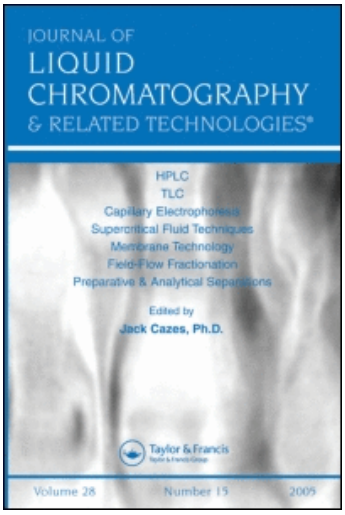

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STUDY OF AcOH/HCOOH/H_2O/$CHCL_3$ SOLVENT SYSTEM APPLICATION TO THE SEPARATION OF TWO LARGE AND HYDROPHOBIC FRAGMENTS OF BACTERIORHODOPSIN MEMBRANE PROTEIN BY CENTRIFUGAL PARTITION CHROMATOGRAPHY

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ABSTRACT

Centrifugal Partition Chromatography has been found useful for the separation of C_1 and C_2, two large hydrophobic peptides resulting from the chymotryptic cleavage of the membrane protein Bacteriorhodopsin. For this purpose the biphasic system AcOH/HCOOH/H_2O/$CHCl_3$ has been studied and the role of the two main parameters, i.e. Acetic acid and Formic acid, clearly demonstrated.

INTRODUCTION

Bacteriorhodopsin, the purple membrane contained in the halophilic bacteria *Halobacterium halobium*, is a hydrophobic membrane protein containing 248 amino-acids (MW 26,000). It contains one molecule of

all-trans-retinal as the chromophore, covalently linked through a protonated Schiff base, and functions as a light-driven proton pump. The polypeptide chain is arranged into seven α-helices which are largely embedded in the lipid bilayer. The amino-acid sequence has been determined by both protein and gene sequencing. Proteolytic degradations of purple membrane have been extensively carried out and led to various fragments. Chymotryptic cleavage of bacteriorhodopsin produced two fragments C1 and C2 which have been separated by gel permeation chromatography and reverse phase high pressure liquid chromatography.

In this paper we have used centrifugal partition chromatography (CPC) to separate and purify these two large and hydrophobic polypeptide fragments. Membrane proteins are known to be very hydrophobic and insoluble in many solvents, and have a marked tendency to aggregate, which is not surprising in view of their natural environment, namely a lipid bilayer. Various studies of chromatographic properties of membrane proteins have been reported [1-4]; recovery is strongly dependent on both columns and solvent systems. Since no solid stationary phase is involved in CPC, it should be a good alternative for separation and purification of membrane proteins.

Separation of hydrophylic proteins has been performed by CPC using two polymeric aqueous phases systems [5], while separation of short hydrophobic peptides (less than 20 amino-acid residues) by countercurrent chromatogaphy has been recently reported [6]. CPC separation of C1 (177 amino-acid residues, including 22 aromatic and 91 aliphatic) and C2 (71 amino-acid residues, including 10 aromatic and 33 aliphatic) opens a new field for applications of CPC.

METHODS

Samples :

Bacteriorhodopsin C1 and C2 fragments were prepared according to published procedures [7].

The fragment C1 has the following amino-acid sequence :

Gly-Gly-Glu-Gln-Asn-Pro-Ile-Tyr-Trp-Ala-Arg-Tyr-Ala-Asp-Trp-Leu-Phe-Thr-Thr-Pro-Leu-Leu-Leu-Leu-Asp-Leu-Ala-Leu-Leu-Val-Asp-Ala-Asp-Gln-Gly-Thr-Ile-Leu-Ala-Leu-Val-Gly-Ala-Asp-Gly-Ile-Met-Ile-Gly-Thr-Gly-Leu-Val-Gly-Ala-Leu-Thr-Lys-Val-Tyr-Ser-Tyr-Arg-Phe-Val-Trp-Trp-Ala-Ile-Ser-Thr-Ala-Ala-Met-Leu-Tyr-Ile-Leu-Tyr-Val-Leu-Phe-Phe-Gly-Phe-Thr-Ser-Lys-Ala-Glu-Ser-Met-Arg-Pro-Glu-Val-Ala-Ser-Thr-Phe-Lys-Val-Leu-Arg-Asn-Val-Thr-Val-Val-Leu-Trp-Ser-Ala-Tyr-Pro-Val-Val-Trp-Leu-Ile-Gly-Ser-Glu-Gly-Ala-Gly-Ile-Val-Pro-Leu-Asn-Ile-Glu-Thr-Leu-Leu-Phe-Met-Val-Leu-Asp-Val-Ser-Ala-Lys-Val-Gly-Phe-Gly-Leu-Ile-Leu-Leu-Arg-Ser-Arg-Ala-Ile-Phe-Gly-Glu-Ala-Glu-Ala-Pro-Glu-Pro-Ser-Ala-Gly-Asp-Gly-Ala-Ala-Ala-Thr-Ser

C1 contains 177 amino-acid residues, has a molecular weight about 19075, and contains the carboxyl terminus of the protein.

