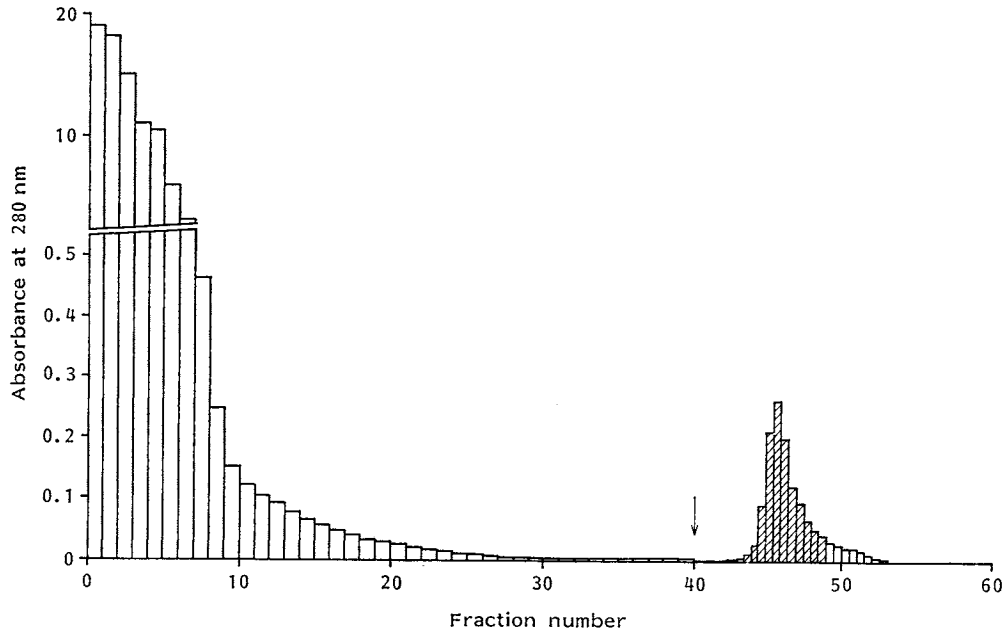
Other Methods

Polyacrylamide gel disc electrophoresis in the presence of SDS was carried out as described previously[1]. Ouchterlony double diffusion analysis[6] was carried out at room temperature. Protein concentration was determined by the method of LOWRY *et al.*[7] with bovine serum albumin as the standard.

Results and Discussion

Immunoaffinity chromatographic procedure was carried out at 4°C. CE (10 mg protein/ml) was applied onto the immunoadsorbent column with recycling for 16 hours at a flow rate of 20 ml/hour. Unadsorbed materials were then washed out with at least 10 bed volumes of the starting buffer at a flow rate of 60 ml/hour. The material adsorbed was eluted with freshly prepared lysine - KOH buffer, pH 11.0 containing 0.14 M KCl. The eluate was collected in 2.5-ml fractions and immediately neutralized with the same volume of 0.1 M Tris-acetate buffer, pH 6.3 containing 0.2 M glycine. A typical elution pattern is shown in Fig. 1. The eluted fractions containing protein, as determined by the optical density of the fractions at a wavelength of 280 nm, were pooled and then concentrated using an UK-10 ultrafilter. The filtrate was dialyzed against DULBECCO's phosphate buffered solution, pH 7.3. The protein containing fraction thus obtained was subjected to physico-chemical and biological analyses. After elution, the column was washed thoroughly with 5 bed volumes of 0.1 M acetate buffer, pH 4.0 containing 0.5 M NaCl, followed by 5 bed volumes of 0.1 M Tris-HCl buffer,

Fig. 1. Single-step isolation of SAGP from a crude extract of the Streptococci on the immunoadsorbent affinity column.



Arrow indicates change to the elution buffer. The hatched area containing SAGP was pooled, and concentrated.

