Journal of Chromatography, 324 (1985) 173–180
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17 509

USE OF A UREA AND GUANIDINE-HCl–PROPANOL SOLVENT SYSTEM TO PURIFY A GROWTH INHIBITORY GLYCOPEPTIDE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(Received December 20th, 1984)

SUMMARY

Reversed-phase high-performance liquid chromatography was used to purify an inhibitory glycopeptide where resolution and recovery were enhanced by using urea or guanidine-HCl–isopropanol–water as a solvent system. Isopropanol alone or other solvent systems that have been proposed for such purification steps were not effective in eluting hydrophobic proteins from the reversed-phase column. The application of the urea or guanidine-HCl solvent systems in the separation and purification of membrane proteins, and other hydrophobic macromolecules, could greatly enhance recovery and efficiency of purification.

INTRODUCTION

The purification of peptides and proteins from biological preparations usually requires the combination of several high-resolution liquid chromatographic procedures. Following the introduction, over the past several years, of suitable reversed-phase high-performance liquid chromatography (RP-HPLC) procedures, rapid selective separations of a large variety of polypeptides and proteins have become possible. Selectivity in these separations is essentially based on the hydrophobic character of the polypeptides[1].

Although numerous solvent systems have been designed for hydrophilic proteins, there is little available information on solvent systems for hydrophobic proteins and polypeptides, especially macromolecules derived from cell membranes. The hydrophobic proteins often separate poorly because of complications associated with their insolubility[2], denaturation[3], or irreversible adsorption to reversed-phase matrices[4] which are manifested in the memory effect[5].

We previously have reported the isolation and partial purification of a growth inhibitory glycopeptide, obtained from bovine cerebral cortex cell surfaces, that can inhibit protein synthesis and cell growth of 'normal' but not transformed cells[6,7]. These glycopeptides may be naturally occurring growth regulators that mediate their

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effect by cell–cell contact in a manner similar to that proposed by Dulbecco and Stoker[8]. This report presents the results of studies on the development of a new solvent sysem that enhances the recovery of hydrophobic proteins and polypeptides with RP-HPLC that could not be eluted with various solvent systems that had been used previously to elute hydrophobic proteins. This new solvent system also can be used to eliminate or reduce the memory effect with RP-HPLC columns.

EXPERIMENTAL

Isolation and purification of the glycopeptide inhibitor

The glycopeptide cell growth inhibitor was isolated from bovine cerebral cortex cell surfaces by mild pronase treatment as described previously[6,7]. The macromolecules released by mild proteolysis were purified by ethanol precipitation, chloroform–methanol (2:1, v/v) extraction, and DEAE ion-exchange chromatography using 0.4 *M* sodium chloride, in sodium acetate buffer (pH 5.0), to elute the inhibitor from the column.

Cell culture

BALB/c 3T3 cells (American Type Culture Collection, Rockville, MD, U.S.A.) were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) (Flow Laboratories, McLean, VA, U.S.A.) containing 10% calf serum (Colorado Serum, Denver, CO, U.S.A.) at 37°C in a humidified atmosphere of carbon dioxide–air (5:95).

Protein synthesis assay

The protein synthesis inhibitory assay has previously been described[6,7]. Briefly, 25 μl of material to be assayed was added to $3 \cdot 10^5$ 3T3 cells suspended in 100 μl of DMEM–Hepes (pH 7.1). The tubes were covered and incubated at 37°C for 45 min. Then 2.0 μCi of [^{35}S]methionine in 10 μl of DMEM–Hepes (pH 7.1) were added, and the cells were reincubated at 37°C for 1 h. After incubation the cells were washed, pelleted by centrifugation, and lysed with deionized water containing 0.1 *N* sodium hydroxide. The macromolecules were precipitated with an equal volume of 20% ice-cold trichloroacetic acid and the precipitates were collected by centrifugation. The precipitates were resuspended in distilled water, resolubilized with alkaline water, and reprecipitated with 20% ice-cold trichloroacetic acid. The resulting precipitates were collected by centrifugation and resolubilized in alkaline water. An aliquot was used to determine radioactivity by liquid scintillation, and another aliquot was used for cell protein determination.