The fragment C2 has the following amino-acid sequence :

Glu-Ala-Gln-Ile-Thr-Gly-Arg-Pro-Glu-Trp-Ile-Trp-Leu-Ala-Leu-Gly-Thr-Ala-Leu-Met-Gly-Leu-Gly-Thr-Leu-Tyr-Phe-Leu-Val-Lys-Gly-Met-Gly-Val-Ser-Asp-Pro-Asp-Ala-Lys-Lys-Phe-Tyr-Ala-Ile-Thr-Thr-Leu-Val-Pro-Ala-Ile-Ala-Phe-Thr-Met-Tyr-Leu-Ser-Met-Leu-Leu-Gly-Tyr-Gly-Leu-Thr-Met-Val-Pro-Phe

C2 contains 71 amino-acid residues, has a molecular weight of about 7772, and originates from the amino terminus.

HPLC analysis :

HPLC analysis has been used to monitor the CPC separation. The retention of peptides by reversed phase supports can be estimated by the value of the Rekker's fragmental constant [8] (146 for C1 and 65 for C2), or more efficiently by calculating Sasagawa' retention constants [9], namely $\log(1+\Sigma D_i)$ values, where D_i is the retention constant of the peptide amino-acid i. For C1 and C2, these values are respectively 7.62 and 6.82, meaning that despite the length difference between these two fragments (177 and 71 amino-acids), the retention times will be close.

Best results have been obtained with a 12.5 x 0.4 cm I.D. column packed with Nucleosil 300-7 Protein RP (Macherey Nagel, Düren, GFR),

using a linear gradient of 0.1 M H_3PO_4 in ethanol/water as eluent (for 40 to 100% ethanol within 20 min; flow rate 0.7 ml/min).

Chromatogaphy was performed with a system comprising a Perkin Elmer chromatography station including a Series 4 pump controlled by a PE 7000 laboratory computer, a Rheodyne 7125 injector and a Kratos Spectroflow 773 variable wavelength detector (monitoring wavelength 215 nm).

Despite the good properties of the Nucleosil column, we noticed some absorption of C1 and C2. In order to prevent contamination to occur between runs, the columns was washed after each gradient elution by injecting pure formic acid while the mobile phase was 0.1 M H_3PO_4 in 100% ethanol; this was followed by a blank injection which showed that no C1, C2 or bacteriorhodopsin remained absorbed on the column, even after a highly concentrated injection of either of three compounds.

CPC separations :

A centrifugal partition chromatography apparatus (Model CPC LLN) manufactured by Sanki Engineering (Nagaokakyo, Kyoto) was used. It consisted of a continuous flow centrifuge containing 6 cartridges (type 250 W; total volume 128 ml) made of monochlorotrifluoroethylene resin, a constant flow pump (LBP II type triple plungers), a valve connection unit (FCU II) linked to a 4 ml PTFE sample loop injector, an electric power unit (PCB II), a fixed wavelength UV detector (254 nm), a fraction collector (Buchler) and a recorder (Fisher).

The solvent systems were mixtures of glacial acetic acid (Amend, Irvington, N.J.), formic acid (Aldrich, Milwaukee, WI), water (Burdick Jackson, Muskegon, MI), and chloroform (hplc grade Fisher, Fairlawn, N.J.). Fractions of 2 - 10 ml were collected and the presence of C1 and C2 was checked by hplc, with or without prior concentration depending on the experiment.

RESULTS AND DISCUSSION.

AcOH/HCOOH/H_2O/$CHCl_3$ systems.

Some preliminary studies has been performed to find a suitable solvent system which would have the following characteristics : good

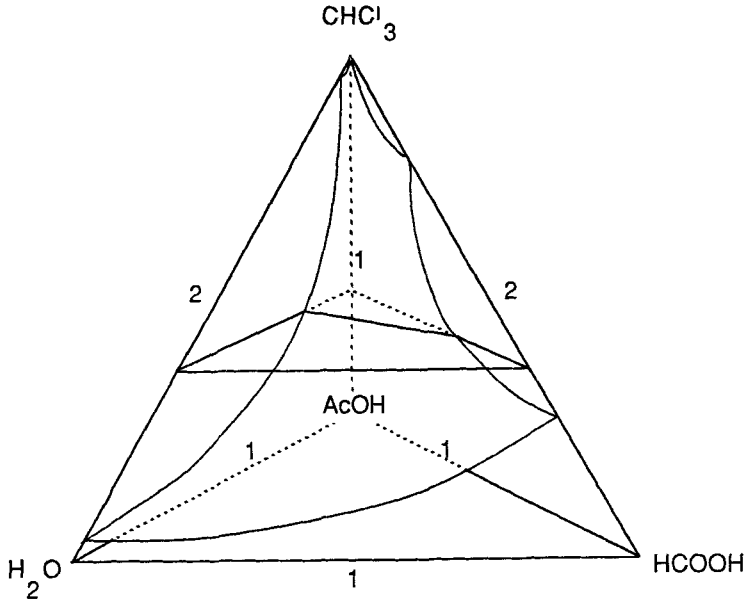

FIGURE 1 : Tetrahedric representation of the four solvent systems used for CPC experiments.
The internal surface separates the two zones (i.e. the upper biphasic and the lower monophasic). The middle triangle corresponding to 44% $CHCl_3$ has been chosen for this study.

hydrodynamic properties, density difference between the two phases compatible with CPC, and overall good solvation properties of both phases for C1 and C2. Indeed it is well known that membrane proteins or peptides derived from membranes are particularly insoluble, except in very acidic media such as 40% formic acid, or become pseudo soluble upon addition of surfactants, the later being incompatible with CPC.