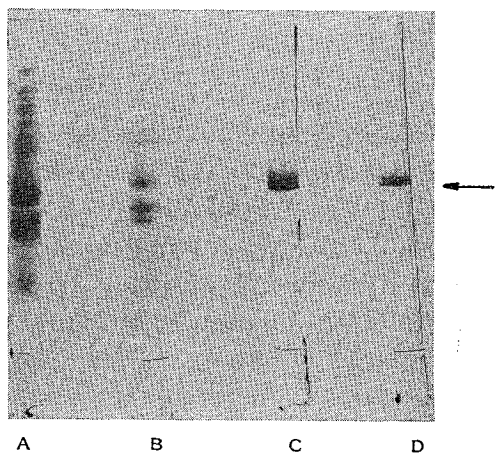
pH 8.0 containing 0.5 M NaCl, and re-equilibrated with the starting buffer. The affinity column could be repeatedly used at least 10 times without loss of the ability to adsorb SAGP.

A portion of CE, unadsorbed material and the eluted fraction from the column were reduced in the phosphate buffer containing 1% SDS and 5% 2-mercaptoethanol at 100°C for 3 minutes, and electrophoresed on 7.5% polyacrylamide gel column (0.5×6 cm) in the presence of 0.1% SDS. At the same time, SAGP purified by the previous method (a multi-step procedure) was run as a control. The fraction eluted from the column was shown to have a relative mobility value corresponding to that of SAGP purified by a multi-step procedure (Fig. 2).

Ouchterlony double diffusion analysis showed a single precipitin line between the eluted fraction and anti-SAGP serum (Fig. 3). The precipitin line was fused with the line which was formed between SAGP purified by a multi-

Fig. 2. SDS-polyacrylamide gel electrophoresis of the fractions from the immunoaffinity column.

A: Starting material (CE), B: unadsorbed fraction passed through the column, C: fraction eluted from the column, D: SAGP purified by a multi-step procedure.



Gels were stained with Coomassie Brilliant Blue R-250. Arrow indicates the 50,000 daltons region.

step procedure and anti-SAGP serum. This demonstrates that the fraction eluted from the column was antigenically identical with SAGP. On the other hand, the unadsorbed material did not form any precipitin band with anti-SAGP serum, indicating that the SAGP in CE was largely adsorbed to the immunoadsorbent.

The growth-inhibitory effect of CE, unadsorbed material and eluted SAGP on L1210 and THEL cells is shown in Fig. 4. The IC_{50} value of the eluted SAGP was almost one-tenth that of CE, suggesting that the eluted SAGP was purified with a 10-fold increase in the cell-growth inhibitory activity by a single-step procedure using immunoadsorbent chromatography. The unadsorbed material exhibited no or low growth-inhibitory activity on L1210 and THEL cells. Table 1 shows the growth-inhibitory activity of SAGP eluted from the immunoaffinity column on L1210 and THEL cells in comparison with that of SAGP purified by a multi-step procedure. The cell-growth inhibitory activity of SAGP

Fig. 3. Agar gel precipitin pattern of fractions from the immunoadsorbent affinity column against anti-SAGP rabbit serum.

A: 3 μl of anti-SAGP rabbit serum, B: 3 μl of CE (7 mg protein/ml), C: 3 μl of unadsorbed fraction (2.6 mg protein/ml), D: 3 μl of fraction eluted from the column (500 μg protein/ml), E: 3 μl of SAGP purified by a multi-step procedure (400 μg protein/ml).

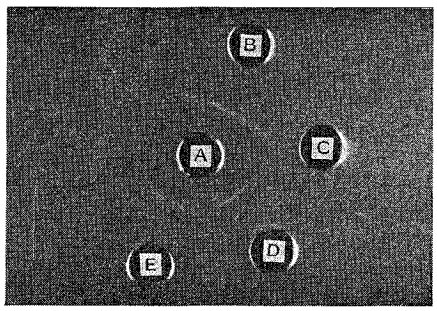


Fig. 4. The growth-inhibitory activity of the fractions from the immunoadsorbent affinity column on THEL (A) and L1210 (B) cells in culture.

○ Starting material (CE), □ unadsorbed fraction, △ fraction eluted from the column.