Stability of the glycopeptide inhibitor in the solvents

The glycopeptide was incubated with the solvents at room temperature for 1 h. After incubation the solvents were evaporated to dryness under a stream of nitrogen gas and the remaining biological activity was assayed as described above. When urea and guanidine-HCl were used the sample was incubated at room temperature for 1 h and dialyzed exhaustively against distilled water. The glycopeptide was concentrated by lyophilization and assayed for biological activity.

Reversed-phase high-performance liquid chromatography

RP-HPLC was carried out with a DuPont Model 8800 gradient controller, a DuPont variable-wavelength detector set at 280 nm, a DuPont chromatographic pump, a Rheodyne 100-μl loop injector, a Spectra-Physics Model SP 4100 computing integrator system, a LKB 2112 Redirac fraction collector, and a Vydac C-4 column (25 cm × 4.6 mm I.D.) from the Separation Group, Hesperia, CA, U.S.A.

The mobile phase solvents were HPLC grade from Burdick & Jackson (Muskegon, MI, U.S.A.). Urea, guanidine-HCl, and trifluoroacetic acid (TFA) were from Pierce (Rockford, IL, U.S.A.). All solvents were filtered and degassed prior to use.

Volumes of 100 μl of DEAE-purified glycopeptide inhibitor were generally injected and separations were performed at room temperature. Other operating conditions are described in the figure legends. Column eluates were collected in 1.0-ml fractions, and those fractions that corresponded to the peaks on the chromatogram ware pooled, dialyzed exhaustively against water, concentrated by lyophilization, and assayed for inhibitory activity as previously described[6,7]. The protein concentration of each peak was measured with the Coomassie blue assay[9] (Pierce) using sperm whale myoglobin as a protein standard.

RESULTS AND DISCUSSION

One problem encountered in the purification of membrane and membrane-associated proteins with RP-HPLC is the quantitative elution of the more hydrophobic macromolecules. The recovery of these proteins is often so low that it causes a strong memory effect which is difficult to eliminate and leads to high back-pressures in the columns. Various solvent systems have been proposed for eluting hydrophobic proteins from RP-HPLC columns[2,10,11]. Although these solvent systems proved successful in some cases, their effectiveness cannot be generalized. We have developed a new solvent system that is capable of eluting hydrophobic proteins from RP-HPLC columns that could not be eluted with the solvent systems previously proposed.

Initially we investigated the effect of three solvents on the biological activity of the bovine glycopeptide inhibitor as described in the Experimental section. Acetonitrile and ethanol were used at 100% and isopropanol at 50% in water (v/v). The pH of the solvents was adjusted to 3.0 with TFA. The results indicated that the inhibitory activity was retained and the inhibitor was stable in these three solvents (data not shown).

The effect of the solvents alone on the protein synthesis of 3T3 cells was also measured. A 1-ml volume of freshly prepared solvents was evaporated to dryness and the activity of the dried residue was measured as described. Double-distilled water was adjusted to pH 3.0 with TFA and used as a control. A residue from the acetonitrile, even though the reagent was the purest HPLC grade, gave 20% inhibition of 3T3 cell protein synthesis whereas isopropanol and ethanol did not inhibit cellular protein synthesis (Table I). Although we have not identified the substance in acetonitrile that inhibited proteins synthesis, Tan[12] has reported that an acidic solution of acetonitrile is unstable and undergoes hydrolysis to acetamide and acetic acid. More importantly, this observation showed that the use of acetonitrile caused complications in the biological assays and it was not used for a solvent to purify the glycopeptide inhibitor. Although acetonitrile has been used as a RP-HPLC solvent

TABLE I

EFFECT OF SOLVENTS ON 3T3 PROTEIN SYNTHESIS

The pH of the solvents were adjusted to 3.0 with TFA, 1.0 ml of each solvent was evaporated to dryness and resuspended in 100 μl of HKM buffer. The samples were then tested for protein synthesis inhibitory activity with 3T3 cells as described in the Experimental section. The results are expressed as the average and experimental range of triplicate determinations.

Solvent	*[^{35}S]Methionine incorporated (cpm)*	*Inhibition (%)*
Water (control)	37 290 ± 10	–
Acetonitrile	30 140 ± 10	20
Ethanol	37 090 ± 20	0
Isopropanol	37 220 ± 20	0

in the purification of growth factors[13,14] and other cell growth inhibitors[15], our observations suggest that one must use caution when the resulting samples are dried and assayed in biological systems. Isopropanol was chosen as the solvent in further studies because it did not interfere with 3T3 cell protein synthesis assays and isopropanol has a higher hydrophobicity per unit volume than ethanol[16].