Since HCOOH and $CHCl_3$ are slightly miscible, the biphasic solvent system n-BuOH/HCOOH/H_2O/$CHCl_3$ (10/40/10/40) contains 92.4 % of HCOOH in the upper aqueous phase in which C1 and C2 are found. On the contrary, with the system AcOH/H_2O/$CHCl_3$, C1 and C2 are found in the lower phase. We therefore adopted the biphasic solvent system AcOH/HCOOH/H_2O/$CHCl_3$ to search for an optimal distribution. This system can be represented by the tetrahedron shown in Fig.1, where

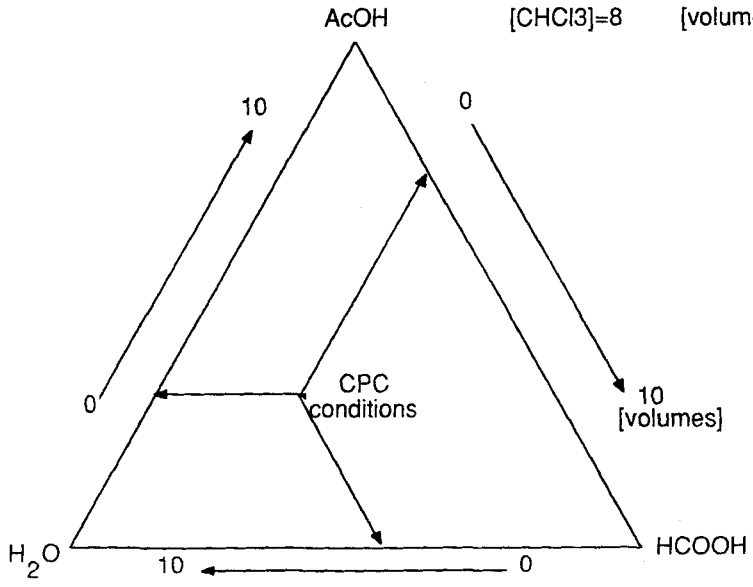

FIGURE 2 : Reduction of a quarternary system to a pseudo 3 component system. This triangle corresponds to the $CHCl_3$ level of 44%, the percentage of the other three components is read on this ternary diagram.

each solvent occupies a summit; only 2 of the 6 edges correspond to mixtures of partly immiscible solvents, namely, $H_2O/CHCl_3$, and $HCOOH/CHCl_3$. The internal surface dividing the tetrahedric volume into biphasic and monophasic zone has been roughly estimated by determination of some of its points (Fig.1). For CPC experiments, we have to remain above this surface, while the monophasic zone could be used for other purposes, such as size exclusion chromatogaphy. The triangle corresponding to 44% $CHCl_3$ (independent of what the remaining 56% might be) has been chosen for CPC experiments. Its biphasic zone corresponds to systems where the upper to lower phases ratio remain approximately 1, the density difference between the two phases being in the range 0.13 to 0.25 g cm^{-3}. Using this triangle we can now define any quaternary system with a constant $CHCl_3$ level (44%). The percentage of the other three components is read on the side of this ternary diagram (Fig.2).

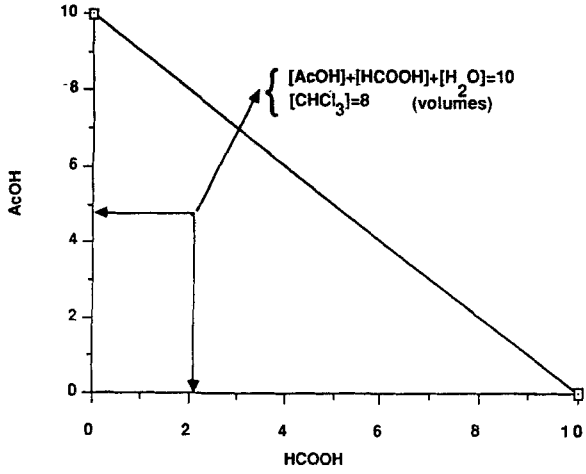

FIGURE 3 : Reduction of a quarternary system to a pseudo 2 component system. AcOH and HCOOH are the main governing parameters for the partitioning of the tested solutes.

This representation is very useful to compare or to make solvent systems, but cannot easily lead to graphic display with computers, which need orthogonal representations. Fig.3 shows the second simplification step for system representations. HCOOH and AcOH volumes are, respectively, the abscissae and ordinates of this orthogonal representation, the volumes of H_2O and $CHCl_3$ being determined by the simple relation :

$$AcOH + HCOOH + H_2O = 10$$
$$CHCl_3 = 8$$

AcOH and HCOOH have been selected because they are the main governing parameters for the partitioning of the tested solutes. With this representation we can easily use computers to go from percentages or volumes to graphic display and *vice versa* .

Partition of C1 and C2.