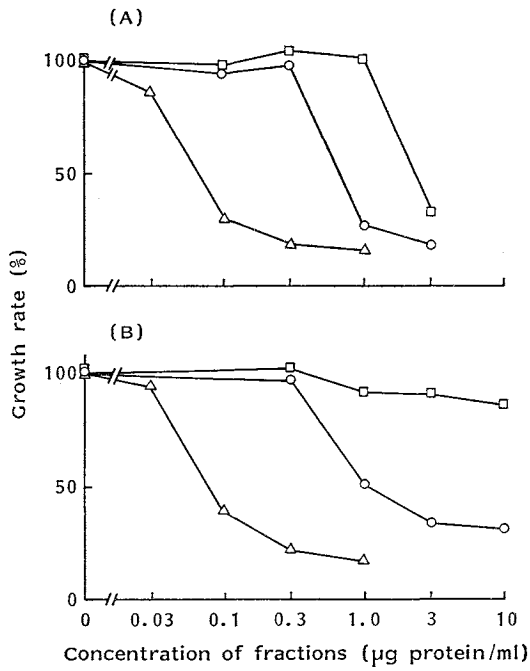


Table 1. Comparison of the cell-growth inhibitory effect of SAGP purified by the immunoadsorbent affinity chromatography with that of SAGP purified by a multi-step procedure.

Target cells	SAGP purified by	Growth rate (%)			
		Final concentration of SAGP (μg protein/ml)			
		0.03	0.1	0.3	1.0
THEL	Immunoadsorbent affinity chromatography	91.0±5.1*	35.5±4.5	22.0±4.9	19.0±1.0
	Multi-step procedure	82.0±12.6	28.5±2.9	22.0±3.2	17.5±3.6
L1210	Immunoadsorbent affinity chromatography	91.0±9.2	44.8±8.0	24.5±2.2	20.5±3.6
	Multi-step procedure	96.5±10.5	40.5±1.5	25.0±3.9	20.3±1.9

* Mean±SE (n=3).

purified by each method was almost the same. In the preliminaly experiment, the antitumor effect of SAGP purified by immunoadsorbent chromatography was demonstrated in tumor-bearing mice. Namely, the mice were inoculated ip with Ehrlich ascites carcinoma cells on day 0. SAGP was injected ip into 3 mice at a dose of 500 μg/mouse/day on day 1 to 4. The 5 control mice received 0.9% (w/v) NaCl solution alone. As a result, two of 3 mice administered SAGP were alive at day 60, the termination of the experiment, whereas the control mice died in less than 24 days.

Thus, the SAGP bound to the immunoadsorbent was eluted in a pure form without loss of the biological activity. In immunoaffinity chromatography, some typical eluants; acids, bases and high concentrations of chaotropic ions or protein denaturants have been employed. We have tried to elute the bound material with bases (50 mM sodium bicarbonate - 0.3 M NaCl buffer, pH 11.0, 50 mM NH_4OH buffer, pH 10.0 containing 20% glycerol), acids (0.1 M glycine-HCl buffer, pH 3.0) and chaotropic ions (3.5 M KI, 1.0~2.5 M NaI, 3 M $MgCl_2$ in the starting buffer)[8]. SAGP eluted with those eluants had low activity in the cell-growth inhibitory effect. SAGP seems to be denatured by exposure to extreme conditions for a long time. The labile nature of SAGP might be due to partial dissociation of SAGP into the subunits. However, several observations indicate that the dissociation of the multisubunit proteins could be readily reversed by removing the denaturants or reactivating in an appropriate condition[9]. Therefore, eluants which minimize dissociation of SAGP should be chosen and also the material eluted should be returned to the native state immediately. Reproducible results were achieved using 5 mM lysine-KOH buffer, pH 11.0 containing 0.14 M KCl as an eluant.

In terms of yield, the present method provided about 5 mg of SAGP from 20 ml of CE (10 mg protein/ml). The previous method yielded about 0.5 mg of SAGP per 20 ml of CE. Furthermore, the purification of SAGP by the present method takes only 3 days, whereas the previous method by a multi-step procedure takes at least 1 month. For larger preparations of SAGP, it would be convenient to repeat applications of CE to the same column and if possible, to prepare a larger column for bulk purification.

In conclusion, the immunoaffinity chromatographic procedure is a greatly improved method for purification of SAGP with higher yield in a shorter time. The new method will facilitate further research into the antitumor mechanisms of Streptococci[10,11] or streptococcal (Su strain) antitumor preparation; OK-432[12].

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