We found that most of the glycopeptide inhibitor was not retained on the RP-HPLC column and eluted at the breakthrough peak (Fig. 1). The blank solvent run that followed elution of the brain material did not elicit a 'ghost' peak, indicating that all proteins bound to the column had been eluted in the initial run. However, measurements of protein recovered indicated that some of the material was retained on the column and could not be eluted with isopropanol alone (Table II). Neither the solvent system of Tarr and Crabb[10] (acetonitrile–2-propanol, 3:1) nor the solvent

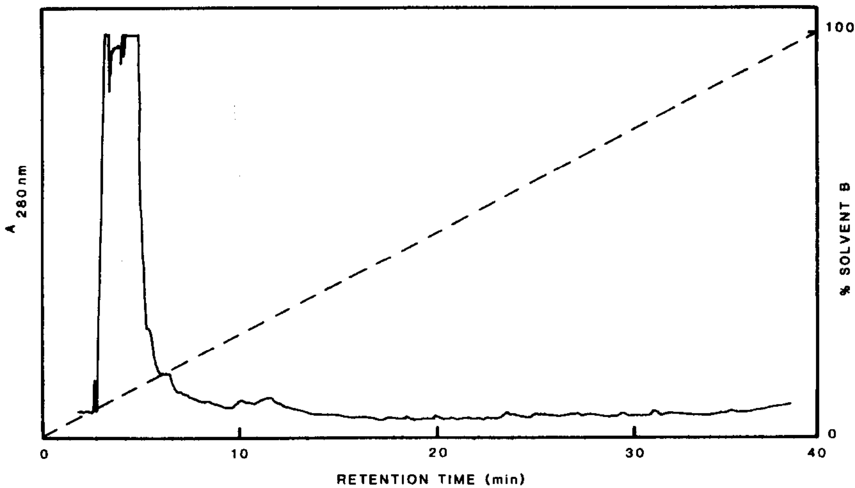

Fig. 1. Elution profile of the glycopeptide inhibitor. Conditions: solvent A, TFA in water (pH 3.0); solvent B, isopropanol–water (50:50) (pH 3.0). Linear gradient, 0–100% solvent B over 40 min; detection wavelength, 280 nm; flow-rates, 1 ml/min; injection volume, 100 μl; temperature, ambient.

TABLE II

THE EFFECT OF SOLVENTS ON THE RECOVERY OF BIOLOGICAL ACTIVITY AND PROTEIN FROM HPLC COLUMNS

The data represent the averages of triplicate determinations.

Solvent	*Recovery (%)*	
	*Inhibitory activity**	*Protein***
Isopropanol	93	71
3.0 *M* Urea–isopropanol–water	94	93
3.0 *M* Guanidine-HCl–isopropanol–water	96	96

* One inhibitory unit was arbitrarily defined as the amount of glycopeptide that gave 25% inhibition of protein synthesis with $3 \cdot 10^5$ 3T3 cells.

** Protein determinations were based on the Bradford[9] Coomassie blue assay as described in the Experimental section.

used by Power *et al.*[11] (acetonitrile–1-propanol, 1:1) was sufficient to elute the retained proteins. We chose not to use the formic acid solvent systems that have been proposed by Heukeshoven[2] owing to the anomalous side-reactions between formic acid and proteins[2,17].

Urea and guanidine-HCl have been known to be effective reagents to disaggregate protein aggregates and to disrupt hydrophobic interactions. We, therefore, examined the ability of urea and guanidine-HCl, in 50% isopropanol–water, to elute the hydrophobic proteins from the RP-HPLC column. Approximately 80 μg of the glycopeptide inhibitor preparation were injected into the column and the macromolecules were eluted with a linear gradient of 50% isopropanol in water (pH 3.0)