As shown in Fig.4, several experimental points have been tested in order to partition C1 and C2 in the AcOH/HCOOH/H_2O/$CHCl_3$ solvent systems. The upper line is the limit between the biphasic and the

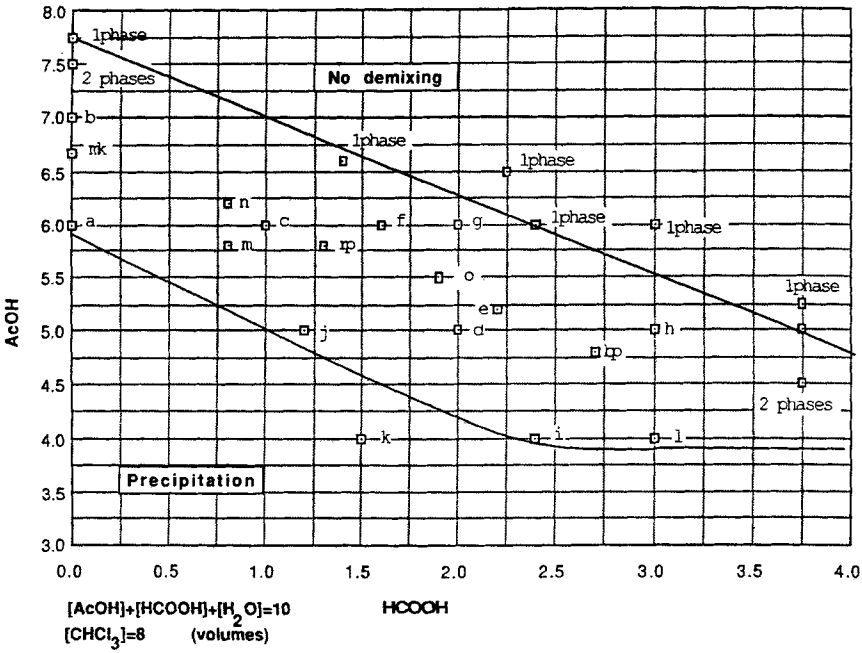

FIGURE 4 : Some experimental points tested during this study. Upper line separate biphasic and monophasic zones. Precipitation of C1 and C2 occur below the lower line.

monophasic zones. The lower line limits the zone under which attempts to dissolve C1 and C2 fail because of partial or total precipitation : hplc chromatograms of upper and lower phases corresponding to points i,j,k,l are flat or show low concentrations of C2 and no concentration of C1.

Within these two limits the partition of C1 and C2 between the lower and the upper phases varied progressively from 100% in the lower (a) to 100% in the upper phase (f,g,h). Table 1 summarizes these results while Fig.5 to 8 show the corresponding chromatograms.

It appears from Table 1 and Fig.4 that both AcOH and HCOOH move C1 and C2 from the $CHCl_3$-rich lower phase to the H_2O-rich upper phase, and that HCOOH has a stronger effect than AcOH. The relative ratio of C1 and C2 in these two phases is reversed when going from HCOOH-poor systems (b,m) to HCOOH-rich systems (d,e).

TABLE 1 : C1 and C2 HPLC PEAK AREAS AND THEIR RATIO IN AcOH/HCOOH/H_2O/$CHCl_3$ SYSTEMS UPPER PHASE.

Peak area for C2(hplc)			Peak area for C1(hplc)		C2(U/(U+L))	C1(U/(U+L))
Point	Upper phase	Lower phase	Upper phase	Lower phase		
a	0	5510695	0	2105160	0	0
b	807871	2115718	528946	4726667	0.28	0.10
c	6911881	10961241	5663582	31763238	0.39	0.15
d	2019415	1034335	1471118	332188	0.66	0.82
e	1778973	546565	4708380	547480	0.76	0.90
f	2067758	0	3263966	0	1	1
h	5076845	0	10757379	0	1	1
i	slightly soluble		nonsoluble			
j	nonsoluble		nonsoluble			
k	nonsoluble		nonsoluble			
l	slightly soluble		nonsoluble			

Unweighed sample of C1+C2 was dissolved in 1ml of the biphasic system, shaken during two minutes and centrifuged at 11000 rpm for 4 min; 20 µl were injected for hplc analysis.

Two factors may explain the stronger effect of HCOOH than AcOH : (i) HCOOH (pKa=3.75) is stronger than AcOH (pKa=4.75), resulting in a better protonation of C1 and C2; (ii) HCOOH is predominantly in the upper phase, and the corresponding salts (C1 and C2 formates) will be strongly solvated by water, resulting in larger variations of the partition coefficients with HCOOH content. The reversed relative ratio of C1 and C2 in the upper phase with increasing concentration of HCOOH is mainly governed by the protonation state of C1 and C2. The calculated isoelectric point [10] for C1 and C2 are, respectively, 4.4 and 7, showing that C2 is fully protonated by AcOH but not C1; consequently the C2 ratio in the upper phase is larger than the C1 ratio in HCOOH-poor systems. On the contrary, full protonation of C1 occurs with increasing concentration of HCOOH, leading to 10 charged residue for C1 against 4 for C2. Since the overall hydrophobicities, i.e., $\log(1+\sum D_i)$ are similar, the larger difference in protonation state result in a stronger solvation of C1, compared to C2, by the H_2O-rich upper phase.

We may now define two methods for the separation of C1 and C2 by CPC.