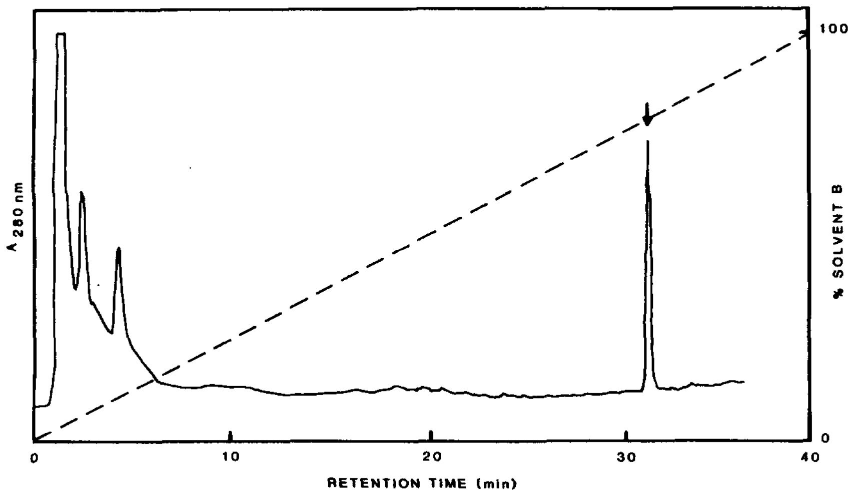

Fig. 2. The effect of urea on the elution profile of the glycopeptide inhibitor. Conditions: solvent A, TFA in water (pH 3.0); solvent B, 3.0 *M* urea in isopropanol–water (50:50) (pH 3.0). Other conditions as in Fig. 1. The position of the hydrophobic protein is marked by an arrow.

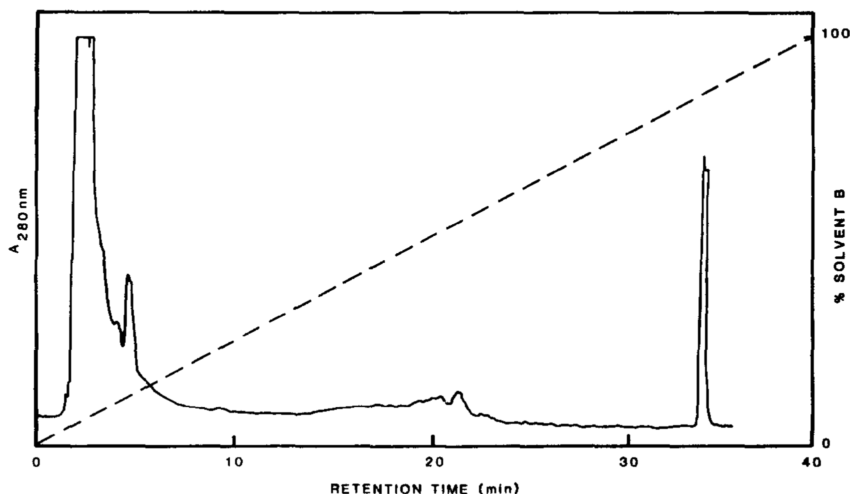

Fig. 3. The effect of guanidine-HCl on the elution profile of the glycopeptide inhibitor. Conditions: solvent A, TFA in water (pH 3.0); solvent B, 3 *M* guanidine-HCl in isopropanol-water (50:50) (pH 3.0). Other conditions as in Fig. 1.

containing either urea or guanidine-HCl as described in the figure legends.

Initially we tested urea and guanidine-HCl at 1.0 *M* concentrations and the chromatographic patterns of the inhibitor were similar to those obtained with 50% isopropanol and water (Fig. 1). Further elution of the column with 6.0 *M* urea or guanidine-HCl produced ghost peaks and indicated that protein was still retained. We found, however, that 50% isopropanol-water, containing 3.0 *M* urea (Fig. 2) or