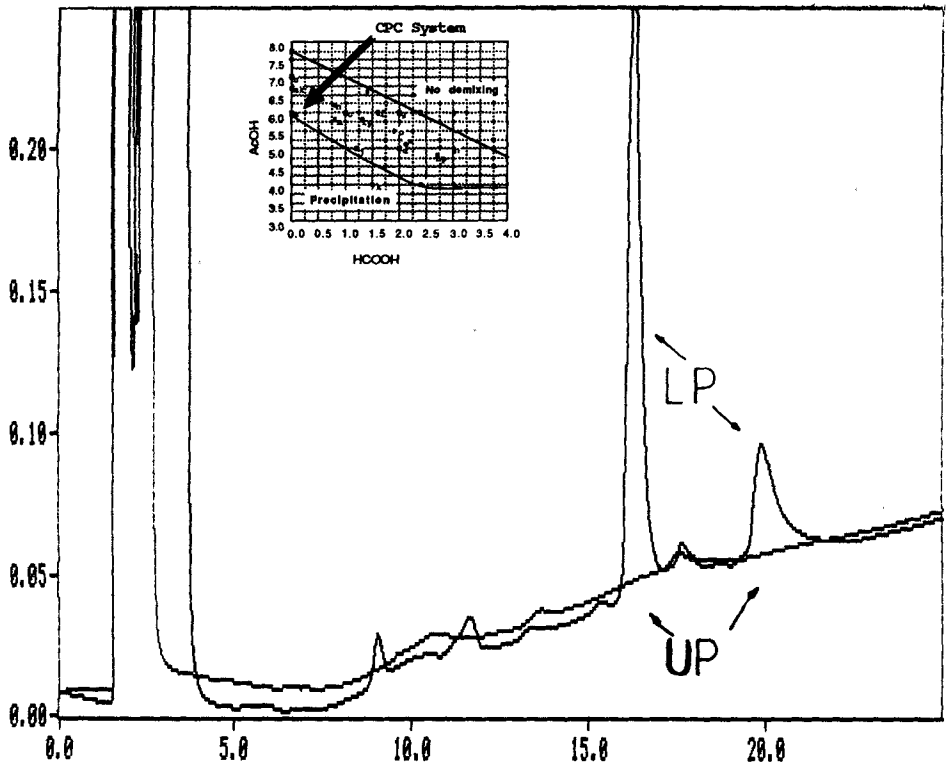

FIGURE 5 : hplc chromatograms of upper and lower phases for point a :
AcOH/HCOOH/H_2O/$CHCl_3$: 33.3/0/22.3/44.4
hplc conditions :
column : 12.5 x 0.4 cm I.D.
packing : Nucleosil 300-7 Protein RP
mobile phase : 0.1 M H_3PO_4 in water/ethanol; 40 to 100% ethanol in 20 min
Flow rate : 0.7 ml/min
Detection : UV at 216 nm
C2 : tr≈ 16 min; C1 : tr≈ 20 min

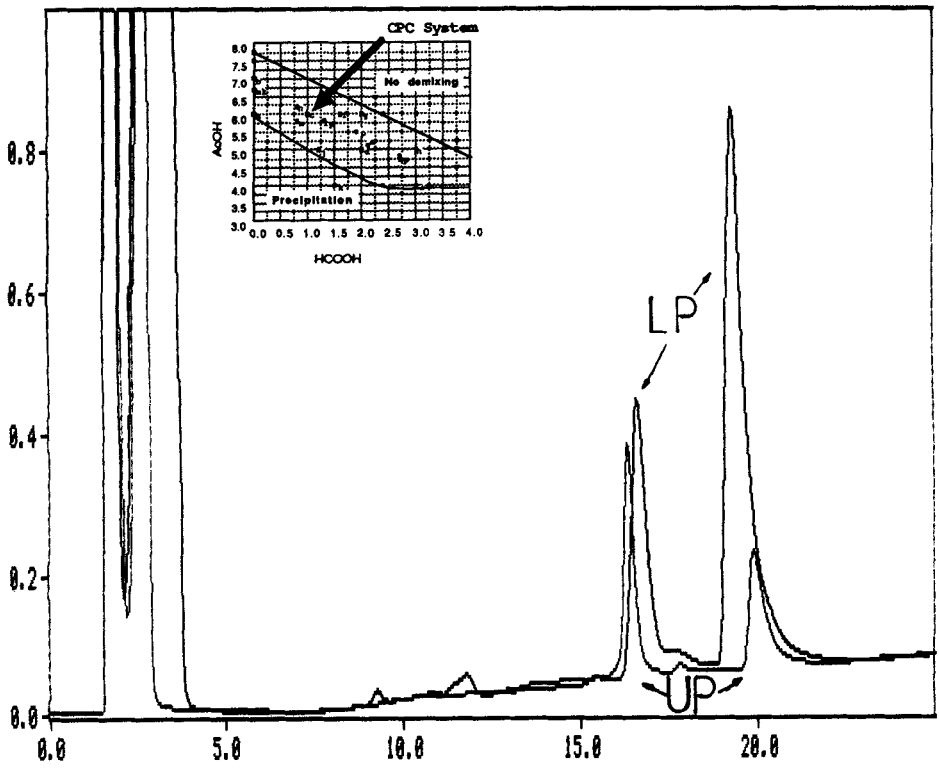

FIGURE 6 : hplc chromatograms of upper and lower phases for point c : AcOH/HCOOH/H_2O/$CHCl_3$: 33.3/5.6/16.7/44.4
Same hplc conditions as in Fig.5

a) HCOOH poor systems : C1 and C2 are preferentially in the lower phase, and if the upper phase is the mobile phase, C2 will be eluted first, as in reversed phase chromatogaphy.

b) HCOOH rich systems : C1 and C2 are preferentially in the upper phase, and if the lower phase is the mobile phase, C2 will be eluted first, as in cation exchange chromatogaphy.

These two methods have been tested, and only one was successful.

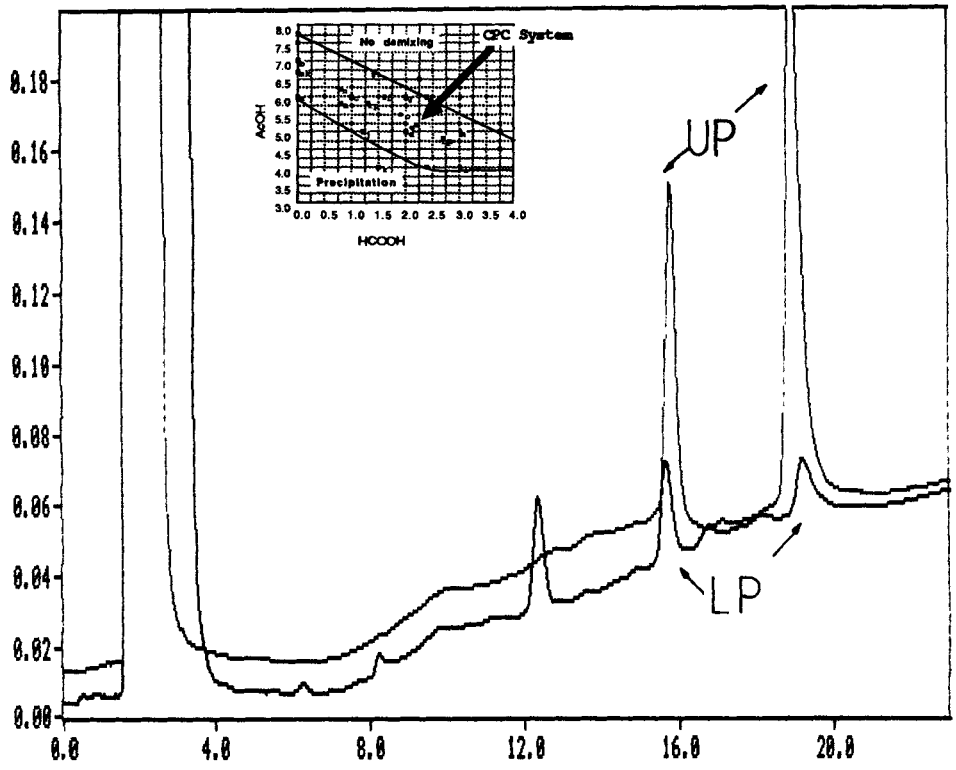

FIGURE 7 : hplc chromatograms of upper and lower phases for point e : AcOH/HCOOH/H_2O/$CHCl_3$: 28.9/12.2/14.5/44.4
Same hplc conditions as in Fig.5

CPC using HCOOH poor systems.

The two biphasic solvent systems shown below and corresponding to points m and n have been tested;

%	AcOH	HCOOH	H_2O	$CHCl_3$
m	32.2	4.5	18.9	44.4
n	34.4	4.5	16.7	44.4

The upper phase (mobile phase) was pumped through the lower stationary phase at 1 ml/min in the ascending mode. The rotation speed