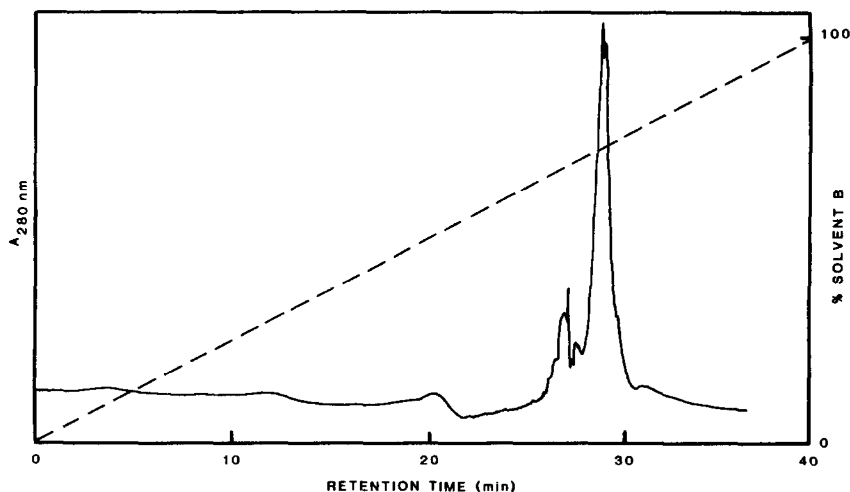

Fig. 4. Effect of urea on the recovery of column-bound hydrophobic protein. The column was first developed as described in Fig. 1, and after the run the column was equilibrated with solvent A. The column was then developed with 3 *M* urea in isopropanol-water (50:50) as described in Fig. 2, and the sensitivity of the detector was set at twice that of previous figures.

guanidine-HCl (Fig. 3), effectively eluted the more hydrophobic proteins from the RP-HPLC columns. The retention of the hydrophobic protein was shorter with 3 *M* urea than with 3.0 *M* guanidine-HCl. In these latter cases further elution with 6.0 *M* urea or guanidine-HCl in 50% isopropanol–water did not result in ghost peaks being eluted.

In addition to being able to elute all hydrophobic proteins from the RP-HPLC column with urea and guanidine-HCl (greater than 90% recovery), the glycopeptide inhibitor was found to be active in these solvent systems and still eluted with the breakthrough peak (Table II). Furthermore, if the glycopeptide inhibitor preparation was first eluted with 50% isopropanol–water, and then washed with 3.0 *M* urea in 50% isopropanol–water, the hydrophobic material that was retained on the HPLC column could be released (Fig. 4). Under these conditions the retention time of the hydrophobic proteins was somewhat shorter than when urea was used in the original solvent (Fig. 2). These more hydrophobic molecules, however, did not have biological inhibitory activity when tested with the 3T3 protein synthesis assay (data not shown).

It is clear from the chromatograms that the glycopeptide inhibitor is a relatively hydrophilic molecule. The acidic pH was important in these solvent systems since Tanford[18] has suggested that proteins that do not contain disulfide bonds, but do contain cysteine, may form disulfide bonds after denaturation and lead to aggregation or even precipitation. An acidic pH can circumvent this complication. At this time we don't know the mechanism by which urea and guanidine-HCl aid in the elution of hydrophobic proteins from the RP-HPLC column. These substances may favor the exposure of non-polar groups in the interior of the hydrophobic protein since aqueous solutions with urea and guanidine-HCl are better solvents than water for non-polar molecules. Studies on the transfer of hydrocarbons, used as models for amino acid side-chains from water to 7.0 *M* urea or 4.0 *M* guanidine-HCl show a favorable ΔG for the process[19]. Although the transfer requires energy at room temperature, the transfer is accompanied by a positive entropy change that overcomes the unfavorable enthalpy.

CONCLUSIONS

The hydrophobic nature of many proteins associated with cell membrane structures can cause special problems in RP-HPLC owing to their limited solubility, aggregation, and binding in an irreversible fashion to column matrices. By incorporating urea or guanidine-HCl, at various concentrations in isopropanol and water, we found that resolution and recovery of the hydrophobic proteins in our inhibitor preparations were enhanced. These data indicate that the glycopeptide inhibitor is a relatively hydrophilic molecule, stable at acidic pH, and is not irreversibly inactivated by the use of urea or guanidine-HCl in the RP-HPLC solvent system. It was also demonstrated that, although pure acetonitrile is volatile, the acidic solution of acetonitrile after evaporation interfered with the biological assays as a result of a residue that was not volatile. The results reported here may provide a useful approach to RP-HPLC or hydrophobic interaction chromatography of membrane-associated proteins, viral capsid proteins, or other hydrophobic polypeptides.

ACKNOWLEDGEMENTS

We thank Sherrie Nash and Heideh Fattaey for their excellent technical assistance. This study was supported by grants CA27648 and CA31531 from the National Cancer Institute, National Institutes of Health, and the Kansas Agricultural Experiment Station. This is contribution No. 85-221-J from the Kansas Agricultural Experiment Station, Kansas State University.

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