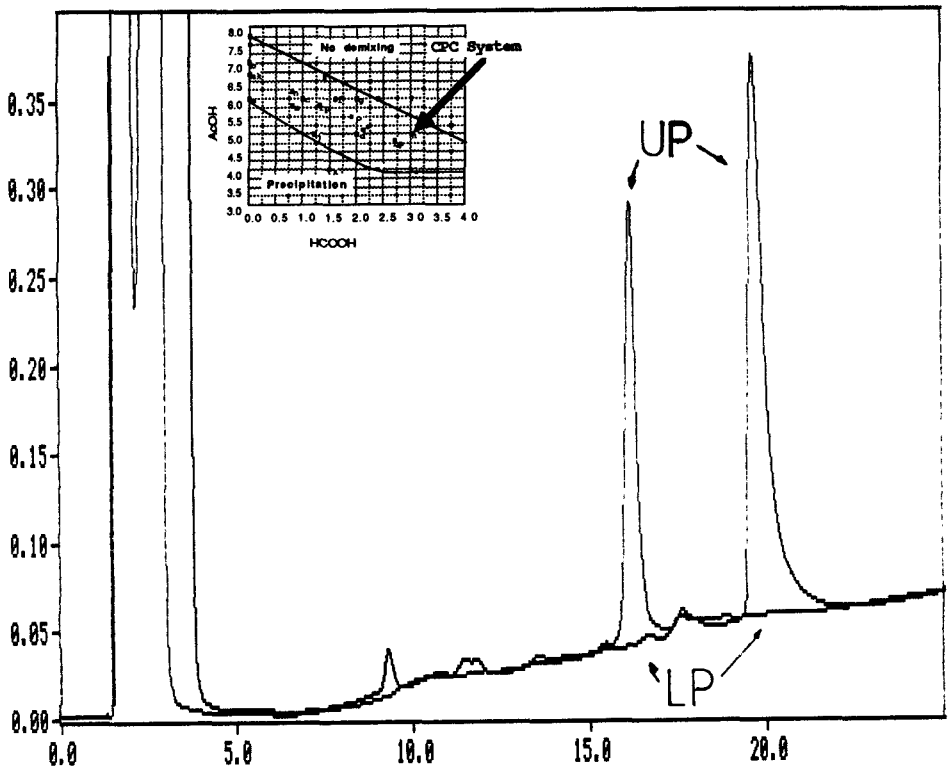

FIGURE 8 : hplc chromatograms of upper and lower phases for point h : AcOH/HCOOH/H_2O/$CHCl_3$: 27.8/16.7/11.1/44.4
Same hplc conditions as in Fig.5

was adjusted to 800 rpm which stabilized the pump discharge at 55 Kg/cm^2. The volume occupied by the mobile phase was 40 ml (m) and 60 ml (n). Crude mixtures of C1 and C2 (m: 13 mg, n: 10 mg) in 4 ml of 1/1 upper + lower phase (m) or 4 ml of upper phase (n) were injected after the mobile phase started to elute from the centrifuge. Although C1 and C2 partition in this biphasic system was in favor of the lower phase, thus predicting a good retention of the peptides by the stationary phase, the peptides were eluted rather quickly; moreover, although the first fractions were rich in C2 and the latest in C1,

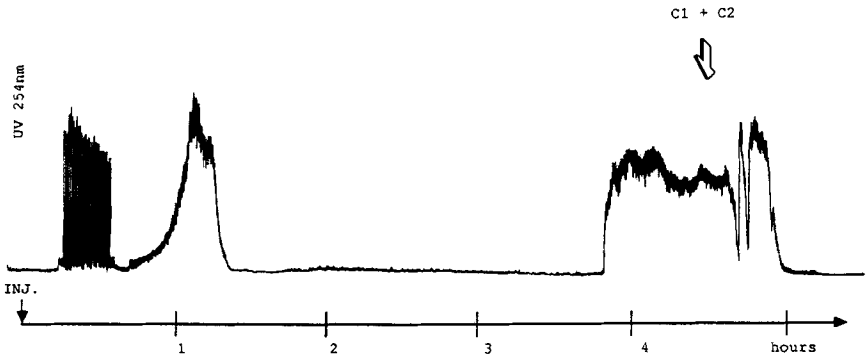

FIGURE 9 : CPC trace during experiment at point m :
AcOH/HCOOH/H_2O/$CHCl_3$: 32.2/4.5/18.9/44.4
Mobile phase :upper phase (ascending mode)
Flow rate :1 ml/min; rotational speed : 800 rpm
Sample : 13 mg of C1 + C2 in 2 ml of upper and 2 ml of lower phase. C1 and C2 come out with a substantial amount of stationary phase, which is the main cause of the observed perturbation.

they are not well-separated. The corresponding trace of the CPC UV detector (254 nm)(Fig.9) shows a strong perturbation during the elution of C1+C2, and the collected fractions contained a substantial amount of the stationary phase. It seems that C1 and/or C2 acted as surfactants; the resulting emulsion is eluted more rapidly than C1 and C2 would have been if this surfacting effect did not occur, thus cancelling the process of separation.

CPC using HCOOH rich systems.

The two biphasic solvent systems shown below, corresponding to points e and o, have been tested;

%	AcOH	HCOOH	H_2O	$CHCl_3$
e	28.9	12.3	14.4	44.4
o	30.6	10.6	14.4	44.4

The lower phase (mobile phase) was pumped through the upper stationary phase at 1 ml/min in the descending mode. The rotation speed was

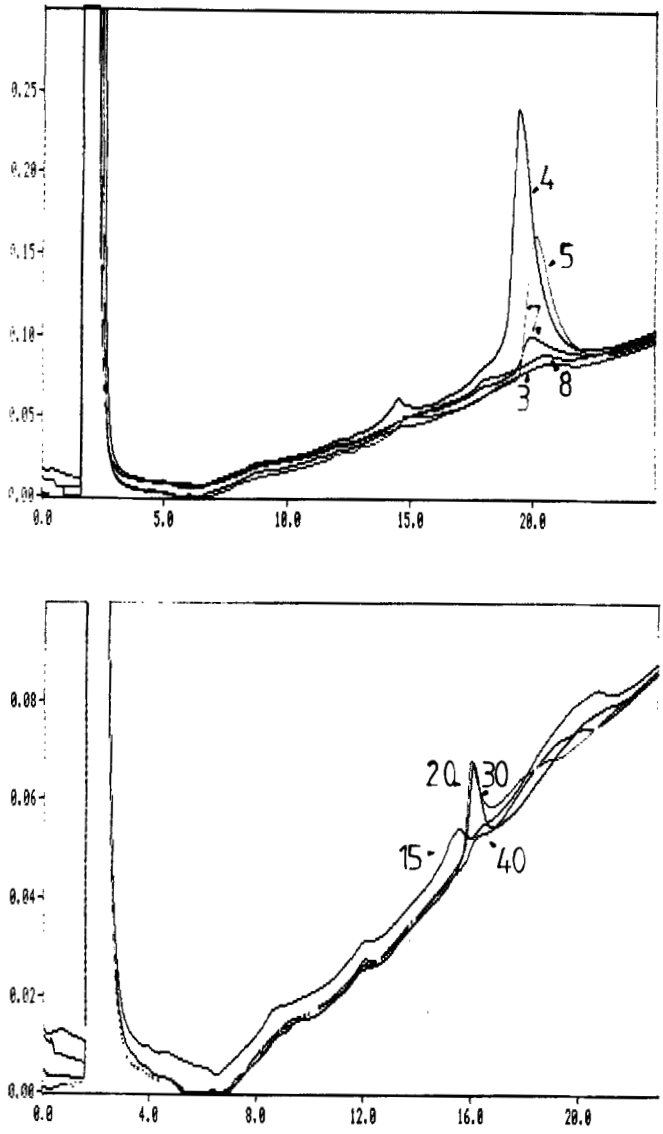

FIGURE 10 : hplc analysis of collected fractions during run at point o : AcOH/HCOOH/H_2O/$CHCl_3$: 30.6/10.6/14.4/44.4
Sample : 20 µl of the 2 ml fractions indicated by numbers.

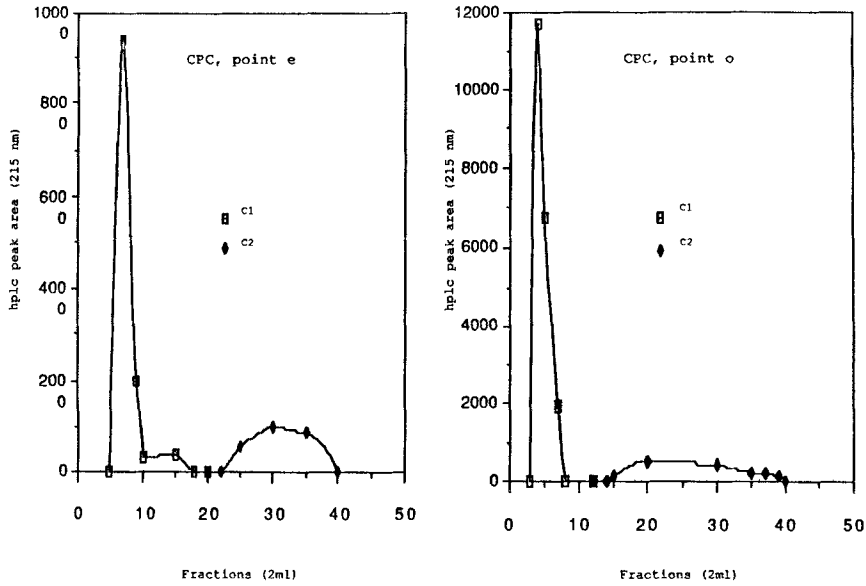

FIGURE 11 : Reconstituted CPC chromatograms of runs at points e and o, from peak areas found by hplc.

adjusted to 800 rpm which stabilized the pump discharge at 55 Kg/cm^2. The volume occupied by the mobile phase was about 40 ml. Crude mixtures of C1 and C2 (e: 12 mg; o: 11 mg) in 4 ml lower phase were injected after the mobile phase started to elute from the centrifuge.

After discarding the first 100 ml containing lipophilic impurities, recycling was performed for 20 hrs (e) and 10 hrs (o); the solvent flow direction was then reversed and C1 and C2 were eluted using the ascending mode. Fractions of 2 ml were collected and analyzed by hplc. Fig.10 shows the hplc analysis of fractions collected during run "o"; fragment C1 is collected in fractions 4-7 and fragment C2 in fractions 20-40. Chromatograms reconstituted from the peak areas of these fractions (Fig.11) show that C1 and C2 separation was excellent; furthermore, the amount of solvent needed for elution was very small, 8 ml for C1 and 30-40 ml for C2.

In both experiments, the recycling mobile phase as well as the total amount of the phase remaining in the centrifuge after elution of C1 and C2 were evaporated and analyzed by hplc; none of the residues contained C1 or C2.

CONCLUSION.

Solvent systems AcOH/HCOOH/H_2O/$CHCl_3$ have proved to be very useful for the separation of the two large and hydrophobic peptides C1 and C2 from the membrane protein bacteriorhodopsin. Physical properties such as viscosities and relative densities are fully compatible with CPC, allowing a good retention of the stationary phase in both ascending and descending modes. The two main parameters AcOH (fully miscible in H_2O and $CHCl_3$, pKa 4.75), and HCOOH (fully miscible in H_2O only, pKa 3.75) allow one to control the partitioning of the peptides from 100% lower phase to 100% upper phase, and to control their charge state which affects their relative affinities for both phases. Moreover, solubilities of the studied peptides are excellent in the two phases, thus eliminating the occurrence of precipitation during the CPC process. This solvent system is recommended for the isolation and purification of other membrane proteins or peptides as well.

ACKNOWLEDGEMENTS.

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